Proc. Natl. Acad. Sci. USA
Vol. 90, pp. 11548–11552, December 1993
Genetics

Hepatic gene therapy: Efficient gene delivery and expression in primary hepatocytes utilizing a conjugated adenovirus–DNA complex

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Communicated by Kenneth M. Brinkhous, September 3, 1993

ABSTRACT **Receptor-mediated endocytosis is an effective method for gene delivery into target cells. We have previously shown that DNA molecules complexed with asialoglycoprotein can be efficiently endocytosed by primary hepatocytes and the internalized DNA can be released from endosomes by the use of a replication-defective adenovirus. Because the DNA and virus enter target cells independently, activity enhancement requires high concentrations of adenoviral particles. In this study, adenoviral particles were chemically conjugated to poly(L-lysine) and bound ionically to DNA molecules. Quantitative delivery to primary hepatocytes was achieved with significantly reduced viral titer when the asialoorosomucoid–poly(L-lysine) conjugate was included in the complex. The conjugated adenovirus was used to deliver a DNA vector containing canine factor IX to mouse hepatocytes, resulting in the expression of significant concentrations of canine factor IX in the culture medium. The results suggest that receptor-mediated endocytosis coupled with an efficient endosomal lysis vector should permit the application of targeted and efficient gene delivery into the liver for gene therapy of hepatic deficiencies.**

Targeted delivery of DNA to specific cell types by receptor-mediated endocytosis is one of the growing number of delivery systems that are suitable for gene therapy (1–10). Specific ligands such as asialoglycoprotein and transferrin have been used as DNA–protein complexes for DNA delivery *in vitro* (11–15) and *in vivo* (16–19). Cellular internalization of DNA–protein complexes occurs by receptor-mediated uptake into endosomes, but the internalized DNA is degraded after fusion of endosomes with lysosomes (20). The capabilities of replication-defective adenovirus (ADV) to lyse the endosome gives a dramatic enhancement of gene expression by receptor-mediated DNA delivery (20). The method, however, requires ADV titers of 10^4 particles per cell, which has cytopathic effects (20). Targeted delivery of DNA can be enhanced by coupling DNA directly to the ADV, which significantly reduces the number of viral particles used but still maintains high levels of gene expression. The modification of ADV for this purpose has been achieved by attaching polylysine to the viral particle by antibodies (21), or chemically (22), permitting DNA attachment directly to the particle. The chemical procedures developed thus far are difficult to use in that multiple steps are required for the modification to add poly(L-lysine) (PLL) to the outside of the particle.

To achieve efficient DNA delivery at lower ADV titers that are not toxic to cells but still achieve high-level gene expression, we have modified the ADV by covalently attaching PLL directly to the viral particle with a water-soluble carbodiimide. This simple chemical modification results in a modified virus that is (*i*) capable of efficient DNA delivery at lower ADV titers, (*ii*) is not cytopathic, and (*iii*) is still capable of efficient endosomal lysis. The efficiency of DNA delivery using complexes containing conjugated ADV and asialoorosomucoid–poly(L-lysine) (ASOR-PLL)–DNA complexes, results in levels of activity that are greater than those achieved with nonconjugated ADV. As a result, the combination of liver-targeting capability of the ASOR-PLL–DNA with the endosome lysis ability of conjugated ADV into one complex has led to efficient delivery of the canine factor IX (cFIX) cDNA into mouse hepatocytes. The high levels of cFIX production achieved *in vitro* could potentially be applied for gene delivery into the liver of hemophilia B dogs for phenotypic correction of the genetic deficiency.

MATERIALS AND METHODS

ADV Modification and DNA Complex Formation. ADV (d1312) was a gift from T. Shenk (Princeton University) and grown in 293 cells. ADV was purified by double banding on CsCl gradients (23) and then dialyzed against 2× filtered Hepes-buffered saline (HBS = 150 mM NaCl/20 mM Hepes·NaOH, pH 7.3). The concentration of the virus was determined by UV-spectrophotometric analysis and either stored in 10% (vol/vol) glycerol at −20°C or further modified for DNA complex formation. Freshly isolated ADV (1.4×10^{11} particles) was combined with PLL (Sigma, 20.5 kDa) at 16 μM, along with 1-ethyl-3-(3-dimethylaminopropyl)carbodiimide (EDC) at a final concentration of 130 μM (low EDC) or 2.6 mM (high EDC) in 4 ml. After incubation on ice for 4 h, the unreacted components were removed by ultracentrifugation (150,000 × *g*) for 18 h on a CsCl gradient at a CsCl concentration of 1.35 g/ml. The ADV was then either dialyzed against 2 M NaCl and then stored at −20°C in 10% glycerol or stored in 10% glycerol.

The DNA used in complex formation was either the plasmid pCMV/βGal, which contains the β-galactosidase (β-gal) gene under the control of the cytomegalovirus (CMV) enhancer and promoter, or the plasmid pCMV/cFIX, constructed as follows. The cFIX cDNA and Kozak translation sequence constructed as described (24) was cloned into the *Xho* I–*Cla* I sites of the spCMV plasmid (1). The *Xho* I–*Sal* I fragment containing the CMV promoter-enhancer and factor IX cDNA was then cloned into the *Xho* I site of the N2 retroviral vector (1) in the forward orientation. All plasmid DNA was purified by banding twice in CsCl gradients.

The modified ADV-PLL–DNA complexes were made in a two-step procedure. The first step involved the addition of 10 μg of DNA in 250 μl of HBS to the ASOR-PLL conjugate in 250 μl, with continuous mixing, followed by incubation at room temperature for 30 min. The ASOR-PLL conjugate was

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Abbreviations: ASOR, asialoorosomucoid; PLL, poly(L-lysine); ADV, adenovirus; EDC, 1-ethyl-3-(3-dimethylaminopropyl)carbodiimide hydrochloride; cFIX, canine factor IX; β-gal, β-galactosidase.

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synthesized and purified (25). Sufficient ASOR-PLL conjugate to neutralize 75% of the charge on the DNA molecule was used. The second step involved the addition of the modified ADV, in 330 μl of HBS, to the DNA–ASOR-PLL mixture, with continuous mixing, followed by incubation for an additional 30 min at room temperature. After complex formation, the complexes were either analyzed by electron microscopy or added to the hepatocytes for analysis.

Analysis of Complex Uptake and Expression in Primary Hepatocytes. All media used for hepatocyte isolation and growth were purchased from GIBCO. Hepatocyte isolation from 10- to 12-week-old C57BL/6 mice was done by collagenase perfusion (26). The hepatocytes were plated at 3×10^5 cells per well in 6-well Primaria plates (Falcon) and grown in 2 ml of low glucose complete medium [low glucose Dulbecco's modified Eagle's medium (DMEM)/10% (vol/vol) fetal calf serum/10 mM Hepes/0.5% minimum essential medium amino acids/2 mM glutamine/penicillin (100 units/ml)/streptomycin (100 μg/ml)]. All uptake studies in hepatocytes were done within 2–4 h after the hepatocytes were plated, allowing the hepatocytes to attach to the surface. Before adding the DNA complexes to the hepatocytes, the complete medium was removed and replaced with 1 ml of low glucose incomplete medium (low glucose DMEM containing 5 mM Ca^{2+} and 2% fetal calf serum). The modified ADV–DNA complexes were incubated with cells in incomplete medium for 2 h at 37°C in a CO_2 incubator, after which the medium was removed and replaced with 1 ml of SUM medium (1) and the incubation continued at 37°C in a CO_2 incubator.

The analysis of β-gal activity was done by staining the cells, using 5-bromo-4-chloro-3-indolyl β-D-galactoside as a substrate (27). Factor IX levels were analyzed from the supernatants of cultured hepatocytes that were incubated in SUM medium for various times as described (24), utilizing an ELISA-based assay. Protein concentration in cell extracts was determined by the BCA Micro-Protein assay (Pierce).

Electron Microscopic Analysis of Modified ADV–DNA Complexes. The ADV-PLL–DNA complexes were examined by electron microscopic analysis of negatively stained samples (28). Briefly, the samples were added to Formvar-coated electron microscopy copper grids and allowed to incubate for 1 min. The solution was then removed with tissue paper and a drop of uranyl acetate was added. After a 1- to 2-min incubation, the solution was removed as before. The grids were allowed to dry for 30 min and then viewed under a Philips EM410 transmission electron microscope.

RESULTS

Receptor-Mediated Endocytosis and ADV-Mediated Endosomal Release of DNA. The ASOR in the ASOR-PLL–DNA complex functions as a receptor ligand to target the DNA to hepatocytes, and the PLL functions to attach DNA to ASOR through ionic interactions. To achieve efficient cellular delivery of DNA without subsequent lysosomal degradation, the complex can be coincubated with a replication-defective ADV (Fig. 1*A*). A fraction of the DNA toroids and the ADV is internalized in the same endosome, leading to the release of the DNA from the endosome, escaping lysosomal degradation. To reduce the viral titers while enhancing the efficiency of DNA delivery, the ADV can be coupled directly to the DNA complex (Fig. 1*B*). As a result, the ASOR-PLL–DNA–PLL-ADV complex is cointernalized, through the ADV receptor or the ASOR receptor (Fig. 1*b*). After cointernalization, the ADV causes endosomal lysis and results in the release of the ASOR-PLL–DNA complex into the cytoplasm.

Ultrastructure of ADV-PLL–DNA Complexes. The ultrastructure of the ADV-PLL–DNA complexes was determined by electron microscopy. When the ASOR-PLL conjugate is combined with DNA in the proper ratio to achieve charge neutralization on the DNA molecule, a DNA toroid results

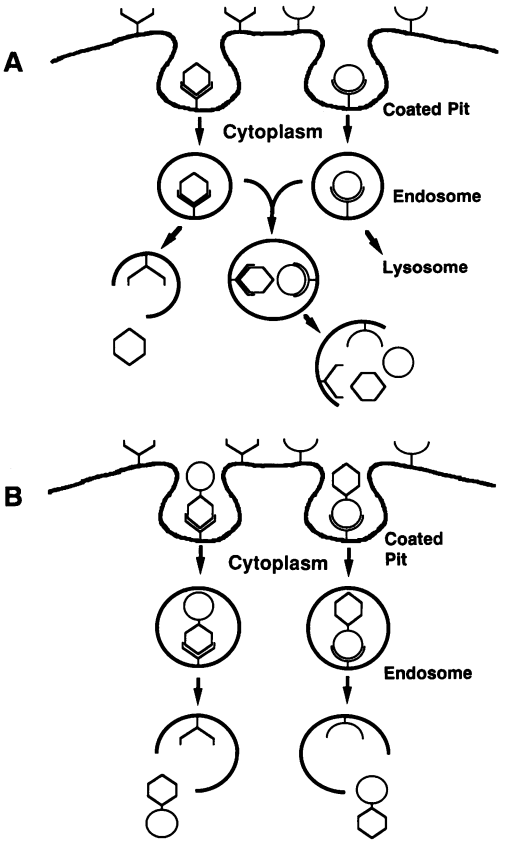

FIG. 1. Uptake of ASOR-PLL–DNA complexes and ADV-PLL–DNA complexes into cells. (*A*) Coincubation of the ADV with the ASOR-PLL–DNA results in the binding and uptake of each component through its own receptor and requires cointernalization into the same endosome. (*B*) In conjugated form, the binding and uptake of ADV-PLL–DNA complexes can occur through either the ASOR receptor or the ADV receptor. Circles, ASOR-PLL–DNA complex; hexagons, ADV.

(Fig. 2*A*). After conjugation with PLL, the ADV particle retains its natural structure (Fig. 2*B*). When the PLL-ADV is incubated with DNA toroids, complexes between DNA toroids and ADV particles are formed. When PLL is conjugated to the ADV particle at low concentrations of EDC and used in DNA complex formation, the viral particles are linked to DNA toroids in limited numbers. The coupling of a DNA toroid with one to three ADV particles usually occurs in the coupling procedure and gives a complex that is <200 nm (Fig. 2*C*). When PLL is conjugated to ADV under high EDC concentrations and then used for complex formation, however, multiple viral particles bound to the DNA toroids are frequently observed. These complexes are >200–300 nm in diameter (Fig. 2*D*).

Dose–Response Effect of ADV–DNA Complexes on Primary Hepatocytes. To analyze the ability of the ADV-PLL–DNA complexes to deliver DNA, primary mouse hepatocytes were used as recipient cells to measure gene expression. Primary hepatocytes (3×10^5 cells) were incubated with increasing titers of ADV, ranging from 0 to 10^3 particles in free and conjugated forms. When the cells were incubated with ASOR-PLL–DNA complexes along with free ADV, the percentage of cells that express β-gal was 9% when 10^3 particles per cell was used (Fig. 3*A*). Previously, we have shown that the amount of free virus that is needed to transduce 100% of cells is 10^4 particles per cell (25). In contrast, when ADV conjugated with PLL at low EDC concentrations was used at 10^3 ADV particles per cell, the percentage of β-gal-positive cells reached 100% (Fig. 3*B*). When ADV conjugated with PLL at high EDC

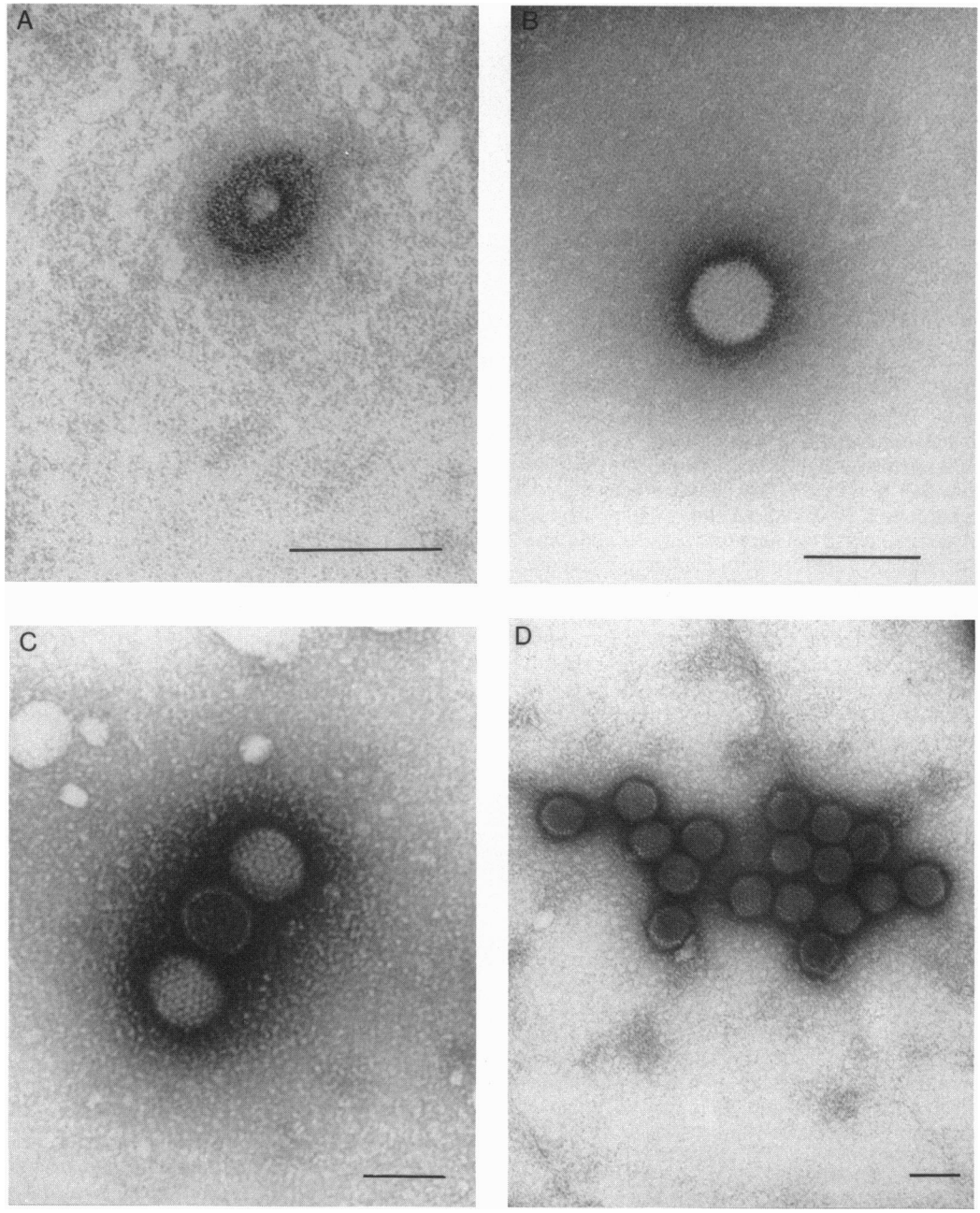

FIG. 2. Ultrastructural analysis of ASOR-PLL–DNA and ADV-PLL–DNA complexes by electron microscopy. (*A*) The plasmid pCMV/β-gal in toroid form. (*B*) Replication-defective ADV D1312. (*C*) ADV-PLL–DNA complexes made with ADV that was modified with low EDC concentrations. (*D*) ADV-PLL–DNA complexes made with ADV that was modified with high EDC concentrations. (Bars = 100 nm.)

concentrations was used, 80% of cells stained blue at 10^3 particles per cell (Fig. 3*C*).

To determine the percentage of cells that internalized DNA specifically through the ASOR receptor, the same dose–response analysis was done as before, but in the presence of a 1000-fold excess of free ASOR. The cells incubated with ASOR-PLL–DNA complexes and free ADV showed a decrease in the percentage of cells expressing β-gal to <2%, when 10^3 particles per cell was used (Fig. 3*A*), demonstrating that most of the DNA uptake specifically occurred through the ASOR receptor. Competition of the complex containing ADV conjugated with PLL at low EDC concentrations showed a minimal decrease in the percentage of β-gal-positive cells from 100% to ≈80% at 10^3 ADV particles per cell (Fig. 3*B*). This result shows that the majority of the DNA uptake of this complex into recipient cells was through the ADV receptor. When ADV conjugated with PLL at high EDC concentrations was used, however, the percentage of cells staining blue decreased from 80% to <30%, when 10^3 particles per cell was used (Fig. 3*C*), indicating that the majority of the DNA delivered by this complex occurred through the ASOR receptor.

To further determine the basis for uptake by these complexes, the ADV-PLL–DNA complex was made with PLL instead of the ASOR-PLL conjugate and then incubated with cells. This complex, when used to delivery DNA in the presence of free ADV, resulted in <2% of the cells staining positive for β-gal (Fig. 3*A*). When the low EDC modified ADV was used, the percentage of blue cells was 80% at 10^3 particles per cell (Fig. 3*B*). When this was done with the high EDC modified ADV, the percentage of blue cells was 37% at 10^3 particles per cell (Fig. 3*C*). The results were in complete

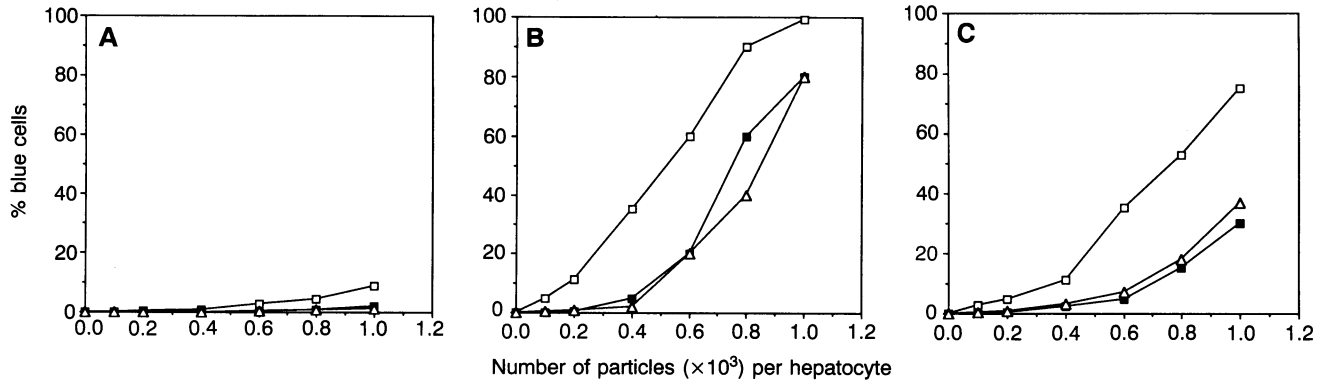

FIG. 3. Dose–response curves of ADV-PLL–DNA complexes for gene delivery and expression in primary mouse hepatocytes. Approximately 3×10^5 hepatocytes were incubated with increasing titers of nonconjugated ADV plus 0.3 μg of pCMV/β-gal in toroid form (*A*) or with increasing titers of ADV in complex form (0.3 μg of DNA) that was modified with low EDC (*B*) or with high EDC (*C*). □, Dose–response analysis performed without competitor; ■, dose–response analysis performed in the presence of a 1000-fold molar excess of ASOR; △, dose–response analysis using complexes that contain PLL instead of the ASOR-PLL conjugate.

agreement with the competition experiment, suggesting that the residual uptake is due to other interactions between the ADV and the cells (29). Analysis of the cytopathic effect of the complexes on the cells after a 96-h incubation showed no toxic effects when either the low or high EDC modified ADV-PLL–DNA complexes were used at 10^3 particles per cell.

Expression of cFIX in Primary Mouse Hepatocytes. To quantitatively compare the levels of gene expression achieved with the ADV-PLL–DNA complexes with those achieved with the free ADV, the complexes were used to deliver a cFIX cDNA into primary mouse hepatocytes (Fig. 4). ADV modified with high EDC concentrations was used to deliver the DNA, since the complexes made with this conjugated ADV deliver the DNA primarily through the ASOR receptor. No cFIX activity was observed when the ADV alone was used (lane 2). When DNA in toroid form was used along with free ADV at 10^3 particles per cell, the level of cFIX increased to 0.032 μg per 10^6 cells per 24 h (lane 4). In contrast, when the conjugated ADV was used to deliver DNA at 10^3 particles per cell, the levels of cFIX increase to 0.79 μg per 10^6 cells per 24 h (lane 5). This represents a 25-fold enhancement of cFIX expression over that achieved with the complex and free ADV at the same titer.

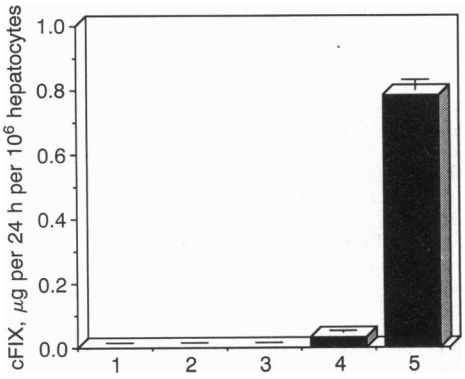

FIG. 4. Factor IX expression after DNA delivery to primary hepatocytes by ADV-PLL–DNA complexes. Bars: 1, primary mouse hepatocytes; 2, mouse hepatocytes incubated with ADV at 1×10^4 particles per cell; 3, hepatocytes incubated with ASOR-PLL–DNA complex, made with 0.3 μg of pCMV/cFIX; 4, hepatocytes incubated with ASOR-PLL–DNA complex (made with 0.3 μg of pCMV/cFIX) and 1×10^4 ADV particles per cell; 5, hepatocytes incubated with ADV-PLL–DNA complex (made with 0.3 μg of pCMV/cFIX and the high EDC modified ADV at 1×10^3 ADV particles per cell. Supernatants from cells were analyzed for factor IX expression 24 h after the initial DNA delivery.

DISCUSSION

The coupling of DNA toroids to ADV permits efficient gene delivery into target cells, without cytotoxic effect. The simple chemical conjugation of PLL to ADV by EDC allows DNA toroids to be bound directly to viral particles and still be 100–300 nm. The compact nature of the complex and the endosomal lysis ability of the ADV allows for efficient internalization into hepatocytes, as seen by the high number of cells that express β-gal after incubation with the ADV-PLL–DNA complex. The ability to reduce the titer of the ADV by an order of magnitude has eliminated the cytopathic effect, based on the lack of cell death with prolonged incubation of the hepatocytes. Although the titer of the ADV is decreased, the level of cFIX expression that occurs when DNA is delivered by the ADV-PLL–DNA complex results in levels that approach 1 μg per 10^6 cells per day. This is 25 times the amount of expression that occurs with free ADV combined with ASOR-PLL–DNA at the same titer.

We have shown (25) that DNA can be delivered efficiently *in vitro* to primary hepatocytes with the help of a nonconjugated ADV and that the DNA can be delivered in sufficient quantities to allow for the complete replacement of phenylalanine hydroxylase (PAH) activity in PAH-deficient hepatocytes. Although this delivery works efficiently *in vitro*, it is not suitable for delivery *in vivo*, since it depends on a chance event that the ASOR-PLL–DNA complex and the ADV are internalized in the same cell and in the same endosome. The ADV-PLL conjugate increases the efficiency of the delivery system since the internalization of the DNA toroid is accompanied by the viral component.

The modification of the ADV with low EDC does not affect the ability of the ADV to interact with cells, since ADV-PLL–DNA complexes made with the low EDC modified ADV give β-gal expression in 100% of the cells. Since competition with free ASOR decreases the number of cells expressing β-gal to only 80%, the majority of DNA that is delivered occurs through the ADV receptor. This is also in agreement with the results of complexes made with PLL only, in that 80% of the cells express β-gal at 10^3 particles per cell. Complexes made with the high EDC modified ADV show lower levels of cells expressing β-gal, which may be due to the modification of the ADV, such that its interaction with the cell through its own receptor is reduced and allows delivery to occur primarily through the ASOR receptor. This has been confirmed by the competition with free ASOR, which results in a decrease in positive staining cells from 80% to 30%. It is also in agreement with the delivery of DNA by complexes made with PLL only, which shows 37% of the cells staining positive at 10^3 particles per cell. The fact that

the percentage of cells expressing β-gal cannot be competed to <30% indicates that the complex must be entering the cell through the ADV receptor. This result is similar to those found by Micheal *et al.* (30) in that the use of a blocking antibody against the fiber protein of the ADV still resulted in 20–25% of the ADV binding and being internalized into the cell. Analysis by Wickham *et al.* (29) has also identified that the ADV coat proteins interact with two receptors, one for attachment and one for internalization. Since the modification presented here is a general addition of the PLL to the viral coat, we have not determined which portion of the virus has been modified. As a result, the high EDC modification may change the normal route that the virus uses for entry into the cell. Since the modification results in two types of modified ADV, DNA delivery is not limited to the liver and hepatocytes, but may also include other cells and organs. The low EDC modified ADV could be used to efficiently deliver DNA into cells that have ADV receptors. In contrast the high EDC modified ADV can be used to deliver DNA to a specific cell type by using a cell-specific ligand. Gao *et al.* (16) have shown that the modified ADV can be used for delivery to the airway epithelium, through both the ADV receptor and the transferrin receptor.

The procedure involved in making the ADV-PLL–DNA complexes that is presented here is simple. As a result these complexes are more advantageous for gene delivery than recombinant retrovirus and ADV vectors. Recombinant viral vectors are limited by the size constraints that are placed upon the packaged nucleic acid and also have safety concerns that have been worked out for retrovirus, but have been worked out to a lesser degree for ADV. Targeted DNA delivery systems, while being simple to make and relatively safe, do suffer from fundamental problems. (*i*) The DNA delivered does not persist for long periods of time without any means to promote persistence, such as a surgical partial hepatectomy (18). (*ii*) In the *in vivo* setting the size of the complex, which is a minimum of 100–300 nm, may be a limitation. The use of these complexes for hepatic delivery may be limited by the diameter of the fenestrations in the liver (31). The ADV-PLL–DNA complex must be <200 nm to reach the hepatocyte *in vivo*.

Further improvements of DNA–protein complexes should address the major limitations of the system, such as DNA plasmids that replicate or integrate to promote persistence of the DNA. Also, the size limitation of the complex designed for liver targeting could be altered by replacing the lysis ability of ADV with proteins or peptides that perform a similar lysis process (32). This last point is important in that there is the potential of constructing completely synthetic vehicles for gene delivery that would make the system much more attractive for pharmaceutical development.

The ADV-PLL–DNA complexes are capable of generating efficient levels of expression as we have shown here for cFIX, which is in the range of 0.5–1 μg per 10^6 hepatocytes per day and comparable to recombinant retroviral vectors for factor IX gene delivery and expression (1). By using a recombinant retroviral vector for cFIX gene delivery to hemophilia B dogs, we have reported (24) that cFIX as low as 2–5 ng/ml of plasma in the treated dogs results in partial phenotypic correction of hemophilia B (24). Thus, the DNA delivery system could potentially lead to similar levels of factor IX gene expression. If successful, it could also be used to treat other metabolic disorders secondary to hepatic deficiencies, such as phenylketonuria, familial hypercholesterolemia, etc. In summary, we have shown that the ADV can be conjugated to PLL by a simple chemical modification procedure that enables its direct coupling with DNA toroids. These ADV-PLL–DNA complexes result in efficient DNA delivery to cells at lower ADV titers that are not toxic to the cell but still allow high-level gene expression. The method shows great promise for efficient gene delivery to the liver for the future correction of hepatic disorders by gene therapy.

We thank X.-H. Chen, F. Leland, and D. Turner for their technical assistance. This work was supported in part by National Institutes of Health (NIH) Program Project Grants DK 44080 and HL 27341 and NIH Grant CA 41424. R.J.C. is the recipient of National Research Service Award GM 14597-01/01, and S.L.C.W. is an Investigator of the Howard Hughes Medical Institute.

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