Primary human fibroblasts and a series of cell lines (A549, BNL CL.2, H225, NIH 3T3 and Rat-1) are efficiently transfected by using positively charged complexes of plasmid DNA and transferrin-polylysine or polylysine in the presence of glycerol (1 molar to 1.8 molar, depending on the cell type). An increase in gene expression of up to several-hundredfold (compared to complexes without glycerol) is obtained if the transfection mixture is incubated with the cells for 3–4 h at 37°C. This simple method has been used for transient expression of luciferase, β-galactosidase and interleukin-2, and also for the generation of stably transfected cells.

INTRODUCTION

The delivery of genes into mammalian cells has become a powerful tool in molecular and cellular biology during the last 30 years. The different methods of introducing DNA into cells can be divided into two groups: namely, viral and non-viral. Besides physical methods, like microinjection, electroporation and particle bombardment, non-viral methods include calcium-phosphate precipitation; DEAE-dextran-mediated transfection; lipofection using cationic lipids like DOTAP®, Lipofectamine® and Transfectam®; dendrimer-mediated transfection (16); and receptor-mediated gene delivery systems (35,38, for review 7). The major advantage of these systems over viral vectors is their ease of use and the lack of length limitation of the DNA to be delivered (up to 650 kb; 19).

Receptor-mediated gene delivery systems in many cases use polylysine (pLys) to condense plasmid DNA. A polylysine-conjugated ligand is incorporated into the DNA complexes to provide a cell-binding domain. A transferrin-polylysine conjugate (TfpLys) has been commonly used because of the widespread expression of the transferrin receptor (17). One of the limiting steps of this system is the release of internalized DNA particles from the endosome to the cytoplasm. Endosome-disrupting compounds such as inactivated adenovirus (9,10,12,37,39) or peptides (26,36,41) have therefore been included in the DNA complexes. Since these reagents are expensive and laborious to prepare, we sought other compounds that might ensure release of DNA.

Here we report a new and simple method to deliver reporter gene constructs using the endocytotic pathway. Incubation of cells with DNA/TfpLys or DNA/pLys complexes in the presence of glycerol resulted in a substantially enhanced transfection efficiency in a variety of primary cells and cell lines. Addition of bafilomycin A1, a specific inhibitor of the vacuolar proton pump (3,40), or chloroquine (6,30), a weak base accumulating in acidic vesicles, further improved the glycerol effect in some cell lines.

MATERIALS AND METHODS

The plasmid pCMVL, coding for the Photinus pyralis luciferase gene under control of the cytomegalovirus enhancer/promoter, has been described (27). pCMVB (22), pRSVneo (15) and pGShIL-2 tet (4) have also been described. Plasmid DNA was prepared by a Triton® X-100 lysis procedure as described (8). The second centrifugation step was extended from 4 hours to an overnight run, which significantly reduced lipopolysaccharide (LPS) contamination. Endotoxin content was measured by the limulus amebocyte lysate assay (BioWhittaker, Walkersville, MD, USA). LPS content of plasmids used in transfection experiments was below 0.05 units/µg DNA. An alternative efficient method to remove LPS is described in Reference 5.

Human transferrin-polylysine conjugates TfpLys (molar ratio of transferrin to polylysine of 1:1.2) were prepared according to Reference 35 and are commercially available (Bender Med Systems, Vienna, Austria). Polylysine hydrobromide (pLys, dp 250; Sigma Chemical, St. Louis, MO, USA) was dissolved in water at a concentration of 10 mg/mL and used without further purification. Glycerol (ca. 87%), ethylene glycol, diethylene glycol, polyethylene glycol (PEG) 1000, PEG 6000, DEAE-dextran (500 kDa) and polyethyleneimine (800 kDa) were from Fluka Chemie (Buchs, Switzerland). Glycerol stock solution was changed regularly to avoid a toxicity occasionally observed with older
batches. Threitol was from Aldrich (Milwaukee, WI, USA). Lipofectamine® was obtained from Life Technologies (Gaithersburg, MD, USA), DOTAP® was purchased from Boehringer Mannheim (Mannheim, Germany) and Transfectam® was a generous gift from J.-P. Behr (Université Louis Pasteur, Strasbourg-Illkirch, France). Chloroquine and bafilomycin A₁ were obtained from Sigma Chemical.

Cell Culture

All media and calf sera were from Life Technologies. Primary human fibroblasts were prepared from a human skin specimen. Human melanoma cell line H225 was kindly provided by S. Schreiber and G. Stingl (University of Vienna, Austria). NIH 3T3 (murine fibroblasts), Rat-1 (rat fibroblasts), BNL CL.2 (mouse embryonic liver cells), A549 (human lung carcinoma cells), KG-1 (human acute myeloid leukemia cells), THP-1 (human acute monocytic leukemia cells) and K562 (human chronic erythroid leukemia cells) were obtained from ATCC (Rockville, MD, USA). M07e (human acute megakaryoblastic leukemia cells) were obtained from DSM (Braunschweig, Germany). H225 cells were cultured in RPMI 1640/10% FCS/2 mM glutamine/antibiotics. M07e were cultured in RPMI 1640/10%FCS/2 mM glutamine/antibiotics/10 U/mL recombinant human IL3 (R&D Systems, Minneapolis, MN, USA). NIH 3T3 cells, A549 cells, Rat-1 cells and primary human fibroblasts were cultured in DMEM/10% FCS/2 mM glutamine/antibiotics. BNL CL.2 cells were cultured in high-glucose DMEM/10% FCS/2 mM glutamine/antibiotics. With adherent cells, 10 000 to 200 000 cells per well (50 000 cells for primary human fibroblasts) were plated in 6-well plates (Nunc, Roskilde, Denmark) the day before transfection. Suspension cells (M07e, KG-1, THP-1 and K562) were split 1:3 the day before transfection and plated at a density of 50 000 cells per well on a 24-well plate on the day of transfection.

Figure 1. Glycerol titration. 10 000 cells (H225)/well were transfected with complexes consisting of 1.5 µg pCMV-L, 0.2 µg pLys and 1.5 µg Tf-pLys in the presence of 10% (vol/vol) glycerol. After the indicated time points, the medium was replaced by fresh medium. Values indicate total luciferase activity of the cells and are the means of duplicates. Note that in this experiment streptavidylated polylysine was used instead of pLys, which has no effect on reporter gene expression (see also Figure 4).

Figure 2. Time course. 100 000 cells (H225)/well were transfected with complexes consisting of 1.5 µg pCMVL, 0.2 µg StplLys and 1.5 µg Tf-pLys in the presence of 10% (vol/vol) glycerol. After the indicated time points, the medium was replaced. Luciferase activity was measured 24 h later. Values are the mean of duplicates and represent luciferase activity per well.

Figure 3. β-Galactosidase staining of cells. H225 cells (A), A549 cells (B) and primary human fibroblasts (C) were transfected with complexes consisting of 3 µg plasmid DNA and 4 µg Tf-pLys in the presence of the optimal amount of glycerol. Histological staining was performed 2 days after transfection and resulted in ≈ 30% positive H225 cells (A), ≈ 10% positive A549 (B) and ≈ 8% positive primary human fibroblasts (C).
**Transfection**

Complexes were formed by incubating 3 µg plasmid DNA in 75 µL HBS (20 mM HEPES pH 7.3, 150 mM NaCl) with 0.4 µg pLys in 75 µL HBS. After a 30-min incubation at room temperature, the indicated amounts of TfpLys or pLys in 75 µL HBS were added and incubated for another 30 min. Alternatively, 3 µg DNA in 75 µL HBS were mixed with the indicated amounts of TfpLys or pLys in 75 µL HBS and incubated for 30 min. Glycerol was added to the complexes to give the indicated vol/vol percentage in the final transfection medium (e.g., 10% means that 200 µL of the commercial 87% solution were added to a final volume of 2 mL). Chloroquine (100 µM) and bafilomycin A₁ (200 nM) were added where indicated. Medium was added to give a total volume of 2 mL, and half of this transfection mixture was added to each of two wells of a 6-well plate. The actual time point of the addition of glycerol is not critical as long as glycerol is present during the transfection period; complexes that were mixed in the presence of glycerol were equally efficient. After 4 h the medium was replaced. Luciferase activity was assayed 24 h after transfection. Each experiment was done several times; absolute values varied within an order of magnitude depending on plasmid batch and the cells’ history.

When other compounds were used in the transfection, they were added at the indicated percentages (wt/vol or vol/vol) from concentrated stock solutions. When glucose was used, complexes were mixed in 2 M glucose and adjusted with the appropriate volume of medium to give a final concentration of 1 M or approximately 700 mM, respectively. In the case of mannitol, complexes were mixed in 0.9 M mannitol.

The osmotic lysis procedure was performed according to Reference 24 with DNA/TfpLys complexes mixed in hypertonic medium (medium/0.5 M glucose/10% PEG 1000). Cells were then washed twice with hypertonic medium (medium/water = 6/4) and kept hypertonic for 3 min before they were further cultured in normal medium.

For lipofection 3 µg of plasmid DNA were diluted into 700 µL serum-free medium; the indicated amounts of cationic lipids were added with 700 µL of serum-free medium and incubated for 15 min at room temperature. One charge equivalent of cationic lipid is the quantity required to neutralize all the negative charges carried by the phosphates of the plasmid (3 µg of DNA correspond to 9.1 nmol of negative charge). Seven-hundred microliters of this mixture were added to each of two wells. After 4 h the medium was replaced with medium containing 10% FCS.

For generating stable clones, 300000 H225 cells were plated in a T25 flask (Nunc). For transfection, the linearized reporter plasmid (pCMVL, pCMVβ or pGShIL2-tet; linearized with SpeI, XmnI or XmnI, respectively) was mixed with XmnI-linearized pRSVneo at a 10:1 ratio (6 µg reporter plasmid/0.6 µg pRSVneo) in 100 µL HBS. TfpLys (7.5 µg) in 100 µL HBS were added and incubated for 30 min at room temperature. The mixture was added to the cells with medium containing 10% glycerol. After 4 h the complex/medium mixture was replaced with fresh medium. Selection was started after 60 h with medium containing 3 mg/mL geneticin (G418; Sigma Chemical). After five days the G418 concent-

---

**Figure 4. Effect of different compounds on gene transfer.**

A: H225 (100000 cells/well) were transfected with complexes consisting of 3 µg pCMVL, 0.4 µg pLys and 3 µg TfpLys in the presence of the indicated compounds at the indicated concentrations. Osmotic lysis procedure was performed for 20 min as described in Materials and Methods. Relative luciferase activities are shown (values obtained in the presence of 10% glycerol are set as 100%) and are the means of duplicates. 100% corresponds to 5.6 × 10⁶ light units/10⁵ cells. B: Primary human fibroblasts (50000 cells/well) were transfected in the presence of indicated compounds. Relative expression levels are shown. 100% corresponds to 1.6 × 10⁶ light units/5 × 10⁴ cells.
tration was reduced to 1 mg/mL. The cells were passaged in medium containing G418 at 1 mg/mL.

Assays

Luciferase assays were performed as described (35). Briefly, the cells were harvested in 250 mM Tris-HCl pH 7.3, 0.5% Triton® X-100, and the luciferase activity was measured from an aliquot of the supernatant. Values are given as total light units per transfection and are the means of duplicates or triplicates.

β-Galactosidase staining was performed according to Reference 21 using the chromogenic substrate 5-bromo-4-chloro-3-indolyl-β-D-galactopyranoside (X-gal).

For the determination of interleukin-2 (IL2) expression of stable clones, 400000 cells/well were plated in a 6-well plate. After 24 h the medium was replaced by 2 mL fresh medium. After another 24-h incubation, IL2 in the supernatant was measured with ELISA according to the manufacturer’s recommendations (Bender Med Systems). Cell number was determined by counting the cells after trypsinization. IL2 values are given as units IL2/24 h/10^6 cells.

RESULTS

Transgene Expression from TfpLys or pLys Condensed Plasmid DNA Is Enhanced in the Presence of Glycerol

We found that increasing concentrations of glycerol, when present during transfection with DNA/TfpLys/pLys complexes, enhanced reporter gene expression in human melanoma cells (H225) up to more than 100-fold (Figure 1), with an optimum of 10% glycerol (1.15 M). Concentrations below 4% did not increase reporter gene
expression, whereas concentrations of 15% or higher were toxic to H225 cells.

To further optimize the transfection conditions, we analyzed the length of time cells must be exposed to glycerol to obtain DNA delivery. Highest expression levels were obtained when cells were incubated with the complex-glycerol for three to four hours (Figure 2). Incubations longer than four hours led to a decrease in reporter gene expression, probably due to toxic effects.

We also determined the transfection efficiency using the lacZ reporter gene and found that about 30% of H225 cells stained positive (Figure 3A) when transfected under these optimized conditions.

Ethylene Glycol Is Able to Substitute for Glycerol

Other compounds chemically similar to glycerol, such as ethylene glycol, diethylene glycol, ethanol, 1,3-propanediol, PEG 1000, PEG 6000, threitol, mannitol, sucrose and glucose, were also tested for their ability to enhance transfection. None of these substances considerably increased reporter gene expression under the conditions tested, except for ethylene glycol, which was 30% to 50% as effective as glycerol at the same molarity (Figure 4A). All the other substances were toxic at concentrations well below 1.15 M (= 10% glycerol). A similar result was obtained when primary human fibroblasts were transfected in the presence of some of these substances (Figure 4B).

We also tried to introduce DNA complexes using the osmotic lysis of pinosomes according to a protocol for the delivery of proteins (24). This protocol did not mediate gene expression with H225 or BNL CL.2 cells, even after extended incubation (up to 3 h) in hypertonic medium (Figure 4A and data not shown).

The effect of glycerol was dependent on its presence during transfection; neither pre- nor postincubation with glycerol enhanced reporter gene expression to any significant extent (Figure 4A). Therefore, osmotic shock mechanisms seem very unlikely.

A Variety of Adherent Cell Lines Can Be Transfected Using Glycerol

We extended the investigation of glycerol-enhanced transfection to different cells and cell lines. In several cases, such as NIH 3T3, Rat-1, BNL CL.2, primary human fibroblasts, A549 and some primary human melanoma

Table 1. Effect of DNA Complex Composition, Glycerol and Additional Drugs on Transfection

<table>
<thead>
<tr>
<th>Cell</th>
<th>Complexa</th>
<th>Glycerol</th>
<th>Relative Luciferase Expression (%)b</th>
<th>Drug</th>
</tr>
</thead>
<tbody>
<tr>
<td>H225</td>
<td>A</td>
<td>0</td>
<td>0.7</td>
<td>-</td>
</tr>
<tr>
<td></td>
<td>A</td>
<td>0</td>
<td>0.3</td>
<td>1% EtOH</td>
</tr>
<tr>
<td></td>
<td>A</td>
<td>0</td>
<td>4</td>
<td>Chloroquine</td>
</tr>
<tr>
<td></td>
<td>A</td>
<td>0</td>
<td>0.8</td>
<td>Bafilomycin A1</td>
</tr>
<tr>
<td></td>
<td>A</td>
<td>10</td>
<td>100</td>
<td>-</td>
</tr>
<tr>
<td></td>
<td>A</td>
<td>10</td>
<td>200 (±20)</td>
<td>1% EtOH</td>
</tr>
<tr>
<td></td>
<td>A</td>
<td>10</td>
<td>300 (±200)</td>
<td>Chloroquine</td>
</tr>
<tr>
<td></td>
<td>A</td>
<td>10</td>
<td>240 (±70)</td>
<td>Bafilomycin A1</td>
</tr>
<tr>
<td></td>
<td>B</td>
<td>10</td>
<td>50 (±6)</td>
<td>-</td>
</tr>
<tr>
<td></td>
<td>C</td>
<td>10</td>
<td>100 (±40)</td>
<td>-</td>
</tr>
<tr>
<td></td>
<td>D</td>
<td>10</td>
<td>70 (±20)</td>
<td>-</td>
</tr>
<tr>
<td>NIH 3T3</td>
<td>A</td>
<td>0</td>
<td>0</td>
<td>-</td>
</tr>
<tr>
<td></td>
<td>A</td>
<td>12.5</td>
<td>100</td>
<td>-</td>
</tr>
<tr>
<td></td>
<td>B</td>
<td>0</td>
<td>0</td>
<td>-</td>
</tr>
<tr>
<td></td>
<td>B</td>
<td>0</td>
<td>35 (±10)</td>
<td>Bafilomycin A1</td>
</tr>
<tr>
<td></td>
<td>B</td>
<td>12.5</td>
<td>200 (±20)</td>
<td>Chloroquine</td>
</tr>
<tr>
<td></td>
<td>B</td>
<td>12.5</td>
<td>350 (±50)</td>
<td>Bafilomycin A1</td>
</tr>
<tr>
<td></td>
<td>B</td>
<td>12.5</td>
<td>80 (±50)</td>
<td>Chloroquine</td>
</tr>
<tr>
<td></td>
<td>C</td>
<td>0</td>
<td>0</td>
<td>-</td>
</tr>
<tr>
<td></td>
<td>C</td>
<td>12.5</td>
<td>1000 (±80)</td>
<td>-</td>
</tr>
<tr>
<td></td>
<td>D</td>
<td>0</td>
<td>0</td>
<td>-</td>
</tr>
<tr>
<td></td>
<td>D</td>
<td>12.5</td>
<td>260 (±30)</td>
<td>-</td>
</tr>
<tr>
<td></td>
<td>B</td>
<td>10</td>
<td>200 (±20)</td>
<td>-</td>
</tr>
<tr>
<td></td>
<td>B</td>
<td>10</td>
<td>1000 (±280)</td>
<td>Bafilomycin A1</td>
</tr>
<tr>
<td></td>
<td>A549</td>
<td>A</td>
<td>0</td>
<td>0</td>
</tr>
<tr>
<td></td>
<td>A</td>
<td>15</td>
<td>100</td>
<td>-</td>
</tr>
<tr>
<td></td>
<td>B</td>
<td>0</td>
<td>0</td>
<td>-</td>
</tr>
<tr>
<td></td>
<td>B</td>
<td>15</td>
<td>150 (±20)</td>
<td>-</td>
</tr>
<tr>
<td></td>
<td>B</td>
<td>15</td>
<td>170 (±10)</td>
<td>Chloroquine</td>
</tr>
<tr>
<td></td>
<td>C</td>
<td>15</td>
<td>170 (±20)</td>
<td>-</td>
</tr>
<tr>
<td></td>
<td>D</td>
<td>15</td>
<td>60 (±10)</td>
<td>-</td>
</tr>
<tr>
<td></td>
<td>Primary</td>
<td>4xA</td>
<td>0</td>
<td>0</td>
</tr>
<tr>
<td></td>
<td>Human</td>
<td>4xA</td>
<td>0</td>
<td>100</td>
</tr>
<tr>
<td></td>
<td>Fibroblasts</td>
<td>4xC</td>
<td>0</td>
<td>0</td>
</tr>
<tr>
<td></td>
<td>Fibroblasts</td>
<td>4xC</td>
<td>13</td>
<td>50 (±10)</td>
</tr>
</tbody>
</table>

aComplex composition: A: 1.5 µg DNA/0.2 µg pLys/1.25 µg TfpLys. B: 1.5 µg DNA/1.5 µg TfpLys. C: 1.5 µg DNA/0.2 µg pLys/1 µg pLys. D: 1.5 µg DNA/1.2 µg pLys.
bExpression levels are normalized to the light units obtained with complex A in the presence of glycerol (set to 100%). 100% corresponds to average expression levels of 1.5 × 10^7 light units/10^5 cells (H225); 1.5 × 10^6 light units/10^5 cells (NIH 3T3); 1.0 × 10^7 light units/10^5 cells (A549); or to 1.2 × 10^6 light units/10^5 cells (primary human fibroblasts). Values (± standard deviation) are shown.
cultures, reporter gene expression was strongly enhanced by glycerol (Table 1 and Figure 3, B and C). However, several cell lines, including the suspension cells K562, M07e, KG-1 and THP-1, gave expression levels barely above background when transfected in the presence of glycerol.

Using primary human fibroblasts, we compared the efficacy of the method with other transfection protocols. Adenovirus/polylysine complexes turned out
to work most efficiently, followed by glycerol and Lipofectamine. DEAE dextran-mediated transfection did not work well in our hands (Figure 5).

Influence of Complex Composition and Transfection Conditions on Transient Transgene Expression

Successful gene transfer was found to correlate with the overall charge of the complex. A charge titration using increasing amounts of TfpLys showed that at least a slight positive charge excess is necessary for good reporter gene delivery (Figure 6). Reporter gene expression in H225 cells was not dependent on the presence of transferrin when these positively charged complexes were used (Figure 7). Relative expression levels of complexes formed with DNA/TfpLys or DNA/pLys, however, varied when different cell lines were transfected. DNA/pLys complexes work far less efficiently than DNA/TfpLys on BNL CL.2 cells (data not shown), but work with the same high efficiency as DNA/TfpLys complexes on NIH 3T3 and H225 cells (see Table 1). Increasing the complex concentration by reducing the volume of the transfection medium from 1 mL to 700 µL further augmented the transfection level by twofold (data not shown). Replacement of polylysine by a basic DNA-binding peptide [Mu (1)] or proteins [HMG (14,34) and histone H4] strongly reduced gene transfer (data not shown), indicating that polylysine is an essential component of this system.

To understand the effect of glycerol on transfection efficiency, we included ethanol, which is known to destabilize membranes (13), in our investigation. Addition of 1%–1.5% ethanol further enhanced transfection levels twofold (Table 1). Another compound, lecinthin, which affects membrane fluidity (33), had no effect (data not shown).

Evaluation of the combination of the components used in this system has shown that maximal reporter gene expression for H225 was obtained when DNA complexes (1.5 µg DNA/1.5 µg TfpLys/0.2 µg pLys) were added to the cells in medium containing 10% glycerol and 1.5% ethanol in a total volume of 700 µL.

Glycerol Does Not Augment Gene Transfer Mediated by Cationic Lipids or Polyethyleneimine

Using 1 to 6 charge equivalents of DOTAP or Transfectam per DNA in the presence of 10% glycerol does not change the transfection efficiency of the lipids in H225 cells (Figure 8). The presence of glycerol did not enhance the efficacy of a new gene transfer agent, polyethyleneimine (2, data not shown).

Expression of IL2 in Primary Human Fibroblasts

In order to test the expression of a secretable gene product, 200,000 primary human fibroblasts were transfected for 4 h using 10% or 15% glycerol with a complex consisting of 6 µg pGShL2/0.9 µg pLys/6 µg TfpLys. The medium was replaced every 24 h after transfection, and IL2 was determined from the supernatant after 2, 3 and 6 days. IL2 levels peaked at 3 days posttransfection (2800 U [10% glycerol], 1600 U [15% glycerol]), and it was still detected in the sample at day 6 (200 U [15% glycerol]).

Stable Integration of Plasmid DNA Using Glycerol-Enhanced Transfection

Glycerol was also used to generate stable clones in the human melanoma isolate H225. The genes used were luciferase, lacZ and hIL2. Luciferase expression of the stably-transfected pooled cells was 200000 light units/10⁶ cells. IL2 levels of six single clones varied between 0.5 U and 220 U per 10⁶ cells and 24 h (average = 110 U; single clones produced 0.5 U, 31 U, 70 U, 75 U, 125 U, 177 U and 220 U). These values were in the same range of cells stably transfected using Lipofectamine. Cells stably transfected with the lacZ gene were analyzed by staining glutaraldehyde-fixed cells with X-gal. About 70% of the cells showed intensive staining (data not shown).

DISCUSSION

The natural uptake mechanism of endocytosis/pinocytosis has been exploited for gene transfer (35,38). These transfection systems use polylysine (pLys) to condense plasmid DNA. A ligand-polylysine conjugate is incorporated in DNA complexes to provide a cell-binding domain. One of the limiting steps of the system is the release of internalized DNA particles from the endosome to the cytoplasm. This bottleneck has been overcome by including endosome-disrupting agents, such as inactivated adenoviruses (9,10,12,37,39) or peptides (26,36,41), in DNA complexes. We were looking for other ways to ensure the release of DNA.

Several reports (11,23,28) describe the interaction of glycerol with cellular membranes. We show here that glycerol is able to enhance reporter gene expression in a variety of primary cells and cell lines when present during transfection with ligand-polylysine/DNA complexes. We tested a series of other compounds; however, only ethylene glycol was able to substitute for glycerol to any great extent. Depending on the cell line, the amount of glycerol ranged from 1.15 to 1.75 M (10% to 15% [vol/vol] of the commercial 87% solution).

It has long been known that transfection efficiency can be enhanced by applying an osmotic shock with glycerol or dimethyl sulfoxide (DMSO) (18,25,29). However, these methods are different in that glycerol is added to the cells after the transfection at a concentration between 10% and 15% for only up to 3 min. This enhancement was usually in the range of about 5-fold compared to up to 1000-fold with our protocol. We have good indication that glycerol does not predominantly act osmotically: First, incubation of the cells with glycerol after transfection did not augment gene expression more than 5-fold. Second, despite the fact that negatively charged complexes were taken up by the cells (data not shown), these complexes did not mediate gene delivery, indicating that the complex composition plays a crucial role. Third, glycerol promotes far higher transfection levels than other compounds at comparable osmolarity. The well-known effect of glycerol on membranes could be responsible for the action. However, glycerol at high concentrations may also exert some osmotic effects.
In some cells, TfpLys is not necessary for efficient gene transfer and can be replaced by unmodified polylysine. This suggests that complexes are internalized by receptor-mediated endocytosis as well as adsorptive endocytosis induced by polylysine, as seen with many other substances including proteins (32) and oligonucleotides (20).

Polylysine seems to synergize with glycerol: The charge titration experiments show that an excess of polylysine is necessary to augment gene transfer. Polylysine, which has the ability to interact with membranes (31,32), could not be replaced by other DNA-binding proteins.

In summary, we have developed a simple protocol to transfect mammalian cells that works especially well for primary human fibroblasts. In its simplest version, only the inexpensive reagents polylysine and glycerol are required. The system can be used for both transient and stable transfections. In some cell types, the addition of glycerol to DNA/(Tf)pLys complexes results in reporter gene expression almost as high as that obtained using adenovirus-enhanced "transferrinfection." For optimal transfection efficiency, one has to optimize several parameters including the amount of polylysine or TfpLys, the overall positive-charge excess, the percentage of glycerol, the incubation time of complexes with cells and the amount of DNA.

ACKNOWLEDGMENTS

Antoine Kichler received a fellowship from Association pour la Recherche contre le Cancer (ARC). This work was supported by a grant from the Austrian Science Foundation (FWF). We thank H. Kirlappos for technical support. We are grateful to Dr. Hartmut Beug, Dr. Matt Cotten and Ciaran Morrison for careful review of the manuscript and helpful discussions.

REFERENCES


Received 16 June 1995; accepted 17 October 1995.

Address correspondence to:
Ernst Wagner
Boehringer Ingelheim R& D Vienna
Dr. Boehringergasse 5-11
A-1121 Vienna, Austria