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Transferrin–polycation conjugates as carriers for DNA uptake into cells

(transferrinfection/transferrin receptors/polylysine/protamine/chicken erythroblasts)

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ABSTRACT **We have developed a high-efficiency nucleic acid delivery system that uses receptor-mediated endocytosis to carry DNA macromolecules into cells. We accomplished this by conjugating the iron-transport protein transferrin to polycations that bind nucleic acids. Human transferrin, as well as the chicken homologue conalbumin, has been covalently linked to the small DNA-binding protein protamine or to polylysines of various sizes through a disulfide linkage. These modified transferrin molecules maintain their ability to bind their cognate receptor and to mediate efficient iron transport into the cell. The transferrin–polycation molecules form electrophoretically stable complexes with double-stranded DNA, single-stranded DNA, and modified RNA molecules independent of nucleic acid size (from short oligonucleotides to DNA of 21 kilobase pairs). When complexes of transferrin–polycation and a bacterial plasmid DNA containing the gene for *Photinus pyralis* luciferase are supplied to eukaryotic cells, high-level expression of the luciferase gene occurs, demonstrating transferrin receptor-mediated endocytosis and expression of the imported DNA. We refer to this delivery system as "*transferrin*fection."**

All actively metabolizing cells require iron that is taken up by the cells as a transferrin–iron complex by means of receptor-mediated endocytosis (the transferrin cycle; refs. 1–3). To exploit this ubiquitous and efficient transport mechanism for introducing DNA into cells, conjugates of chicken transferrin (conalbumin) or human transferrin with DNA-binding polycations were synthesized. We used protamine, a small, naturally occurring, arginine-rich DNA-binding protein (4) and synthetic polylysines of different degrees of polymerization (≈90, 270, or 450 L-lysine monomers). As target cells for DNA delivery, avian erythroblasts transformed with conditional *erbB* oncogenes (temperature sensitive v-*erbB* or c-*erbB* with or without ligand) were selected because these cells display a particularly active transferrin cycle when induced to differentiate (5, 6). By using this system the transferrin–polycation conjugates were shown to function as high-efficiency iron transporters and as carriers to deliver a gene to the living cell. Polylysine–asialoglycoprotein conjugates have been used similarly to target DNA to liver cells, as reported by Wu and Wu (7, 8).

MATERIALS AND METHODS

Synthesis of Transferrin–Polycation Conjugates. Transferrin was coupled to polylysine as described (9) by ligation through disulfide bonds after modification with the bifunctional reagent succinimidyl 3-(2-pyridyldithio)propionate (SPDP; Pharmacia). The transferrin–protamine conjugates were prepared in a similar manner, except that the less reactive protamine (which lacks lysine amino groups) was modified under nonaqueous conditions.

3-(2-Pyridyldithio)propionate-Modified Transferrin 1. A solution of 120 mg (1.5 μmol) of chicken transferrin (conalbumin, iron free; Sigma) in 3 ml of 100 mM sodium phosphate buffer (pH 7.8) was subjected to gel filtration on a Sephadex G-25 column. To the resulting 5-ml solution was added 200 μl of 15 mM ethanolic solution of SPDP (3.0 μmol) and the solution was vigorously mixed. After 1 hr at room temperature, purification by further Sephadex G-25 gel filtration yielded 6 ml of a solution of 1.4 μmol of transferrin modified with 2.8 μmol of dithiopyridine linker. Modification of human transferrin (iron-free; Sigma) was done identically.

3-Mercaptopropionate-Modified Polylysine 2. Poly(L-lysine)s of different molecular weights were used—namely, those with average chain length of 90, 270, or 450 lysine monomers ($pLys_{90}$, $pLys_{270}$, or $pLys_{450}$; hydrobromide; Sigma). Both unlabeled and fluorescent-labeled polylysines were used; fluorescent labeling with fluorescein isothiocyanate (Sigma) was performed in sodium bicarbonate buffer (pH 9) for 3 hr. A gel-filtered solution of 1.1 μmol of $pLys_{90}$ (equivalent to 20 mg of hydrobromide salt) in 1.2 ml of 75 mM sodium acetate buffer was brought to pH 8.5 by adding sodium bicarbonate buffer. One hundred and eighty microliters of a 15 mM ethanolic solution of SPDP (2.7 μmol) was added and then vigorously mixed. One hour later 200 μl of 1 M sodium acetate (pH 5) was added; after gel filtration with 20 mM sodium acetate, the solution contained 1.0 μmol of $pLys_{90}$ with 2.3 μmol of dithiopyridine linker. Twenty-three milligrams (150 μmol) of dithiothreitol in sodium bicarbonate buffer was added, and the solution was kept under argon at pH 7.5 for 1 hr; the pH was adjusted to 5.2 by adding sodium acetate buffer, and after gel filtration (Sephadex G-25) a solution of 0.97 μmol of $pLys_{90}$ modified with 2.2 μmol of mercaptopropionate linker (2.25 linkers for each $pLys_{90}$ chain) was obtained. By the same procedure, modification of 0.33 μmol of $pLys_{270}$ (equivalent to 19 mg of hydrobromide salt) with 1.9 μmol of SPDP gave a 0.27-μmol product of $pLys_{270}$ containing 1.3 μmol of mercapto groups (4.8 linkers for each $pLys_{270}$ chain); modification of 0.20 μmol of $pLys_{450}$ with 2.3 μmol of SPDP gave a 0.19-μmol product of $pLys_{450}$ containing 2.1 μmol of mercapto groups (11 linkers for each $pLys_{450}$ chain).

Conjugation of Transferrin with Polylysine. Transferrin–$pLys_{270}$ conjugates were prepared by mixing 1.0 μmol of modified transferrin 1 in 100 mM phosphate buffer (pH 7.8) with 0.14 μmol of modified $pLys_{270}$ 2 (in 20 mM sodium acetate buffer) under argon. After 18 hr at room temperature the reaction mixture was diluted with water to a volume of 10 ml and fractionated 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

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Abbreviations: SPDP, *N*-succinimidyl-3-(2-pyridyldithio)propionate; $pLys_{90}$, $pLys_{270}$, and $pLys_{450}$, polylysine of average chain length of 90, 270, or 450 lysine monomers.

3 M sodium chloride]. The excess of uncoupled transferrin was eluted first; the product fractions were eluted during the gradient between 30% and 50% buffer B and were pooled into three conjugate fractions: transferrin–$pLys_{270}$ A with a molar ratio of transferrin to polylysine of 5.5:1, transferrin–$pLys_{270}$ B with a ratio of 3.4:1, and transferrin–$pLys_{270}$ C with a ratio of 1.8:1. Conjugates were obtained in an overall yield of 0.23 μmol of transferrin with 0.075 μmol of $pLys_{270}$. Transferrin–$pLys_{90}$ conjugates were synthesized in an analogous way: starting with 1.2 μmol of transferrin 1 and 0.45 μmol of mercapto-modified $pLys_{90}$ 2, conjugates transferrin–$pLys_{90}$ containing 0.32 μmol of transferrin with 0.18 μmol of $pLys_{90}$ were obtained. Transferrin–$pLys_{450}$ conjugates were prepared in a similar manner starting with 1.2 μmol of transferrin 1 and 71 nmol of mercapto-modified $pLys_{450}$ 2. Purification of the reaction mixture occurred by gel permeation chromatography [see Fig. 2*b*; Pharmacia Superose 12 column; 1 M guanidine chloride (pH 7.3)] and after dialysis (as described below) yielded conjugate transferrin–$pLys_{450}$ containing 0.40 μmol of transferrin and 38 nmol of $pLys_{450}$.

SDS/gel electrophoresis [10% SDS/8% polyacrylamide (10), followed by Coomassie blue staining] of transferrin–polylysine samples after pretreatment with 2-mercaptoethanol shows only transferrin bands, whereas in nonreduced samples only more slowly migrating bands of the conjugates are visible. All conjugates were dialyzed against a 20 mM Hepes (pH 7.3) buffer containing 100 mM sodium chloride; iron incorporation was performed by adding 6–12 μl of 100 mM iron citrate buffer (containing sodium bicarbonate, adjusted to pH 7.8) for each mg of transferrin content [as described (2, 11)].

3-Mercaptopropionate-Modified Protamine 2. To a solution of 20 mg (3 μmol) of protamine trifluoroacetate salt [prepared by ion-exchange from salmon protamine (the same as salmine) sulfate salt; Sigma] in 2 ml of dimethyl sulfoxide and 0.4 ml of isopropanol containing 2.6 μl (15 μmol) of ethyldiisopropylamine, a solution of 30 μmol of SPDP in 250 μl of isopropanol and 250 μl of dimethyl sulfoxide was added in several portions within 1 hr. After 3.5 hr at room temperature, the solution was evaporated in high vacuum and taken up in 0.5% acetic acid/10% methanol. Gel filtration [Sephadex G-10; 0.5% aqueous acetic acid/10% (vol/vol) methanol] after lyophilizing yielded 16 mg (2.5 μmol) of protamine acetate salt modified with 2.5 μmol of dithiopyridine linker. Reduction of 1.75 μmol of protamine (containing 1.75 μmol of linker) with 16 mg of dithiothreitol in a sodium bicarbonate buffer (pH 7.5) for 1 hr under argon, followed by pH adjustment to 5.2 and gel filtration [Sephadex G-10, 20 mM sodium acetate buffer (pH 5.2)] produced a solution of protamine **2** modified with 1.6 μmol of mercaptopropionate linker.

Conjugation of Transferrin with Protamine. Reaction of the protamine solution **2** described above (1.6 μmol of linker) with 1.35 μmol of transferrin **1** (modified with 3.1 μmol of dithiopyridine linker) and purification by cation-exchange chromatography (see Fig. 2*a*) as described for the transferrin–polylysine conjugates gave four sequentially eluting product fractions: transferrin–protamine A–D containing 90 nmol, 320 nmol, 240 nmol, or 120 nmol of modified transferrin with increased amounts of protamine (as shown in the SDS gel, see Fig. 3; 10% SDS/8% polyacrylamide, Coomassie blue staining). Dialysis and iron incorporation was performed as for the transferrin–polylysine conjugates.

Quantitative Assays. Polylysine content of fractions was determined spectrophotometrically by ninhydrin assay or for fluorescein isothiocyanate-labeled polylysine by absorption at 495 nm. The amount of dithiopyridine linkers in modified transferrin or polylysine was determined after reducing an aliquot with dithiothreitol by measuring the absorption of released pyridine-2-thione at 340 nm. The amount of free mercapto groups was determined by using Ellman's reagent (12) and measuring at 412 nm. Transferrin content of fractions was determined by UV measurement at 280 nm and correction (where necessary) of the value by subtracting the corresponding UV absorption of fluorescein isothiocyanate, dithiopyridine, or buffer at 280 nm.

RESULTS

Synthesis of Transferrin–Polycation Conjugates. Transferrin and protamine or polylysine were ligated by disulfide bonds (Fig. 1 and *Materials and Methods*). First, dithiopyridine groups were introduced into both apotransferrin and protamine or polylysine by means of SPDP (9). For the less reactive protamine, which contains no lysine amino groups, modification of the trifluoroacetate salt in dimethyl sulfoxide/isopropanol quantitatively substituted one linker per protamine molecule. The dithiopyridine groups in protamine or polylysine were further reduced to give the free sulfhydryl compounds **2** (Fig. 1 and *Materials and Methods*), which, upon mixing with modified transferrin 1, reacted to give the desired conjugates. After purification (Fig. 2), iron was incorporated into all conjugates by adding iron citrate buffer containing bicarbonate.

Dissociation of Transferrin–Polycation Conjugates. The disulfide linkages of transferrin–polycation conjugates are sensitive to reducing conditions. When samples are pretreated with 2-mercaptoethanol, a transferrin band appears in an analytical SDS gel (Fig. 3*b*) in all lanes after Coomassie blue staining (the free protamine or polylysine moiety is not seen because these positively charged groups do not migrate into the gel). Without reduction of the disulfide bonds, the polylysine conjugates do not fully enter the gel because of their positive charge density and their high molecular weight and are therefore partially lost upon staining (data not shown). The protamine conjugates, having lower molecular weight, show the expected, intermediate electrophoretic mobility (Fig. 3*a*).

Formation of Complexes Between Transferrin–Polycation Conjugates and DNA. Complexes of transferrin–polycation with DNA were formed by mixing diluted solutions of DNA (30 μg/ml or less) with the transferrin–polycation conjugates. Dilution of the DNA and the use of phosphate-free buffer (phosphates decrease solubility of the conjugates) were found to be essential to prevent precipitation of the DNA complexes. DNA binding to the polycation conjugates at physi-

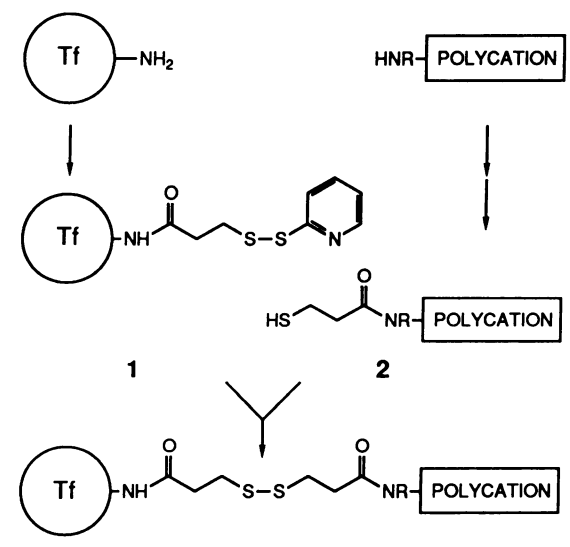

FIG. 1. Synthesis of transferrin (Tf)–polycation conjugates. The polycation is poly(L-lysine) or salmon protamine.

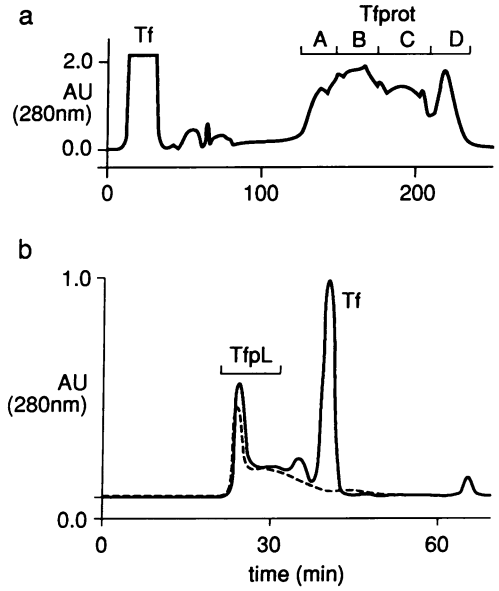

FIG. 2. Purification of transferrin (Tf)–polycation conjugates. (*a*) Cation-exchange chromatography of transferrin–protamine (Tfprot) conjugates. The excess of uncoupled transferrin elutes from the column first; the conjugates are retained on the column due to their positive charges but elute with a salt gradient (Tfprot fractions A–D between 1 M to 2 M NaCl on a Mono S column). (*b*) Gel permeation chromatography of the larger transferrin–$pLys_{450}$ (TfpL) conjugates (Superose 12 column; ---, fluorescence, 520 nm). AU, absorbance units.

ological ionic conditions was confirmed by gel mobility-shift assay using ^{32}P-labeled λ DNA cut with *Eco*RI/*Hin*dIII (Fig. 4). Furthermore, we find that the transferrin–polylysine and transferrin–protamine conjugates associate avidly with DNA and RNA oligonucleotides as short as 19 nucleotides as well as with single-stranded DNA and elicit bandshifts similar to those seen in Fig. 4 (unpublished results).

Transferrin–Polycations Function as Efficient Iron Transporters in Differentiating Chicken Erythroblasts. Chicken erythroblasts transformed with conditional *erbB* oncogenes can be induced to differentiate into erythrocytes *in vitro* by "switching off" oncogene function (5, 6). This *in vitro* differentiation crucially depends on high levels of iron-saturated transferrin (13) as well as on a highly active transferring cycle (2) because inhibition of either arrests differentiation, leading to cell disintegration (2, 13). Thus, this cell system allowed us to assay whether the transferrin–polylysine or transferrin–protamine conjugates and respective DNA complexes could functionally replace native iron transferrin.

Epidermal growth factor receptor erythroblasts (H.B., unpublished results and ref. 6) were induced to differentiate by removing epidermal growth factor and adding a partially purified erythroid growth factor preparation devoid of transferrin (REV-factor, refs. 13 and 14). After incubation in medium devoid of avian transferrin for 18 hr, different amounts of iron-saturated transferrin or transferrin–polycation conjugates were added to the cells (before or after complexing them with plasmid DNA). After 1, 2, and 3 days, cellular differentiation was determined by cytocentrifugation and histochemical staining (5, 14) or by quantitative hemoglobin assay (13). The results show that two different transferrin–polylysine conjugates (transferrin–$pLys_{90}$ or transferrin–$pLys_{270}$) as well as the transferrin–protamine B conjugate could functionally replace native transferrin in mediating rapid iron transport into differentiating erythrocytes—their specific activity being ≈1.5- to 2-fold lower (Fig. 5 and Table 1). Table 1 shows a comparable, low percentage of disintegrated cells, a high percentage of late reticulocytes and erythrocytes, and high hemoglobin content with polycation conjugates. Complexing DNA to transferrin–$pLys_{270}$ and transferrin–protamine did not grossly alter biological activity of the conjugates (Table 1 and data not shown). When either polylysine or protamine was mixed with an appropriate amount of iron citrate and added to the cells instead of transferrin conjugates, the cells failed to differentiate and disintegrated, similar to the controls incubated without transferrin (data not shown). Taken together, both types of transferrin–polycation conjugates were nearly as efficient in mediating iron transport as was native transferrin, such transport depending on the transferrin moiety of the respective complexes.

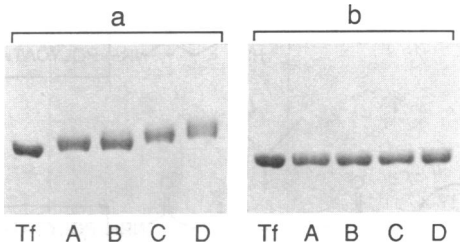

FIG. 3. SDS/gel electrophoresis of transferrin–protamine conjugates. (*a*) Transferrin (Tf) and transferrin–protamine A–D samples. (*b*) Same samples but pretreated with 2-mercaptoethanol.

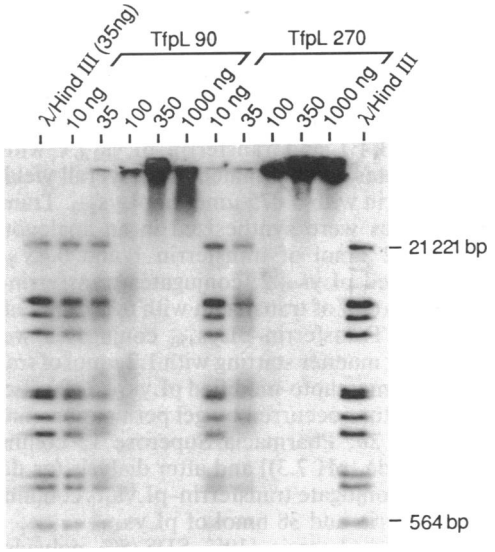

FIG. 4. Gel shift of DNA with transferrin–polylysine (TfpL). DNA binding of transferrin–polylysine was confirmed by gel mobility-shift assay with *Eco*RI/*Hin*dIII-cut λ DNA fragments {between 21,221 and 564 base pairs (bp), ^{32}P-labeled at the 3′ end by filling in the overlay with Klenow enzyme and [^{32}P]dNTP}. To each sample with 1 μl (35 ng) of DNA, 3 μl of a 100 mM Hepes (pH 7.9) buffer containing 1 M sodium chloride was added, and the samples were mixed with increased amounts (10 ng–1000 ng of transferrin–polylysine) of transferrin conjugates in 11 μl of aqueous solution, resulting in final sodium chloride concentration of 200 mM. Electrophoresis on 1% agarose gel with 1× TAE (40 mM Tris acetate/1 mM EDTA, pH 8) running buffer was done at 50 V (45 mA) for 2.5 hr; the gel was dried, and autoradiography proceeded for 2 hr at −80°C with XAR film (Kodak).

Transferrin–Polylysine and Transferrin–Protamine Conjugates Mediate Uptake of DNA into Cells and Expression of a Luciferase Gene. Having shown that the various transferrin–polylysine (transferrin–protamine) conjugates are still biologically active in that they deliver iron to the cell, we next tested whether the conjugates mediate uptake and expression

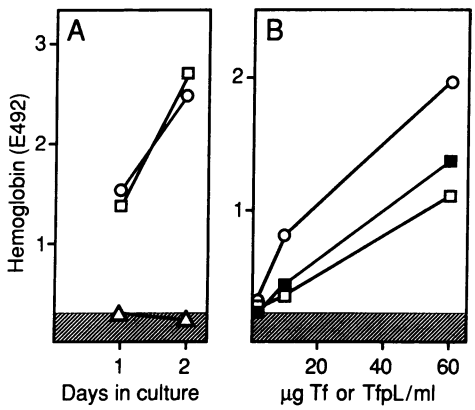

FIG. 5. Erythroid differentiation mediated by transferrin–polylysine (TfpL) conjugates. (*A*) Erythroblasts transformed by a retrovirus containing the human epidermal growth factor receptor together with a temperature sensitive v-*myb* oncogene were generated as described elsewhere. These cells were propagated at 37°C in colony forming unit-E medium (15) with epidermal growth factor at 20 ng/ml. After two washes in colony forming unit-E medium, the cells were seeded at 42°C into 14-mm dishes at 1×10^6 cells per ml in 0.6 ml of transferrin-free differentiation medium (16) supplemented with insulin at 1 μg/ml and partially purified erythroid growth factor (REV factor) at an optimum concentration (ref. 13; 1:5000 dilution for the batch used) without additions (△), with iron-saturated transferrin (○), or with iron-saturated transferrin–$pLys_{270}$ conjugates (□, 100 μg/ml each). After incubation as indicated, hemoglobin content was measured photometrically in 100-μl aliquots (see ref. 13). (*B*) To analyze erythroid differentiation as a function of transferrin (Tf) (or transferrin–polylysine) concentration, cells were seeded as in *A* into medium containing the indicated amounts of iron-saturated transferrin (○), transferrin–$pLys_{90}$ (□), or transferrin–$pLys_{270}$ (■) and analyzed photometrically after 2 days. Hatched area in *A* and *B*, hemoglobin content of transferrin-free controls (mean of four determinations).

of DNA into cells. An expression plasmid encoding the firefly luciferase gene (17) was used as a reporter gene to monitor efficient gene transfer and expression. The luciferase-encoding plasmid DNA pRSVL was mixed with avian transferrin–polylysine (or transferrin–protamine) conjugates to form the transferrin–polylysine–DNA (transferrin–protamine–DNA) complexes, and the mixture was added to avian HD-3 erythroblasts (5). After 24-hr incubation, cell extracts were prepared and analyzed for luciferase enzyme activity. Fig. 6 shows that the transferrin–polylysine conjugates, indeed, mediate uptake of luciferase-encoding plasmid DNA because luciferase enzyme activity is detected in extracts from these cells. Using a constant amount of DNA (3 μg of pRSVL) and increased amounts of transferrin–polylysine conjugates (1–30 μg) in the complex formation reaction increases the luciferase activity detected in the cells (Fig. 6). The same result holds for transferrin–protamine conjugates (18). We found that the efficiency of DNA import into cells by means of the transferrin cycle was enhanced considerably by treating human K-562 cells with agents affecting endosomal/lysosomal pH, such as chloroquine (19). When this modified procedure was applied to the K-562 cell system, significant luciferase gene expression (0.9×10^6 light units of 3 μg of plasmid DNA and 1.2×10^6 light units at 10 μg of DNA per 3×10^5 cells) was seen in these cells (19).

DISCUSSION

We have presented protocols for the synthesis of covalent conjugates of apotransferrin and two types of polycation peptides, polylysine of various lengths or protamine (Fig. 1). Typically, one $pLys_{90}$ moiety becomes linked to 2 transferrin molecules; one $pLys_{450}$ becomes linked to 10 transferrin molecules (in general 1 transferrin per 50 lysines). One to three protamine molecules link to one transferrin molecule.

The conjugates exhibited interesting properties when iron was incorporated into the apotransferrin moiety. They were recognized and transported by receptor-mediated endocytosis in avian erythroblasts, although with somewhat reduced affinity as compared with unmodified transferrin (18). Furthermore, these conjugates were rapidly taken up into endosomal-like intracellular vesicles, as visualized by fluorescence microscopy (18). By using a more stringent bioassay for the iron-transport protein—i.e., the ability to sustain erythroid differentiation *in vitro*—which depends on a particularly efficient performance of the transferrin cycle (2, 13), the conjugates exhibited 50–75% of the specific activity as compared with native iron–transferrin complex. Thus, the concatenation of several transferrin molecules with a single polylysine chain followed by complex formation of several of these conjugates with DNA to achieve approximate electroneutrality (see below) apparently did not impede their rec-

Table 1. Erythroid differentiation in response to medium additions

Exp.	Medium addition: Transferrin	Medium addition: Transferrin–polylysine	Medium addition: DNA	Differentiation parameter: Cell no., $\times 10^6$/ml	Differentiation parameter: Hemoglobin, ε_{492}	Differentiation parameter: Dead cells*, %	Differentiation parameter: Mature cells*, %	Differentiation parameter: Immature cells*, %
1	–	—	—	2.56	0.259	56	<1	44
	+†	—	—	3.72	1.997	3	73	1
	–	Transferrin–$pLys_{90}$‡	—	3.67	1.105	5	54	8
	–	Transferrin–$pLys_{270}$‡	—	3.30	1.366	11	60	4
2	–	—	pRSVL†	1.24	0.280	ND	ND	ND
	+§	—	pRSVL	5.22	2.459	ND	ND	ND
	–	Transferrin–$pLys_{90}$§	pRSVL	4.46	2.265	ND	ND	ND
3	–	—	—	2.10	0.222	79	<1	21
	+§	—	—	2.55	1.369	6	72	0
	–	Transferrin–$pLys_{90}$§	—	2.64	1.016	10	56	7
	–	Transferrin–protamine§	—	2.76	1.055	9	72	4

Erythroid differentiation was monitored either by photometric hemoglobin assay (see Fig. 5, ref. 13), by counting in a Coulter counter, or by cytocentrifugation followed by staining with neutral benzidine (ref. 14; to detect hemoglobin ε_{492}) plus histological dyes (Diff Quik; ref. 14). ND, not done.

*Percentages of dead (disintegrated) cells, mature cells (late reticulocytes plus erythrocytes), and immature cells were determined as described (2, 14).

†Final DNA concentration, 10 μg/ml.

‡Final concentration of transferrin and transferrin conjugates, 60 μg/ml.

§Final concentration of transferrin and transferrin conjugates, 100 μg/ml.

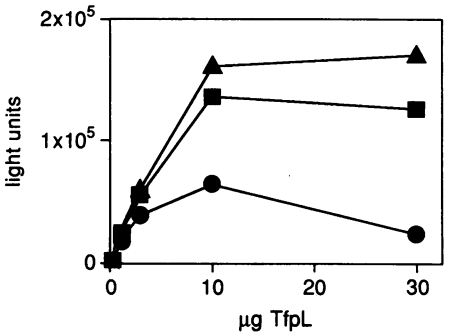

FIG. 6. Expression of luciferase gene in HD-3 cells after transferrin–polylysine (TfpL)-mediated gene transfer. For transferrin–polylysine DNA complex formation 0–30 μg of transferrin–polylysine conjugate in 250 μl of H_2O was added to 3 μg of luciferase encoding plasmid DNA (pRSVL, ref. 17) contained in 250 μl of 0.3 M NaCl. This mixture was added to 10^7 HD-3 cells (5) growing in 10 ml of standard growth medium (EBM + H, ref. 5). Twenty-four hours later cell extracts were prepared and analyzed for luciferase activity (17). Bioluminescence was measured with the Clinilumat counter (Berthold, Wildbach, F.R.G.). Transferrin–$pLys_{90}$ (●), transferrin–$pLys_{270}$ A (▲), transferrin–$pLys_{270}$ B (■).

ognition and use by the transferrin receptor-mediated endocytosis machinery.

Because of its cationic properties, the transferrin–polylysine (or transferrin–protamine) conjugate could bind DNA avidly. As shown in the band-shift experiments of Fig. 4, the transferrin–polylysine conjugates yield a band shift with both small and large *Eco*RI/*Hin*dIII restriction fragments of λ phage DNA without size discrimination. We suspect that several conjugates are involved in complex formation when interacting with long DNA molecules, thus increasing the overall molecular weight of the complex. One can calculate for the transferrin–$pLys_{270}$ conjugate that a 3:1 mass ratio of conjugate to DNA approximately neutralizes the nucleic acid negative charge by the positively charged polylysine. Consistent with this calculation, nearly complete mobility retardation of the DNA occurred at this ratio and above; the optimum DNA transfection into avian erythroblasts (assayed by luciferase activity) also was obtained at this ratio (see ref. 18).

In a single growing erythroblast, as many as 10^4 transferrin molecules are taken up each minute by the $>10^5$ receptors located at the cell surface (1). Ample evidence indicates that transferrin receptor-mediated endocytosis is the mechanism by which our transferrin–polylysine and transferrin–protamine conjugates enter the cell (this paper; see also refs. 18 and 19). The capacity of the transferrin–polylysine or the transferrin–protamine complexes (E.W., unpublished results) to bind and transport high-molecular weight DNA into cells could be shown most clearly by using the circular expression plasmid pRSVL (≈7000 base pairs). We demonstrated that the DNA transported into cells was expressed as active luciferase enzyme (Fig. 6). With our preliminary protocol, expression levels were still lower than those obtained with DNA transfection into the same cells using a DEAE-dextran transfection method optimized for avian erythroblasts (18, 19); this is not too surprising because no attempts have yet been made to increase release of ingested DNA from endosomal vesicles in these cells. As will be described (18, 19), use of agents affecting endosomal and lysosomal pH (e.g., chloroquine) helps to increase the efficiency of DNA uptake and expression.

In conclusion, we have developed a DNA transfection protocol, *transferrin*fection, in which we subvert a natural iron-uptake mechanism to transport DNA. The very same transport system can be used to introduce oligonucleotides into cells (unpublished results). We showed that at least a portion of the pRSVL molecule reaches the cell nucleus, where it can be expressed. In contrast to standard transfection protocols, which often cause appreciable cell death, we found that transfection by means of transferrin-mediated endocytosis is more physiological in nature, as no such cell death is seen and erythroid differentiation proceeds normally in the presence of the transferrin–polylysine complexes used to import DNA into cells.

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