Methods in Enzymology

Volume 217

Recombinant DNA

Part H

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[42] Receptor-Mediated Transport of DNA into Eukaryotic Cells

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General Introduction

DNA-mediated gene transfer into eukaryotic cells has proved to be a powerful approach for the analysis of the molecular and cellular biology of cell differentiation, growth, and carcinogenic transformation. Many techniques have been developed for the introduction of genes: calcium phosphate precipitation,[1,2] electroporation,[3,4] liposome packaging of DNA,[5-7] with[8-10] or without specific targeting of the liposomes, DOTMA-mediated lipofection,[11,12] microinjection,[13] the use of polycations such as

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[12] C. E. Holt, N. Garlick, and E. Cornel, *Neuron* **4,** 203 (1990).
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Polybrene,[14] DEAE-dextran,[15-17] lipopolyamine,[18] and polyornithine[19] as well as asbestos-mediated[20] or direct transfer of genes from bacteria to eukaryotic cells[21] or direct microinjection of tissue.[22,23] Genes have also been transferred by particle bombardment.[24] In none of these procedures has the mechanism of DNA uptake into the nucleus been thoroughly investigated.

There are two basic natural mechanisms for the uptake of substances in the eukaryotic cell. Small molecules such as amino acids, monosaccharides, and most ions are transported directly across the plasma membrane into the cytoplasm. Macromolecules such as peptides and proteins are taken up by the process of receptor-mediated endocytosis. This uptake is made possible by macromolecule-specific receptors that are found on the surface of the cell membrane. Once the macromolecules are bound, ligands and receptors are clustered and reach the coated pits on which the clathrin-associated membrane is pinched off as a coated vesicle. After removal of the clathrin the vesicles fuse with and become part of the acidic endosomes. Ligand and receptors can have different fates. They can either be destroyed in a degradative pathway, or brought back to the cell surface in a recycling pathway. Receptors include, among many others, those targeted to low-density lipoprotein (LDL),[25] transferrin,[26,27] asialoglycoproteins,[28] gp120 envelope protein of the human immunodeficiency virus (HIV),[29-31] and diphtheria toxin.[32,33]

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[17] O.-R. B. Choi and J. B. Engel, *Cell* **55,** 17 (1988).

[18] J.-P. Behr, B. Demeneix, J.-P. Loeffler, and J. P. Perez-Mutul, *Proc. Natl. Acad. Sci. U.S.A.* **86,** 6982 (1989).

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Because of the trafficking of the receptors, their internalization[27,28,34] occurs frequently. Because there are several hundred thousand receptors per cell, at least for some ligands, this rapid trafficking leads to high turnover numbers per cell. Thus, it has been calculated that each of the 100,000 to 500,000 asialoglycoprotein receptors on hepatocytes transports and internalizes as many as 200 ligand molecules per day.[28] Similarly, the iron ion-transporting transferrin has high turnover numbers, with an estimated 2×10^4 iron ions transported across the plasma membrane per minute.[35]

The basic principle of all receptor-mediated transport systems for cloned DNA is to subvert the efficient cellular mechanisms of receptor-mediated internalization of proteins in such a way that ligands bound up with DNA are recognized by the receptors and are carried swiftly and efficiently across the plasma membrane into the cell.

Principle of Method

Transferrinfection is the process by which DNA is transferred into cells by transferrin–polycation conjugates based on transferrin-dependent receptor-mediated endocytosis.[36–38] For this, the human iron-transport protein transferrin, or the chicken homolog conalbumin, was covalently linked to DNA-binding protamines or to DNA-binding polylysines of various degrees of polymerization (90 to 450 lysine residues).

Receptor-Mediated Entry of DNA into Cells. When the plasmid DNA in the transferrin–polylysine conjugate (TfpL) complex containing the luciferase gene is supplied to eukaryotic cells, moderate to high levels of expression of the luciferase gene occurs in some cell types, while others

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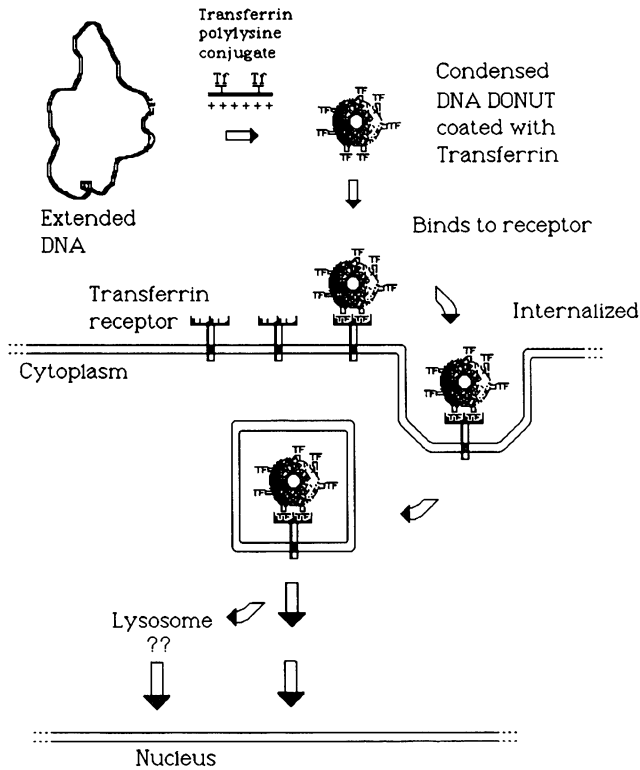
FIG. 1. Diagram summarizing the general principle of transferrinfection. A transferrin–polycation conjugate is prepared. DNA, when incubated with this conjugate, undergoes a polycation-induced condensation to produce a transferrin-coated DNA doughnut. This structure is then supplied to transferrin–receptor cells. Transferrin–receptor interactions result in the internalization of the complex by receptor-mediated endocytosis. By an unknown mechanism, the DNA finds its way to the nucleus, where subsequent expression of genes contained on the DNA ensues.

appear to be refractory (see Table I). The general principle of this method is summarized in Fig. 1.

Several lines of evidence suggest that in K562 cells or chicken HD3 cells receptor-mediated endocytosis of the DNA is involved. Transferrin-fection is competed by addition of iron-loaded transferrin in the cell medium.[37] For K562 cells or HD3 cells polylysine alone is a poor carrier for DNA transfer into the cells.[37,39] Only when linked to the transferrin

[39] E. Wagner, M. Cotten, R. Foisner, and M. L. Birnstiel, *Proc. Natl. Acad. Sci. U.S.A.* **88,** 4255 (1991).

moiety does transferrinfection ensue. That the binding of the iron-containing transferrin to its receptor is a crucial step in transferrinfection is further supported by our finding that protocols that increase the number of transferrin receptors[38] in K562 cells (as determined by Scatchard analysis[40] and our unpublished observations, 1990) also increase transferrinfection.

The binding of transferrin–polylysine and transferrin–polylysine–DNA to cell surface receptors was measured with tritiated compounds whereas the internalization was followed using fluoresceinated transferrin–polylysine–DNA complexes. Binding of the complexes to viable HD3 cells was saturable. The apparent binding constants were similar for transferrin and transferrin–polylysine, suggesting that the polylysine moiety did not severely impede receptor recognition. Finally, fluorescein-labeled transferrin–polylysine–DNA complexes appeared rapidly in numerous small fluorescent vesicles in >95% of the cells, suggesting that the DNA complexes were now in the vesicular (endosomal?) system of the cell.[37]

We observe that, at least for some cell types, transferrinfection is very much enhanced by the addition of chloroquine during the 4-hr duration of transferrinfection, but this requirement appears to vary in a highly idiosyncratic and unpredictable manner from cell type to cell type. We presume that where chloroquine is beneficial, as is the case for K562 cells, this lysosomatropic drug increases the pH in the endosomal and the lysosomal compartments and thus prevents degradation of the reporter DNA during passage from endosomes to the nuclear compartment. But the action of chloroquine is not fully understood and other mechanisms of action are not excluded. Other lysosomatropic substances added with or instead of chloroquine, such as monensin, do not facilitate transferrinfection, but actually interfere with it. NH_4Cl and methylamine are ineffective.

An alternative, and often superior, protocol that allows high-level expression involves treating transferrinfected cells with a defective adenovirus. In cells that can bind and internalize adenovirus (most human and many mouse cell types) the entry of adenovirus involves a membrane disruption as the pH drops in the endosome. We have found that treatment of cells supplied with various ligand–polycation DNAs (e.g., transferrin–polylysine, gp120–polylysine, antibody–polylysine) in the presence of the replication-defective adenovirus dl312 allows enhanced gene expression in the absence of chloroquine (see note added in proof).

Ligands Other Than Transferrin. DNA can be targeted to other receptors by choosing different ligand–receptor systems. Thus, it has been

[40] E. Mattia, K. Rao, D. Shapiro, H. Sussman, and R. Klausner, *J. Biol. Chem.* **259**, 2689 (1984).

possible to target the cell membrane protein CD4 in CD4-positive lines with conjugates formed between polylysine and anti-CD4 monoclonal antibodies or polylysine and recombinant gp120 (the envelope protein of HIV; a kind gift of Genentech, South San Francisco, CA). Other receptors were targeted with anti-CD7 monoclonal antibodies and asialofetuin (unpublished observations, 1992). A related procedure using asialoorosomucoid–polylysine conjugates has been successfully used to deliver DNA, via the asialoglycoprotein receptor, to hepatocytes, both in tissue culture and in rats.[41,42]

Quantitative Aspects. High DNA concentrations during transferrinfection are desirable but the DNA concentration in stock solutions cannot exceed 20–30 μg/ml because of the limited solubility of the TfpL–DNA complexes. We find that plasmids of 13 kb work as well as plasmids of 6 kb. We are currently testing alternate DNA packaging schemes to increase the size of plasmid that can be reliably transfected. (See note added in proof.[5])

Quantitative Southern analysis revealed that initially, after a few hours of incubation, on the order of 5000 ("episomal") DNA plasmids per cell became associated with K562 cells. One or 2 weeks later the number of DNA molecules was reduced by a factor of about 1000. In the case of K562 cells, virtually all cells take up DNA (see above) and close to 100% of the cells express the reporter gene (see Ref. 37 and below). Especially where chloroquine is not required, there is no cell death during transferrinfection. This means that the procedure can be repeated several times on the same cell culture and in this way the amount of DNA that is transferred into the cells can be increased.[37]

Where there is no selection for the transferred gene, expression after transferrinfection is transient. Expression of the Rous sarcoma virus long terminal repeat (LTR)-driven luciferase expression plasmid (pRSVL) in K562 cells, after reaching a maximum of expression around 18 to 24 hr, decays with a half-life of days when measured in the cell culture as a whole. When the pRSVL construct is presented to K562 cells in the context of a dominant control region of the globin gene cluster,[43] expression is maximal after day 2 and a high level persists in the cell culture as a whole for about 2 to 3 weeks. This protracted expression may render the establishment of stable cell lines unnecessary in some distances. When transfection of a selectable marker (e.g., neomycin phosphotransferase)

[41] C. Wu, J. Wilson, and G. Wu, *J. Biol. Chem.* **264**, 16985 (1989).
[42] G. Y. Wu and C. H. Wu, *J. Biol. Chem.* **263**, 14621 (1988).
[43] P. Collis, M. Antoniou, and F. Grosveld, *EMBO J.* **9**, 233 (1990).

is performed on K562 cells about 0.5 to 1% of the cells form colonies of stable transformants.

Methods

Synthesis of Transferrin–Polylysine Conjugates

The initial conjugate synthesis[36,39] involved the modification of one to two amino groups on the transferrin molecule with the reactive bifunctional reagent N-succinimidyl-3-(2-pyridyldithio)propionate (SPDP), followed by the ligation to the similarily modified polycations (polylysine or protamine) through the formation of disulfide bonds (see Conjugation Method 1, below). We have also synthesized transferrin–polylysine conjugates that contain a specific ligation through modification of the transferrin carbohydrate moiety.[44] The two terminal exocyclic carbon atoms of the sialic acids within the carbohydrate chains of human transferrin are selectively removed by periodate oxidation.[45] The oxidized aldehyde-containing form of transferrin was used for coupling to the amino groups of polylysine. The junction that results from aldimine formation was stabilized by reduction with sodium cyanoborohydride to the corresponding amine linkage (see Conjugation Method 2, below). There are a number of advantages of the carbohydrate linkage procedure compared to the SPDP procedure. The carbohydrate method has the advantage of being less time consuming. It is useful for scaling up for the preparation of large quantities of conjugates and it generates conjugates having defined polycation–transferrin ligation sites with "nature-derived" carbohydrate spacers.

Preparation of Conjugates

General Procedures

Quantitative Assays. The polylysine content of fractions was estimated spectrophotometrically by the ninhydrin assay and, in the case of fluorescein isothiocyanate (FITC)-labeled polylysine, by absorption at 495 nm. The amount of dithiopyridine linkers in modified transferrin was determined, after reduction of an aliquot with dithiothreitol, by an absorption measurement of released pyridine-2-thione at 340 nm. The amount of free

[44] E. Wagner, M. Cotten, K. Mechtler, H. Kirlappos, and M. L. Birnstiel, *Bioconjugate Chem.* **2,** 226 (1991).
[45] T. Kishimoto and M. Tavassoli, *Anal. Biochem.* **153,** 324 (1986).

mercapto groups was determined using Ellman's reagent[46] and measurement at 412 nm. Transferrin content of the fractions was determined by ultraviolet (UV) measurement at 280 nm and correction (where necessary) of the value by subtraction of the corresponding UV absorption of FITC, dithiopyridine, or buffer at 280 nm.

Conjugation Method 1: Transferrin–Polylysine Conjugate Synthesis through Disulfide Linkages

Conjugation method 1 has been used for the preparation of conjugates of transferrin or conalbumin with various poly(L-lysines),[36] poly(D-lysine), poly(L-arginine) (unpublished observations, 1990), salmon sperm protamine,[36] and a synthetic protamine analog[39]; conjugates of poly(L-lysines) with rgp120[47,48] have been synthesized (unpublished observations, 1990), as well as poly(L-lysine)–antibody conjugates [with anti-CD4, anti-CD7 (unpublished observations, 1990)] and with asialofetuin (unpublished observations, 1990). A related procedure for the synthesis of asialoorosomucoid poly(L-lysine) conjugates has been described by Wu and Wu.[42]

Transferrin–poly(L-lysine) conjugates with polylysines of an average chain length of 200 or 450 lysine monomers (pL200, pL450) have been synthesized as described[36,39]: coupling of transferrin to polylysine was performed by ligation via disulfide bonds after modification with the bifunctional reagent succinimidyl-3-(2-pyridyldithio)proprionate (SPDP; Pharmacia, Piscataway, NJ).

3-(2-Pyridyldithio)propionate-Modified Transferrin. A solution of 100 mg (1.25 μmol) of human transferrin (iron free, Sigma, St Louis, MO) in 3 ml of 100 mM N-2-hydroxyethylpiperazine-N'-2-ethanesulfonic acid (HEPES) buffer, pH 7.9, was subjected to gel filtration on a Sephadex G-25 column. To the resulting 5-ml solution, 260 μl of a 10 mM ethanolic solution of SPDP (2.6 μmol) was added with vigorous mixing. After 1 hr at room temperature, purification was performed by a further Sephadex G-25 gel filtration to give 6 ml of a solution of 1.1 μmol transferrin modified with 2.1 μmol dithiopyridine linker.

3-Mercaptopropionate-Modified Polylysine. Poly(L-lysine) of different molecular weights was used, namely those with an average chain length of 200 or 450 lysine monomers (pL$_{200}$ or pL$_{450}$ hydrobromide; Sigma). Both unlabeled and fluorescent-labeled polylysines were used; fluorescent labeling with FITC (Sigma) was performed in sodium bicarbonate buffer,

[46] G. L. Ellman, *Arch. Biochem. Biophys.* **82**, 70 (1959).
[47] L. Lasky, J. Groopman, C. Fennie, P. Benz, D. Capon, D. Dowbenko, G. Nakamura, W. Nunes, M. Renz, and P. Berman, *Science* **233**, 209 (1987).
[48] L. Lasky, G. Nakamura, D. Smith, C. Fennie, C. Shimasaki, E. Patzer, P. Berman, T. Gregory, and D. Capon, *Cell* **50**, 975 (1987).

pH 9, for 3 hr. A gel-filtered solution of 0.57 μmol pL_{200} (FITC labeled) in 3 ml 20 mM sodium acetate buffer was brought to pH 7.9 by the addition of 300 μl of 1 M HEPES buffer and 204 μl of a 10 mM ethanolic solution of SPDP (2.04 μmol) was added with vigorous mixing. One hour later 500 μl of 1 M sodium acetate, pH 5, was added; after gel filtration with 20 mM sodium acetate the solution contained 0.54 μmol pL_{200} with 1.86 μmol of dithiopyridine linker. The solution was brought to pH 7 by addition of HEPES buffer, and 36 mg dithiothreitol (DTT) was added. The solution was kept under argon at pH 7.5 for 1 hr. The pH was adjusted to 5.2 by addition of 400 μl 3 M sodium acetate buffer. After gel filtration (Sephadex G-25, 14 × 180 mm column, 15 mM sodium acetate, pH 5.0) a solution of 0.50 μmol pL_{200}, which was modified with 1.84 μmol mercaptopropionate linker, was obtained. Following the same procedure, modification of 0.20 μmol pL_{450} with 0.70 μmol SPDP gave a product of 0.18 μmol pL_{450} with 0.57 μmol dithiopyridine groups; treatment with DTT and isolation gave 0.175 μmol pL_{450} modified with 0.56 μmol mercapto groups.

Conjugation of Transferrin with Polylysine. $TfpL_{200}$ conjugates were prepared by mixing 1.06 μmol modified transferrin (see 3-(2-Pyridyldithio)-propionate-Modified Transferrin, above) in 100 mM HEPES buffer, pH 7.9, with 0.20 μmol modified pL_{200} (see previous section) in sodium acetate buffer under an argon atmosphere. The reaction mixture was kept for 18 hr at room temperature. $TfpL_{450}$ conjugates were prepared in a similar manner starting with 0.61 μmol modified transferrin and 0.12 μmol mercapto-modified pL_{450}.

Both $TfpL_{200}$ and $TfpL_{450}$ were isolated from the reaction mixture by cation-exchange chromatography [Pharmacia Mono S column HR 10/10; gradient elution, buffer A: 50 mM HEPES (pH 7.9) and buffer B: buffer A plus 3 M sodium chloride]; it was essential for the recovery of the polycation conjugates to add sodium chloride to the reaction mixture (final concentration, 0.6 M in case of $TfpL_{200}$ or 1 M in case of $TfpL_{450}$ conjugates) before loading the column and to start the gradient at this salt concentration. The excess of uncoupled transferrin was eluted first. The product fractions were eluted at salt concentrations around 1.4 M with $TfpL_{200}$ or around 2 M salt with $TfpL_{450}$. The $TfpL_{200}$ product fractions were pooled into three conjugate fractions, A–C, with increasing polylysine : transferrin ratio. The $TfpL_{450}$ conjugates were separated into four fractions, A–D. After dialysis against HBS [20 mM HEPES (pH 7.4), 150 mM NaCl], conjugate fractions were obtained with overall yields of 80% ($TfpL_{200}$, containing 39 mg conjugated transferrin) or 64% ($TfpL_{450}$, containing 20 mg conjugated transferrin). The yields are based on equivalents of transferrin in the product relative to equivalents of mercapto groups in the modified polylysine starting material.

Conjugation Method 2: Transferrin–Polylysine Conjugate Synthesis through Carbohydrate Modification

Conjugation method 2 has been applied to the synthesis of transferrin–polylysine conjugates,[44] for the conjugation of rgp120 to polylysine (unpublished observations, 1992), and for the conjugation of transferrin to a synthetic protamine analog or to the DNA intercalator ethidium homodimer.[44] However, the method could not be used for the preparation of conalbumin–polylysine conjugates,[36,44] probably due to the absence of sialic acid residues in the conalbumin carbohydrate.[49]

A solution of 102 mg (1.28 μmol) of transferrin (human, iron-free; Sigma) in 3 ml of a 30 mM sodium acetate buffer (pH 5) was subjected to gel filtration on a Sephadex G-25 (Pharmacia) column. This gel-filtration step serves to remove low molecular weight contaminants that interfere with the modification steps. The resulting 3.8-ml solution was cooled to 0° and 80 μl of a 30 mM sodium acetate buffer (pH 5) containing 4 mg (19 μmol) of sodium periodate was added. The mixture was kept in an ice bath in the dark for 90 min. For removal of the low molecular weight products, a further gel filtration (Sephadex G-25, 30 mM sodium acetate buffer, pH 5) was performed and yielded a solution containing about 82 mg (1.03 μmol) of oxidized transferrin {monitoring: UV absorption at 280 nm and ninhydrin assay; the oxidized form that contains aldehydes in contrast to unmodified transferrin, gives a color reaction on staining with anisaldehyde reagent [a sample is dropped on a silica gel thin-layer plate, dried, immersed into p-anisaldehyde/sulfuric acid/ethanol (1/1/18, v/v/v) and followed by drying and heating]}. The transferrin solution was added to a solution containing 0.50 μmol of fluorescently labeled poly(L-lysine) with an average chain length of 300 lysine monomers [derived from 34 mg hydrobromide salt (Sigma) after labeling with 130 μg of fluorescein isothiocyanate in sodium bicarbonate buffer (pH 9) for 3 hr and subsequent gel filtration] in 4.5 ml of 100 mM sodium acetate (pH 5) with vigorous mixing at room temperature. The pH of the solution was brought to 7.5 by addition of 1 M sodium bicarbonate after 20 min; to the mixture, four portions of 9.5 mg (150 μmol) of sodium cyanoborohydride were added at 1-hr intervals. Purification proceeded in the same fashion as described in conjugation method 1: after 18 hr of reduction, 1.9 ml of 5 M sodium chloride was added to bring the solution to an overall salt concentration of about 0.75 M. The reaction mixture was loaded on a cation-exchange column (Pharmacia Mono S HR 10/10) and was fractionated with a sodium chloride gradient from 0.75 to 2.5 M with a constant content of 25 mM

[49] J. Williams, *Biochem. J.* **108**, 57 (1968).

HEPES (pH 7.3). Some transferrin protein (about 30%) together with a weak fluorescence activity eluted in the flow-through; the major amount of fluorescent conjugate eluted between 1.35 and 1.9 M salt and was pooled into three fractions. After dialysis twice against 2 liters of 25 mM HEPES (pH 7.3), these yielded (in order of elution) fraction A, containing 19 mg (0.24 μmol) of transferrin modified with 80 nmol of polylysine; fraction B, containing 27 mg (0.34 μmol) of transferrin modified with 150 nmol of polylysine; and fraction C, containing 5 mg (62 nmol) of transferrin modified with 80 nmol of polylysine. The overall yield of these conjugates based on transferrin was 50%, based on polylysine (62%).

Storage of Conjugates and Iron Incorporation for Transferrin Ligands

Transferrin conjugates, unless used immediately, can be stored after shock-freezing (liquid nitrogen) at $-20°$ for up to 12 months in the iron-free form. Before iron incorporation, samples (about 0.5 to 1.5 mg) are brought to physiological salt concentration (150 mM) by the addition of sodium chloride; the iron incorporation is performed by the addition of 4 to 8 μl of 10 mM iron(III)–citrate buffer (containing 200 mM citrate and adjusted to pH 7.8 by sodium bicarbonate addition) per milligram transferrin content. The iron-loaded conjugates are used for DNA complex formation as described (see *Conjugate DNA-Complex Formation*). To limit the deterioration of the conjugates that often occurs on several freeze–thaw cycles, the conjugates are divided into convenient small aliquots, shock-frozen, and kept at $-20°$. In general, iron-incorporated samples maintain their transfection activity for 2–3 months if repeated freeze–thaw cycles are avoided.

Application of Conjugates

Choice of Cells

We have tested a variety of cells for their ability to be transfected with transferrin–polycation conjugates. These results are summarized in Table I. The cell types fall into three categories based on the luciferase activity obtained. Luciferase expression appears to be a good general indicator of gene expression. We have tested other parameters of gene transfer such as RNA production by both class II and class III polymerase, generation of β-galactosidase protein, generation of *tat* trans-activation with an HIV LTR system, and generation of stable cell lines expressing neomycin phosphotransferase. The values obtained are consistent with luciferase values.

TABLE I

TRANSFERRINFECTION WITH VARIOUS CELL TYPES

Cell type	Efficiency of transfection[a]
Cells that work well (>10^5 light units/10^6 cells)	
Human K562 cells	+
Human Ewing's sarcoma EW-2 cells	+
Chicken HD3 erythroblasts	−
Chicken REV-NPB4 lymphoblasts	−
Cells that work moderately well (5 × 10^3 to 10^5 light units/10^6 cells)	
Human HeLa cells	−
Human HepG2 cells	+
Human H9 cells	+
Hamster CHO cells	−
Mouse Ehrlich ascites	+
Monkey COS cells	+
Rat H4IIEC3 cells	+
Rat IA cells	−
Chicken EGFR-myb erythroblasts	−
Chicken normal bone marrow cells	−
Cells that work poorly (<10^3 light units/10^6 cells)	
Human primary lymphocytes	
Human U937 cells	
Human Kurkatt cells	
Human CCRF CEM cells	

[a] +, Works only with chloroquine; −, works without or with chloroquine.

Also indicated in Table I is the requirement of each cell type for chloroquine (100 μM) during the 4-hr transfection period.

Preparation of Cells, Treatment to Enhance Transferrin Receptor Levels

Transfection of Suspension Cultures. In general, exponentially growing suspension cultures are collected by centrifugation and suspended in fresh medium (with or without serum; see below) at one-third to one half of their saturating density. For example, K562 cells are grown in suspension in RPMI 1640 medium [Cat. No. 079-03018P (GIBCO—Bethesda Research Laboratories, Gaithersburg, MD), plus 2 g sodium bicarbonate per liter] plus 10% (v/v) fetal calf serum (FCS) 100 units/ml penicillin, 100 μg/ml streptomycin, and 2 mM glutamine, reach a density of 500,000 cells/ml. At 12–20 hr before transfection, the cells are placed in fresh medium containing 50 μM desferrioxamine [D 9533 (Sigma); stock solution is dis-

solved in water at 100 mM, and stored in aliquots at $-20°$]. The morning of the transfection, the cells are collected, resuspended in fresh medium containing 10% (v/v) FCS (plus 50 μM desferrioxamine) at 250,000 cells/ml, and placed in a 24-well dish at 2 ml/well.

The desferrioxamine pretreatment serves to raise the transferrin receptor levels approximately fivefold, resulting in an increase in DNA delivery.[38] With some cell types, such as chicken HD3 cells, exponential growth alone produces high transferrin receptor levels that cannot be further elevated by desferrioxamine treatment. An enhancement of gene delivery is observed in HD3 cells only when stationary cultures are treated with desferrioxamine. This parameter should be evaluated when testing a new cell type.

Transfection of Adherent Cells. In general, cells are transfected at 50–60% confluence to ensure that a large proportion of cells are dividing and will continue to divide during the experiment. The cells [e.g., HeLa, Chinese hamster ovary (CHO), HepG2] are plated on day 1 (300,000–600,000 cells/T-25 flask, having an approximately 25-cm^2 surface area) and transfected on either day 2 or day 3. On the morning of the day of transfection, the cells are placed in 5 ml of fresh medium per T-25 flask.

Application of the Transferrin–Polylysine–DNA Complex. Transferrin–polylysine–DNA complexes are prepared as described below. Normally 6–10 μg of DNA is used for a sample of 500,000 K562 cells, or a sample of 1 million HeLa cells. Just prior to transfection, chloroquine, when required, is added so that the final concentration in the cell suspension *plus* the DNA solution is 100 μM. [Chloroquine is obtained from Sigma (C-6628); a stock solution of 100 mM water is stored in aliquots at $-20°$ in the dark]. Transferrin–polylysine–DNA complex (in 500 μl) is then added for a 4-hr incubation at 37°. The DNA-containing medium is then removed, the cells are washed once with 3 ml warmed medium, and then 10 ml of warmed medium is added to the cells. When chloroquine is not required, the cells are simply incubated in the presence of the DNA complex until harvest (additional medium or serum may be added if necessary). Normally the cells are harvested (see Harvesting Cells, below) 18–48 hr later and processed for the luciferase (or other) assay.

DNA Preparation, Precautions, and Quantitation

DNA quality is an important parameter. We normally purify the DNA by two CsCl gradients followed by an RNase and a proteinase K digestion, phenol–chloroform extraction, and ethanol precipitation. Probably the critical parameter is the quantitation of the DNA. The transfection efficiency varies strongly with the DNA: TfpL ratio, therefore errors in DNA quantitation can influence the transfection. RNA can also bind the

polylysine and compete for DNA binding, as well as contribute to the absorbance at 260 nm. It seems important to have a reliable DNA quantitation in the absence of RNA for good transfections.

Plasmid DNA is isolated from the appropriate bacterial strains using a Triton/lysozyme lysis method. The bacterial pellet from a 1-liter overnight culture is suspended in 10 ml of 20% (w/v) sucrose, 50 mM Tris, 10 mM ethylenediaminetetraacetic acid (EDTA), pH 7.5 (solution 1) and placed on ice for 10 min. Then 2.2 ml of freshly made 10-mg/ml lysozyme in solution 1 is added to the sample, and the samples are vortexed and incubated for 10 min on ice. Five milliliters of 0.2 M EDTA, pH 7.0, is added to the sample, and the sample is again vortexed and incubated on ice for 10 min. Ten milliliters of 2% (v/v) Triton X-100, 40 mM Tris, 60 mM EDTA, pH 7.5 is added to the sample; the sample is mixed thoroughly and incubated for 15–30 min on ice. The sample is then centrifuged [45 min, 17,000 rpm, Sorvall (Norwalk, CT) SS-34 rotor, 4°]. To the supernatant (26 ml) is added 28.5 g of CsCl and 0.5 ml of a 10-mg/ml solution of ethidium bromide and the sample is centrifuged for 16 hr at 49,000 rpm at 20° in a Beckman (Fullerton, CA) VTi50 rotor. The supercoiled plasmid band (the lower band) is removed from the gradient with a syringe and needle, transferred to a VTi65 tube, and centrifuged for 4 hr at 65,000 rpm at 20° in a Beckman VTi65 rotor. The plasmid band is recovered from the tube, extracted with CsCl-saturated 2-propanol until the pink color vanishes, and dialyzed overnight against a 1000-fold excess of 10 mM Tris, 1 mM EDTA, pH 7.4 (TE). The DNA is then recovered by ethanol precipitation, treated with 0.2 mg/ml RNase A (37°, 30 min) followed by 0.2 mg/ml proteinase K (56°, 30 min). Following a phenol–chloroform and then chloroform extraction, the DNA is again recovered by ethanol precipitation, dissolved in TE, and quantitated by triplicate absorbance readings at 260 nm. An absorbance at 260 nm of 1 is taken to be 0.05 mg/ml.

To test the requirement for the CsCl purification steps, parallel samples of DNA were prepared. One set was carried through both CsCl gradients, the second set, after bacterial lysis, was purified by two phenol–chloroform and one chloroform extraction, precipitated at room temperature with 0.54 vol 2-propanol (to remove a large portion of the RNA), and then subjected to the RNase A and proteinase K steps (referred to hereafter as *quick* DNA). Analysis of the DNA samples by gel electrophoresis revealed no detectable differences in DNA quality between the CsCl-purified DNA and the quick DNA. The two sets of DNA samples were then transferrinfected into K562 cells and the subsequent luciferase activity was assayed 20 hr later (Fig. 2; methods described below). We find large differences in the luciferase activity obtained. With the plasmid pRSVL (the Rous sarcoma virus long terminal repeat driving the luciferase gene), the CsCl-

Plasmid   Preparation   Light Units

pRSVL	Quick	1	▨ 178305
pRSVL	CsCl	2	███████ 5563190
pCMVL	Quick	3	▨▨▨▨▨ 2502480
pCMVL	CsCl	4	███████████ 15456605

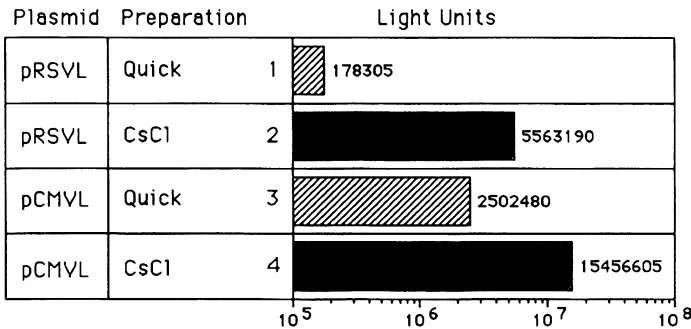10^5 10^6 10^7 10^8

FIG. 2. Influence of DNA quality on transferrinfection. Plasmid DNA was prepared using either a quick lysis technique or by two CsCl density gradients as described in text. Aliquots of 6 μg DNA were transferrinfected into 5 × 10^5 K562 cells (desferrioxamine treated) in the presence of 100 μM chloroquine and harvested for luciferase analysis 20 hr later. The results (plotted as total luciferase light units obtained from each transfected culture) were obtained with either the Rous sarcoma virus LTR-driven luciferase expression plasmid, pRSVL [J. R. de Wet, K. V. Wood, M. DeLuca, D. R. Helinski, and S. Subramani, *Mol. Cell. Biol.* **7**, 725 (1987)], or a plasmid containing luciferase driven by the cytomegalovirus immediate early promoter (pCMVL).

purified DNA gives a 31-fold increase in activity; with pCMVL (the cytomegalovirus promoter driving the luciferase gene) there is a sixfold increase in expression with the CsCl purification.

Conjugate–DNA Complex Formation

Formation of DNA Complexes. Because of its cationic properties, the transferrin–polylysine (or the transferrin–protamine) conjugate binds DNA avidly. In band-shift experiments it has been demonstrated that the transferrin–polylysine conjugates (TfpL) band-shift linear or covalently circular DNA without size discrimination.[36] For each TfpL preparation, one can calculate the mass ratios at which the DNA–transferrin–polylysine mixture reaches electroneutrality. Consistent with this calculation one finds nearly complete gel-mobility retardation of the DNA complex as well as optimal DNA expression for a reporter gene at these ratios (see Ref. 36).

The formation of complexes of transferrin–polycation with DNA is performed by mixing diluted solutions of DNA (30 μg/ml or less) with the transferrin–polycation conjugates. Normally we dilute the DNA (as a 0.5 to 1.5-mg/ml solution in TE) into HBS, with a resulting volume of 330 μl. The dilution of DNA at room temperature and the absence of phosphate in the buffer are essential to avoid precipitation problems. In a second tube, TfpL is diluted into HBS (final volume, 170 μl). The 170-μl TfpL

sample is then rapidly mixed with the 330-μl DNA sample to generate the DNA–TfpL complex ready to add to the cells. The optimum weight ratio of TfpL (as transferrin) to DNA is normally determined in titration experiments in which a constant amount of DNA encoding a reporter gene, 6 μg, is complexed with 12–24 μg of TfpL and tested for transfection efficiency.

Investigations have shown that the polycation–polylysine in the transferrin–polycation conjugates not only serves to attach transferrin to DNA, but also plays a pivotal role in condensing the DNA to toroid-like structures with a diameter of about 80–100 nm,[39] a dimension that resembles the diameter of the coated pits.

We have investigated the role of DNA condensation in transferrinfection efficiency in K562 cells using the luciferase gene (pRSVL; size: 6.2 kb) as a reporter gene. We find that although authentic or synthetic protamines conjugated to Tf bind DNA and function in DNA delivery, no condensed toroid structures can be found by inspection in the electron microscope. Consistent with this lack of DNA condensation we find that the gene transfer is down by at least a factor of 10 as compared to the TfpL system. Polylysine by itself condenses DNA to doughnut-like structures, a finding first reported for λ DNA by Laemmli,[50] but no DNA transfer is observed in K562 cells.[37,39]

The ratio of transferrin to polylysine in the conjugate also influences the efficiency of transferrinfection. After conjugation, fractions with differing transferrin : polylysine ratios can be isolated by chromatography on Pharmacia Mono S columns[39] and their efficiency of transferrinfection tested. Using the luciferase gene as a reporter gene it emerges that conjugation of 1 transferrin per approximately 100 lysine moieties yields optimal compounds for gene transfer experiments.

Conjugate–Free Polycation–DNA Ternary Complexes

With conditions that maximize transferrinfection, a calculated 120 transferrin molecules are associated with each 6 kb of plasmid DNA. We have noted that a large fraction, up to 90% of the TfpL, can be replaced by polylysine or the metabolically stable poly(D-lysine) without impeding doughnut formation or transferrinfection. However, if the number of transferrin molecules per doughnut falls below 10–15, targeting of the complexes to the receptors is apparently no longer possible and transferrinfection is abolished. Condensation of the DNA, with various degrees of success, can be brought about by cations such as histones and protamines, but not with spermidine or spermine (at physiological ionic strength).

[50] U. K. Laemmli, *Proc. Natl. Acad. Sci. U.S.A.* **72**, 4288 (1975).

With the exception of histone H4, a strong correlation between DNA condensation and transferrinfection efficiency has been found.[39]

Transfection, Troubleshooting, Initial Parameters to Vary

The presence or absence of fetal calf serum (FCS) in the transfectioin medium may have an effect on the transferrinfection efficiency. Dependinjg on a particular batch of FCS (different quality and origin), with K562 cells we sometimes find a higher initial transfection rate (day 1) if we omit serum from the medium during the first 4 hr of transferrinfection. However, cells suffer considerably from this serum-free period in the presence of chloroquine. In contrast, more than 99% of cells stay viable when both FCS and chloroquine are included in the transfection experiments; conse-quently at a later time point (day 2) the gene expression of the whole culture is higher.

There are a number of other parameters that can be varied to improve the transfection efficiency. However, the initial parameters that we test when evaluating a new cell type are the use of 100 μM chloroquine and the presence of serum during the 4-hr DNA exposure. In general we use a human transferrin in our conjugates, so that competition with bovine transferrin (in the fetal calf serum) is generally not a problem when trans-fecting human cells. However, the competition effect may be stronger when the human transferrin conjugate is used to transfect mouse cells, rat cells, or cells of other species. Although it should not influence the DNA delivery, the promoter used to drive the indicator gene plays an important role. If the promoter driving the indicator gene has weak activity in the cell type being tested, it will obviously influence the resulting gene expression.

Standard Transfection Protocol for K562 Cells

K562 cells are prepared for transfection (as described in Receptor-Mediated Entry of DNA into Cells, above) at 250,000 cells/ml, and placed in a 24-well dish at 2 ml/well.

Six micrograms DNA in 330 μl HBS (150 mM NaCl, 20 mM HEPES, pH 7.3) is mixed with TfpL conjugate (optimum, e.g., 18 μg in the case of TfpL$_{300}$B) in 170 μl HBS, and after 15 to 30 min at room temperature the mixture is added to K562 cells. Chloroquine is added to the 2-ml cell sample just before adding the 500-μl DNA–conjugate sample (100 μM final concentration; 25 μl of a 10 mM chloroquine solution is added). After a 4-hr incubation at 37°, the cells are washed in prewarmed, fresh medium (no chloroquine) and incubated at 37°. (Normally this is done by gently removing 90% of the medium and replacing it with fresh medium, followed by a repetition after waiting an hour to let the cells resettle.)

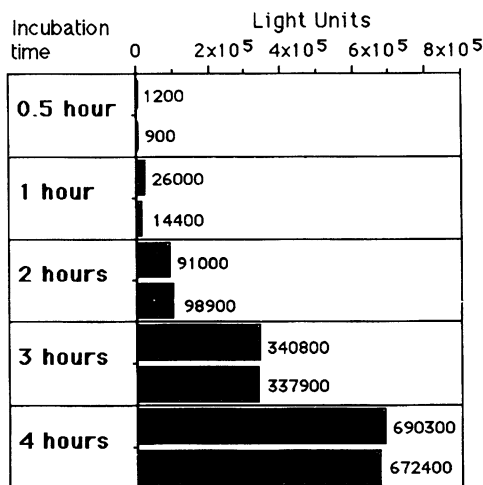An analysis of the optimum time for exposure of K562 cells to

Incubation time	Light Units

FIG. 3. DNA delivery as a function of the time of exposure to transferrin–polylysin–DNA complexes. Transferrinfection was performed with 6 µg pRSVL and 5 × 10⁵ K562 cells (desferrioxamine treated) in the presence of 100 µM chloroquine. The cells were exposed to the DNA for the indicated times, washed with fresh medium, and assayed for luciferase activity 20 hr later.

TfpL–DNA complexes in the presence of 100 μM chloroquine is shown in Fig. 3. K562 cells were incubated with 6 μg pRSVL as a complex with 18 μg of TfpL, in 100 μM chloroquine. After the indicated times, the cells were washed into fresh medium lacking chloroquine. The cells were harvested 20 hr later, and extracts were prepared and analyzed for luciferase activity. We find that the highest levels of expression are obtained when the cells are exposed to the DNA complexes for 4 hr (Fig. 3). Subsequent experiments indicate a decline in cell viability after 5 hr (probably due to the toxicity of the chloroquine) so that in general, when chloroquine is required, the incubation time is limited to 4 hr. In cells where chloroquine is not required (chicken HD3, some HeLa lines) the cells can be kept in constant exposure to TfpL–DNA complexes with no apparent change in cell viability.

Harvesting Cells

The cells are normally harvested for luciferase assay 16 to 20 hr after transfection (see below and Assays for Transfection Efficiency). However, the timing of harvest is an important variable and should be optimized in initial experiments. In general, harvest at 24–48 hr after transfection gives a good yield of activity.
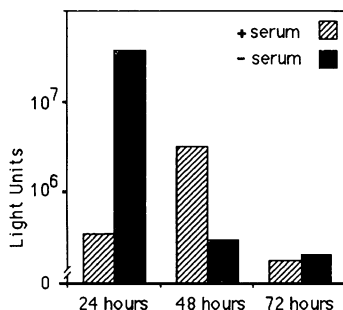
FIG. 4. Luciferase activity as a function of the time of harvest. Transferrinfection was performed with 6 µg pRSVL and 5 × 10⁵ K562 cells (desferrioxamine treated) in the presence of 100 µM chloroquine and in either the presence or absence of 10% (v/v) fetal calf serum. After a 4-hr exposure to DNA, the cells were washed with complete medium and harvested either 24, 48, or 72 hr later. The graph indicates the resulting luciferase activity (in light units).

An analysis of the optimum harvest time for assaying luciferase expression in K562 cells is shown in Fig. 4. K562 cells were transferrinfected with 6 µg of pRSVL in either the presence or absence of 10% (v/v) fetal calf serum. Cells were harvested at various times after transfection, and extracts were made, standardized for protein content, and assayed for luciferase activity (Fig. 4). When transfected in the absence of serum, the yield of luciferase activity peaks at 24 hr and declines by a factor of approximately 10 over the next 72 hr (Fig. 4). The expression pattern obtained when the transfection is performed in the presence of serum is slightly altered, with activity peaking at 48 hr posttransfection (Fig. 4). Note that the plasmid used here, pRSVL has no replication signal. Strikingly different results are obtained when a plasmid with the capacity to replicate, such as an episome, is used [see Fig. 5, fluorescence-activated cell sorting (FACS) analysis].

Assays for Transfection Efficiency

Luciferase Assay

Suspension Cultures. Transfected cell cultures (2–5 ml, 500,000 to 2 million cells) are transferred to 15-ml conical tubes, harvested by centrifugation (1500 rpm, Heraeus minifuge, Kalkberg, Germany), resuspended in 10 ml of phosphate-buffered saline (PBS), again centrifuged, and the cell pellet is transferred to a 1.5-ml Eppendorf tube with 1 ml of PBS. The cells are centrifuged in the Eppendorf 5415 centrifuge (14,000 rpm, 20 sec); the resulting cell pellet is resuspended in 100 µl 0.25 M Tris, pH 7.5.
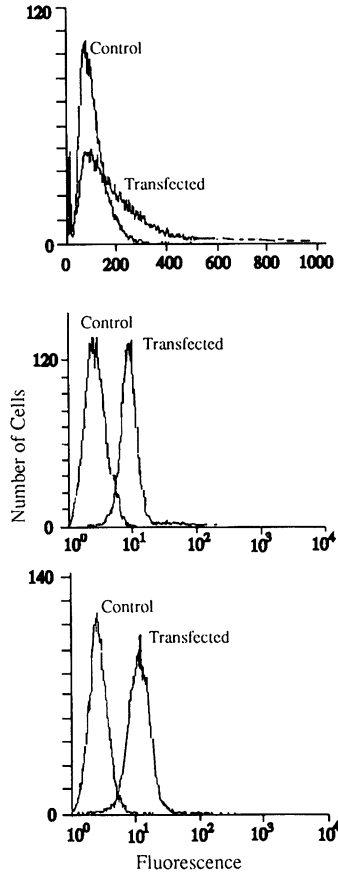
FIG. 5. FACS analysis to determine the proportion of the transfection population that expresses a transfected lacZ gene. Transferrinfection was performed with desferrioxamine-treated K562 cells using (per 5×10^5 cells) 8 μg of pNEBO.LTRlacZ [containing the Epstein-Barr virus (EBV) EBNA1 gene and oriP sequence plus the lacZ gene driven by the HIV LTR] and 2 μg pCMVTat (containing the HIV tat gene driven by the CMV immediate early promoter; both plasmids supplied by Genentech). The transferrinfection was performed for 4 hr in RPMI containing 10% (v/v) FCS, 100 μM chloroquine, and 50 μM desferrioxamine. Cells were harvested at (A) 24 hr, (B) 48 hr, and (C) 1 week posttransfection, loaded with the fluorescent β-galactosidase substrate FDG, and analyzed by FACS.

Adherent Cultures. The medium is removed from the culture (approximately 2 million cells, T-25 flask) and the cells are washed once with 5 ml of PBS. A 1-ml aliquot of PBS is then added to the flask, and the cells are removed from the plastic by gentle scraping with a rubber policeman and transferred to a 15-ml conical centrifuge tube. The flask is washed/scraped

once more with a 1-ml aliquot of PBS. The cells are collected by centrifugation, resuspended in 1 ml of PBS, and transferred to an Eppendorf tube; after centrifugation the resulting pellet is resuspended in 100 μl of 0.25 M Tris, pH 7.5.

Extract Preparation. The cells (suspended in 0.25 M Tris) are subjected to three freeze–thaw cycles (3 min in liquid nitrogen or a dry ice–ethanol bath, 3 min in a 37° heating block). The sample is then centrifuged for 5 min at 15,000 rpm in an Eppendorf 5415 centrifuge and the supernatant (containing the luciferase activity) is carefully removed to a fresh centrifuge tube. Such extracts can be flash frozen with liquid nitrogen and stored at −20° or used immediately for luciferase activity determination. There are reports in the literature that a Triton lysis method can be used to generate luciferase extracts.[51] When we have compared the two methods (freeze–thaw versus Triton) we have found no great difference in the yield of extracted luciferase with K562 cells. However, other cell types may show differences in luciferase extractability.

Determining Luciferase Activity. The luciferase activity in cell extracts is assayed using a Berthold CliniLumat instrument (LB 9502, Wildbad, Germany) as follows:

1. Prepare an assay buffer:

Assay buffer (for 10 ml): 6.85 ml water, 2.5 ml 0.1 M glycylglycine (pH 7.8), 0.5 ml 0.1 M ATP, 0.15 ml 1.0 M MgSO$_4$

This buffer is prepared fresh on the day of assay to avoid deterioration of the ATP.

2. Prepare an injection buffer:

Injection buffer (10 ml): 6.0 ml water, 2.0 ml 1 mM luciferin (in water), 2.0 ml 0.1 M glycylglycine (pH 7.8)

The ATP is from Boehringer Mannheim (Mannheim, Germany; #519 979, M_r 605.2) and a 0.1 M solution is obtained by dissolving 605 mg in 10 ml water. This is stored in aliquots at −20°.

Luciferin is from Sigma (D-luciferin, sodium salt; #L-6882, M_r 302.3). A 1 mM solution is prepared by dissolving 10 mg luciferin in 33 ml water. This is stored in aliquots at −20°.

A luciferase enzyme standard is prepared from luciferase (Sigma, #L-5256) dissolved at 1 mg/ml in 0.1 M glycylglycine, pH 7.8, and stored at −20°. Various dilutions of the enzyme are prepared in 100 mM sodium phosphate (pH 7.5), 10 mM KCl, 1 mM MgCl$_2$, 1 mM DTT containing 20 μg/ml bovine serum albumin (BSA) and are stable at 4° for 1–2 weeks.

[51] O. Schwartz, J. Virelizier, L. Montagnier, and U. Hazan, *Gene* **88**, 197 (1990).

The luciferase assay is performed by adding 1–50 μl of cell extract to 350 μl of assay buffer. The sample is gently mixed (avoid vortexing, which may generate a static charge on the tube) and placed in the CliniLumat instrument. The instrument automatically injects 300 μl of the injection buffer into the sample, measures the light emission, and displays an integrated value for the first 30 sec of light production. In general, 1 μl of 1 μg/ml luciferase gives approximately 10 million light units with these assay conditions.

β-Galactosidase Assay

We have tested three different β-galactosidase assay systems. The *in situ* assay procedure, using the chromogenic substrate X-Gal, has proved to be an insensitive, unreliable indicator of total cell expression with even clonal *lacZ* expressing lines staining in a chimeric pattern (see also Ref. 52). Therefore we describe here a solution assay for *lacZ* activity, useful for determining total population activity, and a FACS assay that is useful for determining what proportion of a transfected population expresses the introduced DNA.

Analysis of Cell Extracts. Extracts are prepared as described (see above) and β-galactosidase activity was assayed as described by Herbomel *et al.*[53] One milliliter of a solution containing 60 mM Na$_2$HPO$_4$, 40 mM NaH$_2$PO$_4$, 10 mM KCl, 1 mM MgCl$_2$, and 50 mM 2-mercaptoethanol, and 0.2 ml of *o*-nitrophenyl-β-D-galactopyranoside [ONPG (Sigma), 2 mg/ml in 60 mM Na$_2$HPO$_4$, 40 mM NaH$_2$PO$_4$] were added to 30 μl of cell extract. The mixture was kept at 37° for 20 min to 1 hr, until a yellow shade was obvious (for the extracts with β-galactosidase activity). The reaction was stopped by adding 0.5 ml of 1 M sodium bicarbonate. β-Galactosidase activity was determined by measuring the absorbance at 420 nm.

Fluorescence-Activated Cell Sorter Analysis. Living cells were loaded with FDG (fluorescein di-β-D-galactopyranoside) by osmotic shock, diluted in staining medium, and processed by FACScan (Becton Dickinson, Paramus, NJ) as described.[54]

Suspension cell cultures (2–5 ml, 1 million cells) are transferred to 15-ml conical tubes, harvested by centrifugation (1500 rpm, 10 min, Heraeus minifuge), and resuspended in 100 μl of SM buffer [PBS plus 10 mM

[52] G. MacGregor, A. Mogg, J. Burke, and C. Caskey, *Somatic Cell Mol. Genet.* **13**, 253 (1987).
[53] P. Herbomel, B. Bourachot, and M. Yaniv, *Cell* **39**, 653 (1984).
[54] G. P. Nolan, S. Fiering, J. F. Nicolas, and L. A. Herzenberg, *Proc. Natl. Acad. Sci. U.S.A.* **85**, 2603 (1988).

HEPES (pH 7.3), and 4% (v/v) FCS]. The samples are transferred into FACS tubes and placed in a 37° water bath; 10 min later 100 μl of pre-warmed 2 mM FDG [Molecular Probes, Eugene, OR; stock solution of 5 mg FDG in 38 μl dimethyl sulfoxide (DMSO)/water (1/1, v/v)] in water is added with mixing and the samples are kept at 37° for exactly 1 min. The FDG loading is stopped by adding 2 ml of ice-cold SM buffer, and 2 μl of propidium iodide (1 mg/ml) is then added. The samples are kept on ice until FACS analysis; analysis is done with a Becton Dickinson FACScan at a fluorescence of 520 nm with live gating.

An analysis of the transfection efficiency of K562 cells using this method is shown in Fig. 5. K562 cells were transfected with a plasmid encoding the *lacZ* gene driven by the HIV LTR mixed with a second plasmid encoding the trans-activator Tat driven by the CMV promoter. This plasmid contains, in addition, the *oriP* region and the *EBNA1* gene, which allow episomal replication of the plasmid. The FACS analysis of these transfected cells indicates a moderate expression of *lacZ* at 24 hr posttransfection (with 20–30% of the cells expressing detectable levels of the enzyme; Fig. 5A) and high expression (70–100% of the cells) at both 48 hr and 7 days posttransfection (Fig. 5B and C). Note that this experiment was performed in the absence of selection; when the same cell population was assayed at 2 weeks posttransfection the cells displayed only background fluorescence, indicating that either the nonepisomal Tat plasmid or the episomal β-Gal plasmid (or both) were lost. The FACS analysis also demonstrated that greater than 95% of the transfected cell population was viable at all times after transfection.

Isolation of Stable Transformants

The isolation of clones from adherent cultures is performed using standard techniques that need not be described here. The determination and isolation of stable transformants of suspension cultures is performed using a methylcellulose cloning procedure. Two to 3 days after transfecting the cells with DNA containing an appropriate selectable marker (e.g., neomycin phosphotransferase), aliquots of the cells are plated in a semi-solid growth medium containing, in addition to their normal requirements, 0.5–1 mg/ml G418 and 20 mg/ml methylcellulose (see the next section). Approximately 14 days later (depending on the cell type) colonies of G418-resistant cells can be picked and transferred to suspension culture. The normal cloning efficiency of K652 cells using this procedure, in the absence of selection, is approximately 60–70% (i.e., when 100 cells are plated, 60–70 G418-resistant colonies are obtained).

Preparation of Methylcellulose Medium. In a 2-liter Erlenmeyer flask (sterile, preweighed) is placed a sterile magnetic stir bar and 460 ml of

sterile water. The water is heated until boiling and then 20 g of methylcellu-
lose (#64630; Fluka, Buchs, Switzerland) is added. The mixture is stirred
for an additional 3 min and then cooled to room temperature.

Powdered medium, sufficient for 1 liter, is placed in a 500-ml graduated
cylinder along with the appropriate quantities of sodium bicarbonate,
penicillin/streptomycin, and other components. The pH is checked, the
volume is adjusted to 500 ml, and this 2× medium is filter sterilized. The
2× medium is then mixed with the cooled methylcellulose solution, the
volume is adjusted to 1 liter, and the medium is stirred at 4° overnight.
The final medium should not be cloudy. This material is stored in 50-ml
aliquots at −20°.

To prepare medium for cloning use, a 50-ml aliquot of the medium is
thawed (normally the medium is removed to 37° the night before it is
needed), mixed with 10 ml of serum, and the volume adjusted to 100 ml
with complete medium lacking serum. The selective agent (e.g., G418) is
added at this step. The material is quite viscous and the most efficient
method of mixing is multiple inversions of a capped tube (10–15 complete
inversions are sufficient for full dispersion of a dye marker).

Plating Cells in Methylcellulose. A 2.5-ml aliquot of the methylcellu-
lose medium is placed in a 5-ml disposable culture tube (measurement
is by comparison to an identical tube containing a measured 2.5-ml
volume). A 50- to 100-μl aliquot of cells is added to the tube, which is
then inverted 15 times to disperse the cells. The contents of the tube
are then poured, in two nearly equal aliquots, into 2.5-cm culture dishes,
of approximately 1 ml/dish. It is nearly impossible to pour the final 0.5
ml of the viscous mixture, and this value is taken into consideration
when aliquoting the cells. The culture dishes are then placed inside a
large, 25 × 25 cm culture plate that contains, in addition, several open
dishes of sterile water that help maintain the humidity during incubation.
The entire assembly is then placed in a 37°/CO_2 incubator. The methyl-
cellulose culture does not immediately attain its full viscosity, so it is
important that the cultures be allowed to "rest" for at least 2 days
after plating before they are moved. After that they can be carefully
examined at 1- or 2-day intervals. Because of the spatially constrained
nature of the cell growth, approximately twofold higher levels of G418
are required compared to suspension cultures. At 2–3 weeks after
plating, colonies can be harvested with a normal 20-μl Pipetman and
transferred to suspension culture (normally into 100 μl medium in a 96-
well dish). With transferrinfection into K562 cells, approximately 0.5–1%
of the transfected cells can be obtained as stable colonies. In other
words, if 10,000 cells are plated at 3 days posttransfection, 2 weeks
later 50–100 colonies are obtained.

Concluding Remarks

We describe here the methods for preparing and using transferrin–polycation and other ligand–polycation conjugates for receptor-mediated DNA transfer. This method, termed transferrinfection, is particularly effective with cell lines derived from the erythroid lineage (e.g., K562 and HD3 cells), most likely due to the high level of cycling transferrin receptor on these cells. In other established cell lines such as HeLa, CHO, Cos, and HepG2, this method works with efficiencies comparable to other transfection techniques. In certain cell types the method works poorly, or not at all. We are currently examining these cells to determine if the problem can be remedied.

In the cells where transferrinfection functions well, the method has the following advantages over other transfection methods:

1. The method is simple to use, once the ligand–polycation conjugates are obtained. Unlike retroviral methods, this technique does not require the generation of special viral constructs.

2. The method can be used with many DNA molecules. We have transfected DNA molecules from 3.5 to 15 kb with great success.

3. The method works with high efficiency. We can demonstrate that, with K562 cells, 70–100% of the transfected cell population both takes up DNA and transiently expresses a marker gene contained on the DNA. We routinely obtain 0.5–1% stable G418-resistant colony formation when transfecting K562 cells.

4. The method is particularly gentle, involving a transferrin–polycation conjugate that the cell binds with nearly the same avidity as unmodified transferrin. FACS analyses of transfected cell populations demonstrate >95% viability at 24 hr, 48 hr, and 7 days posttransfection. This may partly account for the high efficiencies obtained.

Note added in proof: Since submission of this paper there have been major developments in our understanding of the molecular mechanisms and in the procedures of transferrinfection. It is now suggested that the degree of gene transfer into cells is critically dependent on the liberation, and exit into the cytoplasm, of DNA molecules trapped in the endosomal compartment. The exceptional efficiency of gene transfer in K562 cells is

suggested to result from the extremely low endosomal pH in these cells which in turn is caused by a lesion in the Na^+, K^+-ATP regulation of endosomal acidification.[1] In these endosomes, the weak base chloroquine is thought to accumulate to such an extent that it becomes osmotically active leading to disruption of the endosome and liberation of DNA into the cytoplasm.[1]

Adenoviruses escape destruction in the lysosomal compartment because they have developed molecular skills to disrupt the endosome and to gain access to the cytoplasm through which they reach the nuclear compartment. Addition of adenovirus to the DNA-polylysine-transferrin conjugates presumably leads to co-localization of both compounds in the endosome and this results in an at least 1000-fold stimulation of gene transfer and expression in HeLa cells[2] which are relatively poor candidates for the classical transferrinfection even in presence of chloroquine. Most cell lines tested respond to the addition of adenovirus, as long as they contain cell surface receptors for both the virus and for the transferrin.

Where adenovirus receptors are lacking, cells can still be transfected at very high levels after chemical coupling of the adenovirus with the polylysine. Such coupling can be brought about either by reacting the adenovirus with polylysine in the presence of transglutaminase or by biotinylation of the virus and streptavidinylation of the polylysine.[1,3] DNA is then added to the adenovirus–polylysine conjugate and reacted (condensed) by addition of polylysine–transferrin conjugate. In this way ternary complexes arise in which the DNA becomes ionically linked to both adenovirus–polylysine and transferrin–polylysine. These conjugates have a very high capacity for transfering DNA into cells even at low concentration.[3] In this composition DNA is transferred into the cells either via the transferrin or the adenovirus receptor thus extending the application of the technique to a great many cell types. An alternative linkage protocol is to prepare polylysine linked to an antibody directed against the adenovirus.[4] Since in all of these ternary viral-DNA-polylysine complexes the transfected DNA is on the outside of the adenovirus, very large gene constructs can be transfected.[4,5]

Even where the replication deficient adenovirus dl312 is employed, the use of this virus in human or murine cells leads to a noticeable toxicity[5] which can be recognized by cells rounding off and detaching from the petri dish surface. This is the reason for our lack of success in recording high levels of stably transformed cells using the adenovirus supported transfection procedure.

Toxicity can be abolished in one of several ways. First, Ad5 dl312 can be replaced by the chicken CELO adenovirus which is as endosomolytic as Ad5 (unpublished results). Second, it is possible to UV or psoralen inactivate the adenovirus genome.[5] Such inactivated viruses are active in gene transfer and represent a considerable safety feature for biological (and medical) application. Third, endosomolytic synthetic peptides gleaned off the influenza virus

[1] E. Wagner, K. Zatloukai, M. Cotten, H. Kirlappos, K. Mechtler, D. T. Curiel, and M. L. Birnstiel, *Proc. Natl. Acad. Sci. U.S.A.* **89**, 6099–6103 (1992).

[2] D. T. Curiel, S. Agarwal, E. Wagner, and M. Cotten, *Proc. Natl. Acad. Sci. U.S.A.* **88**, 8850–8854 (1991).

[3] K. Zatloukal, E. Wagner, M. Cotten, St. Phillips, Ch. Plank, P. Steinlein, D. Curiel, and M. L. Birnstiel, *Annals of the New York Academy of Sciences* (1992), in press.

[4] D. T. Curiel, E. Wagner, M. Cotten, M. L. Birnstiel, Ch. Li, St. Loechel, S. Agarwal, and P. Hu, *Human Gene Therapy* **3**, 147–154 (1992).

[5] M. Cotten, E. Wagner, K. Zatloukal, St. Philipps, D. T. Curiel and M. L. Birnstiel, *Proc. Natl. Acad. Sci. U.S.A.* **89**, 6094–6098 (1992).

hemagglutinin can be incorporated into DNA–polylysine–transferrin complexes.[6] Such complexes represent virus-free, but virus-like entry vehicles, which work at high efficiency in gene transfer in a number of cells tested in our laboratory, although the efficiency of these vectors is not as yet as high as that obtained with complete adenovirus.

[6] E. Wagner, Ch. Plank, K. Zatloukal, M. Cotten and M. L. Birnstiel, *Proc. Natl. Acad. Sci. U.S.A.* **89,** 7934–7938 (1992).