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Factors Influencing Efficiency and Reproducibility of Polybrene-Assisted Gene Transfer

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Abstract—*A systematic investigation of factors influencing the efficiency of polybrene-assisted gene transfer for both transient and stable foreign gene expression was carried out utilizing NIH 3T3 fibroblasts as prototypic recipients for the plasmid expression vectors pSV2cat and pSV2neo. While transfection cocktail composition and cell density, in addition to polybrene exposure conditions and exogenous DNA concentration, each played an important role, the key determinant to achieving excellent transfection efficiency proved to be the DMSO treatment regimen. Under optimal conditions, the yield of colonies resistant to the neomycin analog, G418, increased linearly at the rate of 10 clones/ng of input (native form I pSV2neo) DNA up to a plasmid concentration of 50 ng, whereupon the dose–response for colony recovery became semilogarithmic. The incidence of stable transformants was doubled by linearization of the vector DNA, whereas the addition of carrier DNA to the transfection cocktail was without effect until present at concentrations above 10-fold molar excess, at which point the efficacy of gene transfer declined rapidly. Combined Southern and dot-blot analyses of transformed cell DNA demonstrated that the polybrene–DMSO procedure led to the stable integration of relatively few copies of the marker gene in each transformant; the actual number varied from 1–3 to 10–15 per host genome, depending on the concentration of pSV2neo DNA added. The potential for the adaptation of this DNA transfection procedure for general use with other mammalian cell types, as well as its technical strengths and weaknesses, is discussed.*

INTRODUCTION

The advent of gene transfer methodologies has provided a powerful tool for the identification, isolation, and characterization of eukaryotic genes (1, 2). However, the efficiency with which foreign genes can be introduced, transiently expressed, or stably inherited depends largely on the cell type chosen to serve as a recipient. Indeed, most mammalian (particularly human) cell types, with the possible exception of certain established rodent and simian cell lines, have remained notoriously refractory to transfection (3–5). As a result, efforts to improve the efficacy of gene transfer and to broaden the spectrum of mammalian cell types amenable to transfection have produced a variety of strategies. These have ranged from the relatively simple scheme of coincubation with either calcium phosphate (6–9) or one of the polycationic reagents, diethylaminoethyl-dextran (DEAE-dextran) (10, 11), poly-L-ornithine (12), or polybrene (13, 14), to the more sophisticated approaches of employing retroviral vectors (15), microinjection (16, 17), electroporation (18, 19), and

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liposome-, protoplast-, or chromosome-mediated gene transfer (4, 20–23).

Polycation adjuvants have been shown to enhance both the adsorption and infectivity of retroviruses (24, 26) and free viral RNAs (27, 28) by serving as electrostatic bridges between the negatively charged virions or viral nucleic acids and the anionic components of target cell membranes. Unfortunately, DEAE-dextran (27) and, to some extent, poly-L-ornithine (12) tend to be deleterious even when present at low concentrations for short exposure times. In sharp contrast, polybrene can be applied to cells over extended periods of time without apparent adverse effects (24, 29). Kawai and Nishizawa have recently reported that the morphological transformation of chicken embryo fibroblasts by cloned Rous sarcoma virus genomic DNA can be significantly enhanced by combining the use of polybrene as an adsorbant in the transfection cocktail with dimethyl sulfoxide (DMSO) as a permeabilization agent for mediating cellular uptake of the bound DNA (14). The optimal regimen required cell monolayers to be exposed first to 30 μg/ml polybrene for 6 h followed by a 4-min treatment with DMSO. These conditions have also been used successfully for transformation of Chinese hamster ovary (CHO) cells (13). However, when applied to transfection of murine NIH 3T3 and several human fibroblast strains, we have found this protocol to be extremely cytotoxic, yielding few, if any, stable transformants.

In view of the inadequacy of the original polybrene–DMSO procedure for our purposes, we systematically set out to define the principle determinants governing the efficiency of the polybrene-assisted gene transfer technique. Using murine NIH 3T3 fibroblasts as archetypic recipients, the present report details a procedure by which foreign genes can be routinely transfected with high efficiency (0.4–0.6%) without compromising cell viability. We also identify parameters deemed critical for the adaptation of polybrene-assisted gene transfer to other mammalian cell types. Our findings confirm and extend two reports, which appeared during the course of our studies, describing the sequential use of polybrene and DMSO to transfect cultured rodent and human cells (13, 30). [A brief description of our findings has been reported elsewhere (31).]

MATERIALS AND METHODS

Plasmids and Their Preparation

Three DNA plasmids were used: pCB6, pSV2cat, and pSV2neo. (The first plasmid was generously provided by a colleague, J.D. Childs; the second was kindly donated by G. Duckworth-Rysiecki, University of Toronto, Toronto, Ontario; and the third was supplied by J. Bell and M. McBurney, University of Ottawa, Ottawa, Ontario.) The molecular structure of each plasmid has been detailed elsewhere (32–34).

Plasmid stocks were propagated in *Escherichia coli* strain HB101 and isolated free of host nucleic acids using the alkaline lysis procedure of Birnboim (35). Each plasmid preparation was further enriched for native form I (covalently closed, circular, supercoiled) DNA molecules by the acid–phenol purification method of Zasloff and coworkers (36). Tritiated thymidine-containing pCB6 DNA (specific activity $\sim 5 \times 10^5$ cpm/μg; kindly provided by a colleague, M. Liuzzi) served as the source of radionuclide-labeled plasmid material for the DNA adsorption assays described below. Each batch of radioactive pCB6 DNA was prepared by (1) propagating the plasmid in HB101 cultures for 18 h in the presence of 20 μCi/ml [*methyl*-^{3}H]thymidine (stock specific activity, 79 Ci/mmol; New England Nuclear, Lachine, Québec) and (2) subsequently isolating ^{3}H-labeled form I plasmid molecules as outlined above, except for the omission of the acid–phenol purification step.

Cells and Their Cultivation

The established mouse fibroblast line NIH 3T3, a gift of E.H. Chang (Uniformed Services Health Sciences Center, Bethesda, Maryland), was grown in monolayer culture in high glucose (4500 mg/liter) Dulbecco's modified Eagle's medium (DMEM) supplemented with 10% fetal calf serum (FCS), 1 mM L-glutamine, 100 units/ml penicillin, and 100 μg/ml streptomycin sulfate (henceforth referred to as growth medium). Cultures were maintained in a 37°C incubator providing a humidified (75–85%) atmosphere of 5% CO_2–95% air, with biweekly replenishment of growth medium. Unless indicated otherwise, all cell culture reagents and media were purchased from GIBCO/BRL Inc. (Burlington, Ontario) and were warmed to 37°C immediately before use.

Stock Solutions: Polybrene and G418

Concentrated solutions (1 mg/ml) of polybrene (1,5-dimethyl-1,5-undecamethylene polymethobromide; Aldrich Chemical Co., Milwaukee, Wisconsin) were prepared by dissolving the solid in calcium- and magnesium-free Hanks' balanced salt solution (HBSS). The preparation was sterilized by filtration through a 0.22-μm syringe tip filter unit, dispensed, in 0.25-ml volumes, into sterile 0.5-ml polypropylene microcentrifuge tubes, and stored frozen (−20°C). [Note: In our experience, polybrene stock solutions prepared in calcium- and magnesium-free phosphate-buffered saline (PBS) were much less effective in DNA transfection experiments than those made up in HBSS. This may be attributed to the higher concentration of phosphate anions in PBS (9.5 mM vs. 0.78 mM in HBSS) which would tend to neutralize the cationic strength of polybrene and in turn reduce its binding potential to both protein and DNA.]

G418 sulfate (Geneticin; GIBCO/BRL) was obtained at potencies ranging from 450 to 600 μg active reagent per milligram lyophilized powder. Stock solutions (100% potency) were prepared by dissolving the lyophilized material at a concentration of 10 mg active G418 sulfate (i.e., 16.7–22.2 mg powder) per milliliter in calcium- and magnesium-free HBSS; each lot was then filter-sterilized, aliquoted, and stored frozen until used.

DNA Adsorption Assay

NIH 3T3 fibroblasts were harvested in late-log phase of growth and seeded at a density of 5×10^5 cells per plate using 60-mm-diam. culture dishes. Approximately 20 h later, the growth medium was aspirated, and the monolayer cultures were washed twice with serum-free medium. The cultures were overlayed with 2.0 ml of fresh growth medium containing 50 ng/ml [^{3}H]pCB6 DNA in combination with varying amounts of polybrene and FCS, and then incubated for appropriate times to permit exogenous form I plasmid DNA–polybrene complexes to adsorb to cell surfaces. The solution was then removed, and each culture was washed twice with 2.5 ml of ice-cold calcium- and magnesium-free PBS to remove any unbound DNA. Next the cells were lysed by addition of 1.0 ml of ice-cold 10% (v/v) trichloroacetic acid (TCA), and the lysate was harvested with a rubber policeman. Residual cellular debris was recovered by washing each dish twice with 5.0 ml of 10% TCA and combining the two washes with the cell lysate. Each pooled sample (totaling 11 ml) was held on ice for 1 h, whereupon the TCA-insoluble radioactive material was collected by vacuum filtration on a Whatman GF/C glass fiber filter disk and quantitated.

Transient Gene Expression Assay

Transfection experiments were carried out, as detailed below, by incubating monolayer cultures of 7.5×10^5 cells (previously seeded in 10-cm-diam. dishes) for 18–20 h with 4.0 ml of growth medium containing 0.5

μg/ml form I pSV2cat DNA and varying concentrations of polybrene. Following permeabilization with 5.0 ml of DMSO solution (4.5 min at 37°C), the cells were rinsed twice with 5.0 ml growth medium and then exposed to 10 mM sodium butyrate (in 10 ml growth medium) for a further 18–20 h to enhance transient expression of the *cat* gene (37). Each culture was next fed fresh growth medium, incubated for a further 16–20 h, harvested in 1 ml PBS using a rubber policeman, and collected by centrifugation (12,000*g* for 2 min at 4°C) in a 1.5-ml capacity microcentrifuge tube. Crude extracts were prepared in 0.1 ml 0.25 M Tris-HCl (pH 7.8) by subjecting cell pellets to three cycles of freeze–thawing. Extraction of the soluble protein fraction containing the CAT enzyme was facilitated by vortexing the broken cells for 30 sec after each cycle. Following removal of the cell debris by centrifugation (12,000*g* for 5 min at 4°C), the supernatant fractions were normalized for total protein (monitored spectrophotometrically at 280 nm) and were assayed for CAT activity according to the procedure of Gorman and coworkers (34).

Optimized Protocol for Polybrene-Assisted Gene Transfer

Adsorption of Foreign DNA. Approximately 20 h prior to gene transfer, mid- to late-log NIH 3T3 cells were seeded at 5×10^5 cells per 60-mm-diam. dish. DNA adsorption to cell monolayers was initiated by replacing the culture medium with a 2.0-ml cocktail consisting of 5.0 μg/ml polybrene and 10 ng/ml form I pSV2neo DNA in fresh growth medium. The cocktail was prepared immediately before use by adding, in succession, prewarmed growth medium, plasmid DNA, and polybrene; brief vortexing was performed after addition of the latter two components. (Note: To avoid irreversible precipitation, polybrene by itself was never mixed directly with DNA.) Dishes were then transferred to a CO_2 incubator, and the pSV2neo DNA was allowed to adsorb to the cells for 16–20 h.

Cell Permeabilization with DMSO. After removal of the polybrene–DNA cocktail, each monolayer culture was gently overlayed with 4.0 ml of growth medium augmented with 15% (v/v) DMSO (Spectranalysed, UV cutoff at 262 nm; Fisher Scientific Co.). The permeabilization solution was distributed uniformly over the cultures by slowly rocking each dish manually for 10 sec, at which time the dishes were returned to a CO_2 incubator. Adequate heat exchange was ensured by placing each dish on the surface of an incubator shelf. The duration of exposure to the permeabilizing solution was carefully controlled, lasting exactly 4.5 min, whereupon the DMSO solution was quickly aspirated, and the cell monolayers were carefully rinsed twice with 5.0 ml of growth medium before the cultures were overlayed with fresh growth medium. (Note: Since the addition of DMSO to prewarmed growth medium was exothermic, the cell permeabilization solution, which was always prepared fresh, was allowed to equilibrate in a 37°C water bath for 15 min prior to use. Both optimal and reproducible results were achieved within a given series of gene transfer experiments by preparing the permeabilization solution in serum-containing medium.)

Cell Recovery and Clonal Selection in G418. Following a 20 to 24-h period to permit recovery from the permeabilization treatment, each culture was reseeded at $2.0–2.5 \times 10^5$ cells per 10-cm dish containing growth medium supplemented with 500 μg/ml active G418. This selective medium was replenished every four days over a two-week period, whereupon the incidence of drug-resistant colonies (representing aggregates of >200 cells) was determined macroscopically after staining with crystal violet.

Southern and Dot-Blot Analyses

Purified high-molecular-weight DNA (5–10 μg), isolated from confluent cultures of pooled G418-resistant clones (38), was cleaved with selected restriction enzymes

(Boehringer Mannheim) under conditions specified by the supplier and resolved by electrophoresis through 0.8% agarose gels. Gels were capillary blotted onto nylon membranes (Hybond-N; Amersham Ltd.) (39) and hybridized at 42°C under stringent conditions (40) to ^{32}P nick-translated pSV2neo DNA ($1–2 \times 10^8$ cpm/μg). Blots were washed under high stringency in 0.1 × SSC (1.5 mM sodium citrate, 15 mM NaCl, pH 7.0), 0.1% SDS at 65°C, and exposed to Kodak XAR-5 film for three to five days at −70°C under DuPont Lightning Plus intensifying screens.

Dot-blot analysis was carried out on serially diluted genomic DNA samples affixed to nylon membranes (Hybond-N) by filtration through a multiwell manifold (Bio-Rad Laboratories) as recommended by Amersham Ltd. (Technical Bulletin PI/162/85/1). Conditions for hybridization of radiolabeled pSV2neo DNA and subsequent autoradiography were identical to those described above for Southern blotting.

RESULTS

Serum Level in DNA Adsorption Cocktail. After conducting pilot experiments to ascertain approximately optimal conditions for polybrene and DMSO treatments, we first examined the effect of various components of the DNA adsorption mixture on the transfection process, paying particular attention to fetal calf serum. Given the polycationic character of polybrene, it was anticipated that the inclusion of FCS, containing an abundance of anionic proteinaceous material, would serve to reduce transfection efficiency simply because these anionic serum constituents would compete with DNA for binding sites created on cellular membranes by polybrene.

Figure 1 presents a comparative profile of DNA adsorption and stable phenotypic transformation for near-confluent monolayers of NIH 3T3 fibroblasts as a function of time of exposure to 5.0 μg/ml polybrene in medium containing either 0.25% (panel A) or 10% FCS (panel B). (Note: Cell monolayers approaching a fully confluent state were used in this one instance to ensure that DNA adsorption measurements would represent DNA–polybrene complexes bound to cells rather than merely DNA–polybrene–serum protein complexes bound to bare regions on the inner surfaces of the dishes.) As illustrated in panel A, adsorption of radiolabeled pCB6 DNA to cell surfaces proceeded rapidly in 0.25% FCS, reaching a maximal level of ~45% input plasmid bound by 6 h. Exposure of cultures to this adsorption cocktail for longer periods turned out to be counterproductive, quickly producing extreme cytotoxic effects. The observed cytotoxicity stemmed from the presence of polybrene, since in its absence cultures could be held for more than 24 h without any appreciable loss of cell viability. Although 0.25% FCS promoted rapid and efficient adsorption of exogenous DNA, transfection with pSV2neo DNA gave rise to relatively few G418-resistant colonies. In contrast, while plasmid adsorption proceeded at a much slower rate in 10% FCS (requiring 24 h to attain a plateau level of ~30% DNA bound; Fig. 1, panel B), the

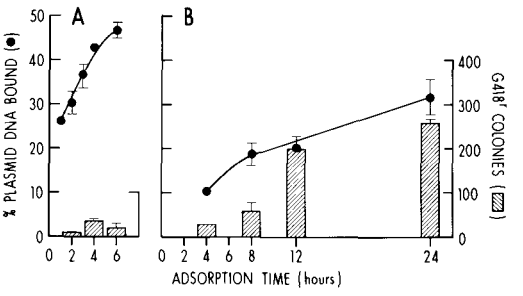

Fig. 1. Comparative kinetics of DNA adsorption and stable phenotypic transformation as a function of time of cell exposure to a DNA adsorption cocktail supplemented with 0.25% (panel A) or 10% (panel B) fetal calf serum. NIH 3T3 cell monolayers were incubated for the indicated times with an adsorption cocktail containing 5 μg/ml polybrene and the appropriate input plasmid (i.e., 50 ng/ml [^{3}H]pCB6 or 10 ng/ml pSV2neo) in culture medium augmented with FCS at low (0.25%) or high (10%) concentration. Monolayer cultures were then assayed for either adsorption of foreign DNA or stable G418-resistant transformants. See Materials and Methods for experimental details.

number of G418-resistant colonies increased steadily over the 24-h period of DNA adsorption, producing ~325 colonies, a yield some 10-fold greater than that attained in low serum. Dose–response experiments substantiated the strong dependency of the overall DNA transfection process on the concentration of fetal calf serum during polybrene treatment (data not shown). Unlike others (6, 41), we found no reason to recommend that the serum be heat-inactivated, nor did we observe variation in the capacity to support efficient gene transfer among different serum lots (12).

Cell Density. The efficiency of stable gene transfer was greatly influenced by the density of the recipient NIH 3T3 fibroblasts at the time of plasmid adsorption (Table 1). The highest yield of drug-resistant colonies was obtained when cells were seeded at 5.0×10^5 per 60-mm culture dish (i.e., $\sim 2 \times 10^3$/mm^2). Accordingly, cells were routinely plated out at this concentration in subsequent experiments.

Polybrene Concentration. Selection of the most effective concentration of polybrene in the adsorption cocktail was based on the following three endpoints: radiolabeled DNA binding, G418-resistant colony formation, and transient gene expression (monitored as percent chloramphenicol acetylated by cell-free extracts, reflecting CAT enzymic expression). Our results (Fig. 2) indicate that the optima for all three endpoints were achieved when DNA was presented to the cells with 5 μg/ml polybrene. Consequently, the polymer was added to the adsorption mixture at this concentration in all subsequent experiments.

In the reports of Kawai and Nishizawa (14) and Chaney et al. (13), maximal transfection efficiency was achieved at shorter DNA incubation times if target cells were coated with polybrene prior to the presentation of DNA. In our hands, precoating NIH 3T3 cells with as much as 15.0 μg/ml polybrene for periods of 4–24 h preceding DNA adsorption was not found to enhance either

Table 1. Effect of Recipient Cell Density during Plasmid Adsorption on Transformation Frequency[a]

Cells/dish ($\times 10^5$)	G418^r Colonies/dish
0.5	3
1.0	19 ± 5
2.0	136 ± 20
5.0	160 ± 16
10	23 ± 13

[a]NIH 3T3 cell monolayers were treated for 20 h with an adsorption cocktail containing 10 ng/ml form I pSV2neo DNA and 5 μg/ml polybrene, permeabilized for 4.5 min with 15% DMSO, and then subjected to selection in G418 as detailed in Materials and Methods. Cell densities at the time of input DNA presentation were confirmed for each series by determining cell counts for three dishes seeded in parallel for this purpose.

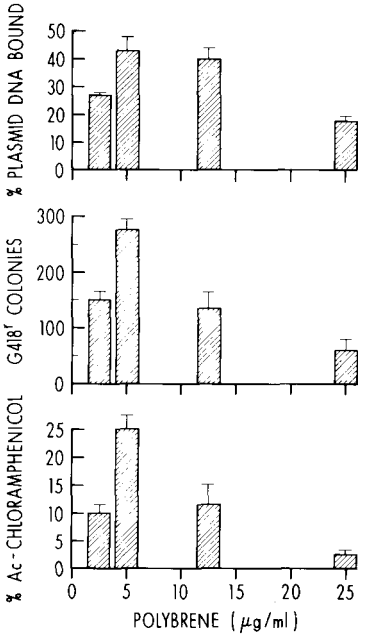

Fig. 2. Comparative profiles of DNA adsorption, stable transformation, and transient gene expression as a function of concentration of polybrene in the DNA adsorption cocktail. NIH 3T3 fibroblasts were exposed for 20 h to adsorption cocktails containing various amounts of polybrene and the appropriate plasmid DNA (i.e., 50 ng/ml [^{3}H]pCB6, 10 ng/ml pSV2neo or 0.5 μg/ml pSV2cat) in medium supplemented with 10% FCS. Cell cultures were then assayed for binding of exogenous DNA, G418-resistant colony formation, and transient expression of CAT enzymic activity as described in Materials and Methods.

the efficiency of DNA binding or the yield of G418-resistant colonies (unpublished data).

DMSO Concentration. Having established optimal conditions for DNA adsorption, we then considered the critical step of cell permeabilization. It is readily apparent from the dose–response curve in Fig. 3 that a maximal yield of almost 200 G418-resistant colonies was achieved when cells were permeabilized in the presence of 15% DMSO. Of particular significance was the finding that as little as a 20% deviation from this maximal concentration (i.e., 12.5% or 17.5% DMSO) led to a 50% reduction in colony recovery. Extension of the permeabilization time beyond 4.5 min did not increase colony yield. Substitution of glycerol (15% in PBS) (11, 42) or polyethylene glycol (8, 43) for DMSO produced radically inconsistent results (unpublished data). Phase-contrast microscopy revealed that gross cytopathic effects were not detectable until cultures were exposed to DMSO concentrations of 17.5%. Cell survival, as judged by colony-forming ability, was also compromised at these concentrations (data not shown). These observations are consistent with the notion that the sharp rise in transformation efficiency occurring between 10 and 15% DMSO can be attributed to a gradual increase in cell permeability, whereas the rapid decline in transformation efficiency at progressively higher concentrations can be ascribed to increased cytotoxicity. This interpretation was further substantiated by our finding that the levels of transient expression of the *cat* gene were highest in cells permeabilized at DMSO concentrations exceeding 20% (Table 2), thus representing conditions under which the influx of pSV2cat DNA approached a maximum. Achieving these higher levels of foreign DNA uptake came, however, at the expense of reductions in cell viability.

Amount of Input DNA. Under conditions in which the major parameters influencing DNA transfection efficiency appeared to be optimal (detailed above), the number of colonies emerging from drug selection was directly proportional to the quantity of form I pSV2neo DNA presented to the cultures (Fig. 4). We were unable to improve the transfection frequency by exposing NIH 3T3 cells to either chloroquine (44) or sodium butyrate (37), but the use of input DNA that had been converted (by BamHI endonuclease digestion) from covalently closed circular struc-

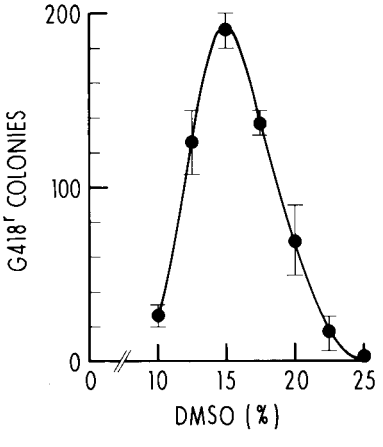

Fig. 3. Effect of DMSO concentration on the yield of stable G418-resistant transformants. NIH 3T3 cells were presented for 20 h with 10 ng/ml pSV2neo plasmid DNA and 5 μg/ml polybrene, permeabilized for 4.5 min in the presence of the indicated amounts of DMSO, and then assayed for the appearance of G418-resistant colonies as detailed in Materials and Methods.

Table 2. Levels of Transient *cat* Expression in NIH 3T3 Fibroblasts Permeabilized with Increasing Concentrations of DMSO[a]

DMSO (%)	Chloramphenicol acetylation (%)
10	6
15	20
17.5	32
20	87
22.5	100
25	100

[a]Monolayer cultures (7.5×10^5 cells/10-cm dish) were exposed for 20 h to an adsorption cocktail containing 0.5 μg/ml form I pSV2cat DNA and 5 μg/ml polybrene, permeabilized for 4.5 min with the indicated amounts of DMSO, and incubated for 16 h in the presence of 10 mM sodium butyrate (to enhance the template activity of the transfected *cat* gene). Finally, the cells were fed fresh growth medium and harvested 20 h later for determination of CAT enzymic activities. Experimental details are given in Materials and Methods.

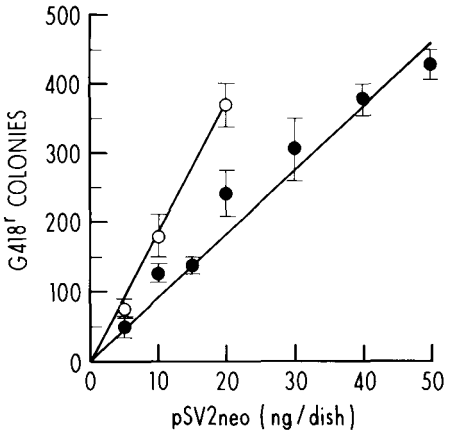

Fig. 4. Yield of G418-resistant colonies as a function of input DNA concentration, using either BamHI-linearized (O) or covalently closed, circular (●) pSV2neo plasmid. NIH 3T3 fibroblasts were transfected under optimal conditions for polybrene-assisted gene transfer, as described in Materials and Methods.

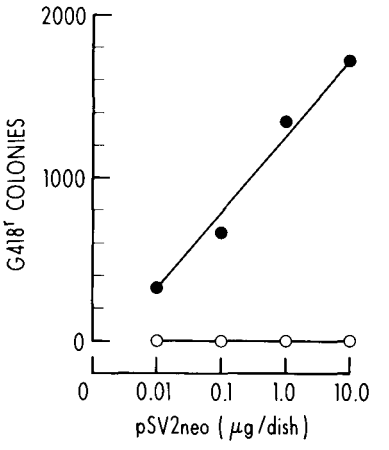

Fig. 5. Extended dose–response curve for polybrene-assisted transfection of NIH 3T3 cells with form I pSV2neo plasmid DNA. Open circles represent cells exposed to polybrene and pSV2neo DNA but not permeabilized with DMSO.

tures to full-length, linear molecules retaining an intact *neo* gene cartridge resulted in some twofold enhancement in colony yield. This latter result is at variance with that of others (8, 13). Figure 4 also demonstrates that linearity in dose–response (i.e., colony recovery) was maintained with concentrations of form I plasmid DNA up to 50 ng/5 × 10^5 recipient cells. When the dose range of plasmid DNA was expanded to 10 μg/dish, a semilogarithmic relationship was observed (Fig. 5), suggesting that the transfection process approached saturation at DNA concentrations in excess of ~100 ng/dish.

In agreement with previous findings (13, 14), data in Fig. 5 also demonstrate that cell permeabilization by DMSO was compulsory for achieving gene transfer; no G418-resistant colonies were recovered if this step was omitted.

In the calcium-phosphate coprecipitation procedure, the inclusion of microgram quantities of carrier DNA in the adsorption mixture is critical for efficient gene transfer (6–9, 45). In contrast, since the adsorption of exogenous DNA in the presence of polybrene appears to be dictated largely by a saturable number of cellular membrane binding sites (see Fig. 2), the addition of supplementary DNA (carrier material or cotransfected, nonselectable genes) would be expected to competitively inhibit the adsorption of marker-containing DNA and thereby reduce the frequency of selectable colonies. Results shown in Fig. 6 are consistent with this expectation. When increasing amounts of pBR322 plasmid DNA were added to the adsorption cocktail with a nonsaturating quantity (10 ng/dish) of

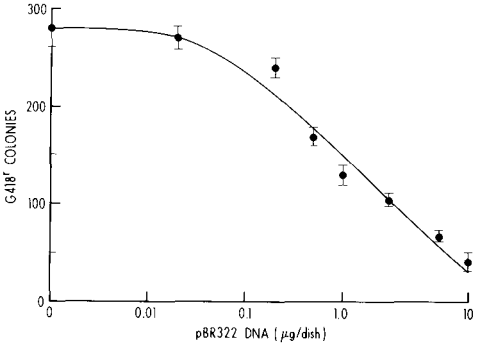

Fig. 6. Cotransfection of pSV2neo DNA with nonselectable pBR322 DNA. NIH 3T3 cells were cotransfected with 10 ng/ml pSV2neo DNA in combination with increasing amounts of carrier pBR322 DNA. See Materials and Methods for details.

pSV2neo DNA, the yield of G418-resistant colonies dropped rapidly when the competitor DNA was present in 10-fold molar excess. To rule out the possibility that the inhibition of the overall transformation process might reflect the transcriptional silencing of *neo* gene expression by negative regulatory elements residing in the cotransfected pBR322 plasmid molecules (46, 47), EcoRI-digested NIH 3T3 genomic DNA was substituted for pBR322 as a source of free carrier DNA; this modification produced similar results (data not shown).

Molecular Characterization of Integrated Neo Genes in Stable Transformants. We next wished to determine both the copy number and physical arrangement of the transfected *neo* gene in populations of drug-resistant cells. To this end, NIH 3T3 fibroblasts were transfected, under optimal condi-

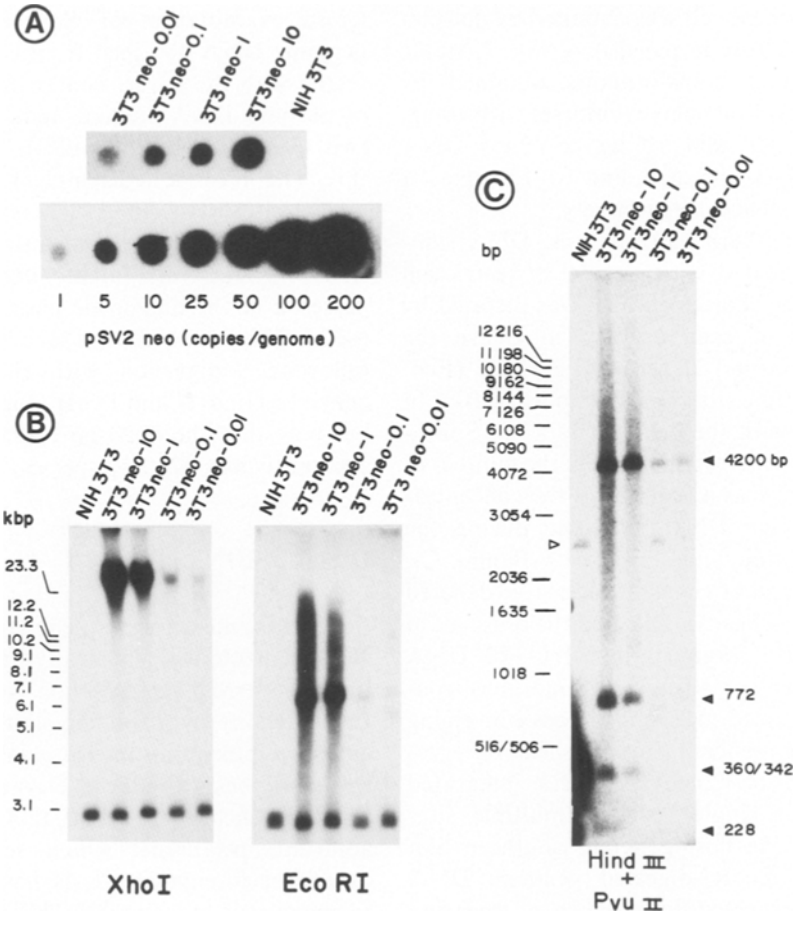

Fig. 7. Identification and genomic organization of pSV2neo sequences integrated in high-molecular-weight DNA isolated from G418-resistant cells. Dot-blot assessment of the number of integrated pSV2neo copies present in the genomes of various G418-resistant NIH 3T3 clones is shown in panel A. In panels B and C, NIH 3T3 cells were transfected with 0.01, 0.1, 1.0, or 10 μg pSV2neo per dish as described. Stable transformants were then selected in G418 and grown into mass culture, after which 10 μg of genomic DNA from each culture were cleaved with the designated restriction endonuclease(s) and processed for Southern hybridization analysis. The electrophoretic positions of DNA size markers (BRL) appear on the extreme left. The open arrowheads indicate the position of a nonspecific band (see text for details). Faint bands corresponding to the smaller size fragments were initially present on the autoradiograms but have faded in the course of photographic reproduction.

tions, with increasing amounts of form I pSV2neo DNA and exposed to G418 for selection of stable transformants. Drug-resistant clones obtained at each DNA concentration were pooled and expanded into mass culture, after which high-molecular-weight DNA was isolated and subjected to conventional Southern and dot-blot analyses using ^{32}P nick-translated pSV2neo DNA as the probe.

Quantitation of the average copy number per transformed cell was obtained by dot-blot analysis. As seen in panel A of Fig. 7, stable G418-resistant transformants obtained by transfection of monolayer cultures with 10 ng, 100 ng, 1 μg, and 10 μg pSV2neo DNA contained 1–3, 3–5, 5–7, and 10–15 copies of plasmid sequences, respectively.

For Southern blot analysis, DNA samples were treated with a panel of restriction enzymes, the choice of which was dictated by the ability of each enzyme to cleave the pSV2neo plasmid at zero (XhoI), one (EcoRI) or multiple sites (HindIII plus PvuII). In agreement with the dot-blot data, all Southern blots revealed an increase in the number of plasmid copies as a function of the concentration of foreign DNA presented during the adsorption step (Fig. 7, panels B and C). [Note: A band of constant intensity (denoted by open arrowheads) indicated the presence of a constitutive fragment in NIH 3T3 DNA which, under our stringency conditions, routinely hybridized to several probes containing pBR322 sequences (unpublished observation).] Had pSV2neo sequences integrated randomly as single units at multiple sites throughout the host genome, Southern blot analysis of EcoRI-digested genomic DNA would be expected to produce a smearing hybridization pattern of roughly uniform intensity along a substantial portion of the blot. Although such a continuum of hybridization bands was detected in transformed cell DNA derived from cultures transfected with microgram quantities of pSV2neo, a major fragment of ~6.6 kbp was prominent in all EcoRI-digested samples, irrespective of the amount of input plasmid DNA used for transfection (Fig. 7, panel B). This would suggest that the majority of transfected plasmid molecules have integrated at a single site as tandem repeats. The size discrepancy between the observed size fragment (~6.6 kbp) and the expected unit length vector fragment (5.7 kbp) can be accounted for by the fact that the molecular weight standards were not run in the presence of restricted genomic DNA. Close examination of Southern blots of genomic DNA obtained from cultures transfected with the lowest concentration (10 ng) of plasmid DNA studied, indicated at least two (XhoI-digested samples), and possibly three (EcoRI-digested samples), distinct integration sites for plasmid sequences.

The integration of the entire *neo* expression cartridge was further verified by the presence of the diagnostic cleavage products (4023, 772, 360, 342, and 228 bp) expected following codigestion with the restriction enzymes HindIII and PvuII (Fig. 7, panel C; but note that the 4023-bp fragment appears aberrantly as a 4200-bp species).

DISCUSSION

Polybrene-assisted gene transfer provides an attractive, technically simple alternative to current transfection schemes (see Introduction) by requiring neither sophisticated equipment nor intricate, labor-intensive manipulations to achieve elevated transfection frequencies (13, 14, 30, this report). The adjuvant, polybrene, which serves as the carrier of foreign DNA, is noncytotoxic to recipient cell monolayers. This is in marked contrast to the widely employed calcium phosphate–DNA coprecipitation method wherein suitability is largely dictated by the target cells' tolerance to high levels of calcium salts in the culture medium (13). Moreover, the polybrene–DMSO procedure is particularly well suited for large-scale gene transfer exper-

iments due to the facile preparation of large quantities of transfection cocktail. By comparison, the preparation of calcium phosphate–DNA coprecipitates requires care (6–9).

The polybrene-assisted gene transfer regimen provides highly reproducible results in any given series of experiments as evidenced by the small standard deviations for data shown here (e.g., see Figs. 2–4). In our experience, this consistency is largely contingent upon the DMSO treatment conditions; hence the cell permeabilization step must be adjusted carefully to minimize cytotoxic effects.

We have utilized NIH 3T3 fibroblasts as archetypic recipients for polybrene-assisted gene transfer to circumscribe parameters deemed critical to the attainment of consistently elevated transfection frequencies and, in the process, have established a useful set of practical guidelines for adaptation of the procedure for use with other cell types. Under optimal conditions, this method produced large numbers of stable phenotypic transformants using only nanogram quantities of purified DNA. In our hands, drug-resistant colonies were produced at a frequency of 10 per nanogram of form I pSV2neo DNA (3.7×10^4/pmol) per 5×10^5 transfected cells up to 50 ng of donor DNA. Moreover, although the relative efficacy of gene transfer declined at DNA concentrations exceeding 100 ng/dish, if we follow the popular convention of normalizing transfection frequency by expressing the incidence as a function of 1 μg of input DNA, the yield extrapolates to a value approaching 10^4 transformants per microgram form I pSV2neo DNA. This figure compares well with that obtained with CHO cells using a similar procedure (13). This unique requirement for minute quantities of foreign DNA promises to make the procedure especially advantageous in studies designed to isolate single-copy genes (13).

The method appears to facilitate the uniform introduction of low numbers of foreign gene sequences within the stably transformed cell population. Polybrene-assisted gene transfer may therefore be of particular benefit when the dosage of the foreign sequences incorporated into host chromosomes must be kept within strict physiological limits. Furthermore, the number of integrated foreign gene copies appears amenable to experimental control by simply varying the amount of exogenous DNA added to the adsorption cocktail. Such is not the case for the calcium phosphate coprecipitation technique, for example, where the quantity of foreign DNA introduced on a per cell basis is highly variable due to the heterogeneous size distribution of calcium phosphate–DNA granules (1, 7, 44). The regimen may also provide an alternate gene transfer strategy for the study of the malignant behavior of tumor cells since Kerbel et al. (48) have recently demonstrated that the process of calcium phosphate gene transfer may itself be associated with profound heritable changes in tumorigenic and metastatic behavior.

The polycation–DMSO technique does, however, suffer somewhat from two minor yet significant drawbacks. One disadvantage is that the cell surface sites for adsorption of polybrene–DNA complexes appear saturable at low DNA concentrations. Consequently, cotransfection of a recessive phenotypic trait with a dominant selection marker (e.g., *neo* gene) may be jeopardized. This potential problem can be overcome by ligating transfecting DNA fragments carrying the sought-after recessive gene(s) to linearized vector molecules (our unpublished observation). The second shortcoming is that peak values of transient *cat* gene expression, although providing a rapid means for determining the polybrene concentration required for maximal transfection efficiency (see Fig. 2), may not be predictive of the optimal DMSO treatment regimen (see Table 2 and reference 42).

Since the potential usefulness of any novel method will be determined ultimately by its general applicability, we have recently

tailored the polybrene-assisted gene transfer regimen to accommodate several SV40-immortalized human fibroblast lines and non-established fibroblast cultures derived from both normal and cancer-prone individuals (manuscript in preparation). Morgan et al. (30) have also recently reported similar success. In short, there is every reason to anticipate that the polycation–DMSO method will prove to be readily adaptable to a broad spectrum of mammalian cell types.

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