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Coupling of adenovirus to transferrin–polylysine/DNA complexes greatly enhances receptor-mediated gene delivery and expression of transfected genes

(gene therapy/endocytosis/endosome/gene transfer/DNA transfection)

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ABSTRACT **We are developing efficient methods for gene transfer into tissue culture cells. We have previously shown that coupling of a chimeric adenovirus with polylysine allowed the construction of an adenovirus–polylysine–reporter-gene complex that transferred the transporter gene with great efficiency into HeLa cells. We have now explored simpler, biochemical means for coupling adenovirus to DNA/polylysine complexes and show that such complexes yield virtually 100% transfection in tissue culture cell lines. In these methods adenovirus is coupled to polylysine, either enzymatically through the action of transglutaminase or biochemically by biotinylating adenovirus and streptavidinylating the polylysine moiety. Combination complexes containing DNA, adenovirus–polylysine, and transferrin–polylysine have the capacity to transfer the reporter gene into adenovirus-receptor- and/or transferrin-receptor-rich cells.**

Gene therapeutic manipulations *in vivo* or *in vitro* require efficient methods of gene transfer in chosen target cells. The classical procedure makes use of retroviruses with which efficacious transfer and expression of genes is usually obtained. We and others (1, 2) have explored the alternative method of transferring genes by receptor-mediated endocytosis (3–5). In particular, we have used the transferrin/transferrin-receptor system to facilitate endocytotic transport of DNA. In some tissue culture cells transferrin–polylysine/DNA conjugates provided a very efficient vector for gene transfer. Thus, after transferrinfection of the erythroleukemic cell line K-562 virtually 100% of the cell population was found to express the transfected reporter gene for a protracted period of days (6).

DNA delivered by receptor-mediated endocytosis suffers from the limitation that endocytosed DNA is trapped in intracellular vesicles and later largely destroyed by lysosomal action. Addition of chloroquine during transfection, preventing acidification of the endosomal and lysosomal compartment, is one measure to ensure better survival and transfer of DNA into the nuclear compartment (4). Simultaneous addition of (replication-deficient) adenoviruses during transfection is another (7). The added adenoviruses are thought to disrupt endosomes upon endocytosis and to admit DNA into the cytoplasm and eventually into the nucleus. The adenovirus-aided transferrinfection was found experimentally to augment gene transfer and expression of a reporter gene by as much as a factor of 1000 in HeLa (7, 8) or BNL CL.2 (8), whereas without adenovirus, transfection efficiency, even in the presence of chloroquine, was moderate to poor for these cell types. For adenovirus-aided transfection (7, 8) the simultaneous presence of adenovirus receptor and transferrin receptor on target cells is a precondition for efficient gene transfer.

We have recently demonstrated that coupling of adenovirus to polylysine/DNA complexes by means of an adenovirus-specific antibody (see Fig. 1*C*) results in efficient transfer of DNA into cells with high levels of adenovirus receptors (9). In this system, colocalization of both virus and polylysine/DNA complex in the endosome is assured. We have now explored simpler, biochemical means of coupling adenovirus to the DNA/polylysine complex and show that such complexes yield virtually 100% transfection of both HeLa cells and murine hepatocytes (BNL CL.2 cells). Furthermore, we describe the delivery of DNA to cells lacking high levels of adenovirus receptors.

MATERIALS AND METHODS

Enzymatic Conjugation of Adenovirus and Polylysine. Two milliliters of a dl312 adenovirus preparation (10^{11} particles per ml) were freed of CsCl by gel filtration (Sephadex G-25) equilibrated in reaction buffer [0.1 M Tris·HCl, pH 8.0/2 mM dithiothreitol/30% (vol/vol) glycerol]. The elution volume was 3.5 ml. Virus solution (1150 μl) was incubated in reaction buffer with 0.5 nmol of guinea pig transglutaminase (Sigma) and 20 nmol of polylysine 290/10 mM $CaCl_2$ in an end-volume of 1.5 ml. After 1-hr incubation at 37°C the reaction was stopped by adding 30 μl of 0.5 M EDTA. Unincorporated polylysine was removed by layering the reaction mixture over CsCl (1.33 g/ml) and centrifuging the solution at 170,000 × *g* for 2 hr. The virus–polylysine conjugate forming an opalescent band was recovered, an equal volume of glycerol was added, and this solution was stored at −70°C. Conjugation of virus and polylysine was confirmed with ^{125}I-labeled polylysine.

Biotinylation of dl312 Adenovirus. Two and four-tenths milliliters of a gel-filtrated adenovirus (dl312; 10^{11} particles per ml) in 150 mM NaCl/5 mM Hepes, pH 7.9/10% glycerol was treated with 10 nmol of NHS-LC-Biotin (sulfosuccinimidyl 6-(biotinamido) hexanoate; Pierce 21335). After incubation for 3 hr at room temperature, the virus was separated from unreacted biotin by gel filtration. An equal volume of glycerol was added, and the solution was stored at −70°C.

Streptavidinylation of Polylysine. Seventy-nine nanomoles (4.7 mg) of streptavidin in 1 ml of 200 mM Hepes, pH 7.9/300 mM NaCl was treated with 236 nmol of SPDP (heterobifunctional reagent; Pharmacia) dissolved in ethanol. After incubation for 1.5 hr at room temperature, 75 nmol of streptavidin modified with 196 nmol of dithiopyridine linker was obtained after gel filtration over Sephadex G-25. Polylysine 290 (75 nmol, average chain-length, 290 residues) was modified with 190 nmol of mercaptopropionate linker as described in ref. 3

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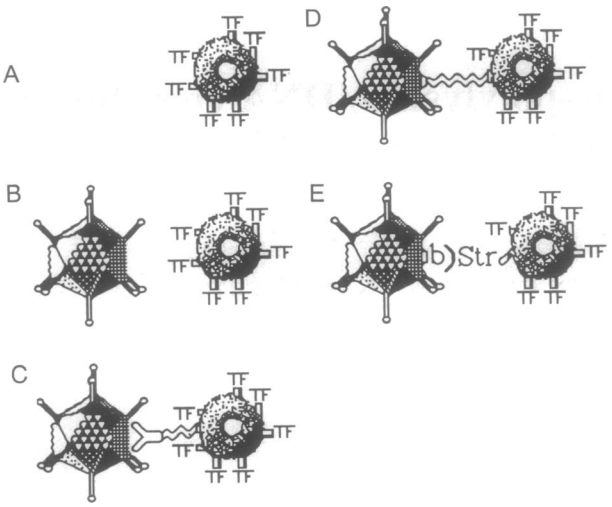

FIG. 1. Schematic drawing of transport complexes. (*A*) transferrin (TF)–polylysine/DNA complex; (*B*) transferrin–polylysine/DNA complex in the presence of inactivated adenovirus; (*C*) transferrin–polylysine/DNA complex conjugated to adenovirus via an adenovirus-directed antibody (ternary complex). (*D*) Polylysine/DNA complex attached to virus–polylysine conjugate generated with transglutaminase. (*E*) Transferrin–polylysine/DNA complex conjugated with adenovirus via a biotin/streptavidin (Str) bridge (ternary complex).

and treated with the modified streptavidin in 2.6 ml of 100 mM Hepes, pH 7.9/150 mM NaCl, under argon overnight.

The conjugates were recovered by chromatography on a Mono S HR5 column (Pharmacia) by salt-gradient elution. The conjugate eluted between 1.2 M and 1.7 M NaCl. The conjugate consisting of 45 nmol of streptavidin and 53 nmol of polylysine was obtained after extensive dialysis against HBS (20 mM Hepes, pH 7.3/150 mM NaCl).

Transfection of Tissue Culture Cells. K-562 cells were grown in suspension in RPMI 1640 medium (GIBCO/BRL, plus 2 g of $NaHCO_3$ per liter)/10% fetal calf serum, penicillin at 100 units per ml, streptomycin at 100 μg per ml/2 mM glutamine, reaching a density of 500,000 cells per ml. At 16 hr before transfection, the cells were placed in fresh medium containing 50 μM desferrioxamine (Sigma). Just before transfection, the cells were collected, resuspended in fresh medium containing 10% fetal calf serum (plus 50 μM desferrioxamine) at 250,000 cells per ml, and placed in a 24-well dish, 2 ml per well. HeLa cells were grown in Dulbecco's modified Eagle's medium/5% fetal calf serum/penicillin at 100 units per ml/streptomycin at 150 μg per ml/2 mM glutamine.

The murine embryonic hepatocyte cell line BNL CL.2 (American Type Culture Collection TIB 73) was grown in "high-glucose" Dulbecco's modified Eagle's medium (0.4% glucose)/10% heat-inactivated fetal calf serum/penicillin at 100 units per ml/streptomycin at 100 μg per ml/2 mM glutamine. HeLa cells and hepatocytes were grown in 6-cm plastic Petri dishes. Transfection was done at a cell density of $\approx 3 \times 10^5$ cells per dish. Before transfection 1 ml of fresh medium containing 2% fetal calf serum replaced the standard culture medium.

Formation of Binary Complexes. Biotinylated adenoviruses (10^{10} particles) were treated with 800 ng of streptavidinylated polylysine in 50 μl of HBS. After 30 min at room temperature, 6 μg of pCMVL-DNA in 170 μl of HBS was added, the mixture was incubated for 30 min, and then 3 μg of polylysine pL300 in 200 μl of HBS was added; after a further 30 min, the solution was used for transfection experiments.

Formation of Ternary Complexes. Ternary DNA complexes containing adenovirus and transferrin were formed as follows: biotinylated adenoviruses (10^{10} viral particles) were mixed with 800 ng of streptavidinylated polylysine. After 30 min at room temperature, the solution was mixed with 6 μg of plasmid DNA in 170 ml of HBS, the mixture was incubated for 30 min, and then 10 μg of transferrin–polylysine TfpL190B (10) in 200 μl of HBS was added; after a further 30 min, the solution was used for transfection experiments.

β-Galactosidase Assay. BNL CL.2 cells were seeded onto coverslips and 24 hr later the cells were transfected with the pCMV-βgal (11) reporter gene. Forty-eight hours later, β-galactosidase was assayed according to Lim and Chae (12).

RESULTS

DNA-Transport Complexes. We have shown (9) that coupling of a chimeric adenovirus with polylysine via an adenovirus-directed antibody allowed the construction of an adenovirus–polylysine–reporter-gene complex that transferred the reporter gene with great efficiency into HeLa cells. We wanted to simplify the procedure by coupling adenovirus with the polylysine moiety using biochemical procedures. One such procedure consisted of covalently linking the two entities, adenovirus and polylysine, through the action of transglutaminase; another consisted of coupling biotinylated adenovirus with streptavidinylated polylysine (Fig. 1, see *Materials and Methods*).

Binary Transport Complexes. To construct binary transport complexes biotinylated adenovirus was combined with streptavidinylated polylysine. Alternatively adenovirus was linked covalently with polylysine through the action of transglutaminase (see *Materials and Methods*). Adenovirus–polylysine conjugate was added to DNA, allowing complex formation between DNA and polylysine, thus neutralizing a known fraction (≈one-fourth) of the negative charges of the DNA. A calculated amount of polylysine was then added to neutralize the remainder of the charges. We refer to the complexes consisting of DNA bound to adenovirus–polylysine conjugate and to polylysine as binary transport complexes.

There are essentially two ways of assembling binary transfer complexes. DNA can be bound to streptavidinylated polylysine and then coupled to biotinylated adenovirus or the adenovirus is coupled to polylysine first and later complexed with DNA. The latter procedure quite clearly yields better results (data not shown), especially at low-DNA input, and, therefore, is the preferred method for assembling both binary and ternary complexes.

Ternary Transport Complexes Containing Transferrin. Adenovirus–polylysine conjugates were prepared as mentioned above. These conjugates were added to DNA, allowing complex formation and neutralization of a fraction (≈one-fourth) of the negative charges of the DNA (see *Materials and Methods*). A calculated amount of transferrin–polylysine conjugates was then added to the complex to neutralize the remainder of the DNA. We refer to complexes consisting of DNA, adenovirus–polylysine, and transferrin–polylysine as ternary complexes (Fig. 1). In principle, such a ternary complex should have the capacity of being endocytosed by binding either to the cellular adenovirus receptors or to transferrin receptors.

Linkage Between DNA Condensates and Adenovirus Greatly Enhances Luciferase-Reporter-Gene Expression. The effect of adenovirus–polylysine linkage on transfection efficiency is clearly demonstrated in Fig. 2, where hepatocytes were incubated with transferrin–polylysine/DNA complexes (Fig. 2, TfpL) in the presence of chloroquine or in the presence of adenovirus (Fig. 2, AdenoV + TfpL). Transferrinfection in the presence of free adenovirus is elevated, showing the typical enhancement of release of transferrin–polylysine/DNA complexes into the cells (8). In slot polylysine–adenovirus/transferrin–polylysine (pLAdenoV/TfpL) adenovirus was

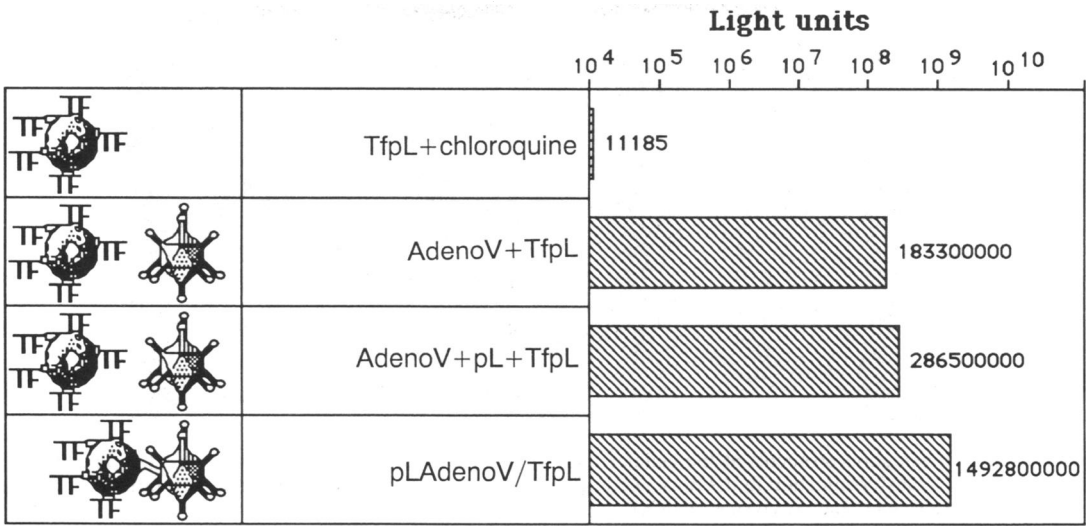

FIG. 2. Comparing transferrinfection, transferrinfection of hepatocytes in the presence of free adenovirus, and transferrinfection with linked adenovirus (ternary complexes). (For explanation see text.)

conjugated with polylysine by means of transglutaminase and was then treated with DNA, neutralizing part of the negative charges of the DNA. Later, transferrin–polylysine was added, neutralizing the remainder of the charges. In this way a ternary complex of adenovirus–polylysine/transferrin–polylysine/DNA was synthesized. As can be seen, an extraordinarily high value of 1.5×10^9 luciferase light units was obtained (or ≈5000 light units per cell). In slot adenovirus plus polylysine plus transferrin–polylysine (AdenoV + pL + TfpL), adenovirus, and polylysine were mixed, as for the transglutaminase treatment. However, to demonstrate the specificity of the transglutaminase-mediated binding of polylysine to the virus, the enzyme was omitted. Then the virus preparation was complexed to the same amount of DNA and transferrin–polylysine as in pLAdenoV/TfpL. In this case the transfection was moderate as in adenovirus plus transferrin–polylysine (AdenoV + TfpL) because in both experiments colocalization of virus and transferrin/DNA is a stochastic process, in contrast to slot pLAdenoV/TfpL, where cointernalization is assured by the physical linkage of virus and DNA in a ternary complex, yielding high level of transferrinfection.

Transfection of K-562 Cells Reveals the Endosomolytic Properties of Adenovirus. The human erythroleukemic cell line K-562 contains ≈150,000 transferrin receptors (13). In the presence of chloroquine, as reported earlier (5), these cells can be transferrinfected at very high level with polylysine–transferrin–reporter-DNA complexes, even in the absence of adenovirus (Fig. 3, TfpL + chloroquine). The same complexes with added free adenovirus, but in the absence of chloroquine, yield relatively poor levels of reporter-gene expression (Fig. 3, TfpL + AdenoV), presumably because K-562 cells like other blood cells (14, 15) have low levels of adenovirus receptors. When the adenovirus is linked to polylysine via biotin/streptavidin bridge and the reporter DNA fully condensed by addition of more polylysine to complete the binary complex (Fig. 3, pLAdenoV/pL), adenovirus-supported transfection reaches intermediate levels. Presumably, the few adenovirus receptors on K-562 cells are

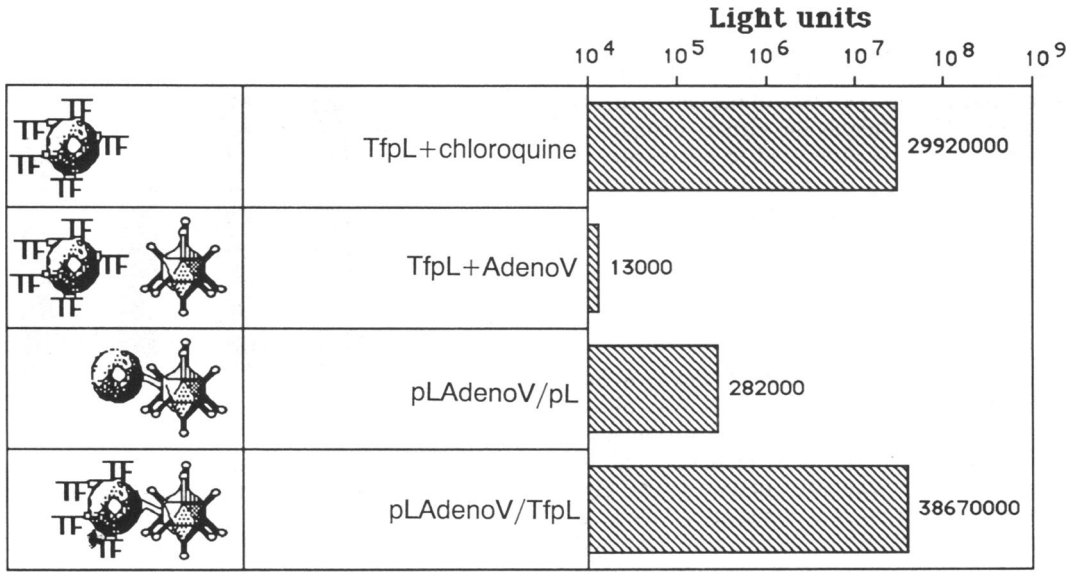

FIG. 3. Comparison of transferrinfection of K-562 cells in the presence of chloroquine, of free adenovirus, of binary complex containing polylysine linked to adenovirus, and of ternary complexes containing transferrin, polylysine, reporter gene, and polylysine-modified adenovirus (for explanation, see text).

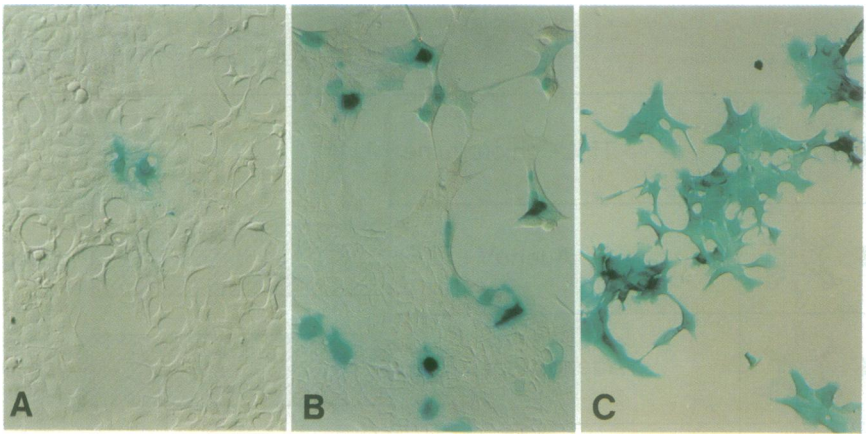

FIG. 4. Comparison of transfection protocols at the cellular level with β-galactosidase as a reporter gene. (*A*) Transferrinfection in the presence of chloroquine. (*B*) Transferrinfection in the presence of free adenovirus. (*C*) Transferrinfection with ternary complexes containing transferrin, polylysine, reporter gene, and polylysine-modified adenovirus (for explanation, see text).

used efficaciously. If, however, the coupled adenovirus–polylysine–reporter DNA is fully condensed and neutralized by addition of polylysine–transferrin to form a ternary complex (Fig. 3, pLAdenoV/TfpL) and the numerous cellular transferrin receptors come into play. The transfection efficiency, owing both to efficient transferrin binding and to the endosomolytic properties of the virus, is increased by at least another two orders of magnitude.

Ternary DNA Complexes Lead to the Expression of the Reporter Gene in Almost 100% of Hepatocytes. The efficacy of these DNA transport complexes was also tested in mouse hepatocytes (BNL CL.2), determining the percentages of the cells that can be reached with our various transfection protocols. A β-galactosidase gene driven by a cytomegalovirus promotor was used as a reporter gene (11). After fixation of the cells β-galactosidase activity was detected according to Lim and Chae (12).

Fig. 4 shows the β-galactosidase assay on mouse hepatocytes after transferrinfection in the presence of chloroquine, transferrinfection in the presence of free dl312 adenovirus, and transfection with ternary, linked (dl312) adenovirus–polylysine–transferrin–reporter-DNA complexes. In the absence of adenovirus, after standard transferrinfection of the reporter DNA, only few cells express the reporter gene. Percentage of transfection is <0.1% (data not shown). When chloroquine is included, percentage is increased to ≈0.2% (Fig. 4*A*). With free adenovirus, ≈5–10% of the cells express the reporter gene (Fig. 4*B*), whereas the ternary complexes with transglutaminase-modified virus lead to expression in most, if not all, cells (Fig. 4*C*). Because the ternary complexes can be used at high dilution, the toxic effect seen with high doses of free (inactivated) adenovirus (8) does not usually arise. But it should be noted that where ternary complexes are deployed at high concentration to reach 100% of the tissue culture cells, a similar toxic effect becomes noticeable (see ref. 8).

Expression of a Transfected Reporter Gene Is Transient but Lasts for Weeks in Nondividing Hepatocytes. A two-thirds-confluent hepatocyte cell culture was transfected similar to Fig. 4*C* with the luciferase-reporter gene pCMVL, and luciferase activity was determined at different time points. Fig. 5 shows that luciferase activity was maximal after 3 days, at which time the hepatocyte cell culture became confluent, and the cells stopped dividing. Expression of the reporter gene persisted in the nondividing cell culture without applying selection for the maintenance of the gene and lasted for at least 6 weeks.

DISCUSSION

At least for some cell types, receptor-mediated transport of DNA, in which polylysine is covalently linked to a ligand and complexed ionically with DNA, has proven a highly effective and physiological means of introducing DNA into cells (3–5). The method using transferrin as a ligand, termed *transferrin*fection, was particularly efficient for K-562 cells, which have many transferrin receptors per cell (13), whereas other cell types were more refractory to this treatment. It can be shown by a variety of techniques (unpublished results) that even in tissue culture cells other than K-562 cells all cells become usually associated with the DNA complexes initially. It appears that an important limitation for gene transfer occurs further down the transport route, in that DNA becomes localized to the endosomal and, eventually, to the lysosomal compartment where the DNA is degraded (for discussion, see ref. 5; our unpublished results). A remedy for this is the simultaneous addition of (replication-defective) adenovirus and polylysine–transferrin/DNA complexes to

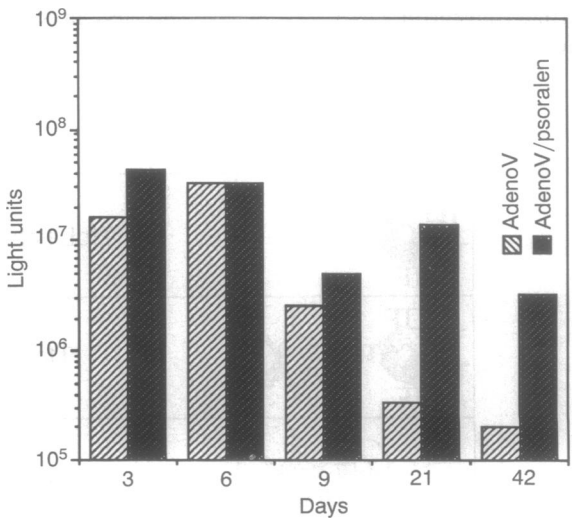

FIG. 5. Long-term persistence of luciferase expression in confluent, nondividing hepatocytes. Ternary transport complexes (polylysine–adenovirus/transferrin–polylysine) were made with polylysine-modified Adenovirus dL312 (AdenoV) and polylysine-modified Adenovirus dL312 further inactivated by treating the virus with psoralen (8). Reporter-gene expression in BNL CL.2 cells persists at high level for at least 6 weeks, especially when psoralen-inactivated adenovirus was used for the formation of the ternary transport complexes.

the cells. In this method, especially at high input, adenovirus and transferrin complexes are colocalized in endosomes. The virus, because of its endosomolytic properties, is thought to destroy the integrity of the endosomal membrane and to effect exit of the colocalized DNA into the cytoplasm (7).

Chloroquine, which enhances transferrin-mediated DNA transfer in K-562 cells to an unusually large degree may, in fact, act in a similar fashion. K-562 cells are unusual in that the minimum endosomal pH observed is 5.4, so low presumably because of the absence of Na^+,K^+-ATPase regulation of endosomal acidification (16). Because of the low pH the K-562 cells are expected to accumulate chloroquine to an unusually high degree, leading to vacuolization and the eventual bursting of the endosome (R. F. Murphy, personal communication) accompanied with the release of the DNA into the cytoplasm. This circumstance may be the reason why transferrinfection in the presence of chloroquine leads to expression of a reporter gene in virtually 100% of the transfected K-562 cells (6).

BNL CL.2 or HepG2 hepatocytes do not respond as well to chloroquine as do K-562 cells, but they can be transfected to a level of 5–10% when exploiting the endosomolytic properties of added replication-defective (7) or chemically inactivated (8) free adenovirus. For HeLa cells nearly 100% of the cells can be transfected with free adenovirus. As we show in this paper, the efficacy for hepatocytes can be still further improved when using ternary DNA complexes in which the reporter DNA is complexed to polylysine–transferrin conjugates and linked to adenovirus. Here, colocalization of the endosomolytic virus and the ligand–receptor complex in the endosome is guaranteed, yielding virtually 100% transfection for a variety of cells, such as BNL CL.2 (Fig. 4) and HepG2 cells. In this instance, both viral and transferrin receptors on the cell surface can act to capture the ternary DNA complexes. However, one can envisage also that DNA ternary complexes can be internalized solely by the action of the cellular ligand–receptor association. Such a situation might be approximated in the experiments of Fig. 3, where ternary DNA complexes containing transferrin gain access to K-562 cells in the main via the transferrin, rather than the adenovirus, receptor.

Ternary complexes transfer DNA, even when presented for transferrinfection at very low levels. Thus, at an input of 30 pg of DNA per 3×10^5 cells 1.8×10^4 light units are obtained (data not shown). At this input there are as few as 60 DNA molecules and 1 plaque-forming unit of virus per cell. This result has to be compared with the less-efficient calcium-precipitation protocol, which uses 2×10^5 DNA molecules per cell (17).

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