

Transferrin–polycation–DNA complexes: The effect of polycations on the structure of the complex and DNA delivery to cells

(polylysine/histones/endocytosis/DNA transfection/gene transfer)

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ABSTRACT We have previously described a gene delivery system based upon the receptor-mediated endocytosis of DNA complexed with transferrin–polycation conjugates. This delivery system has been found to be very effective for both the internalization and the expression of genetic material in cells that have many transferrin receptors. Upon scrutinization of the parameters involved in this method, which we have termed *transferrinfection*, we note two important features of the process: the polycation in polycation–transferrin conjugates, as expected, serves to attach the transferrin moiety to the DNA and, in addition, the polycation functions to condense the DNA into a doughnut structure. Electron microscopic analysis of a range of poorly active to highly active transferrinfection samples reveals a strong correlation between DNA condensation and cellular DNA uptake. Furthermore, we demonstrate that the transfection activity of the DNA complex can be increased by addition of free polycation as long as a sufficient quantity of polycation–transferrin conjugates remains in the complex to ensure its binding to the cellular receptor.

Transferrinfection, the cellular uptake and expression of DNA complexed with transferrin (Tf)–polycation conjugates, has been shown to be based on Tf-dependent receptor-mediated endocytosis (1–3), with the polycation polylysine or protamine acting as the DNA-binding moiety. Consistent with DNA delivery as an endocytotic event, we have demonstrated that the presence of excess free Tf interferes with DNA uptake (2) and that up-regulation of the Tf receptor by agents like desferrioxamine (deferrioxamine) increases the subsequent gene expression in K-562 cells (3). Virtually 100% of such cells take up and express a transferrin-infected reporter gene (unpublished observation).

Our initial studies demonstrated that delivery of DNA occurred maximally by using ratios of Tf–polylysine to DNA that resulted in electroneutrality (1). We chose to examine the composition of DNA complexes that gave maximal transferrinfection in some detail with the hope of learning more about the DNA delivery event. We find that there are at least two important features of a delivery-competent DNA complex: (i) The presence of sufficient polycation in the mixture to ensure full condensation of the DNA molecule into a form that is compatible with endocytosis of the conjugate. We find that we can replace a certain portion of Tf–polycation conjugate with free polycation without compromising DNA delivery, and in certain cases, this replacement can result in enhanced DNA delivery. (ii) The presence of sufficient Tf on the condensed DNA molecule. We must maintain 10–20 Tf molecules per DNA molecule to maintain receptor-mediated gene delivery. When the amount of Tf–polylysine replaced by free polylysine is further increased, DNA delivery falls

drastically to low levels typically seen for pure polycation delivery schemes (4).

MATERIALS AND METHODS

Materials. Polymers of L-lysine with an average chain length of 55, 90, 200, or 450 lysine residues (Lys₅₅, Lys₉₀, Lys₂₀₀, and Lys₄₅₀) and a poly(D-lysine) with an average chain length of 240 lysine residues (DLys₂₄₀) were obtained from Sigma as hydrobromide salts; protamine was obtained as a sulfate salt (from salmon; histone free) from Sigma; histones H1, H3, and H4 (from calf thymus) were obtained from Boehringer Mannheim. Plasmid pRSVL (6.8 kilobases) carrying the luciferase gene has been described (5).

The Tf–poly(L-lysine) conjugates Tf–Lys₂₀₀ and Tf–Lys₄₅₀ were synthesized as described (1) with an improved isolation procedure. It was important for the enhanced recovery of the polycation conjugates from the chromatographic purification [Pharmacia Mono HR 10/10 S column; gradient elution, buffer A (50 mM Hepes at pH 7.9) and buffer B (buffer A plus 3 M sodium chloride)] to add sodium chloride to the reaction mixture before loading the column (final concentration of 0.6 M in the case of Tf–Lys₂₀₀ or 1 M in the case of Tf–Lys₄₅₀ conjugates) and to start the gradient at these salt concentrations. The product fractions were eluted at salt concentrations of ≈1.4 M in the case of Tf–Lys₂₀₀ and ≈2 M in the case of Tf–Lys₄₅₀. After dialysis against Hepes-buffered saline (HBS; 150 mM NaCl/10 mM Hepes, pH 7.3) conjugate fractions were obtained in overall yields of 80% (Tf–Lys₂₀₀) or 64% (Tf–Lys₄₅₀), respectively.

Tf conjugates with a synthetic protamine analogue having the sequence (N to C terminal) Lys-Pro-Arg-Ala-Arg-Arg-Ser-Ser-Ser-Arg-Pro-Val-Arg-Arg-Ser-Ser-Arg-Pro-Ala-Val-Ser-Ala-Arg-Arg-Arg-Ser-Arg-Gly-Gly-Ser-Arg-Arg-Gly-Gly-Gly-Cys were synthesized (C. Plank, M.C., K. Mechtler, H. Kirlappos, and E.W., unpublished results).

Cell Culture and Transfection. All transfections were performed with K-562 cells grown in RPMI 1640 medium containing 10% fetal calf serum, 2 mM glutamine, penicillin (100 units/ml), and streptomycin (100 μg/ml). Unless indicated otherwise, cells were pretreated with 50 μM desferrioxamine for 18–24 hr before transfection. Transfections were performed with 500,000 cells in 2 ml of the above medium in the presence of 100 μM chloroquine. DNA–Tf–polycation complexes were prepared in a 500-μl volume containing 150 mM NaCl and 20 mM Hepes (pH 7.4) by mixing 330 μl of DNA solution with 170 μl of Tf–polycation conjugate solution, allowing it to incubate for 30 min at room temperature, and adding it to the cell culture. After a 4-hr incubation at 37°C, the cells were washed into fresh medium, incubated at 37°C, and harvested for luciferase assay (1, 2) 18 hr later.

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Abbreviation: Tf, transferrin.

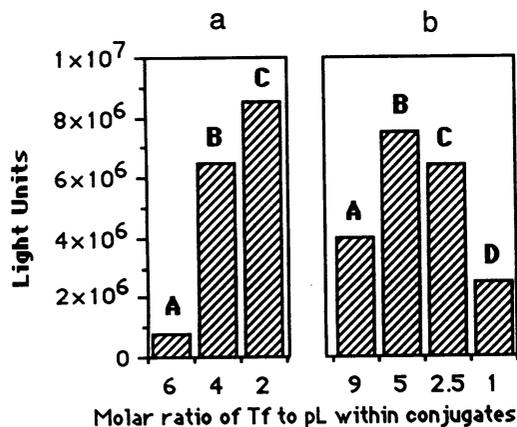


FIG. 1. Influence on the transfection efficiency of the ratio of transferrin coupled to polylysine (pL) within conjugates. (a) Three Tf-Lys₂₀₀ conjugates (fractions A-C) with different average numbers of Tf molecules conjugated to a single polylysine chain were used at optimized amounts (40 μ g for fraction A and 30 μ g for fractions B and C, determined in preliminary titrations; data not shown) for complex formation with 10 μ g of pRSVL plasmid. Complexes were used for gene delivery to 500,000 K-562 cells; aliquots of cell extracts standardized for protein content (about 15–20% of total extract) were assayed for resulting luciferase activity. The activity shown is adjusted to represent total activity from the entire cellular sample. (b) Transfection experiments with four different Tf-Lys₄₅₀ conjugates (fractions A-D) were performed analogous to those in a with the difference that only 6 μ g of pRSVL plasmid was used and mixed with the optimum amount of 18 μ g for fractions A-C and 12 μ g for fraction D.

RESULTS

Polylysine-Rich Conjugates Are Efficient in Delivery of pRSVL Plasmids into K-562 Cells. Various Tf-polylysine

conjugates were synthesized as described (1) with an improved isolation procedure that resulted in higher yields (see *Materials and Methods*). We have tested each conjugate in preliminary titrations and found that all conjugates clearly display an optimum conjugate-to-DNA ratio for DNA delivery as measured by luciferase activity (results not shown). When we compared the optimum luciferase activity values for conjugates varying in Tf-to-polylysine ratios, there was a marked improvement in activity as we went from high Tf-to-polylysine to low Tf-to-polylysine ratios. We found that conjugate fractions with a lower number of Tf molecules attached to a single polylysine chain had an increased transfection efficiency (Fig. 1a). Thus, Tf-Lys₂₀₀ fraction C with a molar ratio of Tf to polylysine chains of 2:1 was 10-fold or more effective than conjugate A with a ratio of 6:1. In experiments with a long chain Tf-Lys₄₅₀ conjugate (Fig. 1b), it was observed that conjugates with a ratio of Tf to polylysine chains of 9:1 were less active than those with a ratio of 5:1; these were comparable in activity to conjugates with a ratio of 2.5:1, whereas at even higher polylysine content (a ratio of 1:1), the activity again decreased. We conclude that conjugates with an average transferrin spacing of \approx 1 Tf per 100 lysine residues in the transferrin-polycation function best.

Complexes of DNA with Tf-Polylysine: Formation of Doughnut Structures. We suspected that as we varied the conjugation ratios between Tf and polylysine we might alter the higher order structure of the Tf-polylysine-DNA complexes and that these structural alterations might influence the DNA uptake by cells. Therefore, we undertook electron microscopy (EM) studies to analyze these complexes. For the EM studies the complex formation of the pRSVL plasmid with Tf-Lys₂₀₀ was performed by using the same conditions (high dilution, 150 mM salt concentration) as in the transfection experiments. EM revealed that in the presence of 6 μ g

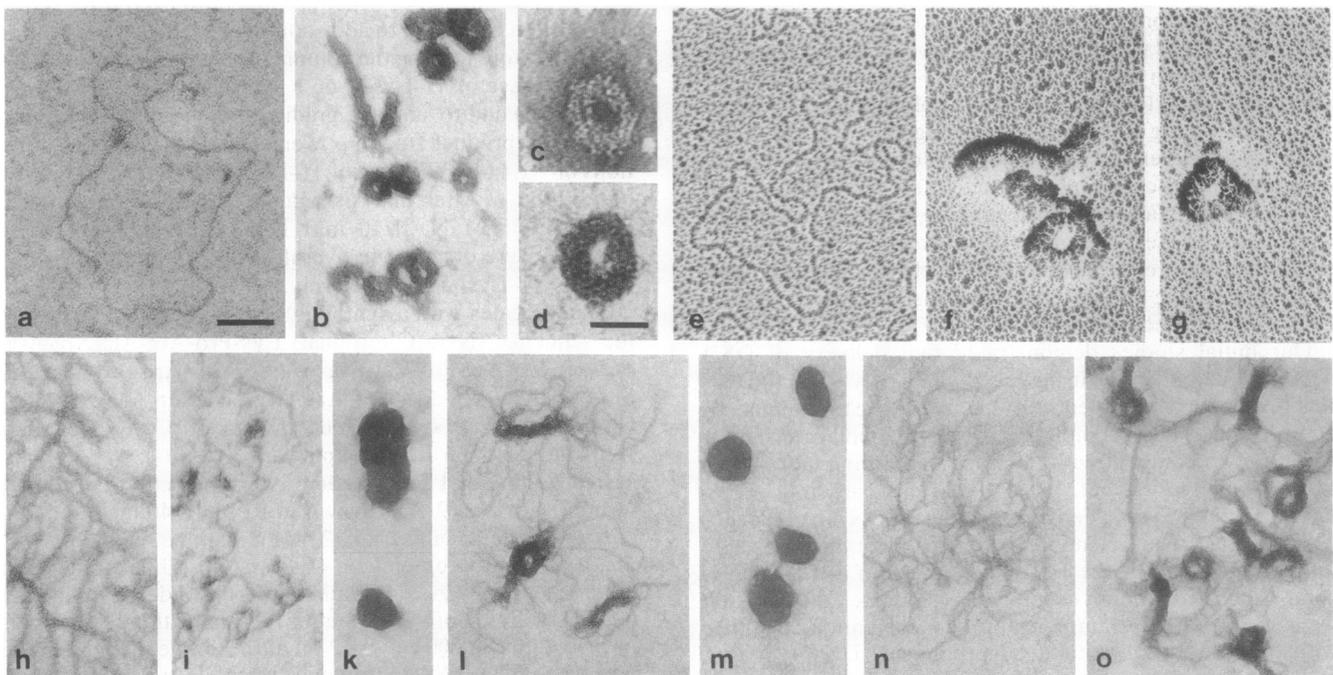


FIG. 2. EM of negatively stained (uranyl acetate; a-d and h-o) and rotary shadowed (e-g) samples of plasmid DNA alone (a and e) or 2 μ g of DNA complexed either with the optimal amount (6 μ g) of Tf-Lys₂₀₀ fraction C (b-d and f), with 1.5 μ g of Lys₂₀₀ (g), with the optimal amount (8 μ g) of Tf-Lys₂₀₀ fraction A (h), with 6 μ g of Tf-synthetic protamine (i), with a 4-fold suboptimum amount (1.5 μ g) of Tf-synthetic protamine and 0.55 μ g of Lys₉₀ (k), with a 4-fold suboptimum amount (1.5 μ g) of Tf-Lys₂₀₀ fraction C (l), with 1.5 μ g of Tf-Lys₂₀₀ fraction C and 0.55 μ g of Lys₉₀ (m), with 5 μ g of histone H4/Tf-Lys₂₀₀ fraction C mixture (4:1) (n), or with 6 μ g of histone H4/Tf-Lys₂₀₀ fraction C (1:1) (o). Complex formation was performed by mixing 2 μ g of pRSVL circular plasmid in 50 μ l of HBS with a 50- μ l solution of various conjugates. For negative staining, samples were processed as described (6). For rotary shadowing, samples in 100 μ l of 150 mM ammonium acetate instead of HBS were supplemented with 2 μ l of 0.3% isoamyl alcohol/0.2% cytochrome c, mixed with equal amounts of glycerol, sprayed onto freshly cleaved mica plates, and shadowed with carbon/platinum essentially as described (7). All samples were viewed in a JEOL 100S electron microscope operated at 80 kV. (Bar in a, representative for a, b, and e-o equals 100 nm; bar in d, representative for c and d equals 50 nm.)

of Tf-Lys₂₀₀ fraction C (see legend to Fig. 1) a profound condensation of 2 μg of plasmid DNA (Fig. 2a) into toroid structures occurred (Fig. 2 b–d). As shown at higher magnification (Fig. 2 c and d), these “doughnuts” with a size of about 80–100 nm in diameter possessed distinct substructures. The condensation was seen independently of whether samples were prepared by uranyl acetate staining or by rotary shadowing as shown in Fig. 2 e and f. However, DNA complexes with the optimal amount of Tf-Lys₂₀₀ fraction A (see legend to Fig. 1) contained less polylysine (0.5 μg of polylysine per 2 μg of DNA) and were less efficient in transferrinfection (Fig. 1a) and were not as well condensed (Fig. 2h). It appears then that one property of the Tf-polylysine–DNA complex particularly efficient in transferrinfection is a condensed structure, which may facilitate the endocytotic event. Note that the coated pit structures through which the Tf/Tf receptor is internalized has an internal diameter in the range of 100 nm (8). The condensation of DNA molecules into units of roughly these dimensions may be important for the efficiency of the transfection process.

Complexes of pRSVL prepared with free Lys₂₀₀ under the same conditions contain similar doughnut structures (Fig. 2g). Therefore, the coupling of Lys₂₀₀ to Tf does not significantly impair this polycation’s ability to condense DNA in the case of the (polylysine-rich) Tf-Lys₂₀₀ fraction C conjugates. There are examples in the literature of the formation of similar condensed complexes of λDNA with polylysine at high salt (1 M) concentration (9) and with spermidine at very low ionic strength (25 mM salt; ref. 10).

We have prepared a series of Tf molecules conjugated to either authentic or synthetic protamines (see ref. 1 and *Materials and Methods*). Although these protamine conjugates function for DNA delivery, their activity is ≈10-fold lower than that of the Lys₂₀₀ conjugates. When we examined DNA complexes with Tf-protamine by EM, we were unable to find condensed doughnut structures (Fig. 2i and data not shown). This is consistent with the idea that full condensation of the plasmid DNA by polycations may be an important factor in efficient DNA delivery to cells.

Various Polycations Can Partially Replace Tf-Polycation Conjugates in Transferrinfection. In a standard experiment at the optimized ratio of Tf-polycation to DNA, each DNA plasmid doughnut contains ≈120 Tf molecules. Whereas the optimal polylysine-to-DNA ratio is important and probably results in electroneutrality and proper condensation of the DNA molecule, such a high number of Tf molecules per DNA may not be necessary for receptor-mediated endocytosis. We determined the minimum number of Tf molecules per DNA molecule in the following series of experiments. Samples were prepared in which an increasing part of the Tf conjugate was replaced by unmodified polycations, keeping the total polylysine content at the predetermined optimum (≈4 μg of polylysine per 6 μg of DNA) (Fig. 3a). We found that transferrinfection efficiency was maintained (or even slightly increased) as we decreased the polylysine–Tf content of the sample and replaced it with either the metabolically stable poly(D-lysine) (Fig. 3a) or with poly(L-lysine) (results not shown). However, we found that as we dropped the Tf content below a calculated 10–15 molecules per DNA molecule, the transferrinfection efficiency dropped precipitously (Fig. 3a, samples 5–8). This suggests that a minimum of 10–15 Tf molecules per DNA molecule are required for adequate targeting of the complex to the cell. If there is no Tf-polylysine in the DNA doughnuts, the gene delivery falls essentially to zero.

A second type of experiment is shown in Fig. 3b in which a constant amount of Tf-Lys₂₀₀ (4.5 μg), which is 4-fold less than the optimum, was mixed with increasing amounts of Lys₉₀ and added to a constant amount of DNA (6 μg). The

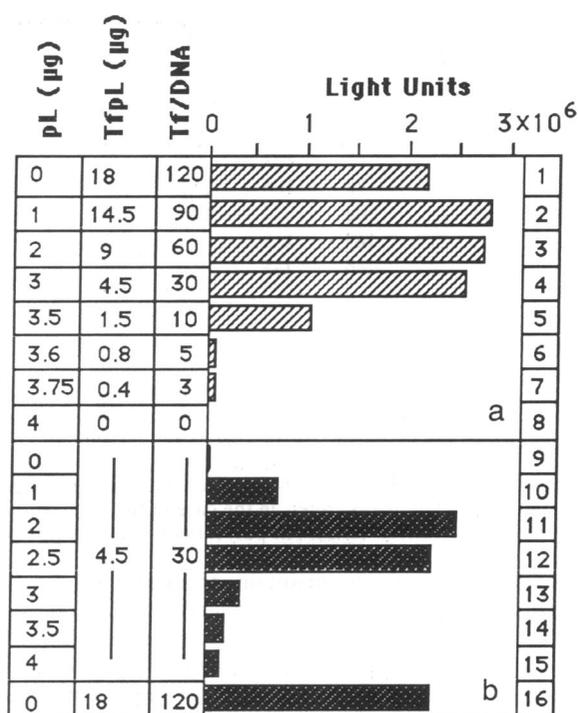


FIG. 3. Partial replacement of Tf-polylysine by polylysine. (a) Bars 1–8 show luciferase gene expression resulting from transferrinfection where the complex formed with 6 μg of pRSVL plasmid DNA and the optimum amount of 18 μg of Tf-Lys₂₀₀ fraction C was gradually replaced by equivalent amounts of dLys₂₄₀. (b) In the experiments shown by bars 9–15, the complex formation of 6 μg of pRSVL was performed with 4.5 μg of Tf-Lys₂₀₀ fraction C to which increasing amounts of Lys₉₀ were added as indicated; bar 16 shows the results from the control experiment with 6 μg of pRSVL and the optimum amount (18 μg) of Tf-Lys₂₀₀ fraction C. Note that the samples in a and b represent two different experiments with different pRSVL and polylysine preparations, which account for the differences in the optimum of the transfections. pL, polylysine; TfPL, conjugates of Tf with polylysine.

transfection efficiency, which was about 100-fold reduced with the suboptimal amount of Tf-polylysine alone, could be restored by the additional Lys₉₀. EM analysis again revealed a correlation between cellular DNA uptake and DNA condensation; the formation of fuzzy structures obtained with suboptimum amounts of Tf-polylysine (see Fig. 2l) could be suppressed by the polylysine addition, which resulted in fully compacted structures (Fig. 2m). We found, however, that when higher amounts of polylysine were added to the system the transfection efficiency decreased again (Fig. 3b, samples 13–15). When these transfection-inefficient complexes were examined by EM, we found that the DNA was fully condensed (data not shown). In this case the decline in DNA delivery might be due to a competitive displacement of Tf-polylysine by polylysine. Thus, at high free polylysine levels, a large fraction of the DNA may occur complexed as Tf-free doughnuts, and this material is not internalized by the cells. Similar experiments with other poly(L-lysine) molecules (Lys₅₅, Lys₉₀, and Lys₄₅₀), with dLys₂₄₀, as well as with the naturally occurring protamine and with histones H1, H3, and H4 showed that these polycations could also restore or even improve the efficiency of DNA delivery (Table 1). On the other hand, spermidine and spermine, which are known to condense DNA only at low ionic strength (11), were not able to restore DNA uptake at the physiological salt concentration of the tissue culture system (results shown in Table 1).

Polycations Can Improve Transferrinfection Efficiency. In the above experiments it was demonstrated that various

Table 1. Relative transfection efficiency of DNA complexed with a mixture of a suboptimum amount of Tf-Lys₂₀₀ fraction C and various amounts of polycations

| Polycation | Amount of polycation, * μg | | | | | | | | | | | | | | |
|---------------------|---------------------------------------|-----|-----|------------|------------|------------|------------|-----|-----|------------|------------|------------|-----|-----|-----|
| | 0 | 1 | 1.5 | 1.75 | 2 | 2.25 | 2.5 | 3 | 4 | 6 | 8 | 10 | 12 | 16 | 32 |
| Lys ₅₅ | 21 | 42 | 103 | 67 | 52 | 131 | <u>149</u> | 142 | | | | | | | |
| Lys ₉₀ | 34 | 78 | 166 | <u>252</u> | 118 | 50 | 39 | 26 | | | | | | | |
| Lys ₄₅₀ | 45 | 102 | | 125 | 176 | <u>210</u> | 60 | 26 | | | | | | | |
| dLys ₂₄₀ | 21 | 169 | 115 | | 268 | | <u>344</u> | 290 | | | | | | | |
| Protamine | 56 | 146 | 148 | | <u>198</u> | | 175 | 130 | 55 | | | | | | |
| Histone H1 | | | | | 96 | | | | 111 | 112 | 84 | <u>178</u> | 133 | 130 | 173 |
| Histone H3 | 47 | | | | 91 | | | | 138 | <u>284</u> | 153 | 115 | 60 | 44 | 30 |
| Histone H4 | 47 | 59 | | | 89 | | | 85 | 122 | 300 | <u>560</u> | 211 | 174 | 57 | 1 |
| Spermine | 47 | | | | 76 | | | | 85 | | <u>90</u> | | | 71 | 63 |
| Spermidine | 47 | | | | <u>76</u> | | | | 62 | | 59 | | | 56 | 61 |

DNA (6 μg) was complexed with a suboptimum amount (9 μg) of Tf-Lys₂₀₀ fraction C and various amounts of polycations. The relative transfection efficiency is the light units obtained, expressed as a percentage of the light units obtained with complexes prepared with the optimum amount (18 μg) of Tf-Lys₂₀₀. The underlined numbers indicate maximal values.

*The amounts of polycation refer, in the case of the polylysines, to hydrobromide salts, to protamine sulfate, and, in the case of spermine and spermidine, to the free bases. All polycations were used as solutions in HBS.

polycations are able to maintain or even improve DNA transport with the already very effective and polylysine-rich Tf-Lys₂₀₀ conjugates. There are two major instances where dilution of the Tf-polylysine conjugates with unmodified polycations seems to result in a large improvement: (i) in cases where the DNA-binding group of the conjugate has lost its ability to condense DNA efficiently (as is the case, for instance, with the protamine-Tf conjugate) and (ii) in cases where the number of Tf receptors is low and may be saturated by a surplus of Tf in the DNA complexes.

We have noted above that conjugates between Tf and a synthetic protamine (see *Materials and Methods*) as well as Tf conjugates with authentic protamine bind to DNA and have significant, but rather low, DNA delivery ability compared to Tf-polylysine conjugates. When DNA complexes with Tf-synthetic protamine or Tf-protamine were examined, no significant condensation of the DNA was observed (Fig. 2i; data not shown). However when portions of the Tf-synthetic protamine conjugate were replaced by free polylysine, we observed an increase of DNA expression up to 25-fold (Fig. 4), and an increased condensation of the DNA was observed by EM (Fig. 2k). This is consistent with the idea that incomplete DNA condensation limits the DNA delivery capacity of the protamine conjugates and improvement of DNA delivery is observed when the condensation is improved.

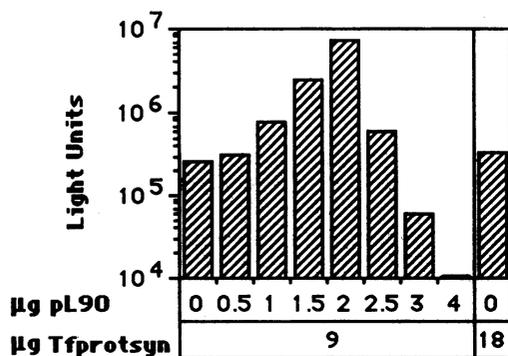


FIG. 4. Improvement of transfection efficiency of Tf-synthetic protamine conjugates by partial replacement with polylysine. Complexes were formed with 6 μg of pRSVL and the indicated mixtures of Tf-synthetic protamine conjugates (Tfprotsyn) with Lys₉₀ (pL90) and used for gene delivery as described in Fig. 1; for comparison, the experiment with the optimum amount (as determined before) of Tf-synthetic protamine conjugate in the DNA complex formation is shown.

We observed that the extent of improvement in the transfection effected by dilution with polylysine varied with the state of the cells. In the above experiments, desferrioxamine had been included to maximize the number of Tf receptors on the K-562 cells (see ref. 3). An experiment similar to that shown in Fig. 3b (4.5 μg of Tf-polylysine and increasing amounts of Lys₉₀) was performed with the exception that desferrioxamine was omitted (Fig. 5). Due to the lower Tf receptor number on these cells (≈ 5 -fold lower by Scatchard analysis; results not shown), the subsequent DNA expression was reduced. By using the standard optimized conditions (18 μg of Tf-Lys₂₀₀ for 6 μg of pRSVL), the DNA expression was about 15-fold lower than that in the presence of desferrioxamine. However, when using these cells with three-fourths of the Tf-polylysine replaced by Lys₉₀, a >8 -fold increase in DNA expression was obtained, reaching a value that was only 1.8-fold reduced compared to the Tf receptor-rich cells. In desferrioxamine-stimulated cells, the same replacement with Lys₉₀ maintains, but does not considerably improve, Tf-mediated DNA uptake.

Improvement in Transfection by Histone H4. We conclude from our experiments that full condensation of plasmid DNA by polycations is one important factor in transfection efficiency. However, when we tested the ability of the natural polycation histone H4 to replace portions of Tf-polylysine, we encountered a second phenomenon. The standard replacement experiment using suboptimum Tf-poly-

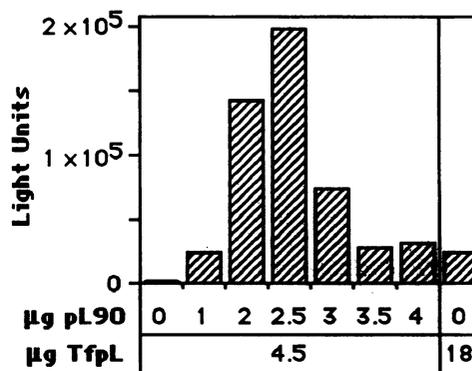


FIG. 5. Partial replacement of Tf-polylysine conjugates by polylysine with K-562 cells that have lower Tf receptor levels. The experiment was performed as in Fig. 3b by mixing pRSVL DNA samples (6 μg each) with various Tf-Lys₂₀₀/Lys₉₀ mixtures; in contrast to other experiments, the desferrioxamine was omitted from the cell culture. pL90, Lys₉₀; Tf pL, Tf-polylysine.

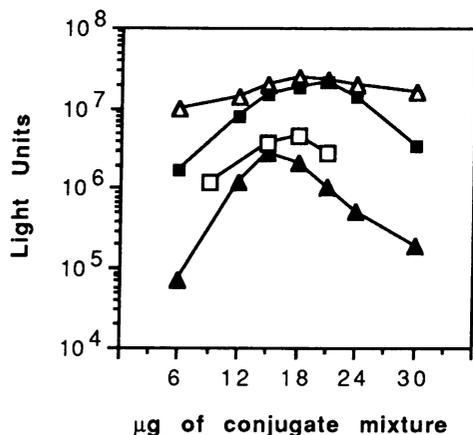


FIG. 6. Transferrinfection with mixtures of histone H4 and Tf-polylysine. Stock mixtures of Tf-Lys₂₀₀ fraction C with 1, 2, or 4 weight equivalents of histone H4 were prepared. Each mixture was used in the indicated amounts for complex formation with a constant amount (6 µg) of DNA. Transferrinfections were performed with these complexes under normal conditions. Δ , Tf-polylysine/H4 = 1:1; \blacksquare , Tf-polylysine/H4 = 1:2; \blacktriangle , Tf-polylysine/H4 = 1:4; \square , Tf-polylysine alone.

lysine and adding increasing amounts of histone H4 showed a 5.6-fold increase in gene activity compared to samples of pure Tf-polylysine at their optimum conjugate-to-DNA ratio (see Table 1). In repeated experiments, we found some variation of the effect from about a 4- to 6-fold increase. To examine this effect in more detail, we designed a new experiment; stock mixtures of the Tf-Lys₂₀₀ fraction C conjugates with 1, 2, or 4 weight equivalents of histone H4 were prepared, and the optimum amounts of each individual mixture for complex formation with a constant amount of DNA was determined in a titration experiment (Fig. 6). The optimum gene activity mediated through the H4/Tf-polylysine (4:1) mixture was comparable to the activity using the Tf-polylysine conjugate alone; however, when these H4-Tf-polylysine-DNA complexes were examined by EM, we found that they displayed only a weak DNA condensation (Fig. 2n). EM of the DNA complexes generated with the optimum amount of H4/Tf-polylysine (1:1) showed stronger condensation (Fig. 2o) but still significantly less than Tf-polylysine-DNA complexes alone. But, surprisingly, the transferrinfection efficiency was increased nearly 7-fold. This suggests that the role of histone H4 in enhancing DNA delivery may be distinct from the condensation effect of other polycations. The high stimulation with histone H4 also seems to be special among the histones we tested in our experiments: histone H1 mediates a <2-fold increase in transferrinfection efficiency and histone H3 mediates an \approx 3-fold increase (see Table 1). A histone mixture of H3 and H4 (mass ratio of 1:1) effected an improvement not greater than the histone H3 alone (results not shown). These effects were considerably lower than with histone H4 alone.

DISCUSSION

Initially we designed Tf-polycation conjugates to be used as DNA-binding transport vehicles for transferrin receptor-mediated delivery of genes into mammalian cells (1). We considered the polycation to be simply a DNA-binding ele-

ment. However, during further experimentation with the efficient Tf-polylysine conjugates we discovered that the polycation, in addition to binding DNA, plays a role in DNA condensation. EM studies of DNA complexes with the most efficient polylysine-rich conjugates revealed a profound collapse of plasmid DNA to small toroid structures with a size of 80–100 nm, a dimension that resembles the diameter of coated pits. This polycation-mediated DNA condensation event, which has been observed in similar fashion in the course of studies on DNA complexes with free polycations such as spermine or polylysine (9, 10), is an important promoting factor in our gene delivery system. We demonstrate here a strong correlation between transferrinfection conditions that generate fully condensed DNA complexes and high DNA delivery activity.

The presence of 100–300 Tf ligands within a single plasmid DNA-conjugate complex represents an unnecessary surplus for binding to the cellular Tf receptors. We show here that the replacement of a large fraction of Tf-polylysine conjugate (up to 90%) by free polylysine does not reduce, but in many cases enhances, DNA delivery (see Fig. 3). Polylysine alone does not function in gene delivery in K-562 cells, although it condenses DNA to toroid structures. Tf conjugates with an inherent low DNA condensation capacity (e.g., Tf-synthetic protamine conjugates) benefit most from polylysine addition in terms of transferrinfection efficiency.

In our experiments we demonstrate that in place of various polylysines, other polycationic compounds (i.e., salmon sperm protamine as well as histone H1, H3, or H4) may be used as complex-promoting components in transferrinfections. EM data of the ternary complexes with histone H4 clearly show that the 4- up to 7-fold improved gene expression is not caused by an increased DNA condensation event, as is the case for polylysine, so that we see here a special effect of histone H4, which needs to be clarified.

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