Biochemical and Functional Characterization of DNA Complexes Capable of Targeting Genes to Hepatocytes via the Asialoglycoprotein Receptor*

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Electrostatic binding of polycations or basic polypeptides to the DNA phosphate backbone has been previously described as a one-step process which results in uncontrolled aggregation and precipitation of the DNA in solution. We describe here a multistep process in which the condensation of DNA in the presence of poly-L-lysine can be controlled to produce particles of discrete size and shape suitable for receptor-mediated gene transfer *in vivo* and *in vitro*. The first step in this process involves the gradual accretion of poly-L-lysine onto the DNA phosphate backbone, until charges are neutralized. The addition of poly-L-lysine to a concentrated solution of DNA in this fashion prevents intermolecular aggregation of the DNA, presumably by promoting the formation of a nucleus of condensation along the length of each DNA molecule. The second stage of the process involves adjusting the ionic strength of the solvent to facilitate the solubilization of compact DNA·poly-L-lysine complexes. Several physical and biochemical parameters have been studied and correlated with the efficacy of DNA/ligand-poly-L-lysine particles in transferring genes to the liver of adult animals by receptor-mediated endocytosis.

Functional genes can be introduced into mammalian cells *in vitro* by a variety of physical methods, including direct microinjection, electroporation, and co-precipitation with calcium phosphate. Most of these techniques, however, are impractical for delivering genes into tissues of intact animals. In contrast, receptor-mediated gene transfer has been shown to successfully introduce DNA into suitable recipient cells, both *in vitro* and *in vivo* (1–12). This procedure involves the formation of a complex between DNA and a polycation (such as poly-L-lysine), which bears a covalently linked ligand moiety specific for a given receptor on the surface of cells in the target tissue. The gene is internalized by the tissue, transported to the nucleus,

and expressed in the cell for varying lengths of time (1, 3, 6, 11). The overall level of expression of the transgene in the target tissue is dependent on several factors, such as the stability of the DNA/ligand·poly-L-lysine complex, the presence and number of specific receptors on the surface of the targeted cell, the receptor-DNA/ligand interaction, endocytosis of the DNA complex and the efficiency of gene transcription in the nucleus of the target cells.

DNA in the nucleus of a higher eukaryote is intimately associated with basic nuclear proteins rich in lysine (*i.e.* histones) or arginine (*i.e.* protamines). The interaction of DNA with these basic proteins is responsible for the control of the condensation process that occurs upon chromosome formation during metaphase and is thought to play a role in the regulation of gene expression. DNA condensation, which occurs naturally in viruses, bacteria, and eukaryote nuclei, has been extremely difficult to reproduce in the laboratory (13, 14). Due to the high negative charge of the DNA phosphate backbone, an increase in the degree of charge neutralization of the DNA theoretically results in extensive condensation and the separation of the DNA phase in the form of insoluble compact structures (15, 16). We have found, however, that the structure and stoichiometry of DNA·polycation complexes in solution can be manipulated by means of the process by which DNA·cationic polypeptide complexes are formed.

Specific complexes of DNA (Ψ-DNA) are formed with cationic homo-polypeptides (poly-L-lysine, poly-L-arginine, or poly-L-or-nithine) after "annealing" both components in a step-down dialysis from NaCl concentrations of 3 to 0.010 M (11, 16). In contrast, direct addition of basic polypeptides to DNA at physiological salt concentrations results in reversible molecular aggregation and the formation of precipitates (7, 17, 18). Shapiro *et al.* (16) elucidated changes in DNA secondary structure in DNA·poly-L-lysine complexes prepared by directly mixing poly-L-lysine and DNA. The physical properties of the resulting soluble complexes were investigated by circular dichroism (CD) and optical rotatory dispersion. A change in the magnitude of the molar residue rotation was found, with a characteristic red shift and a strong negative rotatory transition centered near 269 nm. The average diameter of the molecular complexes of DNA and polycation was 340 nm, with an estimated dry mass corresponding to 70 nucleic acid/polypeptide molecules per particle. The changes in optical activity noted in these studies probably arose from the formation of Ψ-DNA consisting of multiple DNA molecules in a higher order molecular complex (15, 19–21).

Gosule and Schellman (22–25) showed that the condensation of very low concentrations of λ phage DNA (1 μg/ml) by interaction with spermine resulted in compact structures similar to those reported previously by Shapiro *et al.* (16), with the sig-

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nificant distinction that the complexes had a unimolecular structure consisting of a single molecule of DNA condensed to a maximum diameter of about 50 nm. CD analysis indicated that there was no perturbation of the DNA helical conformation (B-DNA) nor was there aggregation of multiple molecules of DNA into higher order complexes.

To be useful for gene therapy, the condensed DNA complex prepared *in vitro* must contain a relatively high concentration of DNA, be stable in the blood, and yet retain the critical structural features necessary for the interaction with the targeted receptor. In order to satisfy these conditions it is important that the DNA be condensed into complexes of a minimum size and not into large, multimolecular DNA complexes (aggregated DNA and Ψ-DNA), which are nonspecifically taken up *in vivo* by macrophages and degraded. We have previously described a procedure to generate DNA-cationic polypeptide complexes of defined size (12 nm) (1). These complexes are capable of introducing functional genes into targeted cells both *in vitro* and *in vivo* by receptor-mediated endocytosis. In the current report we have studied the process by which the DNA is condensed with galactosylated poly-L-lysine using absorption spectrophotometry, turbidity, circular dichroism (CD), and electron microscopy (EM) and correlated with the functional activity of the various DNA-galactosylated poly-L-lysine complexes for receptor-mediated endocytosis into cells *in vitro* and *in vivo*.

EXPERIMENTAL PROCEDURES

Materials—High performance liquid chromatography grade water was used to prepare all solutions (Fisher). DNA-modifying enzymes, nucleotides and 5-bromo-4-chloro-3-indolyl-β-D-galactopyranoside were purchased from Boehringer Mannheim. All chemicals, including poly-L-lysine and α-D-galactopyranosyl phenylisothiocyanate, were obtained from Sigma. The luciferase assay system was obtained from Promega. All media, sera, and antibiotics were obtained from Life Technologies, Inc.

Reporter Genes and Plasmid Preparation—The expression plasmid pGL2 contained the SV40 viral promoter and enhancer ligated to the *Photinus pyralis* luciferase gene, and inserted into the pUC19 vector (Promega). The plasmid pRSV*lacZ* (26) consisting of the Rous sarcoma virus (RSV)[1] promoter linked to the *Escherichia coli lacZ* gene was also used as a reporter. The plasmid pPEPCK-hFIX has been previously described (1) and consists of the PEPCK promoter linked to the cDNA for hFIX. The plasmids were grown in *E. coli* DH5α, extracted, and purified by standard techniques (27). No substantial contamination with bacterial genomic DNA or RNA was present in the plasmid preparations. All plasmid preparations contained less than 30% open circular or linear DNA.

Cells and Cell Culture—HepG2 human hepatoma cells were grown in modified Eagle's medium, supplemented with 5% calf serum and 5% fetal calf serum. Hu-H7 hepatoma cells were grown in RPMI medium supplemented with 10% fetal calf serum. DNA transfection was performed when the cells reached approximately 50% confluence. Fifty microliters of 1.0 M $CaCl_2$ (to a final concentration of 3 mM) was added to the culture medium at the time of transfection. 60-mm plates were transfected using 5 μg of DNA added directly to 3 ml of culture medium. Primary cultures of mice hepatocytes were obtained as described (28). Cells were cultured in 10% fetal calf serum Waymouth's MB medium (Life Technologies, Inc.) and plated in Type I collagen-coated 60-mm plates, and transfected with the DNA complexes containing the RSV-*lacZ* gene 3 days after plating. Calcium phosphate co-precipitation was performed according to the method of Chen and Okayama (29).

Animals—Male Harlan Sprague Dawley rats, weighing approximately 300 g, were anesthetized with ether. Using aseptic techniques, 0.3–0.5 ml of a solution containing 300 μg of DNA-galactosylated poly-L-lysine complex was injected into the caudal vena cava. The rats were killed 2 days after infusion of the DNA and various organs were taken for analysis. The animal research protocol was reviewed and approved

by the Case Western Reserve University Institutional Animal Care Committee.

Production of Galactosylated Poly-L-lysine—Poly-L-lysine was galactosylated as described previously (30). Briefly, 2 mg of poly-L-lysine-HBr (Sigma P-2636 with an average of 236 lysine residues per polypeptide) was reacted with 85 μg of α-D-galactopyranosyl phenylisothiocianate (Sigma G-3266) (5 mg/ml) in a 50% *N,N*-dimethylformamide, 50% water solution. The reaction was adjusted to pH 9 by the addition of one-tenth volume of 1 M sodium carbonate, pH 9, to make a final volume of 2 ml. The tube was shielded from light with aluminum foil and mixed for 16 h at room temperature, then dialyzed using Spectra-Por dialysis tubing (3500 M_r cutoff), against 500 ml of distilled water for 2 days with frequent changes of water (4 changes/day). The reaction is stoichiometric with respect to the limiting amounts of α-D-galactopyranosyl phenylisothiocianate, and resulted in the galactosylation of 0.8–1% of the NH_4^+ groups present in the solution. Ionic exchange chromatography confirmed that the reaction proceeds to completion, since all of the added α-D-galactopyranosyl phenylisothiocianate was conjugated to the poly-L-lysine moiety (data not shown). The amount of galactose derivative present in the galactosylated poly-L-lysine can be calculated from internal standard concentrations of galactose derivative by measuring their absorbance at 254 nm.

Plasmid DNA Preparation—Plasmid DNA was prepared using standard CsCl gradient centrifugation (27). DNA was precipitated twice using one-tenth volume of (v/v) 3 M sodium acetate, pH 7, and 2.5 volumes of -40 °C ethanol. The DNA was re-suspended in 10 mM Tris-HCl, pH 8.0, containing 1 mM EDTA and the concentration of the DNA determined spectrophotometrically. The DNA preparation was treated twice with RNase A + T1. DNA was resuspended to a final concentration of 1.5–2 mg/ml.

Production of Ψ-DNA—Ψ-DNA was obtained essentially as described (17). In brief, 100 μg of DNA in HEPES, 1.5 M NaCl (pH 7.5) was mixed with galactosylated poly-L-lysine in HEPES, 1.5 M NaCl (pH 7.5) at a 1:1 ratio with respect to charge equivalents. Each component had a volume of 250 μl. Thirty minutes after mixing the DNA and poly-L-lysine, the DNA complex was dialyzed against HEPES, 150 mM NaCl (pH 7.5) for 16 h at room temperature.

Formation of Aggregated, Condensed, and Relaxed DNA·Poly-L-lysine Complexes—For animal, electron microscopy and circular dichroism studies, the following basic protocol was used to generate DNA·poly-L-lysine complexes in the indicated structural states. Three hundred micrograms of DNA (200 μl final volume) adjusted to 0.75 M NaCl in a 1.5-ml microcentrifuge tube was vortexed at medium speed, using a VIBRAX shaking apparatus (VIBRAX-VXR, IKA Works Inc., Wilmington, NC). One hundred and twenty microliters of galactosylated poly-L-lysine adjusted to 0.75 M NaCl (200 μl final volume) was added over a period of 1–1.5 h. Five-microliter aliquots were added dropwise every 5 min. This generates a molar ratio of 1 DNA PO_4^- group to 1 lysine NH_4^+ group in the final complex. The solution becomes turbid at the end of the addition process (aggregated DNA complex). Three-microliter aliquots of 5 M NaCl were then added dropwise to the vortexing solution until turbidity disappears as monitored by eye. The addition of NaCl is performed slowly, allowing 5 min between the addition of each aliquot. The structure of DNA-galactosylated poly-L-lysine complexes was monitored either by CD or directly visualized using a JEOL-100C electron microscope. Stepwise addition of 5 M NaCl resulted in the structural changes to the complexes shown in Fig. 6. The formation of highly condensed DNA·poly-L-lysine complexes is complete when the diagnostic CD spectrum of condensed DNA·poly-L-lysine complex is observed (see Fig. 1*D*), and the appropriate structure visualized by EM (see Fig. 6*D*). Further addition of concentrated NaCl results in the formation of relaxed DNA complexes. For subsequent preparations of DNA·poly-L-lysine complex consisting of the same plasmid DNA at the same concentration, the NaCl addition protocol established during the initial condensation reaction can be repeated without CD or EM evaluation; the process is reproducible. However, if different concentrations of DNA or a different plasmid are used, the condensation process should be re-evaluated using CD and EM.

For absorbance and cell culture experiments, the following basic protocol was used to generate DNA·poly-L-lysine complexes. Sixty micrograms of DNA in 350 μl, adjusted to 0.4 M NaCl, is vortexed in a 1.5-ml microcentrifuge tube at medium speed, using a VIBRAX shaking apparatus. Twenty-four micrograms of galactosylated poly-L-lysine in 350 μl adjusted to 0.4 M NaCl is added dropwise over a period of 1–1.5 h in 15-μl aliquots; slowly mixing 15-μl aliquots every 5 min. This generates a molar ratio of 1 DNA PO_4^- group to 1 lysine NH_4^+ group in the final complex. The solution is clear to the eye at the end of the process. Three-microliter aliquots of 5 M NaCl are

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[1] The abbreviations used are: RSV, Rous sarcoma virus; PEPCK, phosphoenolpyruvate carboxykinase; SV40, simian virus 40; hFIX, human factor IX; CD, circular dichroism; EM, electron microscopy; LDL, low density lipoprotein; ASGP, asialoglycoprotein.

then added dropwise to the vortexing solution while monitored at 260 and 400 nm. The addition of salt is performed slowly, allowing 5 min between the addition of each aliquot.

Circular Dichroism—The circular dichroism spectra of the complexes were obtained on a JASCO spectropolarimeter using a 0.1-cm path cell and the ellipticity values (Θ) are given in terms of millidegrees. The ellipticity value for the non-buffered NaCl solutions was subtracted from the experimental values for the DNA·poly-L-lysine complexes.

Electron Microscopy—Aqueous 2% uranyl acetate was used in these studies as a contrast stain. DNA·poly-L-lysine complex preparations were diluted immediately prior to staining to 50 μg/ml in 150 mM NaCl. Samples were applied for 3 min to a copper electron microscope grid containing a carbon film, blotted, and allowed to dry for 30 s. A drop of the staining solution was then applied for 1 min, blotted, washed for 1 s in high performance liquid chromatography grade water and allowed to dry. The grids were examined in a JEOL-100C microscope and plates were exposed to the image at a magnification of $\times$ 50,000. The microscope was calibrated by the use of 87.5-Å spacing of catalase crystals (31).

Assay for Luciferase Activity—Rats injected with the DNA-galactosylated poly-L-lysine complex and control animals were killed and the tissues of interest were perfused *in situ* with 50 ml of cold phosphate-buffered saline (pH 7.5). The tissues were then homogenized using a Dounce homogenizer in lysis buffer (Promega) and incubated at 22 °C for 10 min. The cells lysates were subsequently centrifuged for 5 min at 4 °C, and the protein extracts were analyzed for luciferase activity following instructions of the manufacturer. The lysates were assayed for protein content and the measured integrated light units over a 10-s interval were standardized for protein content. All measurements were performed in triplicate and expressed as an average $\pm$ S.E. (S.E. of the mean).

Assay for β-Galactosidase Activity—Individual cells expressing β-galactosidase were identified following incubation with 5-bromo-4-chloro-3-indolyl-β-galactopyranoside. Forty-eight hours after transfection with either the DNA-galactosylated poly-L-lysine complexes or CaPO$_4$ precipitates, the cells were fixed with a solution of 1% glutaraldehyde in phosphate-buffered saline for 10 min, and then incubated with a solution containing 0.5% 5-bromo-4-chloro-3-indolyl-β-galactopyranoside for 8–12 h at 37 °C. Blue-colored cells were identified by light microscopy. A minimum of 100 cells in tissue culture were counted to determine the percentage of cells expressing β-galactosidase.

Assay for hFIX Concentration—An indirect enzyme-linked immunosorbent assay specific for hFIX protein was utilized to quantitate hFIX protein in the incubation medium of Hu-H7 cells. Microtiter plates were coated overnight at 4 °C with 10 μg of the coating antibody (HFIXα-40, Hematologic Technologies Inc., Essex, VT) in Tris-HCl-buffered saline. The next day the wells were washed twice with 200 μl of Tris-buffered saline, 0.1% Tween 20 and nonspecific binding blocked for 2 h at room temperature using RPMI, 10% fetal calf serum. Media samples (50 μl) from Hu-H7 cultured cells were obtained after administration of the DNA complex solution and applied to the wells. After a 2-h incubation at room temperature, the wells were washed 3 times with 200 μl of washing solution (Tris-buffered saline, 0.1% Tween 20). A 1:1,000 dilution in RPMI of a rabbit anti-hFIX polyclonal IgG (Calbiochem Inc., San Diego, CA) was then incubated for 1 h at room temperature and then washed 3 times with the washing buffer. One hundred microliters of a 1:2,000 dilution in RPMI of a goat anti-rabbit IgG-conjugated horseradish peroxidase (Boehringer Mannheim) was then incubated for 1 h at room temperature and washed 5 times with the washing buffer. One hundred microliters of the substrate (3,3′,5,5′-tetramethylbenzidine) was then added and 30 min later the reaction was stopped using 50 μl of 0.5 M H$_2$SO$_4$. Spectrophotometric readings were taken at 450 nm. Purified hFIX (American Diagnostica Inc., Greenwich, CT) diluted into RPMI, 10% fetal calf serum was used to generate standard curves for each experiment. Absorbance values were linear within the range of concentrations of hFIX obtained in these experiments.

RESULTS

Physical Characterization of Condensed DNA/Poly-L-lysine Particles in Solution Using Circular Dichroism and Electron Microcospy

Electrostatic binding of polycations or basic polypeptides to the DNA phosphate backbone at a 1:1 stoichiometric charge ratio results in uncontrolled precipitation of the DNA when mixing of the DNA and polycation occurs rapidly (15, 16).
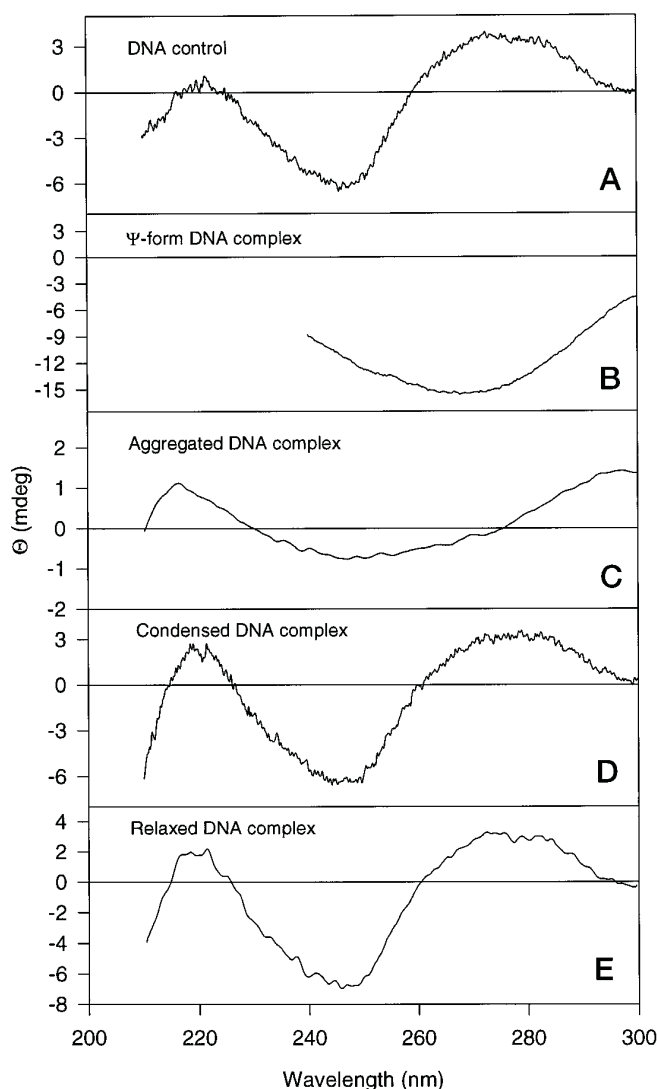
FIG. 1. **Ellipticity *versus* wavelength spectra of DNA-galactosylated poly-L-lysine complexes prepared using different conditions for the preparation of the complex.** CD spectra were taken in a 0.1-cm path cuvette. The DNA was complexed with poly-L-lysine at identical molar ratios of amino to phosphate groups and various CD spectra compared: *A*, standard control for DNA in 1 M NaCl; *B,* Ψ-DNA as observed at a concentration of NaCl at which multimolecular condensation occurs (see Fig. 6); *C*, aggregated DNA shows turbidity and decreased ellipticity; *D*, condensed, unimolecular complexes of DNA; *E*, relaxed DNA complex spectrum. The spectra was taken at equal concentrations of polymer and the signal for the buffer was subtracted in each case. details of the assay are presented under "Experimental Procedures."

Changes in DNA secondary structure associated with the binding of cationic polypeptides to the DNA have been studied using CD (19). With either of two mixing protocols, annealing of both components in a step-down salt gradient (11, 16, 19) or direct mixing of the polycation and the DNA (7, 17), DNA is condensed into ordered particles as indicated by an inversion of the ellipticity maxima at 270 nm observed in the CD spectrum, as compared to that of B-DNA in aqueous solution (Fig. 1*B*). This feature in the CD spectrum is characteristic of Ψ-DNA and indicates chiral packing of multiple DNA helices in solution under charge-neutralized conditions (32). The aberrant optical spectrum is thought to be due to base stacking in the same optical plane, and is observed in solutions of DNA/poly-L-lysine in the absence of turbidity.

To prepare a suitable ligand for the introduction of genes by receptor-mediated gene transfer targeting the asialoglycopro-

TABLE I
Estimated and experimental size of condensed DNA complexes

DNA	Size	Condensed diameter (nm ± S.D.)		
		Electron microscope[a]	Hydrated model (partial specific volume)[b]	Hydrated model (x-ray diffraction density)[c]
	bp			
PEPCK-hFIX	4,500	12.8 ± 1.56	18	22
PEPCK-hOTC	5,300	18 ± 1.83	20	23
SV40-luciferase	5,600	16.95 ± 3.5	20	24
PEPCK-CAT	5,800	16.3 ± 2.56	20	24
CMV-hLDLs	7,400	20.7 ± 2.6	22	26
$\phi 29$[d]	18,000	38[e]	40	47

[a] Measured diameter of at least 10 DNA complexes in a printed photograph ($\times$240,000).

[b] Calculated diameter of a unimolecular DNA complex assuming a condensed sphere. The partial specific volume of Na-DNA was used as 0.5 ml/g (33). The contribution of galactosylated poly-L-lysine at a charge ratio of 1:1 has been added. The molecular mass of DNA was calculated based on an average molecular weight of 6,500 dalton/10 base pairs.

[c] Calculated diameter of a unimolecular DNA complex assuming a condensed sphere. The calculation assumed a hydrated density of 1.25 ± 0.1 g/ml as determined by x-ray diffraction (32). The contribution of galactosylated poly-L-lysine at a charge ratio of 1:1 has been added. The molecular weight of DNA was calculated based on an average molecular mass of 6,500 daltons/10 base pairs.

[d] From the literature (50).

[e] The size of the phage prohead includes the protein out-shell (50).

tein (ASGP) receptor of hepatocytes, we have chemically coupled galactose molecules to the poly-L-lysine moiety by galactopyranosyl phenylisothiocianate derivation of 1% of the ϵ-amino groups present in the poly-L-lysine molecule. The structural features of DNA-galactosylated poly-L-lysine complexes prepared by direct mixing were investigated using EM; we observed condensed DNA particles with diameters ranging from 50 to 200 nm (Fig. 6*B*). Based on the previously described density of Ψ-DNA (32) we have calculated that these heterogeneous DNA particles contain from 5 to 20 plasmid DNA molecules in addition to the poly-L-lysine component. This result indicates that condensation of DNA under the conditions described results in the expected aggregation of multiple DNA fibers into electron dense particles of heterogeneous size, and with optical properties characteristic of Ψ-DNA.

To prevent aggregation of DNA at high concentration in the presence of galactosylated poly-L-lysine, we have developed a novel multistep process in which the polycation is added slowly to a vortexing solution of DNA at high ionic strength. We hypothesize that binding of substoichiometric amounts of galactosylated poly-L-lysine to DNA avoids multimolecular aggregation by forming a nucleus of poly-L-lysine condensation along the length of each molecule of DNA. Further addition of galactosylated poly-L-lysine up to a 1:1 stoichiometric charge ratio results in the formation of aggregates of unimolecular complexes of DNA and galactosylated poly-L-lysine (aggregated DNA complex), that are dispersed in the final step by increasing the ionic strength of the solution with NaCl to a final concentration of approximately 1.1 M. DNA-galactosylated poly-L-lysine complexes formed under these conditions have an average diameter of about 17 nm (see Fig. 6*D*), and have a CD spectrum indistinguishable from that of free B-DNA in solution (compare Fig. 1*D* with 1*A*). We conclude that these conditions prevent the perturbation of DNA secondary structure characteristic of multimolecular chiral packing (Fig. 1*B*).

DNA-galactosylated poly-L-lysine complexes were examined in the EM at different steps during the condensation process. DNA complexes range from 12 to 20 nm in diameter (see Table I) when dispersed at the critical NaCl concentration (for example, Fig. 6*D* shows condensed DNA complexes containing the SV40-luciferase gene). Based on the measured density of compacted DNA (1.25 g/ml; see Table I) or in the partial specific volume of sodium B-DNA (0.5 ml/g; see Table I) (33) in a variety of bacteriophages and Ψ-form DNA species, our data suggests that the observed particles consist of a single unit of DNA plasmid (Fig. 6*D*; see Table I). These DNA complexes are referred to as *unimolecular*. At lower NaCl concentrations the

complexes are more heterogeneous in size, and appear to be formed by multiple unimolecular DNA particles (Fig. 6*C*). These structures, which are associated with a high degree of turbidity in solution, are referred to as *aggregated* DNA complexes.

Continuing addition of NaCl to the solution of condensed DNA·poly-L-lysine complexes results in the exchange of poly-L-lysine from the DNA backbone with NaCl. This results in the liberation of the DNA from electrostatic interaction with the poly-L-lysine moiety and the decompaction of the DNA complex (Fig. 6*E*). The electron micrograph shows a mixed population of free DNA fibers and loosely condensed DNA·poly-L-lysine complexes (see *arrow* in Fig. 6*E*). While these structures are clearly identifiable using EM, there is no characteristic CD spectrum associated with them (Fig. 1*E*). These structures, which are formed when the ionic strength of the solution exceeds a critical concentration, are referred to as *relaxed* DNA complexes.

Characterization of the DNA/Poly-L-lysine Complexes Using Ultraviolet and Visible Spectrophotometry

Since ionic strength is crucial to the multistep condensation protocol, we have employed spectrophotometric techniques to follow changes in the state of aggregation of the DNA complexes upon poly-L-lysine binding as a function of salt concentration. First the turbidity of the solution was determined spectrophotometrically by monitoring absorbance at 400 nm. Fig. 2 illustrates the effect of adding poly-L-lysine to the DNA solution at different starting concentrations of NaCl, on the absorbance at 400 nm. The turbidity increases as the initial concentration of salt is increased (this could be easily confirmed by eye) (Fig. 2), and on further addition of salt, the absorbance at 400 nm decreases to its minimum value. The solutions of DNA condensed at the lowest salt concentration used in our experiments (200 mM) contained precipitated DNA in suspension. At higher initial concentrations of NaCl, the absorbance at 400 nm increases; there were no visible precipitated DNA fibers, although the solution was highly turbid. Solutions containing the DNA·poly-L-lysine complex with different degrees of turbidity were analyzed by EM to visualize the DNA structures formed in each situation. Appropriately condensed, unimolecular DNA complexes were found in both clear and slightly turbid solutions (data not shown). Thus, a loss of turbidity as evaluated by a decrease in absorbance at 400 nm, is not an unequivocal predictor of dispersion of the aggregated DNA complexes.

A more reliable technique for diagnosing the structural transition of DNA-galactosylated poly-L-lysine complexes from ag-
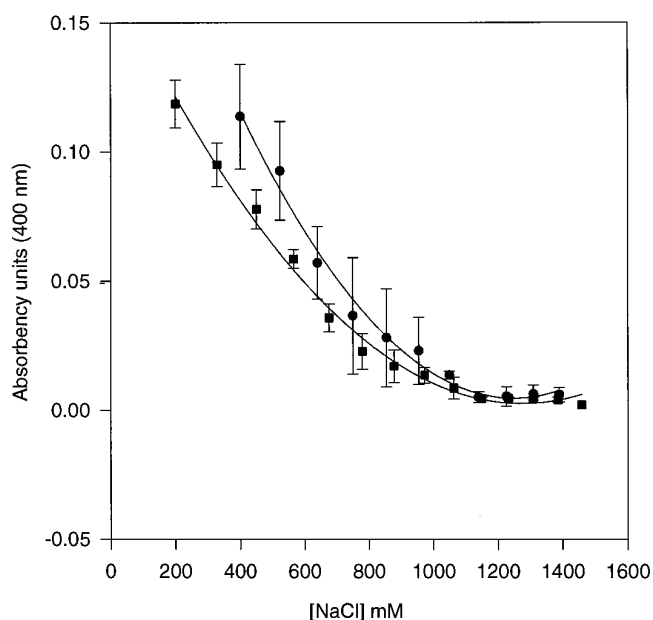
FIG. 2. **Changes in the turbidity of solutions containing DNA·poly-L-lysine complexes as a function of the concentration of NaCl.** A plasmid containing the chimeric PEPCK-hFIX gene was condensed with poly-L-lysine, using the procedure described in detail under "Experimental Procedures." After the addition of poly-L-lysine the absorbance of the solution at 400 nm was determined. Concentrated NaCl was then added stepwise and the absorbance determined. 200 (■]) and 400 mM (●) *initial* concentrations of NaCl were used to generate the figure. Data represents three independent determinations ± S.D.

FIG. 3. **The relationship between changes in the absorbance of the DNA complexes during the condensation process and the biological activity of the DNA.** A plasmid containing the chimeric PEPCK-hFIX gene was condensed with galactosylated poly-L-lysine, using the procedure described in detail under "Experimental Procedures." After the addition of poly-L-lysine the absorbance of the solution at 260 nm was determined. Concentrated NaCl was then added stepwise and the absorbance determined. The initial NaCl concentrations used in the condensation reaction were 200, 400, and 600 mM. Aliquots of the DNA solution were removed at various NaCl concentrations and used to determine the uptake and expression of the gene for PEPCK-hFIX by Hu-H7 cells. The concentration of hFIX in the incubation medium 5 days after transfection was determined by enzyme-linked immunosorbent assay and is plotted on the right axis (*open bars*). The background level of hFIX using this enzyme-linked immunosorbent assay is 0.1 ng/ml. Cells transfected using nongalactosylated DNA·poly-L-lysine complexes produced no hFIX above 0.1 ng/ml (*striped bars*).

gregated to dispersed unimolecular particles with increased concentration of NaCl can be achieved by monitoring the absorbance of the complex at 260 nm. The DNA·poly-L-lysine complex in suspension at the initial concentration of NaCl has only about 20% of the expected absorbance because the aggregated particles do not absorb at 260 nm. The addition of NaCl disperses the DNA·poly-L-lysine complex in suspension and then causes a steep increase in the absorbance (Fig. 3). At this point the solution is clear. The rapid shift in the absorbance of the DNA-galactosylated poly-L-lysine complex at 260 nm correlates with the structural shifts shown in Fig. 6. At a concentration of NaCl (1.1 M) that results in an absorbance at 260 nm which is just below the observed maxima, we noted unimolecular complexes by EM (Fig. 6D). Below 1.1 M NaCl the DNA·poly-L-lysine complex appeared aggregated (Fig. 6C), whereas above 1.1 M the DNA·poly-L-lysine complex appeared relaxed (Fig. 6E). The increased absorbance at 260 nm is indicative of the full solubilization and partial disruption of the DNA complex, whereas at salt concentrations below those resulting in the sharp increase in absorbance at 260 nm, the condensed DNA·poly-L-lysine complex is a colloidal solution with minimal absorbance at 260 nm.

The biological activity of the various DNA complexes formed at salt concentrations spanning the absorbance transition shown on Fig. 3 was determined by transfection of a 4.5-kilobase plasmid containing the promoter from the gene for PEPCK linked to the structural gene for human factor IX (hFIX) (6) into Hu-H7 human hepatoma cells. The PEPCK-hFIX gene was condensed with galactosylated poly-L-lysine to target the asialoglycoprotein receptor present on the Hu-H7 cells. There was increased hFIX production into the culture medium after transfection with DNA complexes at ionic strengths spanning the exponential transition of absorbance values at 260 nm. The maximum level of expression of hFIX was noted at a concentration of 1.1 M. At NaCl concentrations below 1 M and above 1.1 M the level of production of hFIX was
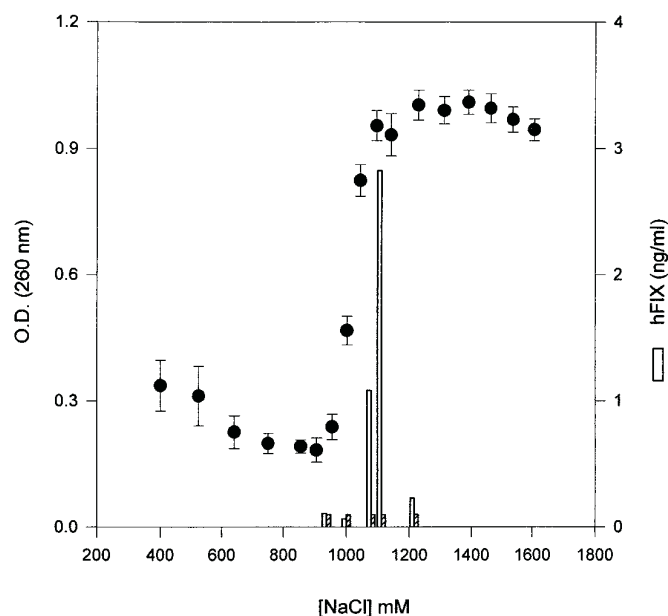
close to basal. As will be discussed in detail below, the concentration of NaCl at which the DNA-galactosylated poly-L-lysine complexes produce maximal levels of hFIX production in Hu-H7 cells correlates with the observation of unimolecular complexes in the EM. We thus conclude that the generation of unimolecular DNA-galactosylated poly-L-lysine complexes with full biological function can be monitored by absorbance at 260 nm and that the formation of a unimolecular complex between the DNA and the galactosylated poly-L-lysine is critical for the uptake and/or expression of the transgene in hepatoma cells.

Variables Involved in the Stable Formation of Condensed DNA in Vitro

DNA Concentration—There is a strong correlation between the concentration of NaCl at which the condensed DNA/poly-L-lysine complex remains stable in solution and the concentration of DNA (Fig. 4, *top*). Various quantities of the 4.5-kilobase PEPCK-hFIX gene were condensed with galactosylated poly-L-lysine sufficient to achieve a 1:1 ratio of negative to positive charges. The final concentration of NaCl in each case was that which gave dispersed unimolecular DNA complexes as observed by EM (Fig. 6D), or exhibited a minimum absorbance at 400 nm.

The linear fit of the data (Fig. 4, *top*) using the least square method is described by the following equation.

$$\log [\text{DNA} (\mu\text{M phosphate}) = 2.52 \times 10^{-3} [\text{NaCl (mM)}] + 0.577; \, r^2 = 0.97$$
(Eq. 1)

Because a logarithmic increase in the concentration of DNA results in a linear increase in concentration of NaCl needed for
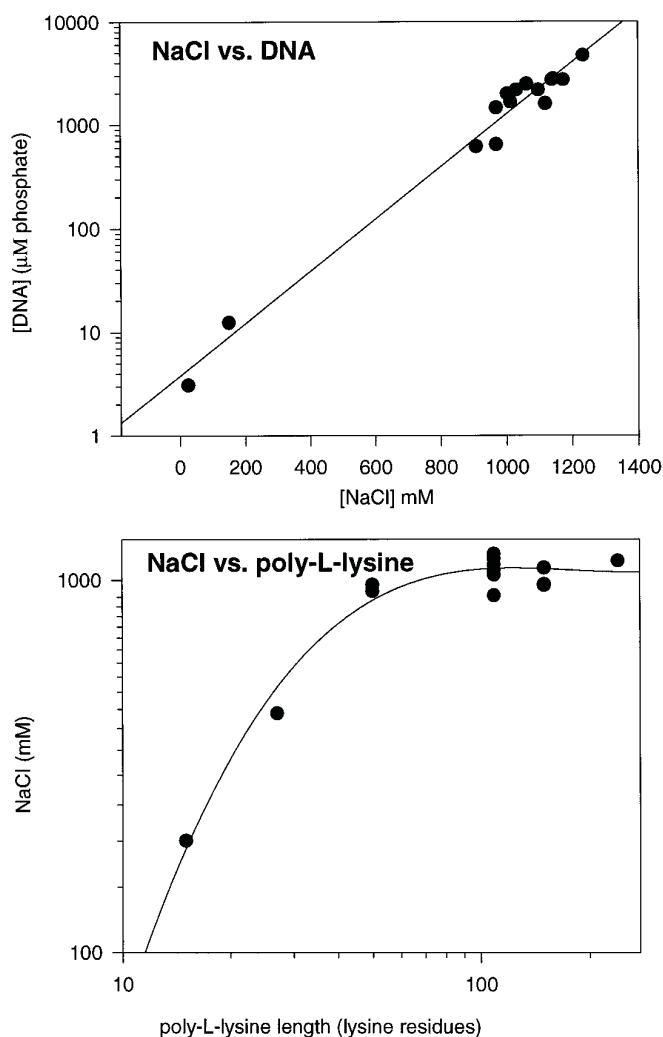
FIG. 4. **The effect of the concentrations of DNA and the length of poly-L-lysine on the concentration of NaCl required for the formation of unimolecular DNA·poly-L-lysine complexes.** This figure presents the results of a number of experiments in which unimolecular DNA·poly-L-lysine complexes were prepared. The formation of unimolecular complexes was determined by EM (see Fig. 6). *Top*, effect of DNA concentration: a variety of plasmids were used at varying concentrations to establish the relationship between DNA and NaCl concentrations in the formation of a condensed DNA complex. *Bottom*, effect of poly-L-lysine length: the length of the poly-L-lysine (lysine residues per polypeptide) was the average molecular weight of various lots of poly-L-lysine obtained from Sigma.

appropriate condensation, there is only a marginal effect on the ionic load in the animal's blood when high concentrations of DNA complex are injected.

DNA Purity—For maximum binding affinity, the DNA was precipitated twice with sodium acetate and 2.5 volumes of −40 °C ethanol (see "Experimental Procedures"). There was no difference in binding affinity of poly-L-lysine for DNA of different topological forms, or for DNA purified using either anionic exchange chromatography or cesium chloride gradient centrifugation (data not shown). However, if the DNA is not prepared as described above, there was a decreased affinity of the poly-L-lysine for DNA, indicated by a decrease in the yield of the precipitated complex formed at various concentrations of poly-L-lysine (34). This may indicate the presence of a contaminant in the DNA preparations with poly-L-lysine binding activity that is eliminated by sequential DNA precipitation.

Poly-L-lysine Length (Lysine Residues per Polypeptide)—The effect of the length of the poly-L-lysine (residues per molecule)

on the concentration of NaCl necessary for the effective condensation of DNA (Fig. 4, *bottom*) was determined using a fixed concentration of various DNA plasmids. A broad range of poly-L-lysine lengths (18–250 lysine residues) were used in these studies, each of which represent the average value of a heterogeneous population of polypeptides determined by low-angle laser light scattering analysis (analysis provided by the manufacturer). The distribution of sizes within a single lot is a source of error in our determinations. Nevertheless, a clear correlation between the average length of the poly-L-lysine and the concentration of NaCl necessary for the condensation of the DNA complex in solution is shown in Fig. 4 (*bottom*). This correlation is a linear function of poly-L-lysine length up to 150 residues, after which the function reaches saturation and there is no increase in the concentration of NaCl needed for the condensation of DNA with longer poly-L-lysine. These data are consistent with cooperative binding between the poly-L-lysine and the DNA phosphate backbone (35). Thus, reducing the length of the poly-L-lysine molecules used to condense the DNA is desirable, since the solution containing the DNA complex will be less hypertonic when injected into the animals.

Functional Characterization of DNA Complexes containing Galactosylated Poly-L-lysine as a Ligand

Ligand Affinity and Endocytosis—The hepatocyte ASGP receptor has an upper size limitation for the uptake of a specific ligand of about 10 (36) or 23 (37–39) nm in diameter, depending on the type of artificial ligand used. This size preference is probably related to the existence of another receptor for galactosylated proteins in the Kupffer cells of the liver (40). The Kupffer cell receptor is very efficient in taking up and degrading galactosylated molecules of larger size *in vivo* and competes for the uptake of the galactosylated DNA complex with the ASGP receptor on the surface of hepatocytes. Thus, the diameter of the ligand-DNA complex must be in the order of 23 nm or smaller to be effective in transferring genes specifically to hepatocytes by receptor-mediated endocytosis via the ASGP receptor (37). Condensed DNA complexes containing galactosylated poly-L-lysine were prepared (Fig. 6D) and used to assess the specificity of binding and internalization of the DNA complex via the asialoglycoprotein receptor present on the surface of HepG2 hepatoma cells in culture. The time course of internalization of the DNA particles was demonstrated by slot-blot hybridization of DNA-galactosylated poly-L-lysine complexes recovered from the culture medium. After 3 h, less than 20% of the input DNA remained in the medium. This process could be blocked by the presence of a 100-fold molar excess of asialo-orosomucoid in the culture media, thus demonstrating first that the DNA complex is inherently stable in the medium over the first 3 h of the assay and second, that the internalization process is specific to the ASGP receptor (Fig. 5).
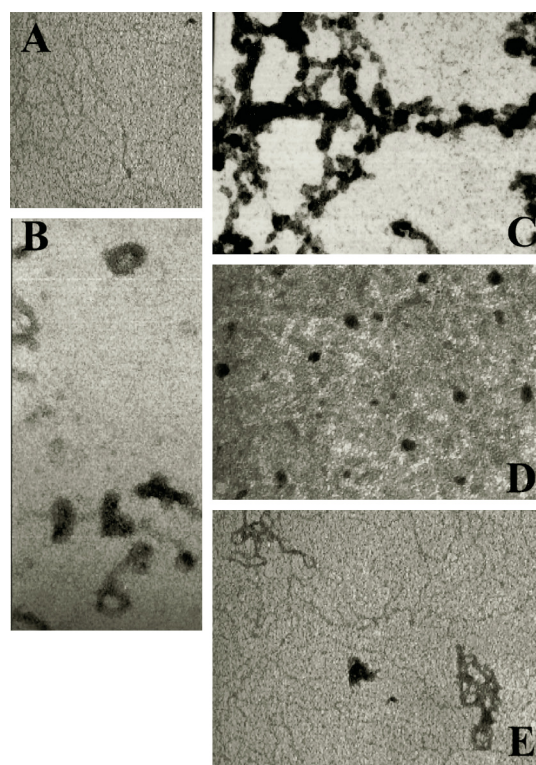
Transfection Efficiency in Vitro—The ability of the condensed DNA-galactosylated poly-L-lysine complex to effectively introduce a fully functional gene into cells *in vitro* was also investigated. Primary cultures of mouse hepatocytes were prepared and placed on type I collagen-coated plates. Cells were transfected using either $CaPO_4$ co-precipitation (27) or with the condensed DNA complex (Fig. 6D). A chimeric gene consisting of the RSV promoter and enhancer elements linked to the structural gene for *E. coli* β-galactosidase was used to evaluate the efficiency of DNA transfection. Forty-eight hours after transfection the cells were fixed and stained *in situ* with 5-bromo-4-chloro-3-indolyl-β-galactopyranoside. The number of transfected cells was 10-fold greater using the delivery system targeting the asialoglycoprotein receptor than using calcium phosphate-precipitated DNA (Fig. 7). Thus, a highly condensed
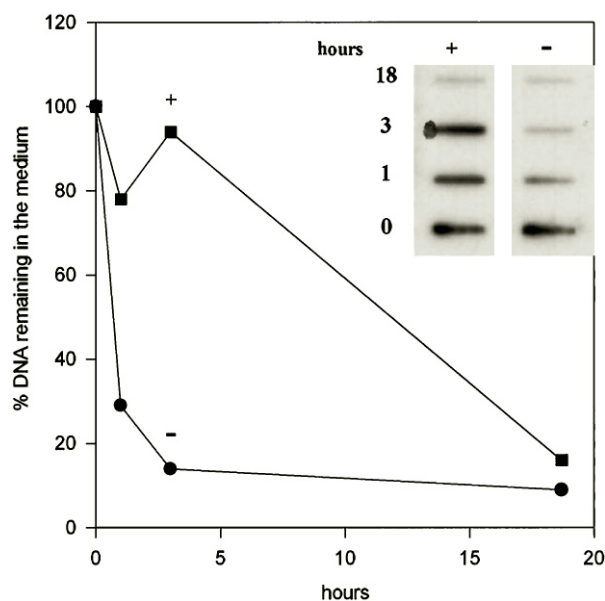
FIG. 5. **Uptake of the galactosylated DNA complex by the ASGP receptor of HepG2 cells; competition for uptake with asialofetuin.** HepG2 cells were grown to confluence and transfected with 10 μg of cytomegalovirus-β-galactosidase complexed to galactosylated poly-L-lysine. The transfections were performed either in the absence (●) of asialofetuin or in the presence of 100-fold molar excess asialofetuin (■). At 0, 1, 3, and 19 h 5 μl of media were collected from the two experimental groups and blotted onto a nitrocellulose membrane with a slot-blot machine (see *inset*). The membrane was hybridized with a ^{32}P-labeled cytomegalovirus-β-galactosidase plasmid probe; 90% of the DNA present is internalized during the first 3 h while the DNA complex internalization is effectively competed by the specific ligand for the ASGP receptor.

FIG. 6. **Structural features of DNA·poly-L-lysine complexes prepared using different conditions.** Electron micrograph of 2% uranyl acetate-stained DNA complexes were prepared as described under "Experimental Procedures." The complexes were fixed and stained within 30 min after their preparation (*A*) uncomplexed DNA (1 μg/ml); *B*, DNA condensed by the method of direct mixing resulting in Ψ-form DNA; *C*, DNA complex prepared at a suboptimal concentration of NaCl (aggregated DNA complex); *D*, DNA complex prepared at an optimal concentration of NaCl (DNA complex condensed); *E*, DNA complex prepared at an above optimal concentration of NaCl (DNA complex relaxed). The *bar* equals 100 nm.

DNA·poly-L-lysine complex, containing galactose as a ligand, can efficiently transfer a functional gene into non-dividing cells.

Specificity and Efficiency of DNA Delivery in Vivo with Various DNA Complexes—To investigate the importance of size and structure of DNA-galactosylated poly-L-lysine complexes on their efficiency for transferring genes *in vivo*, plasmid DNA containing the *P. pyralis* luciferase gene under control of the SV40 promoter and enhancer elements was condensed with galactosylated poly-L-lysine at various concentrations of NaCl, and the structure of the resulting complexes evaluated by CD and EM. The complexes were then injected into rats via the caudal vena cava. When the DNA complex was prepared at a NaCl concentration which is below that required for the formation of dispersed unimolecular DNA complexes (Fig. 6*C*), luciferase was not expressed in the liver and spleen of transfected rats (Fig. 8). In contrast, dispersed unimolecular DNA complexes condensed into particles of about 17 nm in diameter (Fig. 6D) was expressed in the liver and to a lesser extent in the spleen (Fig. 8). Relaxed DNA generated detectable luciferase activity in the lung, spleen, and to a lesser extent the liver of injected rats. The uptake and expression of the complex containing the SV40-luciferase gene in the spleen indicates nonspecific uptake, probably by macrophages. Even appropriately condensed DNA was found to be expressed in the spleen, indicating that there was some nonspecific internalization of the DNA complex in the animal. Possibly, a subpopulation of the DNA complexes that was not apparent in the EM, may have been improperly condensed. These complexes may be unstable after injection into the bloodstream and be taken up by spleen macrophages. We conclude that there is a correlation between the structure of the DNA complex and the efficiency and specificity for receptor-mediated gene transfer by the asialoglycop-

rotein receptor. It is likely that this relationship applies also to the targeting of other endocytic receptors and could be of critical importance for the evaluation of molecular approaches for human gene therapy.

DISCUSSION

Receptor-mediated gene delivery is an attractive alternative to the use of viral vectors for introducing genes into animal tissues. As with any procedure, this technique has its advantages and limitations. Its advantages for use in gene therapy include: 1) the gene delivery vehicle can be customized for a specific target receptor (2, 4, 41); 2) the DNA does not have to integrate into the host cell genome to be expressed; 3) the delivery system is not theoretically limited by the size of the transgene; and 4) the technique does not involve the use of potentially infectious agents. There are also disadvantages which must be overcome before this procedure can be routinely used for human gene therapy. For example, the transgene is not integrated into the host cell chromosomes so that its expression is relatively transient. It will most likely be necessary to subject patients to multiple injections of a gene of interest. The DNA-ligand complexes are difficult to prepare and, until recently, little was known about their structure-function correlation. Finally, there is only a fragmentary understanding of the biological process involved in the transfer of the transgene into the cell and its subsequent expression. These and other features of this system for gene therapy have recently been reviewed in detail (42, 43).
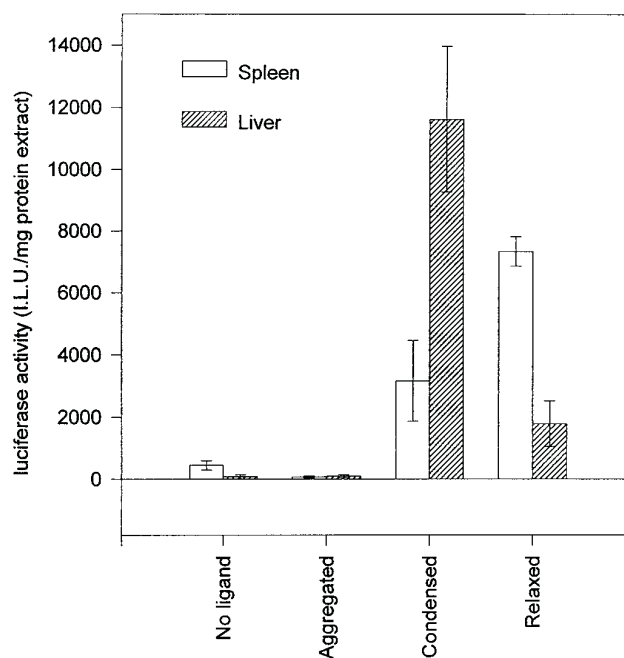
DNA condensation *in vitro* has been widely studied in the
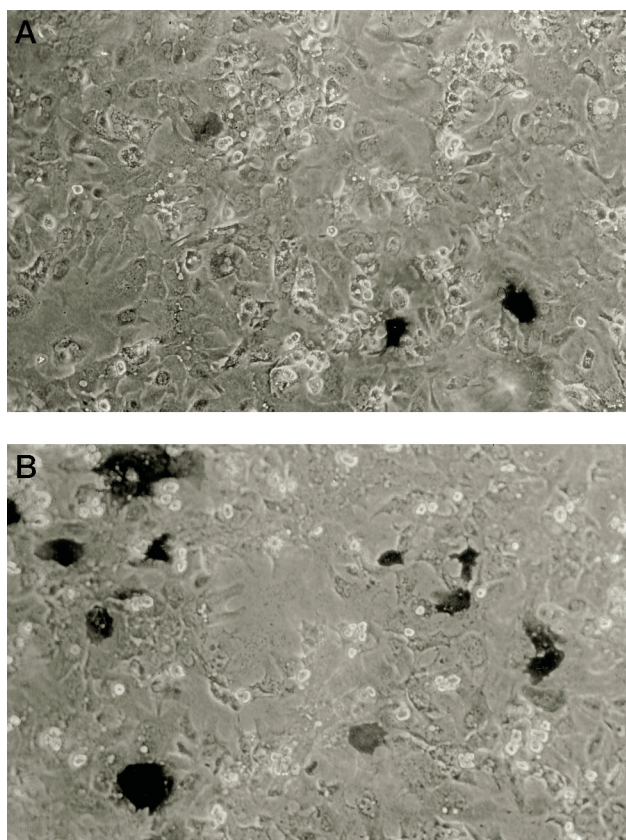
FIG. 7. **Transfection of the gene for RSV-β-galactosidase into primary hepatocytes using calcium phosphate or the galactosylated DNA·poly-L-lysine complex.** Primary culture of mice hepatocytes, grown on collagen-coated plates, were transfected either with $CaPO_4$ co-precipitates (*A*) or with the galactosylated DNA complex (*B*) (corresponding to Fig. 6*D*). Forty-eight hours after transfection the cells were fixed and stained *in situ* with 5-bromo-4-chloro-3-indolyl-β-galactopyranoside for 2 h. Blue cells indicate the presence of β-galactosidase activity.

FIG. 8. **Relationship between the structure of the DNA complex and its function in adult rats.** DNA-galactosylated polylysine complexes were prepared which correspond to various states of condensation/aggregation shown in Fig. 6. The DNA consisted of the SV40 promoter linked to the structural gene for *P. pyralis* luciferase gene. Rats were injected in the caudal vena cava with 300 μg of the various DNA complexes and the activity of luciferase was determined in extracts from the liver and the spleen 48 h after injection. Each *bar* represents the mean ± S.E. for three rats; luciferase activity from rats injected with DNA condensed using non-galactosylated poly-L-lysine are included as controls.

context of chromosome formation and structure. Generally, the condensation of DNA using agents that destabilize the solvent interaction of DNA in solution, such as polyethylenglycol, polyamines (polylysine, spermine, and spermidine), histone H1, etc. results in the formation of toroids and other compact structures as identified spectrophotometrically and by electron microscopy (23, 32, 44–47). Most of these condensed DNA complexes are heterogeneous and consist of multimolecular DNA complexes (*i.e.* Ψ-DNA), however, it is also possible to condense individual molecules of DNA (intramolecular condensation) into unimolecular DNA complexes (23). Since the size and structure of the DNA complex is critical for receptor-mediated gene transfer into target tissues in animals, it is important that the condensation process does not result in the formation of aggregated, multimolecular DNA complexes but rather into unimolecular complexes of a minimum size.

We have designed a multistep protocol for the condensation of DNA with galactosylated poly-L-lysine that appears to result in a stepwise, intramolecular condensation of DNA at high concentrations. The first step in the condensation protocol is the slow addition of the poly-L-lysine while vigorously mixing the DNA solution at high ionic strength. This vigorous mixing is necessary to increase the maximum effective length of the DNA polymer in high salt solutions, thus achieving efficient binding of the poly-L-lysine moiety to the DNA backbone. In addition, rapid mixing avoids localized, high concentrations of poly-L-lysine that result in uncontrolled precipitation of DNA. We hypothesize that the formation of a nucleus of condensation

ensures that the overall process of condensation is intramolecular, since poly-L-lysine binding to DNA is a cooperative process, *i.e.* the binding of the first molecules of poly-L-lysine to the DNA facilitates the binding of subsequent molecules (16, 34, 48). When the charge on the DNA is totally neutralized (charge ratio of 1:1) a turbid solution consisting of a fine precipitate of DNA complexes is produced. When examined using an EM, these precipitates are large aggregates of smaller units that we hypothesize are highly condensed, unimolecular DNA complexes. The equilibrium at low NaCl concentrations is in the direction of the association of the DNA poly-L-lysine complexes with each other to form aggregated complexes. This equilibrium is displaced toward the non-associated state (*i.e.* unimolecular condensed DNA complexes as shown in Fig. 6*D*) at a critical concentration of NaCl. Thus the formation of appropriately condensed, unimolecular DNA complexes requires an extremely careful titration with NaCl. The absorbance of aggregates of unimolecular DNA complexes at 260 nm is about 20% of that expected for a given concentration of DNA in solution, indicating that the particles are not entirely solubilized. The DNA complex can thus be described as a colloid in suspension. However, there is negligible turbidity as determined by absorbance at 400 nm and the optical rotatory spectrum is consistent with that noted for B-DNA (Fig. 1*D*). DNA condensation by the process described here is reversible, since the addition of NaCl beyond the critical concentration at which the DNA complexes are formed results in a steep increase on the absorbance of the DNA complex at 260 nm; this increase in absorbance correlates with the release of the DNA from interaction with poly-L-lysine (see Fig. 6*E*). Thus, absorbance of the DNA complex at 260 nm can be used to diagnose the formation of unimolecular complexes, which can be verified by electron microscopy.

There is a strong functional correlation between the struc-

ture of the various DNA complexes studied and the efficiency of transferring and expressing the DNA contained in the complexes *in vitro* (Fig. 3) and *in vivo* (Fig. 8). However, DNA which has not been condensed appropriately can be transfected into cells in culture by pathways other than receptor-mediated endocytosis. Thus, to test for specific receptor-mediated endocytosis of DNA complexes which include a specific ligand, we routinely use a competition assay which employs an excess of ligand to insure that the DNA complex is actively internalized via the appropriate receptor. The presence of 100-fold molar excess of asialofetuin or asialo-orosomucoid does not compete for the internalization of galactosylated Ψ-form, aggregated and relaxed DNA complexes by the receptor (data not shown); only the uptake of appropriately condensed DNA complexes is altered by the presence of a competing ligand (Fig. 5).

Ligand size is clearly a major factor in achieving proper recognition and internalization of DNA complexes targeted to receptors (36–39, 49). There are several reports of a characteristic ligand size limitation for the ASGP receptor (36–39, 49). Using lactosylated bovine serum albumin conjugated to gold particles of various sizes, Schlepper-Schäfer *et al.* (36) found that the cut-off size for the internalization of ligands of different sizes by liver hepatocytes was 8 nm in diameter (size of gold particles counted inside hepatocytes in EM photographs of histological sections of the liver after injection of ligand). However, the actual size may be larger since the conjugation of lactosylated bovine serum albumin to the gold particle may had changed the size and state of aggregation of the particle. The specific internalization of both an artificial ligand composed of galactosylated high density lipoprotein (larger than 10 nm in diameter) (38) and DNA complexes containing galactosylated poly-L-lysine (about 13 nm in diameter) (1) by the ASGP receptor present in liver hepatocytes has been reported. On the other hand, Kupffer cells of the liver specifically internalize lactosylated low density lipoprotein (LDL) of about 23 nm in diameter (37). Thus, the size limit for the internalization of artificial ligands by the receptor is between 13 and 23 nm in diameter. However, other factors such as the degree of lactosylation of the LDL particles influences the uptake of the ligand by either hepatocytes or Kupffer cells. Bijsterbosch *et al.* (39) found that at a low degree of lactosylation (60 lactose/LDL), the specific uptake of the protein (ng/mg of cell protein for each cell type) was 28 times higher in Kupffer cells than in parenchymal cells. At high degrees of lactosylation (greater than 300 lactose/LDL), the specific uptake in Kupffer cells was 70–95 times that in parenchymal cells (39). Under these conditions, Kupffer cells were, despite their much smaller mass, the main site of uptake. Thus not only the size but also the number of galactose residues on the lactosylated LDL is important for specific uptake by Kupffer and parenchymal cells (39).

We have found EM to be a valuable tool in assessing the structure-function relationships of the various DNA complexes formed during the condensation process (Table I). Despite its value, EM does not provide accurate information on the hydrodynamic size of the DNA complexes in solution because the DNA complex is blotted onto a copper grid for visualization. The hydrodynamic size of the DNA used in our experiments has been calculated, assuming that the DNA is condensed into a minimum size sphere (Table I). The difference between the size of the DNA complex noted by EM and the calculated hydrodynamic size may be due to the dehydration of the DNA complex which occurs upon condensation. Indeed, the size of the prophage Φ29 as determined by EM is 38 nm (50), while the calculated hydrodynamic size ranges from 40 to 46 nm, depending on the method of calculation (Table I). We are currently attempting an in-depth analysis of the size of the DNA com-

plexes in solution using dynamic light scattering and atomic force microscopy. This should provide us with a more critical assessment of the size of the complex in solution and allow us to better correlate the structure of the complex with its biological activity in the animal.

The current paper provides insight into the physical-chemical parameters required for the generation of DNA complexes capable of targeting the liver and which result in the prolonged expression of the transgene. It is our goal to establish this technique on a systematic basis by providing a rigorous set of guidelines which can be followed in order to synthesize a productive DNA complex for gene therapy. The key to this process is the condensation of the DNA-galactosylated poly-L-lysine complex into a compact structure of a size small enough to be recognized and internalized by the hepatic ASGP receptor, while at the same time surviving the journey from the site of injection to the nucleus in a state suitable for transcription. We have found in preliminary studies that genes condensed with non-galactosylated poly-L-lysine are an order of magnitude more efficiently expressed than the same DNA that has not been condensed prior to injection into the muscle of rats. This suggests that condensation of the DNA prior to its use in gene therapy may be of general usefulness for a number of applications and is not limited to receptor targeted gene delivery. In some respects, the DNA complex described in this paper resembles an encapsulated, virus-like particle, in which the capsid protein coats the tightly condensed DNA, rendering it resistant to nuclease digestion prior to its functional association with the transcription machinery in the nucleus of the cell.

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