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Biological Properties of Poly-L-lysine-DNA Complexes Generated by Cooperative Binding of the Polycation*[S]

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Ge Liu‡, Maria Molas‡§, Gregory A. Grossmann‡, Murali Pasumarthy¶, Jose C. Perales§¶, Mark J. Cooper¶, and Richard W. Hanson‡‖

From the ‡Department of Biochemistry, Case Western Reserve University School of Medicine, Cleveland, Ohio 44106-4935 and ¶Copernicus Therapeutics, Inc., Cleveland, Ohio 44106

We have evaluated the effect of NaCl concentration on the mode of binding of poly-L-lysine to DNA and the resulting structural and functional features of the condensed DNA particles using DNA precipitation, DNase I resistance, electron microscopy, and receptor-mediated gene transfer assays. At a high concentration of NaCl and in the presence of excess DNA, poly-L-lysine interacted with DNA cooperatively, fully condensing some of the DNA and leaving the rest of the DNA unbound. At low NaCl concentrations, poly-L-lysine molecules interacted with DNA in a noncooperative fashion, *i.e.* they bind randomly to the whole population of DNA molecules. Cooperative binding of poly-L-lysine to DNA occurred over a narrow range of NaCl concentrations, and the specific salt concentration depended on the length of the poly-L-lysine. The ability of condensed DNA to withstand digestion by DNase I was correlated with the structural features of the condensed DNA as determined by electron microscopy. Using our condensation procedure, cooperative binding of poly-L-lysine to DNA is a necessary prerequisite for the preparation of condensed DNA having a spherical shape and a diameter of 15–30 nm. Condensed DNA, containing galactosylated poly-L-lysine, was evaluated further for the extent and specificity of receptor-mediated gene transfer into HuH-7 human hepatoma cells via the asialoglycoprotein receptor. Efficient receptor-mediated transfection occurred only when condensed DNA complexes had a spherical shape with a diameter of 15–30 nm; asialofetuin, a natural ligand for the asialoglycoprotein receptor, inhibited this process by up to 90%. Our results support the importance of appropriate DNA condensation for the uptake and ultimate expression of DNA in hepatic cells.

The concept of receptor-mediated gene transfer originated from the work of Cheng *et al.* (1), who covalently attached a ligand to DNA. This idea was modified and used more widely for gene delivery by Wu and Wu (2, 3). They introduced poly-L-lysine into the gene transfer system to act as a "bridge" between the DNA and the ligand. After intravenous injection of ligand·poly-L-lysine·DNA complexes targeting the asialoglycoprotein receptor, the transgene was delivered specifically to the liver, in which it expressed transiently. Because receptor-mediated endocytosis is a general cellular mechanism, it can be applied to other specifically localized receptors (4). Since then, several groups have reported similar receptor-mediated gene delivery systems using ligand·poly-L-lysine·DNA complexes to introduce various genes to specific tissues by using different targeting ligands (5–15).

It was proposed that poly-L-lysine has the dual function of condensing DNA after electrostatic binding and providing the attachment site for the liver-targeting ligand (16). Condensation of the DNA into small particles is a crucial step for successful gene transfer. The process of DNA condensation has been examined widely (17–28). For example, if electrostatic interactions between poly-L-lysine and the DNA phosphate backbone occur in a rapid fashion, mixing basic polypeptides with DNA at a stoichiometric charge ratio will result in uncontrolled precipitation of the DNA (18, 20). However, reconstitution (annealing) of the polycation and DNA using NaCl-gradient dialysis or direct mixing of both components at low salt concentrations produces condensed DNA particles with the features of Ψ-DNA (highly ordered toroid-shaped multimolecularly condensed DNA complexes that are mutimolecular with respect to DNA) (14, 18, 20). Ψ-DNA features a circular dichroism spectrum that indicates chiral packing of multiple DNA helices in solution (29), which is thought to be caused by base stacking in the same optical plane. Using sedimentation and light-scattering techniques, Shapiro *et al.* (20) showed that Ψ-DNA·poly-L-lysine complexes exist as roughly toroidal and highly hydrated aggregates of ~170 nm in diameter. Thus, the simple electrostatic mixing of DNA and poly-L-lysine produces DNA particles with various structures. The two modes of binding of poly-L-lysine to DNA have been described: (*a*) poly-L-lysine processively binds to specific DNA molecules in a selective manner (cooperative binding), or (*b*) poly-L-lysine binds to a population of DNA molecules randomly in a nonselective way (noncooperative binding) (18, 19, 23, 26, 28, 30).

In this paper, we evaluated the effect of NaCl on the binding mode of poly-L-lysine to DNA and the resulting structural motifs of the condensed DNA particles using DNA precipitation, DNase I resistance assays, and electron microscopy (EM).[1] The structural features of the condensed DNA are correlated with

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[S] The on-line version of this article (available at http://www.jbc.org) contains the EM images from which data are derived.

§ Current address: Departament de Ciencies Fisiologiques, Universitat de Barcelona, Feixa Llarga s/n, L'Hospitalet 08907, Spain.

‖ To whom correspondence should be addressed: Dept. of Biochemistry, Case Western Reserve University, School of Medicine, Cleveland, OH 44106-4935. Tel.: 216-368-5302; Fax: 216-368-4544; E-mail: rwh@po.cwru.edu.

[1] The abbreviations used are: EM, electron microscopy; ASF, asialofetuin; CMV, cytomegalovirus; PEPCK, phosphoenol pyruvate carboxykinase; hFIX, human factor IX; hOTC, human mitochondrial ornithine transcarbamylase; GalPLL, galactosylated poly-L-lysine; Luc, *P. pyralis* luciferase.

its ability to withstand digestion by DNase I and with its level of expression in HuH-7 cells *in vitro* via the asialoglycoprotein receptor-mediated pathway. These results provide the basis for a better understanding, at the molecular level, of several crucial requirements for the formation of poly-L-lysine·DNA complexes of a minimum size and its importance to allow for a receptor-mediated gene transfer process.

EXPERIMENTAL PROCEDURES

Materials—High performance liquid chromatography-grade water ($W5^{SK}$-4) was used to prepare all solutions (Fisher). All chemicals, including poly-L-lysine, α-D-galactopyranosyl phenyl isothiocyanate, asialofetuin (ASF, A 1908), DNase I (D 4527), and trypsin type I (T 8003) were obtained from Sigma. The luciferase assay system E4530 was obtained from Promega (Madison, WI). The DC protein assay kit was purchased from Bio-Rad. DNA size markers (1-kilobase DNA ladder) and all media such as RPMI 1640, sera, and antibiotics were obtained from Life Technologies, Inc. SigmaScan® and SigmaPlot® software were purchased from SPSS, Inc. (Chicago, IL).

Reporter Genes and Plasmid Preparation—The expression plasmid pCMV-Luc is 6.9 kilobases in length and contains the human CMV viral promoter and enhancer elements ligated to the *Photinus pyralis* luciferase gene. The plasmid pPEPCK-hFIX has been described previously (10, 14) and consists of the phosphoenolpyruvate carboxykinase (PEPCK) gene promoter linked to the cDNA for human factor IX (hFIX). The plasmid pPEPCK-hOTC (5.3 kilobases) is identical to the pPEPCK-hFIX except for the coding region that contains the cDNA for human mitochondrial ornithine transcarbamylase (hOTC) (31). Plasmids were grown in *Escherichia coli* DH5α and purified by double equilibrium centrifugation in CsCl-ethidium bromide gradients (32) after alkaline lysis. The DNA was precipitated twice using one-tenth (v/v) 3 M sodium acetate and 2.5 volumes of ethanol and resuspended in water, and the concentration was determined using an Ultrospec 3000 spectrophotometer from Amersham Pharmacia Biotech. The DNA preparation was treated twice with RNase A and RNase T1 and resuspended to a final concentration of 1.5–2 mg/ml. 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.

Preparation of Condensed DNA Complexes—Our condensation method refers to the procedure of preparing condensed DNA complexes at a fixed NaCl concentration by titrating poly-L-lysine into a solution of DNA. Galactosylated poly-L-lysine (GalPLL) was prepared by galactosylating poly-L-lysine as described previously (14). The input molar ratio of poly-L-lysine to DNA, *r*, was the ratio of moles of lysine to nucleotide (NH_4^+/PO_3^-). Sixty micrograms of DNA in 365 μl of solution, adjusted to specific NaCl concentrations using a 5 M NaCl stock solution, was vortexed at 300 rpm using a VIBRAX shaking apparatus (VIBRAX-VXR, IKA Works, Inc., Wilmington, NC). Twenty-four micrograms of poly-L-lysine or GalPLL (for $r = 1$) in 365 μl of solution containing the identical concentration of NaCl was added dropwise to the corresponding DNA solution at the same salt concentration over a period of 80 min in 5-μl small aliquots. During the processes of titration, samples of DNA complexes with *r* values of 0, 0.25, 0.50, and 0.75 were prepared by titrating DNA with a portion of poly-L-lysine or GalPLL solutions at the same final volumes as those of samples with $r = 1$; the final concentrations of the DNA for all of the samples were identical (82 μg/ml).

DNA Precipitation Assays—DNA precipitation experiments were performed as described by Shapiro *et al.* (20). Varying amounts of poly-L-lysine were slowly added dropwise to vortexing solutions of plasmid DNA. After the titration, all samples were left vortexing at room temperature (25 °C) for 30 min and then centrifuged for 30 min at top speed (12,000 × *g*) in a Sorvall MC12C (DuPont) microcentrifuge. The absorbance of the supernatant fraction at 260 nm was determined using a spectrophotometer.

Specific Activity of DNase I Determined at Various Concentrations of NaCl—DNase I activity was measured by the rapid spectrophotometric method adapted from that originally described by Kunitz and Lindberg (33–35). The method is based on the increase in the absorbance at 260 nm (hyperchromicity) when DNA is degraded by enzymatic catalysis. The bacterial plasmid pPEPCK-hOTC was used as the substrate. A typical assay was performed as follows. Different amounts of enzyme were pipetted into the substrate solution at various concentrations of NaCl containing the DNase I working buffer (200 mM Tris-HCl, pH 7.6, 10 mM $MgCl_2$, and 1 mM dithiothreitol). The total reaction volume was 600 μl. Rapid vortexing ensured the complete mixing of the components. The reaction mixture was then poured into a quartz cuvette and the increase in absorbance at 260 nm was determined at 20-s intervals for 3–5 min using a spectrophotometer. One unit of DNase I activity at a specific NaCl concentration is defined as the amount of enzyme that will produce a ΔA_{260} of 0.001/min at pH 7.6 at 25 °C using bacterial plasmid pPEPCK-hOTC as a substrate.

DNase I Digestion Assay—To evaluate the amount of DNA bound and thus protected by poly-L-lysine during the titration process, samples of DNA complexes with *r* values of 0, 0.25, 0.5, 0.75, and 1 were incubated with 800 units of DNase I. Typically, a DNA·poly-L-lysine complex containing 4.9 μg of plasmid DNA (pPEPCK-hOTC) in a 60-μl solution was mixed with 1 μl of DNase I (800 units) and 7 μl of 10× DNase I working buffer. The final volume was then adjusted to 70 μl with water. The duration of digestion was controlled strictly at 15 min at room temperature. The DNase I was inactivated by adding 5 μl of 0.5 M EDTA to the reaction mixture. Under these conditions free DNA was completely degraded to very small fragments, whereas the poly-L-lysine-bound DNA was protected. The complexed DNA was released from poly-L-lysine·DNA complexes by the digestion of poly-L-lysine with 2.5% (w/v) trypsin solution. An equal volume of the trypsin solution was added to the DNA complex and incubated at room temperature for 40 min. The mixture was extracted twice with phenol/chloroform (1:1) before gel electrophoretic analysis on a 0.8% agarose gel as described previously (32).

Electron Microscopy—Uranyl acetate (0.04%) in methanol was used in these studies as a contrast stain. Samples, either in water or 1 M NaCl or diluted in 150 mM NaCl, were applied for 3 min to a copper electron microscope grid covered with 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, fixed using ethanol, and allowed to air dry. The grids were examined in a JEOL-100C transmission electron microscope, and plates were exposed to the image at a magnification of 20,000–50,000. The microscope was calibrated with the 87.5-Å spacing of catalase crystals (36). The major diameter, minor diameter, area, and perimeter of each condensed DNA particle on the EM images were automatically collected using the software SigmaScan® from the scanned negatives.

Cell Culture and DNA Transfection—A human hepatoma cell line, HuH-7, was maintained as a monolayer culture in RPMI 1640 medium supplemented with 10% fetal calf serum. DNA transfection was performed when cells reached ~30% confluence, *i.e.* 1 day after the seeding of 1.5×10^6 cells into 60-mm plastic dishes. One microliter of 2.5 M $CaCl_2$ was added to each milliliter of culture medium before transfection (to a final Ca^{2+} concentration of 3 mM in the medium). Each plate was transfected by adding a solution containing 4 μg of DNA directly to 2 ml of culture medium. The cells were exposed to the DNA complexes for 1.5 h at 37 °C in the presence or absence of 5 μg/ml of ASF as competitor for binding to the asialoglycoprotein receptor. The medium was then removed, and the cells were rinsed twice with phosphate-buffered saline. Cells were then placed in fresh RPMI 1640 medium supplemented with 10% fetal calf serum and incubated for 2 days prior to the assay of luciferase activity. The luciferase activities of cell lysates were then analyzed as recommended by the manufacturer. The cell lysates were assayed for protein content using the Bio-Rad DC protein assay kit, and the light units over a 10-s interval were standardized for protein content. All measurements were performed at least three times and expressed as the mean ± S.E.

Statistical Analysis—The data in Figs. 7 and Table I were analyzed by one way analysis of variance or unpaired *t* test using SigmaPlot® software.

RESULTS

Effect of NaCl on DNA Condensation—We first assessed the amount of DNA precipitated by poly-L-lysine at various salt concentrations. For GalPLL (poly-L-lysine with 1% of the NH_4^+ groups galactosylated) of various lengths, a set of precipitation assays was carried out to evaluate the effect of salt concentration on the quantitative precipitation of DNA. Using GalPLL of an average length of 256, 92, 57, and 26 amino acids, we found that a 1:1 molar equivalent precipitation (one molar equivalent of positive charge from poly-L-lysine precipitates one molar equivalent of negative charge from DNA) occurred near 1.0, 0.7, 0.6, and 0.3 M NaCl, respectively (Fig. 1). For GalPLL256 at 1.1 M NaCl (Fig. 1*A*), more poly-L-lysine was required to drive the precipitation of DNA by poly-L-lysine to completion. As the

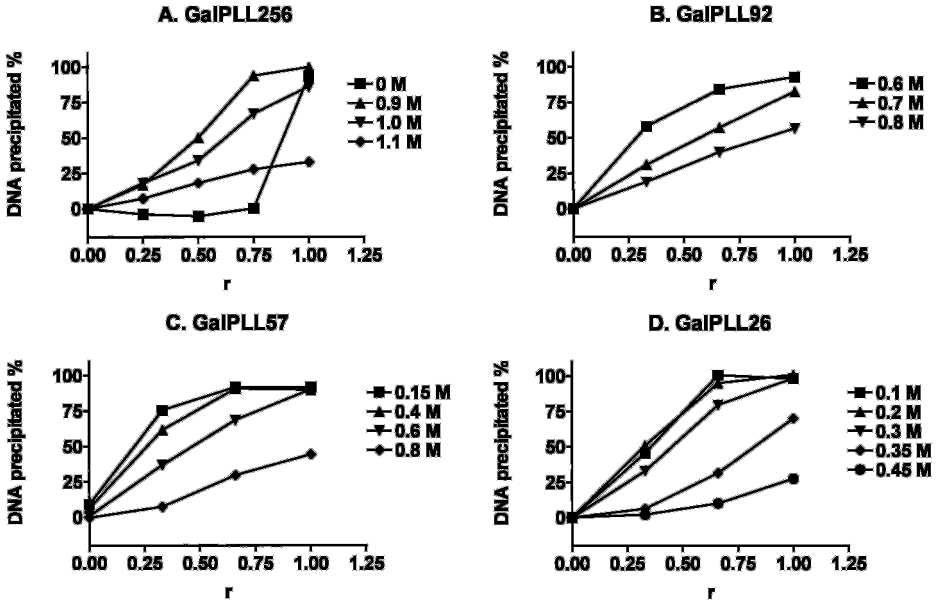

FIG. 1. **DNA precipitation assays using GalPLL of various lengths at different salt concentrations.** Plasmid DNA pPEPCK-hFIX was titrated with various amounts of GalPLL of different lengths at different salt concentrations with continuous mixing on a Vortex agitator. After the titration, all samples were left vortexing at room temperature for 30 min and then centrifuged at 12,000 × *g* for 30 min to remove the DNA·GalPLL complexes. DNA in the supernatant was quantified by measuring absorbance at 260 nm in a spectrophotometer. The percentage of DNA in the pellets is plotted *versus r*, the input ratio of GalPLL to DNA (moles of lysine/nucleotide or NH_4^+/PO_3^- ratio). For each GalPLL of different length, a set of precipitation assays was carried out to determine the salt concentration at which cooperative binding occurred; this is when a 1 molar equivalent of lysine precipitated a 1 molar equivalent of nucleotides. Using GalPLL with 256, 92, 57, and 26 lysine residues, we found that cooperative binding (1:1 equivalent precipitation) occurs near 1.0, 0.7, 0.6, and 0.3 M NaCl, respectively.

salt concentration was reduced to 0.9 M (Fig. 1*A*), a molar equivalent of lysine residues precipitated somewhat more than a molar equivalent of nucleotide (*i.e.* the amount of poly-L-lysine necessary to produce complete precipitation decreases below a 1:1 charge ratio). Similar results were observed when GalPLL of shorter lengths was mixed with DNA at a NaCl concentration that was below the NaCl concentration that gave 1:1 precipitation (Fig. 1, *B–D*). In the absence of NaCl (Fig. 1*A*), there was no decrease of DNA in solution until a threshold concentration of GalPLL256 was reached near the end of the titration. At this point most of the DNA molecules were precipitated by GalPLL256 within a very narrow range of *r* values (0.75–1) (Fig. 1*A*). These curves agree with the results of poly-L-lysine-induced DNA precipitation reported previously (28, 37). Therefore, the concentration of NaCl at which selective binding occurred, *i.e.* when a 1 molar equivalent of lysine precipitates a 1 molar equivalent of nucleotides during the entire titration, varies according to the length of poly-L-lysine and corresponds to the ionic strength at which cooperative association of poly-L-lysine to DNA occurs as suggested by Shapiro *et al.* (20).

DNase I Digestion Assay—Although the NaCl concentrations at which 1:1 precipitation occurs can be determined using the DNA precipitation assay, it does not provide direct information concerning the distribution of poly-L-lysine on the DNA. For this reason, we further evaluated the presence of bound *versus* free DNA in DNA complexes using a DNase I digestion assay. We first determined the specific activity of DNase I (change of absorbance at 260 nm) under 0, 0.2, and 1 M NaCl to ensure that the same specific activity of the enzyme was used in all the experiments. Using an excess of pPEPCK-hOTC plasmid DNA, the digestion process, after an initial lag phase, closely approximates a first-order reaction. At concentrations of NaCl of 0, 0.2, and 1 M the specific activities of DNase I are 4.52×10^4, 2.17×10^3, and 52.9 units/mg of protein, respectively. Thus, the ratio of DNase I specific activities at NaCl concentrations of 0:0.2:1 M is close to 800:40:1.

The DNase I digestion assay was repeated at least three times, and representative results are shown in Fig. 2. In these experiments, plasmid DNA and GalPLL256 were used to prepare DNA·poly-L-lysine complexes of different *r* values at 0, 0.2, and 1 M NaCl (Fig. 2, *A*, *B*, and *C*, respectively). After the preparation, these complexes were digested with 800 units of

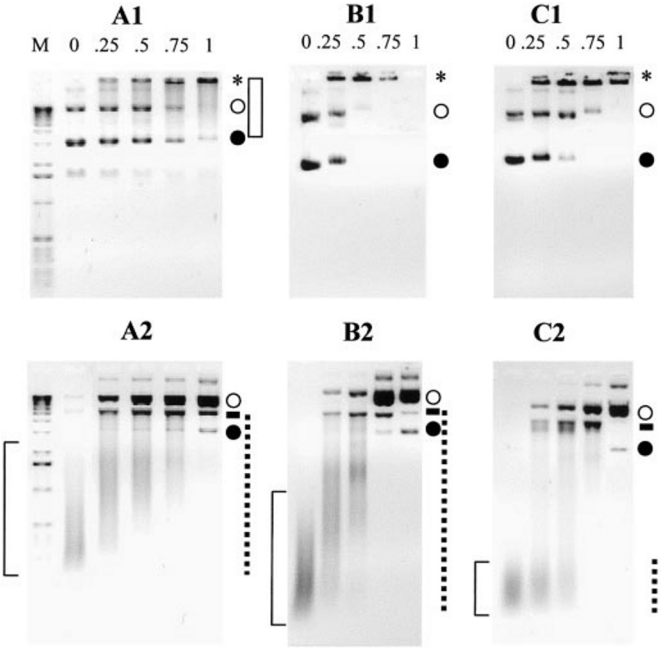

FIG. 2. **DNase I digestion assay of DNA complexes of different *r* values.** Condensed DNA complexes were prepared at 0 (*A1* and *A2*), 0.2 (*B1* and *B2*), and 1 M (*C1* and *C2*) NaCl with plasmid DNA pPEPCK-hOTC and GalPLL256. In each panel, the loading order from left to right is $r = 0$, 0.25, 0.5, 0.75, and 1 except for a 1-kilobase ladder marker (*M*) on the left-most lanes of *A1* and *A2*. *A1*, *B1*, and *C1*, samples of DNA complexes were loaded onto the gel directly after preparation of condensed DNA complexes. *A2*, *B2*, and *C2*, samples of DNA complexes were exposed to DNase I and then treated with trypsin to release the DNA from the poly-L-lysine in the complex. *Asterisks*, DNA complexes retarded in the loading wells; *open circles*, nicked DNA; *short solid bars*, linear DNA; *solid circles*, supercoiled DNA; *left brackets*, the degraded fragments from free DNA; the *open bar* in *A1*, DNA species with slower mobility; *dotted lines*, degraded fragments from DNA·poly-L-lysine complexes at intermediate *r* values.

DNase I. The time course of DNase I digestion was strictly controlled such that most of the free DNA was degraded. DNA samples were then incubated further with trypsin to degrade GalPLL256 to evaluate the amount and form of the protected DNA in the DNA complexes. DNA complexes were analyzed by 0.8% agarose gel electrophoresis either directly (Fig. 2, *A1*, *B1*,

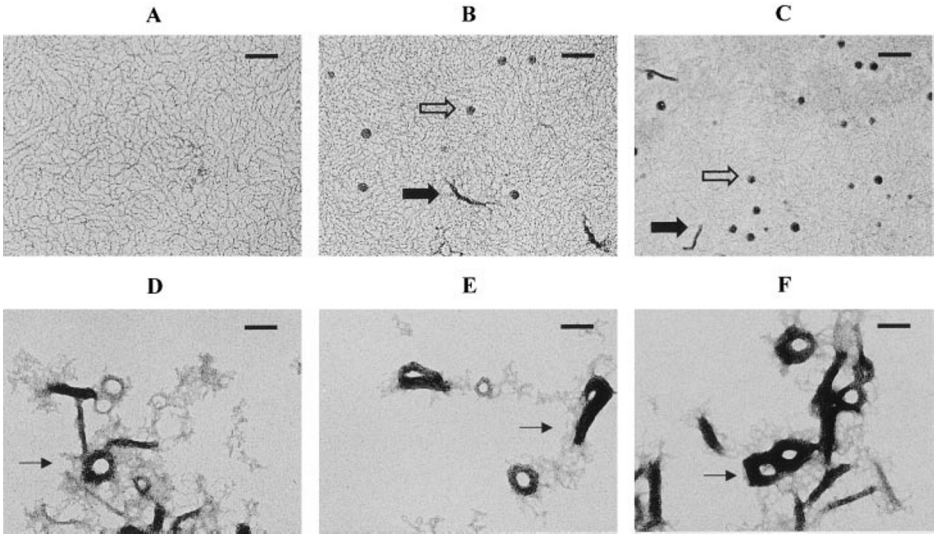

FIG. 3. **Electron micrographs of the structures of condensed DNA complexes prepared at different ionic strengths and poly-L-lysine/DNA ratios.** Electron micrographs of 0.04% uranyl acetate-stained DNA complexes were prepared using plasmid DNA pCMV-Luc and GalPLL256 as described under "Experimental Procedures." Samples were stained within 30 min after their preparation. *A–C*, DNA samples prepared at 1 M NaCl with GalPLL256 from Experiment 1. *D–F*, DNA samples prepared at 0 M NaCl with GalPLL256. *r* values: *A* and *D*, 0.25; *B* and *E*, 0.5; *C* and *F*, 0.75. There was no adjustment of salt concentration before processing samples. *Open arrows*, spherical complexes; *solid arrows*, rodlike complexes (major diameters of ~100 nm); *thin arrows*, aggregated structure including large rods and toroids complexes. The *bars* in all panels represent 100 nm.

and *C1*) or after both DNase I and trypsin digestion (Fig. 2, *A2*, *B2*, and *C2*). Trypsin digestion alone showed that the same amount of DNA could be recovered from GalPLL256·DNA complexes for each experimental set and was used as a loading control (data not shown).

Retardation of DNA by GalPLL256 was observed in all experimental samples (bands labeled with an *asterisk* in Fig. 2, *A1*, *B1*, and *C1*) and can be explained by the neutralization and/or increased size of the GalPLL256·DNA complexes formed. At 0 M NaCl, the DNA complexes were retarded in the electrophoretic field into slower mobility species with increasing *r* values (smeared DNA labeled with an *open bar* in Fig. 2*A1*). DNA was also retarded completely in the well at higher ratios of lysine/nucleotide (bands labeled with an *asterisk* in Fig. 2*A1*). At 0.2 M NaCl (Fig. 2*B1*), free DNA was retarded completely (*asterisk*-labeled band) when the *r* value was 0.5. However, at 1 M NaCl (Fig. 2*C1*), there was a linear correlation between the amount of GalPLL256 ($r = 0$ to $r = 1$) and the amount of DNA completely retarded in the well. Slower mobility species generated from the DNA complexes were barely observed in Fig. 2, *B1* or *C1*.

Removal of poly-L-lysine by trypsin allowed the protected DNA to enter the gel from the loading wells and migrate as free DNA bands (Fig. 2, *A2*, *B2*, and *C2*). The patterns of DNase I digestion indicated that the DNA that was bound to GalPLL and thus protected from degradation by DNase I existed as either nicked (*open circles*), linear, or fragmented DNA. The degradation patterns shown in Fig. 2, *A2*, *B2*, and *C2*, were significantly different. At 0 and 0.2 M NaCl, the degraded DNA (lanes with $r = 0.25$, 0.5, and 0.75) existed as a population of fragmented DNA species (smeared, labeled with *dotted lines* in Fig. 2, *A2* and *B2*) with a larger molecular weight than noted with completely degraded products (smeared, labeled with a *bracket* in Fig. 2). In contrast, the degradation pattern of DNA complexes prepared in 1 M NaCl had the mobility of completely degraded DNA (see *left bracket* in Fig. 2*C2*). Total protection from DNase I degradation (bands labeled with a *closed circle* in Fig. 2) was only observed at higher ratios of lysine/nucleotide ($r = 1$).

Analysis of the Structure of DNA Complexes by Electron Microscopy—Three molecular species can be inferred from our DNase I experiments: free DNA, partially bound DNA (slower mobility), and completely bound DNA (completely retarded). However, the DNase I digestion assay does not provide direct information concerning the possible structural differences among these bound DNA complexes. We therefore carried out nine separate experiments to analyze the structural and functional properties of DNA condensed with poly-L-lysine at *r* values of 0.25, 0.5, and 0.75. The DNA complexes were formed by binding GalPLL256 at 0 and 1 M NaCl and were analyzed using transmission EM. DNA complexes at $r = 1$ routinely aggregated and precipitated; the precipitated DNA broke the supporting carbon film on the EM grids, did not produce consistent results, and were not analyzed further.

The Effect of NaCl on DNA Condensation—Fig. 3 (*A–C*) presents the results of Experiment 1, a DNA condensation experiment that generated small functional particles as an example of the effect of NaCl concentration on the condensation process. All nine experiments will be presented subsequently. DNA condensed by GalPLL256 at 1 M NaCl typically generated two DNA structures: free DNA and condensed DNA complexes. The free DNA appeared as thin fibers, whereas most of condensed DNA complexes were present as small spherical particles (15–30 nm). No free DNA fibers were observed to extend outside the condensed region. The binding of GalPLL256 to DNA occurred in a cooperative fashion, because there were two populations of DNA: either in a free or condensed form. Increasing the concentration of GalPLL256 increased the population of condensed DNA complexes and decreased the concentration of free DNA. The concentration of condensed DNA complex was found to be highest when $r = 0.75$. These spherical particles (15–30 nm) consist of a single molecule of DNA and a range of up to three DNA molecules per particle based on theoretical calculations (14).

The structure of DNA complexes observed by binding of GalPLL256 at 0 M NaCl and at *r* values of 0.25, 0.5, and 0.75 is shown in Fig. 3 (*D–F*). In the absence of NaCl, most of the DNA was bound to GalPLL256 at all ratios of lysine/nucleotide used in this study. With the addition of GalPLL256, large aggregates began to appear from $r = 0.25$ (Fig. 3*D*). Most of these aggregated complexes contained unbound DNA fibers that extended outward from the condensed region. This explains the partially degraded DNA observed in the gel patterns of DNA complexes prepared at low NaCl concentrations (Fig. 2*A2*). The structures observed in the EM demonstrate that the DNA was in a multimolecular partially condensed form at 0 M NaCl. Some these aggregates form large rods and toroids at least several hundred nanometers in diameter. When the *r* values increased, these partially condensed DNA complexes further aggregated (Fig. 3, *E* and *F*). They probably correspond to the protected DNA complexes noted in Fig. 2*A2*.

Analysis of EM of DNA Complexes Using SigmaScan®—We

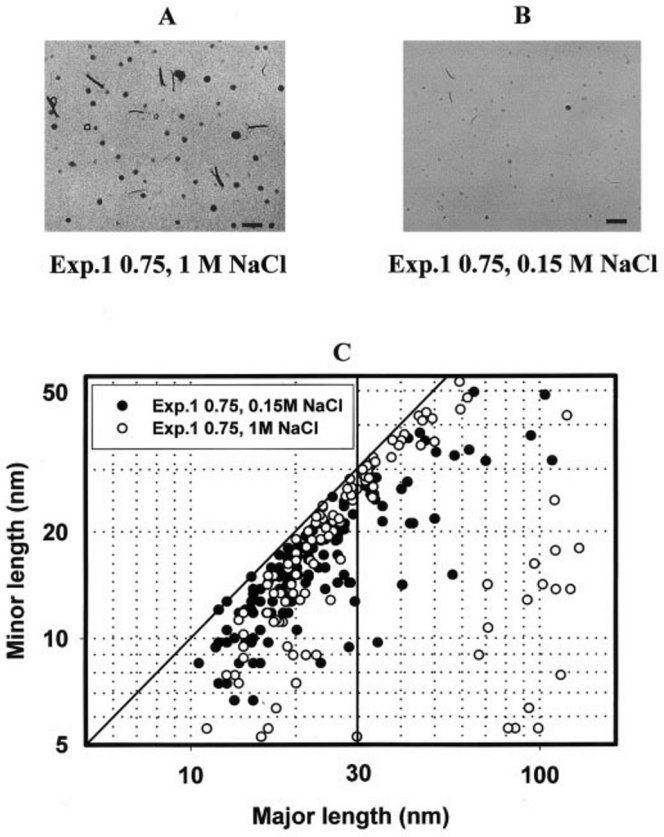

FIG. 4. **The effects of dilution of condensed DNA prepared at 1 M NaCl with GalPLL256 to 0.15 M NaCl.** Condensed DNA complexes were prepared using plasmid DNA pCMV-Luc and GalPLL256 at 1 M NaCl from Experiment 1. Electron micrographs were prepared using these samples within 30 min after their preparation at 1 M NaCl (*A*) or after dilution of the DNA complexes to 0.15 M NaCl for 4 h at room temperature (*B*). The *bars* in *A* and *B* represent 100 nm. Each electron-dense particle on these EMs was measured for its area, major diameter, minor diameter, and perimeter using the software SigmaScan®. The log of the major diameters and minor diameters of these ellipsoidal particles was plotted in *C*. These particles falling on the *45° diagonal line* have a spherical structure. Extended shapes such as rods are located below the *45° diagonal line*. The *vertical line* represents a major length of 30 nm.

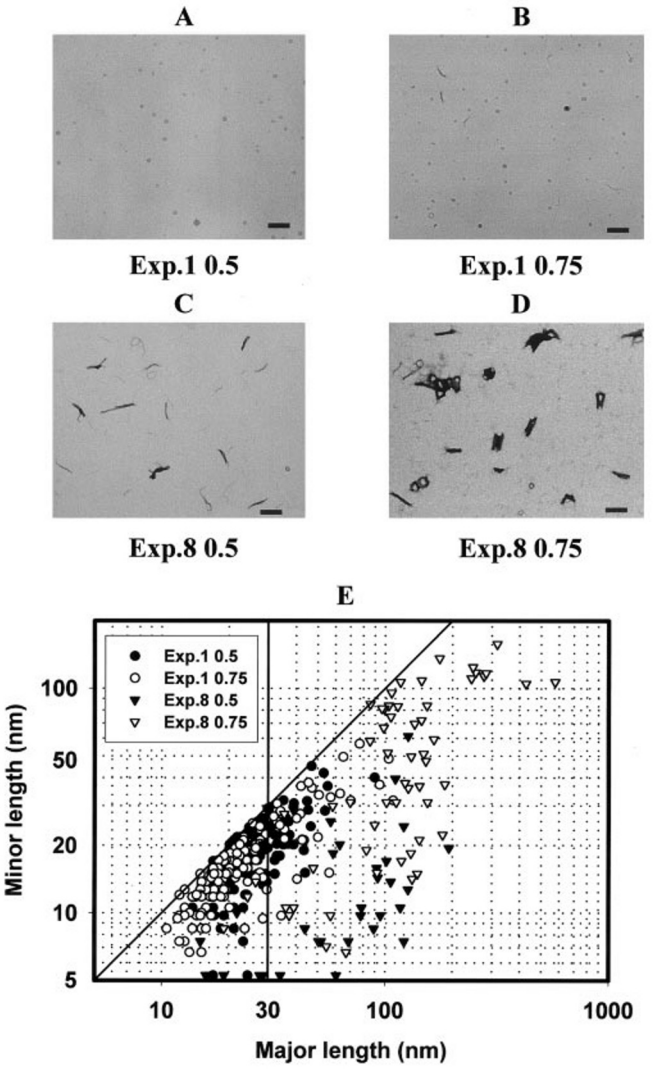

FIG. 5. **Structural analysis of condensed DNA from Experiments 1 and 8.** Condensed DNA was prepared at 1 M NaCl using plasmid DNA pCMV-Luc and GalPLL256 and then diluted to 0.15 M NaCl for 4 h at room temperature before electron microscopy. The SigmaScan® analysis described in the Fig. 4 legend was performed. Condensed DNA complexes with $r = 0.5$ in Experiments 1 (*A*) and 8 (*C*) and $r = 0.75$ in Experiments 1 (*B*) and 8 (*D*) are shown. The *bars* in *A–D* represent 100 nm. *E*, the SigmaScan® plot of the condensed DNA in the micrographs from Experiments 1 and 8.

routinely diluted the DNA complexes from 1 to 0.15 M NaCl prior to analysis by EM or cell transfection. To ensure that the dilution did not alter the structural properties of the DNA complexes, we determined their structures before and after dilution. Samples from Experiment 1 were stained before and after the dilution of the DNA complexes to 0.15 M NaCl for 4 h at room temperature (Fig. 4, *A* and *B*). Each electron-dense particle on these EMs was measured for its area, major diameter, minor diameter, and perimeter using the software SigmaScan®. The scatter plots of major *versus* minor diameters provide a quantitative assay of the size and shape of DNA complexes (Fig. 4*C*). Except for a small percentage (<10%) of rods having a major length of ~100 nm, the change in NaCl concentration did not alter the morphology of the condensed DNA particles; most maintained a diameter of 30 nm or less. Thus, once the condensed DNA complexes were formed, they remained condensed and did not aggregate after being diluted to 0.15 M NaCl for 4 h at room temperature.

We next used SigmaScan® plot to examine the EM negatives from Experiments 1 and 8 at *r* values of 0.5 and 0.75 (Fig. 5). These two Experiments were selected as representative of a DNA condensation that produced small spherical particles and a condensation that produced rods but not small spherical particles. These are the two extremes noted in our DNA condensation studies. After dilution into 0.15 M NaCl for 4 h at room temperature, the DNA in Experiment 1 was present as small spherical particles (30 nm or less), whereas the DNA condensed in Experiment 8 was viewed as rods (major diameters of ~100 nm). Experiment 1 demonstrated a signature profile of particles having a spherical shape (close to the *45° diagonal line*) and a diameter below or near 30 nm (the *vertical solid line*).

All nine independent DNA condensations were analyzed by SigmaScan®, and the data are presented in the scatter plots shown in Fig. 6. The EM images from which these data are derived can be found as supplemental data available with this paper and are not presented in detail here. Assuming condensed DNA is a homogenous population, the major and minor lengths for each experiment are presented as the mean and standard error of the mean (S.E.) for $r = 0.5$ and 0.75. Experiments 1, 7, and 9 showed a signature profile with a spherical shape and a diameter below or near 30 nm at both *r* values. The other experiments produced rodlike DNA complexes with major diameters of ~100 nm. The only exception was Experi-

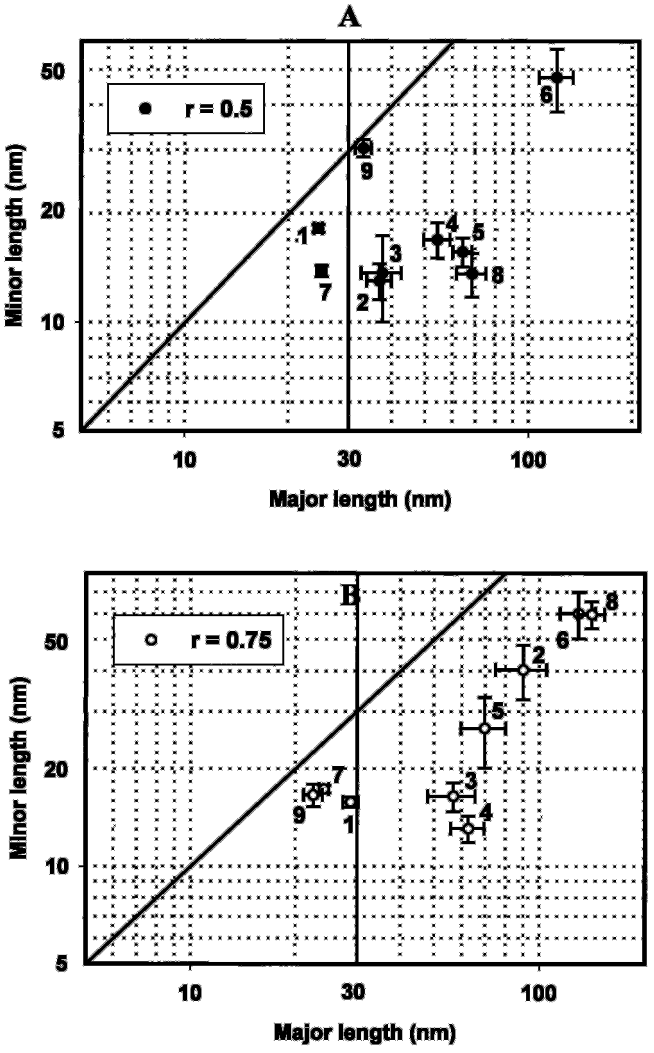

FIG. 6. **Structural analysis of condensed DNA from nine independent experiments.** Condensed DNA complexes were prepared using plasmid DNA pCMV-Luc and GalPLL256 at 1 M NaCl. DNA complexes with $r = 0.5$ or 0.75 were then diluted to 0.15 M NaCl for 4 h at room temperature before electron microscopy. The major and minor lengths are presented as the mean and standard error of the mean (S.E.) for $r = 0.5$ (*A*) and 0.75 (*B*) for these experiments. Experiments 1, 7, and 9 showed a signature profile with the shape of a sphere and a diameter below or near 30 nm (*vertical line*). The other experiments generated particles that were either ellipsoids larger than 30 nm and/or rods (major diameters of ~100 nm) except for Experiment 3 with $r = 0.75$, which generated a mixture of small spherical particles (15–30 nm) and rods (major diameters of ~100 nm). The experiment number is listed in the figure together with the S.E. for the specific experiment (*bars*).

ment 3, in which $r = 0.75$ produced a mixture of two populations: small spherical particles (15–30 nm) and rods (major diameters of ~100 nm). The EM for this experiment is presented in Fig. 7*B*.

Effect of DNA Structure on Its Biological Properties—To determine the effect of the structure of the DNA complexes on the specificity and efficiency of gene transfer, we evaluated the level of expression of pCMV-Luc after transfection into HuH-7 cells in the absence or presence of ASF. DNA complexes with $r = 0$, 0.25, 0.5, and 0.75 were prepared using GalPLL256 in 1 M NaCl and diluted with cell culture medium before transfection. As a natural ligand for the asialoglycoprotein receptor, ASF competes with galactosylated DNA complexes for uptake by HuH-7 cells.

Table I summarizes the transfection results from all nine DNA condensation experiments presented in Fig. 7*A*; the data are expressed as the percentage of inhibition of luciferase activity by ASF. Values less than 0% represent a stimulation of expression of the *CMV-Luc* gene by ASF. Specific inhibition of luciferase activity by ASF was only detected in Experiments 1, 7, and 9 for $r = 0.5$ and 0.75. None of the other DNA complexes produced significant differences with the sole exception of Experiment 3, which produced significant competition at $r = 0.75$. As shown in Fig. 7*B*, the condensed DNA in Experiment 3 with an r value of 0.75 contained a mixture of two populations: small spherical particles (15–30 nm) and rods (major diameters of ~100 nm). The average inhibition of *CMV-Luc* gene expression was ~90 or ~70% for DNA complexes with $r = 0.5$ (Experiments 1, 7, and 9) or 0.75 (Experiments 1, 3, 7, and 9), respectively.

Cell transfection experiments performed on the DNA complexes of Experiments 1, 7, and 9 gave essentially the same results; Experiment 7 is shown in Fig. 7*C*. The *CMV-Luc* gene was only expressed at a marginal level (3-fold above the blank) with DNA complexes at $r = 0.25$. When the r value was 0.5 or 0.75, the luciferase activity was ~400- or 1300-fold higher than background (blank), respectively. The luciferase values for condensed DNA with r values of 0 and 0.25 were significantly different from the luciferase activity of condensed DNA with r values of 0.50 and 0.75 ($p < 0.0001$). The luciferase activity of condensed DNA with an r value of 0.5 was statistically different from DNA with an r value of 0.75 ($p < 0.01$). Thus, the efficiency of transfection was greatest when the condensed DNA had r values of 0.5 and 0.75; at these r values small spherical condensed DNA particles (15–30 nm) were produced.

DISCUSSION

Two types of binding of poly-L-lysine to DNA were noted in this study. Cooperative binding of poly-L-lysine to DNA is a specific feature of poly-L-lysine·DNA complexes prepared at high concentrations of NaCl. Random, noncooperative binding occurs when poly-L-lysine·DNA complexes were prepared at a relatively low NaCl concentration (18, 19, 23, 26, 28, 30). Cooperativity reflects the influence of one bound poly-L-lysine on the binding affinity of a second poly-L-lysine (38). As a result of positive cooperativity, poly-L-lysine condenses a fraction of the DNA at a 1:1 charge ratio but leaves the rest of DNA unbound in the presence of excess DNA. These two modes of DNA condensation are illustrated in Fig. 8.

The characteristics of these two binding processes are illustrated by the results of DNA precipitation (Fig. 1) and electron microscopy (Fig. 3). In the DNA precipitation assays, the bound "DNA precipitate" as described by Shapiro *et al.* (20) can be removed from solution by centrifugation. DNA precipitation assays were used to assess the relationship between the length of poly-L-lysine and the salt concentration that permits 1:1 molar equivalent precipitation (one molar equivalent of positive charge from poly-L-lysine precipitates one molar equivalent of negative charge from DNA). In the presence of excess DNA, the amount of precipitable DNA at various concentrations of GalPLL is affected by the concentration of NaCl (Fig. 1). The concentration of NaCl at which 1:1 molar equivalent precipitation occurred is different for each GalPLL length and corresponds to the ionic strength at which cooperative association of GalPLL to DNA occurs. In contrast, at 0 M NaCl the pattern of DNA precipitation is clearly different. The DNA is entirely precipitated within a very narrow range of GalPLL concentrations, indicating a noncooperative association of these biopolymers that forms multiple molecular aggregates (Fig. 3, *D–F*). These observations are clearly supported by the EM photos in Fig. 3. It is apparent that when GalPLL binds to DNA in a cooperative fashion some molecules of DNA are fully condensed, and the rest of the DNA is unbound; there were

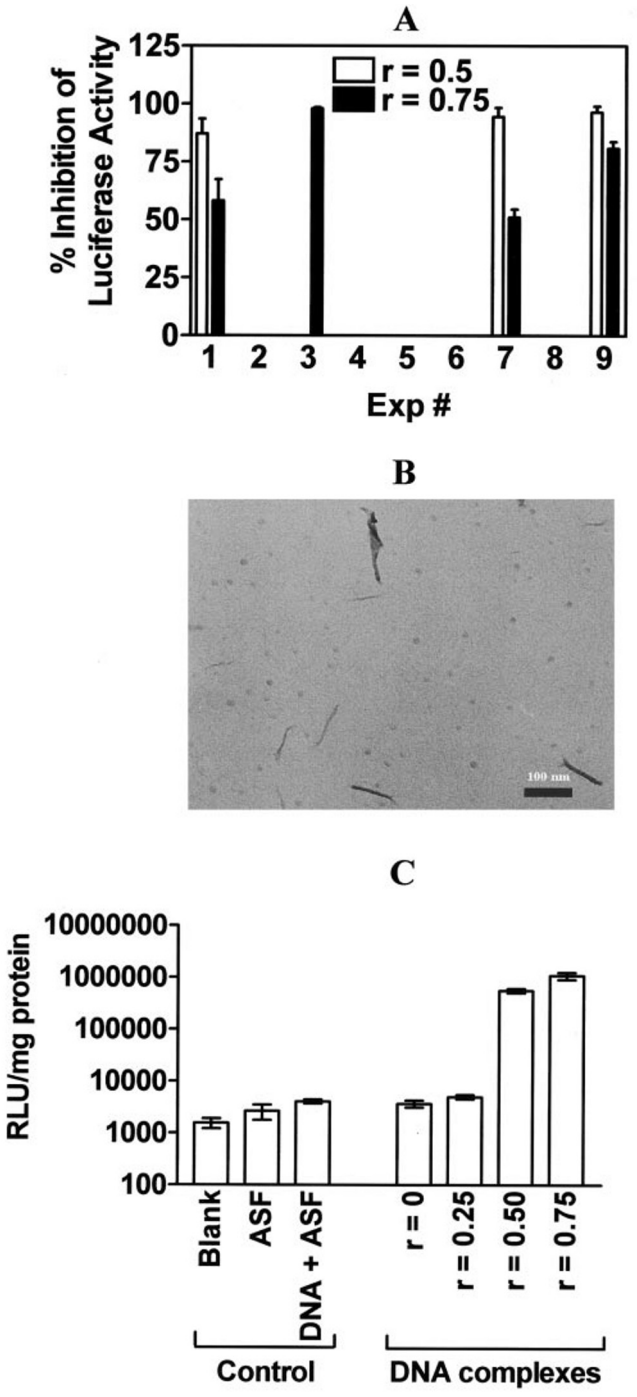

FIG. 7. **Analysis of the extent of uptake via the asialoglycoprotein receptor and determination of the level of expression of the gene for CMV-luciferase using condensed DNA.** *A*, the inhibition of gene transfer by ASF. Condensed DNA complexes were prepared using plasmid DNA pCMV-Luc and GalPLL256 at 1 M NaCl. HuH-7 cells were transfected with DNA complexes of $r = 0.5$ or 0.75 in the absence or presence of a 100-fold molar excess of ASF. *B*, the electron micrographs for condensed DNA with $r = 0.75$ from Experiment 3, which showed a mixture of both small spherical particles (15–30 nm) and rods (major diameters of ~100 nm). *C*, receptor-mediated gene transfer is proportional to the extent of DNA condensation. DNA complexes of $r = 0$, 0.25, 0.5, and 0.75 were prepared using plasmid DNA pCMV-Luc and GalPLL256 at 1 M NaCl from Experiment 7. HuH-7 cells incubated with plain medium, free DNA, and ASF were included as controls for the basal luciferase level. *RLU*, relative luciferase units. The luciferase values for condensed DNA with *r* values of 0 and 0.25 were significantly different from the luciferase activity of condensed DNA with *r* values of 0.50 and 0.75 ($p < 0.0001$). The luciferase activity of condensed DNA with an *r* value of 0.5 was statistically different from DNA with an *r* value of 0.75 ($p < 0.01$).

very few partially condensed intermediate DNA complexes in solution (Fig. 3, *B* and *C*). The initial NaCl concentration is an important factor that determines whether poly-L-lysine will cooperatively condense DNA and therefore is a key parameter that determines the structure of the resultant complexes.

DNase I degradation assays at low *r* values also distinguish between DNA condensed under cooperative or noncooperative conditions (Fig. 2). Under cooperative conditions, the presence of free and fully compacted DNA is inferred by the presence of both nondegraded and completely degraded DNA; intermediates of partially degraded DNA are not present. Noncooperative conditions, however, generate a broad range of DNA fragments, suggesting that poly-L-lysine is binding to multiple strands of DNA without providing full protection to most DNA molecules. Cross-linking of DNA by poly-L-lysine is also inferred by the large aggregates of electron-dense structures observed in Fig. 3, *D–F*.

If the effects of small counterions were not considered, this type of cooperativity violates a basic electrostatic principle that predicts that the cationic polypeptides would bind DNA sites as far away from one another as possible (39). There are two probable explanations for positive cooperativity of the binding of poly-L-lysine to DNA. First, the interaction between poly-L-lysine and DNA could induce a local conformational change in the DNA duplex such as base-tilting, which might provide more accessible binding sites for additional poly-L-lysine molecules (29, 40). Alternatively, the poly-L-lysine molecules could attract one another along the DNA backbone using their terminal carboxyl and amino groups, which favors adjacent interaction of poly-L-lysine with DNA despite the unfavorable electrostatic environment. A model for a complex between basic polypeptides and DNA has been proposed in which the poly-L-lysine wraps and binds the DNA along its minor groove in a β-sheet-like conformation (41).

When condensation occurred at 1 M NaCl, two distinct condensed DNA populations were observed using EM: small spherical particles (with a diameter 30 nm or less) and/or rods (major diameters of ~100 nm). At increasing *r* values, the amount of free DNA decreased, and the amount of fully condensed DNA increased. Interestingly, most DNA complexes in Experiments 1, 7, and 9 had a spherical or ellipsoidal shape with a mean diameter of 20.8 ± 1.0 nm (Fig. 6). The calculated volumes of either spherical particles (15–30 nm) or rodlike complexes (major diameters of ~100 nm) based on EM micrographs indicate that these particles have an average of one molecule of DNA and a range of up to three DNA molecules per complex. These DNA complexes are fully resistant to DNase I digestion, indicating that a high level of protection of the DNA was achieved by its condensation at NaCl concentrations that allow cooperative binding of poly-L-lysine to the DNA.

There are several possible reasons for the variation in the shape of DNA complexes formed at 1 M NaCl. Commercially available poly-L-lysine is a mixture of polymer chains differing in the degree of polymerization. For example, poly-L-lysine with an average length of 256 amino acids contains a range of chain lengths varying from 320 to 140 amino acids (as reported by the manufacturer). For poly-L-lysine molecules of a specified length, there is a specific NaCl concentration at which selective binding to DNA occurs. However, this concentration varies depending on the chain length of the poly-L-lysine used to condense the DNA (Fig. 1). Trace amounts of counterions such as acetate in the condensation solution can influence the shape of DNA complexes (42). These and other as yet poorly understood factors may contribute to the variations in the size and shape of the DNA complexes noted in this study.

To assess the functional relevance of the structural motifs

TABLE I

Summary of the structural and functional properties of condensed DNA complexes from nine independent experiments

For each experiment, the percentage of condensed DNA having a major length less than 30 nm is listed. Values represent the mean ± S.E. for three independent determinations of luciferase activity expressed as relative luciferase units/mg of protein × 10^{-4}. A percentage inhibition less than 0% represents stimulation by the added ASF. N, not statistically significant with $p < 0.05$; *, $p = 0.01$—0.05; **, $p = 0.01$—0.001; ***, $p < 0.001$.

	Experiment no.	Percentage <30 nm	−ASF	+ASF	Percentage inhibition (+/−)	Statistical significance
			Mean ± SEM	Mean ± SEM		
$r = 0.5$	1	83	153.47 ± 22.87	19.97 ± 9.97	87	**
	2	52	17.10 ± 15.57	17.65 ± 16.45	−3	N
	3	47	4.26 ± 4.03	0.63 ± 0.06	85	N
	4	28	0.23 ± 0.12	0.16 ± 0.03	30	N
	5	14	1.28 ± 0.71	6.09 ± 5.55	−376	N
	6	17	154.53 ± 106.20	42.34 ± 17.67	73	N
	7	78	55.55 ± 5.41	3.09 ± 2.20	94	***
	8	22	2.88 ± 0.40	5.01 ± 1.31	−73	N
	9	62	226.12 ± 59.84	8.04 ± 5.88	96	*
$r = 0.75$	1	73	568.22 ± 51.07	237.93 ± 53.01	58	*
	2	35	245.67 ± 223.54	4.08 ± 3.55	98	N
	3	54	33.84 ± 0.27	0.67 ± 0.18	98	***
	4	25	0.54 ± 0.20	0.22 ± 0.02	59	N
	5	13	2.05 ± 0.40	3.49 ± 1.75	−70	N
	6	19	322.26 ± 96.49	412.55 ± 225.27	−28	N
	7	87	107.98 ± 17.83	52.78 ± 3.69	51	*
	8	5	62.93 ± 14.36	69.15 ± 60.25	−10	N
	9	80	461.21 ± 121.15	88.05 ± 13.57	81	*

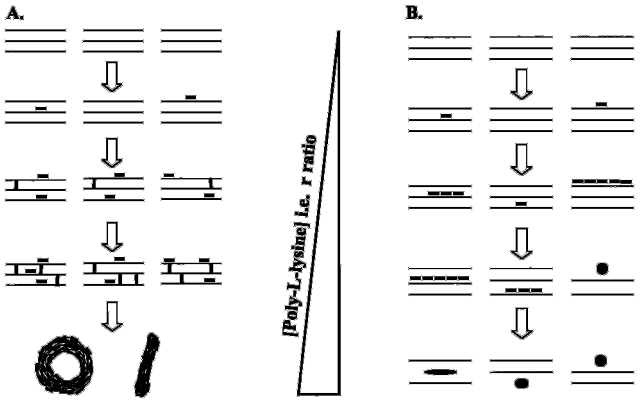

FIG. 8. **Schematic diagram contrasting noncooperative and cooperative binding of DNA and poly-L-lysine.** *A*, noncooperative binding: when poly-L-lysine is mixed with DNA under low salt concentrations, cross-linking of different DNA molecules produces aggregated structures including rods and toroids. *B*, cooperative binding: cooperative binding of poly-L-lysine to DNA involves the initial binding of poly-L-lysine to an individual DNA molecule forming a nucleus of condensation. Subsequent poly-L-lysine molecules prefer to bind to the neighboring sites around this nucleus of condensation on the same DNA molecule. This results in the condensation of DNA into small spheroids (15–30 nm) or ellipsoidal rods (major diameters of ~100 nm). There are two populations of DNA: fully bound small DNA complexes (*spheroids* and *ellipsoidal rods*) and unbound free DNA (*thin lines*). Poly-L-lysine is shown as *small dark bars* bound to DNA.

found in these experiments, we transfected DNA complexes into HuH-7 cells using the asialoglycoprotein receptor as a target for DNA uptake into the cells. Condensed DNA complexes could be taken up by the HuH-7 cell either nonspecifically or specifically via the asialoglycoprotein receptor. Specific uptake of DNA complexes through receptor-mediated endocytosis can be inhibited by competition with excess ligands (for example, ASF). The transfection activities of condensed DNA are listed along with the percentages of condensed DNA with a diameter less than 30 nm (Table I). Receptor-mediated gene transfer only occurred when most of the condensed DNA complexes had a spherical or ellipsoidal shape with a diameter less than 30 nm. Using these condensed DNA complexes, we also observed a correlation between the concentration of DNA complexes and the efficiency of gene transfer (Fig. 7*C*). In contrast to small spherical particles (15–30 nm), rodlike condensed DNA complexes (major diameters of ~100 nm) formed under conditions of positive cooperativity were not expressed in HuH-7 hepatoma cells by receptor-mediated gene transfer. Presumably, the shape of these particles prevented efficient receptor-mediated uptake and/or interfered with efficient cellular trafficking to the nucleus.

In a parallel study, we analyzed the nuclear translocation of condensed DNA particles by determining the level of green fluorescence protein gene expression. DNA preparations were microinjected into the cytoplasm of HuH-7 cells that contained an intact nuclear membrane.[2] To be expressed, the condensed or naked DNA plasmid encoding the green fluorescence protein gene must be transported into the nucleus of these cells. Only condensed DNA complexes of 25 nm or less, which were in the shape of spheroids or short ellipsoids (as noted by EM), were expressed in these cells, most likely because of the size limitations imposed by transport through the nuclear pore complex. Furthermore, DNA complexes prepared at 0 M NaCl also failed to demonstrate receptor-mediated gene transfer (data not shown). This gene transfer result correlated with the presence of Ψ-form DNA and aggregated DNA complexes using formulation conditions that do not favor cooperative binding of poly-L-lysine to DNA (Fig. 3, *E* and *F*). In summary, these results support our general conclusions that specific uptake into the cell via the asialoglycoprotein receptor and the ultimate expression of the transgene depends on the size and shape of condensed DNA complexes.

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