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Receptor-mediated endocytosis of transferrin–polycation conjugates: An efficient way to introduce DNA into hematopoietic cells

(transferrinfection/transferrin receptor/chloroquine/DNA transfection/gene therapy)

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ABSTRACT **Most current gene transfer methods function satisfactorily in specialized systems involving established cell lines but are often not applicable with nonadherent, primary hematopoietic cells, which are notoriously difficult to transfect. To approach this problem, we have investigated an alternative method of gene transfer, "*transferrin*fection," in which DNA complexed to transferrin–polycation conjugates is introduced into cells by receptor-mediated endocytosis [Wagner, E., Zenke, M., Cotten, M., Beug, H. & Birnstiel, M. L. (1990) *Proc. Natl. Acad. Sci. USA* 87, 3410–3414]. We show here that transferrin–polylysine and transferrin–protamine, when complexed to plasmid DNA containing a luciferase reporter gene, is efficiently bound and moved into avian erythroblasts by endocytosis. Successful transfer and expression of the luciferase reporter gene depends on specific interaction of the transferrin–polylysine–DNA complex with the transferrin receptor and occurs in a significant fraction (>95%) of the cells. Gene transfer efficiency by transferrinfection is lower than with an optimized DEAE-dextran transfection method but reaches similar efficiencies when the cells are treated with chloroquine. Because the procedure in the absence of chloroquine is completely nontoxic to cells, a constant expression level of transferred genes may be maintained by repeated additions of transferrin–polylysine–DNA complex. In addition, the usefulness of transferrinfection for gene transfer into primary hematopoietic cells is demonstrated.**

Many techniques are available to introduce and express foreign DNA in eukaryotic cells (1–4). However, most procedures function well mainly with adherent cells and/or established cell lines and are often associated with severe cytotoxic side effects. In particular, most available methods proved to be ineffective or too toxic with nonestablished hematopoietic cells. Only recently, specific protocols employing electroporation or DEAE-dextran have met with some success in such cells (5, 6). We have therefore studied the utility of receptor-mediated endocytosis as an alternative means to introduce and express DNA in hematopoietic cells, thus avoiding cytotoxic effects. A similar approach has been used by Wu and Wu (7) who employed the asialoglycoprotein receptor to deliver DNA to liver cells.

In a recent communication (8) we have described transferrin–polycation conjugates that efficiently bind DNA of widely different size and can replace native iron transferrin as an efficient iron transporter into the living cell. Because transferrin receptors are elevated on the surface of extensively proliferating or neoplastic hematopoietic cells (for review, see ref. 9), we attempted to use these transferrin–polycation conjugates to efficiently introduce DNA into cells via the transferrin cycle (a method termed "*transferrin*fection"). Pilot experiments described in the previous communication, showing the principal feasibility of the transferrin–polylysine gene transfer approach (8), encouraged us to study its usefulness with hematopoietic cells in more detail, by using both established cell lines and primary hematopoietic cells of avian origin.

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MATERIALS AND METHODS

Transferrin–Polylysine and Transferrin–Protamine Conjugates and Recombinant Plasmids. The origin of polylysine (Sigma), protamine (Sigma), and chicken transferrin (ion-saturated conalbumin; Sigma) and the synthesis of transferrin–polylysine and transferrin–protamine conjugates have been described (8). The pRSVL plasmid DNA (10) as well as the pRSV-βGal and pB-SK$^-$ DNAs (Stratagene) were prepared by using the Triton-X lysis procedure (11) followed by cesium chloride/ethidium bromide equilibrium density gradient centrifugation, destaining with 1-butanol, and dialysis against 10 mM Tris·HCl, pH 7.5/1 mM EDTA.

Cells and Cell Culture. The temperature-sensitive v-*erbB*-transformed chicken erythroblast cell line HD3 (12) as well as the lymphoid (REV_T) transformed clone REV NPB4 (13) were grown in standard growth medium (EBM + H; ref. 12) at a density of 1–2 × 10^6 cells per ml at 37°C and 5% CO_2. A primary clone of erythroblasts transformed by a retrovirus expressing the human epidermal growth factor receptor together with a temperature-sensitive *myb* oncogene (EGFR-ts-*myb*) was generated as will be described elsewhere (ref. 14 and H.B., unpublished work). The cells were grown in CFU-E medium (15) in the presence of 20 ng of epidermal growth factor per ml. Normal bone marrow cells were prepared as described (16) and enriched for immature erythroid cells by centrifugation through Percoll at 1.072 g/cm^3 and removal of cells adherent to plastic (17).

Transferrin–Polylysine–DNA or Transferrin–Protamine–DNA Complex Formation and Gene Transfer Reaction. In a typical complex formation reaction 10 μg of transferrin–polylysine or transferrin–protamine conjugate in 250 μl of H_2O was added to 3 μg of pRSVL plasmid DNA contained in 250 μl of 0.3 M NaCl (added while agitating). Phosphate buffers should be avoided because precipitates form. By using the above conditions, up to 100 μg of transferrin–polylysine or transferrin–protamine conjugate and 30 μg of plasmid DNA could be used per 500 μl of final volume without precipitation of the transferrin–polylysine–DNA or transferrin–protamine–DNA complexes. After 30 min at room temperature, the transferrin–polylysine–DNA or transferrin–protamine–DNA mixture was directly added to 5–10 × 10^6 HD3 cells growing at 0.5–1 × 10^6 cells per ml in standard

Abbreviation: FITC, fluorescein isothiocyanate.

growth medium (see above) and incubated for 16–48 hr. Other cell types were treated similarly, except being grown in their respective media [see above; CFU-E medium plus REV factor (13) was used for the normal bone marrow cells].

Preparation of Tritiated Transferrin–Polylysine. Tritiated transferrin–polylysine was prepared by conjugation of tritiated polylysine to transferrin in a similar fashion as described (8). Tritiation of poly(L-lysine) (hydrobromide salt, $M_r \approx$ 18,000; Sigma) was performed by treatment with formaldehyde and tritiated sodium borohydride (18).

Uptake of Transferrin–Polylysine–DNA Complexes into Intracellular Vesicles. Avian erythroblasts (HD3; ref. 12) were incubated with transferrin-free differentiation medium (8) at 37°C for 18 hr. After addition of transferrin–polylysine conjugates [labeled with fluorescein isothiocyanate (FITC) at the polylysine moiety (8)] that had been complexed with DNA in some of the experiments, cells were further incubated for 18 hr. Cells were cytocentrifuged onto slides, fixed with a mixture of 3.7% formaldehyde (prepared from paraformaldehyde; ref. 19)/0.02% glutaraldehyde, washed with phosphate-buffered saline (PBS), mounted in Mowiol 4.88 (Hoechst) (19), and examined using a Zeiss Axiophot fluorescence microscope. FITC-labeled goat antimouse antibody (0.1 mg/ml) was used in the controls. For quantitative determination of FITC–transferrin or FITC–transferrin–polylysine–DNA, cells were incubated with the respective transferrin–polylysine preparation for 6 hr at 37°C, washed three times in cold PBS/bovine serum albumin, and processed for quantitative fluorescence activated cell sorter analysis in a Becton Dickinson FACScan.

Luciferase Assay. Aliquots from cultures receiving transferrin–polylysine/transferrin–protamine–DNA complexes were harvested by centrifugation (8 min, 1500 × *g*, 4°C), cell extracts were prepared (10), and bioluminescence was measured with the Clinilumat counter LB 9502 (Berthold, Wildbach, F.R.G.). For determination of the percentage of cells expressing DNA, the cells were allowed to take up transferrin–polylysine–pRSV-βGal complexes or, as controls, transferrin–polylysine–pB-SK$^-$ complexes prepared as above for 36 hr. Cells were then loaded with fluorescein di-β-D-galactopyranoside (Molecular Probes) by osmotic shock, diluted in staining medium, and processed in a Becton Dickinson FACScan exactly as described (20).

RESULTS AND DISCUSSION

Efficient Binding and Internalization of Transferrin–Polylysine–DNA Complexes in Avian Hematopoietic Cells. Binding of transferrin–polylysine and transferrin–polylysine–DNA to cell-surface receptors was measured with tritiated compounds (21), whereas internalization was followed by using fluoresceinated transferrin–polylysine. Fig. 1*A* shows that both transferrin–polylysine and transferrin–polylysine–DNA complexes bind to viable HD3 cells in a saturable fashion. Apparent binding constants calculated from these data were 22 nM for transferrin–polylysine and 43 nM for transferrin–polylysine–DNA complexes. Although somewhat higher, these K_d values (which rather represent on-rates than real dissociation constants; ref. 21) were still in reasonable agreement with those determined for native transferrin (15 nM; I. Killisch, P.S., K. Roemisch, H.B., and G. Griffith, unpublished data).

To study internalization, transferrin–polylysine conjugates containing a polylysine moiety labeled with fluorescein (8) were complexed with DNA and added to HD3 cells (see *Materials and Methods*). Numerous small fluorescent vesicles were detected in >95% of the cells, in both the presence and absence of DNA (Fig. 2 *B* and *C* and data not shown). In controls using either transferrin plus an unrelated fluorescent antibody or fluorescent antibody alone, no fluorescent vesicles were observed (Fig. 2*A* and data not shown). These

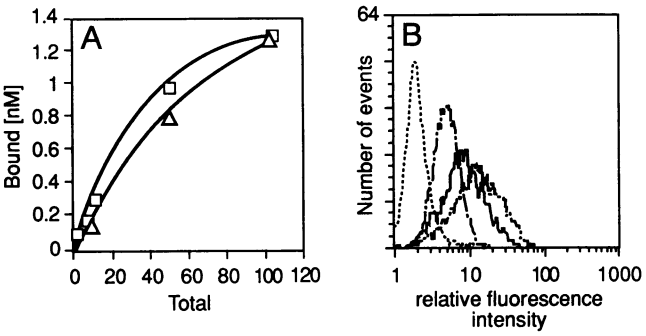

FIG. 1. Binding and internalization of transferrin–polylysine or transferrin–polylysine–DNA by HD3 cells. (*A*) Tritiated transferrin–polylysine$_{90}$ (□) or tritiated transferrin–polylysine$_{90}$–DNA (△) were assayed for specific binding to the transferrin receptor of HD3 cells, as described. (*B*) HD3 cells were incubated with FITC–transferrin at 40 μg/ml (-▪-), FITC–transferrin–polylysine$_{270}$ at 50 μg/ml (—), FITC–transferrin–polylysine$_{270}$ plus pB-SK$^-$ DNA at 50 μg/ml and 16 μg/ml, respectively (-▪▪-), and binding buffer (···) for 6 hr and processed for fluorescence-activated cell sorter analysis as described.

observations were confirmed by FACSCAN analysis of HD3 cells allowed to internalize FITC–transferrin–polylysine or transferrin–polylysine–DNA for 6 hr. With both transferrin–polylysine and transferrin–polylysine–DNA, essentially all cells showed >10-fold increase in relative fluorescence intensity (Fig. 1*B*), indicating that the transferrin–polylysine–DNA complexes are internalized by >95% of the cells.

Optimized Procedure for Transferrinfection of Avian Erythroblasts. To establish an optimal ratio of transferrin–polylysine or transferrin–protamine versus DNA in the complex formation reaction a constant amount of transferrin–polylysine or transferrin–protamine conjugate (10 μg) was mixed with an increasing amount of luciferase-encoding plasmid DNA (0.1–10 μg of pRSVL) and incubated with HD3 cells; the cells were analyzed for the luciferase enzyme activity produced. Fig. 3*A* shows that maximal luciferase enzyme activity was achieved when 3 μg of DNA per 10 μg of transferrin–polylysine and 0.3–1 μg of DNA per 10 μg of transferrin–protamine conjugate were used. By assuming that all the transferrin–polylysine–DNA or transferrin–protamine–DNA complexes formed were identical, this represents a molar ratio of 25 transferrin–polylysine molecules (or 75 transferrin–protamine molecules, respectively) per molecule of plasmid DNA. It appears, therefore, that in this complex the DNA is completely covered by transferrin–polylysine or transferrin–protamine molecules at a transfer-

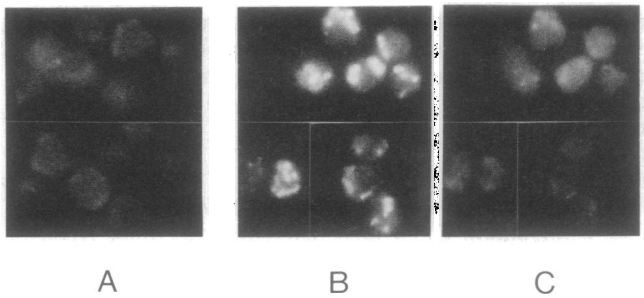

FIG. 2. Uptake of transferrin–polylysine into intracellular vesicles. Viable HD3 cells were incubated with FITC–transferrin–polylysine$_{270}$ (*B*) or, as a control, with FITC-labeled goat anti-mouse IgG (*A*) for 18 hr, cytocentrifuged onto slides, and processed for fluorescence microscopy as described. (*C*) The same field as in *B* viewed under conditions for tetramethylrhodamine (TRITC)-fluorescence to rule out nonspecific vesicle fluorescence due to fixation (H.B., unpublished work).

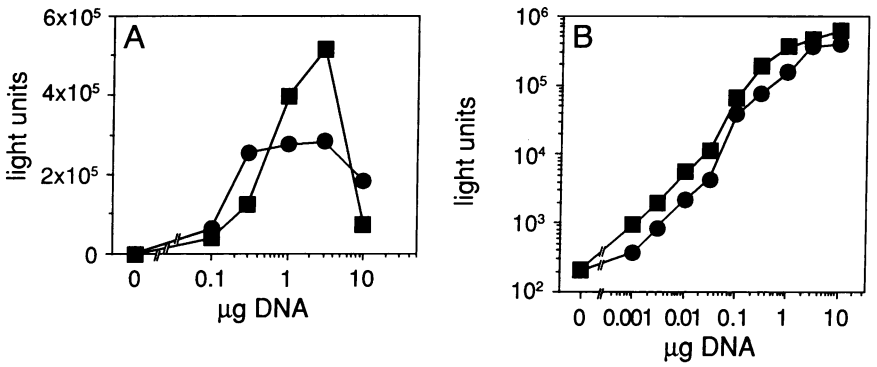

FIG. 3. Optimal conditions for complex formation and sensitivity of transferrinfection. (*A*) For complex formation 0–10 μg of luciferase-encoding plasmid DNA (pRSVL; ref. 10) were mixed with 10 μg of transferrin–polylysine$_{270}$ (■) or transferrin–protamine conjugates (●), respectively, and used for transferrinfection of HD3 cells. Cell lysates were then assayed for luciferase enzyme activity (8) and expressed as light units per 10^7 cells. (*B*) With an optimized transferrin–polycation/DNA ratio in the complex formation reaction (3 μg of transferrin–polylysine$_{270}$ conjugate or 10 μg of transferrin–protamine conjugate per 1 μg of pRSVL DNA, respectively) 1 ng–10 μg of pRSVL DNA were transferrinfected to 10^7 HD3 cells. After 24 hr extracts were analyzed for luciferase activity as in *A*.

rin–polylysine/DNA or transferrin–protamine/DNA ratio that probably results in electroneutrality.

With such an optimized transferrin–polylysine/DNA or transferrin–protamine/DNA ratio for complex formation we have determined the sensitivity of this gene transfer system by altering the amounts of transferrin–polylysine–DNA complex added. Less than 1 ng of luciferase-encoding plasmid DNA per 5×10^6 HD3 cells still yielded a detectable signal (Fig. 3*B*). Apparent saturation of the system was obtained at 2 μg of plasmid DNA complexed with 6 μg of transferrin–polylysine or 20 μg of transferrin–protamine conjugate, respectively; higher doses failed to further increase the luciferase activity measured (Fig. 3*B*).

Dependence of Gene Transfer by the Transferrin–Polylysine–DNA (and Transferrin–Protamine–DNA) Complexes on Specific Interaction with the Transferrin Receptor. Next we studied whether and to what extent uptake of the transferrin–polycation–DNA complexes into cells occurred by means of specific, receptor-mediated endocytosis. For this analysis, two approaches were used (Fig. 4*A* and data not shown). First, luciferase activities obtained by using polylysine–DNA complexes were close to the background activity of the assay. Similarly, mixtures of polylysine and native transferrin with DNA did not lead to luciferase gene expression significantly above background. It appears therefore that transferrinfection is dependent on a covalent complex of both transferrin and polylysine.

In the second type of experiment an excess of native, free transferrin was added to a constant amount of transferrin–polylysine–DNA complexes to compete for transferrin binding to its cognate receptor. Fig. 4*A* shows that free transferrin in the medium efficiently competed for the transferrin–polylysine-mediated DNA uptake, as witnessed by a 95% reduction of luciferase enzyme activity. Therefore, we conclude that transfer of the transferrin–polylysine–DNA complexes into cells depends on the transferrin receptor. This notion is further confirmed by experiments in which the abundance of transferrin receptors on the surface of HD3 cells and a human leukemia cell line has been modulated by various means, leading to the expected changes in transferrin–polylysine-mediated gene transfer efficiency (data not shown and ref. 22).

To study which fraction of the cells actually express plasmid DNA administered by transferrin–polylysine, HD3 cells were incubated with transferrin–polylysine–pRSV-βGal complexes. Expression of this reporter gene was then studied

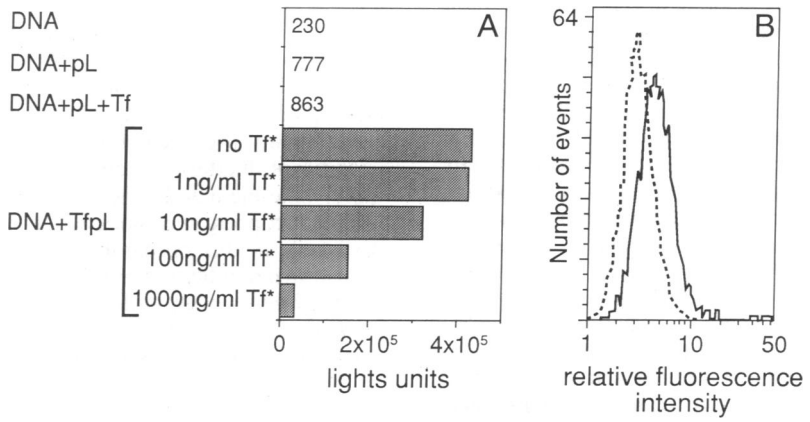

FIG. 4. Uptake of transferrin–polylysine–DNA complexes occurs via the transferrin receptor. (*A*) Three micrograms of pRSVL DNA without polycations (DNA), plus 2 μg of polylysine$_{270}$ (DNA + pL), plus 2 μg of polylysine$_{270}$ and 10 μg of iron-saturated transferrin (Tf) (DNA + pL + Tf), or plus 10 μg of transferrin–polylysine$_{270}$ conjugate (DNA + TfpL) were mixed in typical complex formation reactions. After transferrinfection to 10^7 HD3 cells, cell extracts were prepared and analyzed for luciferase activity. Transferrinfection of transferrin–polylysine–DNA complexes (DNA + TfpL) was performed in the absence of exogenous transferrin (no Tf*) and in the presence of exogenous free transferrin at 1, 10, 100, and 1000 ng/ml in the culture medium (1 ng–1000 ng/ml Tf*). Average values of three independent experiments (the values of DNA, DNA + pL, and DNA + pL + Tf are given in numbers) are shown. (*B*) HD3 cells were transferrinfected in absence of chloroquine with transferrin–polylysine–pRSV-βGal (—) or, as a control, by transferrin–polylysine–pB-SK$^-$ (---) and analyzed for β-galactosidase expression by fluorescence-activated cell sorter analysis.

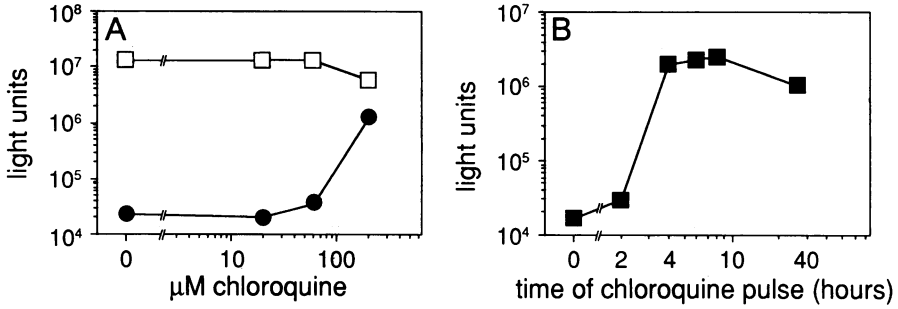

FIG. 5. Chloroquine enhances the efficiency of transferrinfection. (*A*) Three micrograms of pRSVL DNA was transfected to 2×10^7 HD3 cells without and with 20, 60, and 200 μM chloroquine using a DEAE-dextran protocol optimized for chicken hematopoietic cells (ref. 6; □). Similarly, 3 μg of pRSVL DNA complexed with 10 μg of transferrin–polylysine$_{270}$ conjugate were transferrinfected to the same number of HD3 cells in the absence and presence of chloroquine (●). Twenty-four hours later cell extracts were analyzed for luciferase enzyme activity (the values for 10^6 cells are shown). (*B*) Transferrinfection of 3 μg of pRSVL DNA (complexed with 10 μg of transferrin–polylysine$_{270}$ conjugate) to 2×10^7 HD3 cells was performed both in the absence (0 hr) and presence of chloroquine (200 μM). After 2, 4, 6, and 8 hr of transferrinfection in the presence of chloroquine, cells were extensively washed, seeded in fresh growth medium and incubated for a total of 24 hr. In addition, cells were treated with chloroquine (200 μM) for the entire transferrinfection period (24 hr). Luciferase activity was determined as described for *A*.

at the single-cell level by FACS analysis (20) after introducing the β-galactosidase substrate fluorescein di-β-D-galactopyranoside. The unimodal distribution of cells containing fluorescein derived from fluorescein di-β-D-galactopyranoside due to β-galactosidase enzyme activity suggests that a large fraction of the cells exhibited expression of the β-galactosidase reporter gene (Fig. 4*B*).

Chloroquine Augments the Efficiency of Transferrin–Polylysine-Mediated Gene Transfer. To obtain information on the efficiency of the transferrin–polylysine-mediated gene transfer as compared with conventional methods, we introduced the pRSVL plasmid into HD3 cells by DEAE-dextran transfection, using a protocol optimized for avian erythroid cells (6). In comparison with this method, transferrinfection was consistently found to be 50- to 100-fold less efficient (Fig. 5*A*). It should be noted, however, that the DEAE-dextran protocol was quite toxic, always killing 30–40% of the transfected cells, whereas no cytotoxic effects were observed using the transferrin–polylysine method.

To test whether the relatively low efficiency of DNA transfer by transferrin–polylysine or transferrin–protamine conjugates could be due to trapping and/or degradation of the DNA within the endosomal/lysosomal pathway, chloroquine, a drug known to alter lysosomal pH and thus to inhibit lysosomal degradation of macromolecules (23), was added to cells simultaneously with the transferrin–polylysine–DNA complexes. With this treatment, the gene transfer efficiency (as measured by luciferase activity) was highly elevated and approached levels similar to those seen for DEAE-dextran transfection, which by itself could not be further increased by chloroquine (Fig. 5*A*). The disadvantage of the chloroquine procedure, however, was the appearance of a cytotoxic effect, especially at prolonged exposure of the cells and at high concentrations of chloroquine. We therefore tried to add the chloroquine in a pulsed fashion, which largely avoided cytotoxicity. By using increasingly shorter times of incubation with chloroquine we found that its application for 3–4 hr was sufficient for maximum gene transfer activity (Fig. 5*B*).

Transient Expression of DNA After Transferrin–Polylysine-Mediated Gene Transfer. Because the transferrin–polylysine gene transfer method was not deleterious to cell proliferation, the effect of transferrin–polylysine–DNA complexes applied to cells over an extended period of time was tested. In this experiment, the same concentration of cells (1×10^6 cells per ml) were incubated for 1–4 days with or without daily readdition of transferrin–polylysine–DNA complexes. At various time periods, aliquots were analyzed for luciferase enzyme activity. In the cultures with repeated administration of transferrin–polylysine–DNA complexes, a relatively high level of luciferase gene expression (100,000–200,000 light units per 10^7 cells) was seen in the absence of chloroquine, which remained essentially constant over the period of investigation (data not shown). No cytotoxic effects were observed during this time. In contrast, when cells were loaded with transferrin–polylysine–DNA complexes only once for 24 hr, the luciferase activity declined 10- to 20-fold between day 2 and day 4 (data not shown). These results suggest that although expression of the luciferase gene introduced into cells by the transferrin–polylysine method is transient, a constant high expression of transferred genes can be maintained by repeated addition of transferrin–polylysine–DNA complex, a feature not offered by the other known DNA-transfer methods.

Transferrinfection of Primary Avian Hematopoietic Cells. To test the suitability of transferrinfection for gene transfer into primary avian cells, EGFR-ts-*myb* erythroblasts, REV_T transformed lymphoblasts, and erythroid cells enriched from normal bone marrow were subjected to transferrinfection with transferrin–polylysine–pRSVL complexes. In primary erythroblasts, gene transfer by the transferrin–polylysine method was ≈30- to 50-fold less effective as in HD3 cells (Table 1). However, the efficiency of DEAE-dextran transfection was reduced in these cells to a similar degree (data not shown). Expression of the pRSVL plasmid DNA was not restricted to the erythroid lineage because it was also observed in transformed lymphoid cells (REV NPB4, Table 1).

In freshly prepared, normal bone-marrow cells, low but significant luciferase activity was seen after transferrin–polylysine-mediated gene transfer (Table 1), possibly due to a large proportion of noncycling, transferrin-receptor negative cells in this preparation (24). Our results demonstrate that transferrinfection can be applied for gene transfer into primary cells, even without prior cultivation *in vitro*.

With the conditions used so far for transferrinfection, expression of the DNA introduced is transient. However,

Table 1. Transferrinfection of primary hematopoietic cells

Cell type	pRSVLuc expression, light units/10×10^6 cells
HD3 ebl	5×10^5
EGFR-*myb*-ebl	1.4×10^4
REV-NPB4 lymphoblasts	1.7×10^5
Normal bone-marrow cells	6.6×10^3, 7.8×10^3

The various cell types were transferrinfected in the absence of chloroquine. ebl, Erythroblast.

even when using a vast excess of transferrin–polylysine–DNA or transferrin–protamine–DNA complexes (10-fold above the physiological concentration of native transferrin), no cytotoxic effects of the compounds have been observed, thereby enabling their repeated administration both at high concentration and over an extended period of time.

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