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DNA Complexing with Polyamidoamine Dendrimers: Implications for Transfection

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DNA and polyamidamine (PAMAM) dendrimers form complexes on the basis of the electrostatic interactions between negatively charged phosphate groups of the nucleic acid and protonated (positively charged) amino groups of the polymers. Charge neutralization of both components and subsequent increases of the net positive charge of the complex result in changes in the physicochemistry and biological properties of the complexes. The formation of soluble, low-density and insoluble, high-density complexes was analyzed using UV light absorption and measurements of radioactive labeled DNA. Formation of high molecular weight and high-density complexes depended mainly on the DNA concentration and was enhanced by increasing the dendrimer−DNA charge ratio. Electrostatic charge related effects (attraction or repulsion of charged particles) appeared to be modulated by the generation of dendrimer (size of the polymer). With the progressive increases in the dendrimer−DNA charge ratio (above 20), an increase in the amount of low-density, soluble complexes was observed. Functional analysis revealed that the great majority (>90%) of transfection is carried by low-density, soluble, complexes which only represent approximately 10−20% of total complexed DNA. The ability of the dendrimer to complex and form aggregates with DNA is crucial for efficient transfection and the function of the complexed DNA.

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INTRODUCTION

Formation of a complex between DNA and a synthetic carrier appears to be the initial and possibly the critical parameter for nonviral gene delivery. A number of synthetic vector systems, including polylysines, cationic liposomes, cationic polymers, or various types of block copolymers, have been utilized for the transfer of DNA molecules into cells (*1*−*3*). All these cationic systems have been shown to self-assemble with plasmid DNA expression vectors. In certain conditions, the interaction of DNA with polyvalent cations results in compaction of extended DNA structures, that then aggregate and precipitate from the solution (*4*−*6*). Physicochemical properties of the formed complexes depend mainly on the type of cationic agent used (*7*−*9*). Among cationic polymers, dendrimers have recently emerged as a novel synthetic carrier for DNA transfer (*10*−*12*). Starburst PAMAM dendrimers are nanoscopic polyamidoamine polymers with a molecular architecture characterized by the regular dendritic branching and radial symmetry (*10*, *13*, *14*). The perfectly spheric topological structure and homogeneity of these polymers is achieved by the ordered assembly of polymer subunits in concentric, dendritic tiers surrounding an initiator core. Physical and chemical properties of dendrimers are an outgrowth of their shape as well as the presence of charged groups on the surface. High positive charge density in these molecules is due to the presence of protonized primary amine groups on the surface of the molecules. Together with high solubility in water, these characteristics have led to the use of these polyamines to mediate efficient DNA transfer. Dendrimers appear to be highly efficient for in vitro transfection and are not cytotoxic (*12*, *15*). Other evidence suggests that these polymers are not immunogenic or carcinogenic, enhancing their potential as vectors for an in vivo gene transfer system (*16*).

The alterations of DNA structure caused by complexation with dendrimers and the function of the complexed genetic material are yet to be clarified. In other trivalent or higher valency cationic polyamines, nucleic acid complexation affects DNA structure and physicochemical properties both in vitro (*17*−*19*) and in vivo (*20*, *21*). In particular, high molecular weight cationic polymers such as polylysine, protamine, spermidine, spermine, and histones are known to complex and condense DNA and alter transcription efficiency in vivo (*17*, *22*−*24*). Complexing DNA with dendrimers leads to changes in transcription efficiency in vitro, but also increases DNA survival upon delivery in vitro and in vivo (*15*, *25*). Thus, the physicochemical properties and biological function of dendrimer−DNA complexes appear to be an important issue for the development of artificial, self-assembling systems for gene transfer (*17*, *26*).

Our previous studies suggested that transfection efficiency and functionality of DNA complexed with dendrimers may depend on the structure, size, and charge density of the polymer (*12*, *17*). The defined structure of dendrimers and the ability to alter a single parameter in the structure of the molecule make these polymers unique reagents to study the interaction between DNA and polycations. Structural studies on dendrimer-complexed condensed DNA may reveal the geometric and stoichiometric interactions relevant for the in vivo function. We therefore set out to examine the interactions occurring during DNA−dendrimer complex formation for use in in vitro transfections. This focused our work on

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complexes formed in DNA concentrations up to 1 mg/mL and in salt-free, water solutions. In the present studies, we characterize the processes involved with the formation of DNA−dendrimer complexes and the implications of this phenomenon for gene transfer.

MATERIAL AND METHODS

Dendrimers. The synthesis of Starburst PAMAM dendrimers has been previously described in detail (*10, 11*). In these studies we have used dendrimeres with ethylenediamine (EDA) as a tetravalent polymerization initiator core. Generations (G) 4, 5, 6, 7, and 9 EDA core dendrimers have molar masses of 14 215, 28 826, 58 048, 116 493, and 467 162 kDa, respectively; the number of surface charges (amine groups) is 64, 128, 256, 512, and 2048.

Preparation of Polycation−DNA Complexes. Methyl alcohol stocks of dendrimers were diluted to an appropriate concentration in water and stored at 4 °C. Dendrimer−DNA complexes were formed by incubating the two components together in 100 μL of double distilled water for a minimum of 10 min at room temperature (RT) unless indicated otherwise. Ratios of dendrimer to nucleic acids were based on the calculation of the electrostatic charge present on each component, the number of terminal amine groups on a dendrimer vs the number of phosphate groups in the nucleic acid. For example, a G7 EDA dendrimer has approximately 2.65×10^{15} positive charges (protonized surface amine groups $NH3^+$)/μg (1 μg); the number of bases in a microgram (1 μg) of DNA is 1.71×10^{15}, resulting in approximately 1.71×10^{15} negative charges (ionized P, of phosphodiester groups, in pH 7)/1 μg of DNA. To obtain the theoretical charge ratio equal 1 (neutralizing conditions), 0.65 μg of this dendrimer is mixed with 1 μg of DNA. Similar calculations and mixing were used to make DNA complexes with the spermidine and spermine (1.8×10^{24} and 2.4×10^{24} positive charges/ mol, respectively). Charge ratios are presented as positive equivalents of the cationic component to negative charge equivalents of the nucleic acid components, as recommended in Nomenclature for Synthetic Gene Delivery Systems (*27*).

DNA for Precipitation Studies. Herring sperm DNA (from Promega or Gibco BRL-LifeTechnologies) preparations of sonicated double stranded DNA with a particle size ranging 100−1000 kb, where the majority of DNA fragments ranged 600−800 bp, were utilized in most of the precipitation studies. Plasmids pGL-2 control (Promega, 6 kb), pBK-RSV (Stratagene, 4.4 kb) and pCMV-Luc (*11*) were amplified in *E. coli* XL1Blue cells and isolated by double cesium chloride gradient (*28*) to ensure the purity of the DNA preparation. Single-stranded deoxyribonucleotide 27 base long oligonucleotide was synthesized using a DNA/RNA synthesizer (Model 394, Applied Biosystems) and purified by HPLC in the Biomedical Core Facility of the University of Michigan.

Analysis of Polycations−DNA Interactions. A number of polycations, such as dendrimers, spermidine, or spermine, were mixed with DNA samples in double distilled water, gently vortexed, incubated for 10 min RT and centrifuged at 11750*g* in an Eppendorf centrifuge for 5−7 min unless indicated otherwise. The supernatant and pellet were separated and the amount of DNA present in supernatant, denoted as "low density", was analyzed by spectrophotometry of DNA absorption at 260 nm or by measurement of the radioactivity of ^{32}P-labeled DNA. For UV absorbance measurement, an aliquot (100 μL) of the supernatant was diluted in water up to 400 μL. For the radioactive assay, 2.0−5.0 ng of tracer DNA, either 27 bp oligonucleotide or herring sperm DNA 5′-end labeled with ^{32}P, was added to the nonlabeled DNA. After centrifugation the radioactivity in supernatants was determined by counting in a Wallac 1409 Liquid Scintillation Counter.

Cells and Media. COS-1 and Rat2 cells were maintained in D-MEM medium (Gibco BRL) with 5% −10% FCS (Hyclone), 1% penicillin-streptomycin and 2 mM L-glutamine. D5 cells were cultivated in RPMI 1640 (Life Technologies) supplemented with 10% fetal calf serum, 1% penicillin-streptomycin, 2 mM L-glutamine, 50 μM 2-mercaptoethanol, 1 mM nonessential amino acids). All cell lines were incubated at 37 °C in 5% CO_2.

Transfection Methods. Transfections with dendrimer−plasmid DNA complexes were performed and analyzed using assays for luciferase activity expression from pCMV-Luc reporter plasmid (*15*). Indicated amounts of pCMV-Luc DNA were mixed with dendrimers at a variety of dendrimer to DNA charge ratios (ranging 1−50) and were then allowed to complex for 5−10 min at RT. The 24-well plates seeded 24 h before the transfection with approximately 2×10^4 cells/well, were washed once with serum-free media. The dendrimer−DNA complexes were then added, and transfection was carried for 3 h at 37 °C. After transfection, mix containing the complexes was washed out of the cells and standard growth media was added for incubation. The cells were harvested 24 h later and assayed for the expression of luciferase. To further analyze transfectional properties of complexes, dendrimer-complexed DNA was fractionated by centrifugation at 8160−11750*g* in water or using 0 to 2% and 0 to 5% step glycerol gradients. For the transfections 25 μL of the solution containing DNA−dendrimer complexes either not fractionated (suspension) or after centrifugation (low density) was added to 200 μL of serum-free media in each well of cells. Transfections and posttransfectional incubations proceeded as above. Luciferase activity was determined by measuring the light emission from 10 μL of cell lysate incubated with 2.35×10^{-2} μmol of luciferin substrate (Promega, Technical Bulletin no. 101). Light emission was measured in a relative light units (RLU) using chemiluminometer LB96P (EG&G Berthold), and adjusted to the protein concentration of the sample. The protein concentration in the cell lysates was measured in a standard protein assay (DC protein assay, Bio-Rad Richmond, CA).

Statistical Analysis. Statistical analysis was performed using Systat 5.2 software for Macintosh. Mean and standard deviations of the samples were determined and differences between them were analyzed by ANOVA.

RESULTS

Complexing with Dendrimers Results in DNA Precipitation. Formation of dendrimer complexes with supercoiled plasmid DNA, single- and double-stranded oligonucleotides, and linear forms of high molecular weight DNA has been previously demonstrated by retardation of electrophoretic mobility as well as by electronmicroscopy. Binding of DNA to dendrimer changes the molecular structure of DNA and results in condensation and aggregation of DNA particles (*12, 15, 17*). To examine the general physicochemical properties of dendrimer−DNA complexes and the changes in solubility of DNA upon interaction with dendrimers, several studies were performed. Complexes of dendrimer/herring sperm DNA at charge ratios ranging 0.01−100 were formed in 100 μL of water containing 10 μg of DNA (concentration

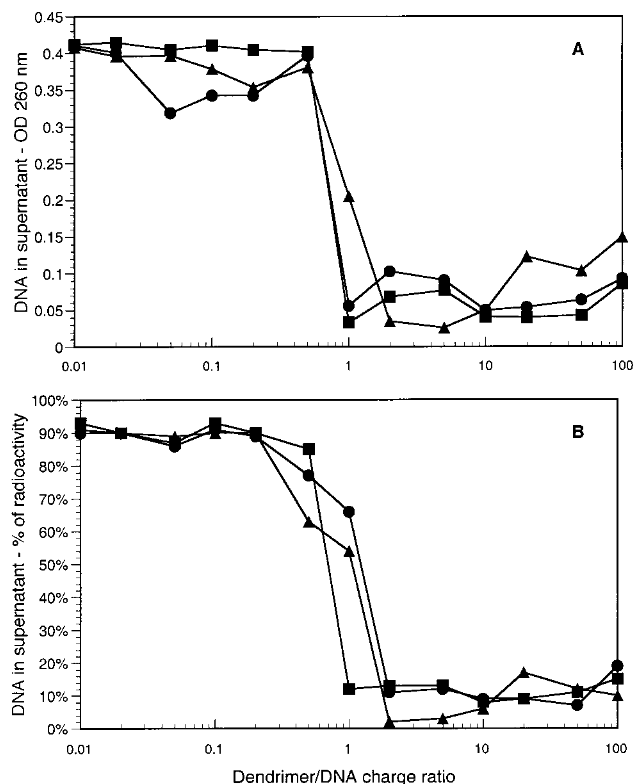

Figure 1. The formation of insoluble DNA aggregates with different generations of EDA core dendrimers. Comparison of the data obtained with spectrophotometric assay of DNA absorption at 260 nm (A) and measurement of radioactive DNA (B) present in supernatants after separation of precipitated complexes and soluble DNA fractions. EDA dendrimers G5 (■), G7 (●), and G9 (▲).

0.1 μg/μL) and increasing amounts of G 5, 7, and 9 EDA dendrimers. All complexing was performed in the absence of other ions in double-distilled water. DNA remaining in supernatant after centrifugation was analyzed using DNA absorption at 260 nm (Figure 1A) and by measuring the presence of radioactivity in the supernatant (Figure 1B). A simple spectrophotometric assay was possible because dendrimers, in the concentrations used in the experiments, have very low absorption across the broad spectrum of wavelengths (including the 260 nm maximum of DNA absorption; data not shown). The course of the precipitation curves in Figure 1 illustrates that both methods of measurement can accurately reflect formation of DNA precipitate. At 0.1 μg/μL concentration, DNA is completely neutralized at dendrimer−DNA charge ratio of 1 when complexed with three different generations of EDA core dendrimer (*12*, *17*). Approximately 95% of these complexes precipitate and the transition of DNA from soluble to condensed form seemed to be abrupt. Charge ratios lower than 1 (e.g., 0.5), did not result in a significant formation of the precipitate, and increasing of the dendrimer−DNA charge ratio (to 10−15) did not further enhance the precipitate formation. However, complexing at a charge ratio of 20 or greater resulted in higher DNA content in the soluble fractions reaching approximately 10−20% (of the total DNA). This phenomenon seems to be somewhat dependent on the generation of dendrimer used (Figure 1B). We also observed increased UV light absorption at these ratios (up to 25−30%), suggesting the generation of low-density particles containing both DNA and dendrimers (Figure 1A).

DNA Concentration Effects. We have studied the effect of DNA concentration on the formation of dendrimers−DNA complexes using plasmid pGL-2 DNA and G7 EDA. A broad range of DNA concentrations (from 0.01 to 1 μg/μL) was mixed with dendrimer at increasing concentrations from 0.0065 to 26 μg/μL (Figure 2). Plasmid DNA at the lowest concentration of 0.01 μg/μL did not form insoluble complexes with G7 EDA dendrimers at any polycation concentration or charge ratio (for this DNA concentration, analyzed dendrimer−DNA charge ratios are 1−4000). Increasing the DNA concentration to 0.1−1 μg/μL and complexing with 3.25 μg/μL of G7 EDA resulted in a 50% of DNA present in precipitates. Complexing DNA at a concentration of 1 μg/μL with G7 EDA at the charge ratio of 10 (concentration 6.5 μg/μL) resulted in the formation of 95% aggregated dendrimer−DNA complexes. The formation of the precipitate increased as the DNA concentration grew from 0.01 to 1 μg/μL, and less dendrimer and a lower dendrimer−DNA charge ratio were sufficient to generate insoluble aggregates in this conditions (Figure 2). We have observed that further increases of DNA concentration resulted in resolubization of the complexes, particularly at greater than 10 dendrimer−DNA charge ratios (data not shown).

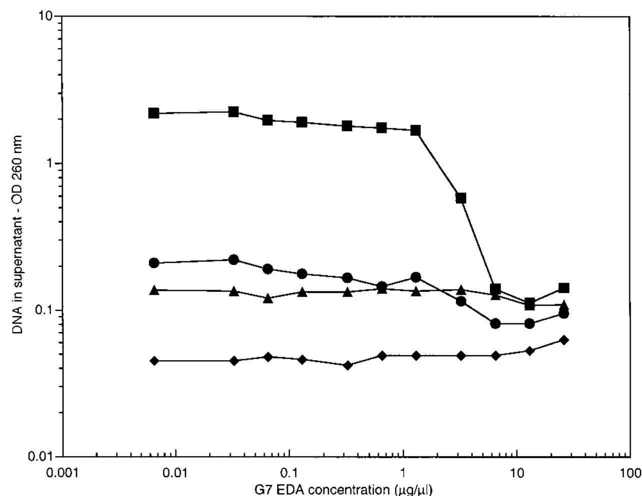

Figure 2. Effect of DNA concentration on the formation of aggregated dendrimer−DNA complexes. Note that a logarithmic scale is used for dendrimer concentration (μg/μL). DNA concentrations: (■) 1 μg/μL, (●) 0.1 μg/μL, (▲) 0.05 μg/μL, (◆) 0.01 μg/μL.

Dendrimers Form Complexes with Various Forms of Nucleic Acid. G7 EDA dendrimer formed complexes with 27 base oligonucleotides, herring sperm DNA (average length of DNA particle 600−800 bp) or pGL2−the latter, a supercoiled, covalently closed 6 kb plasmid (Figure 3). All DNA preparations were prepared at 0.1 μg/μL concentration and mixed at the appropriate charge ratio with G7 EDA. To increase the capability to detect differences in dendrimer−DNA complex formation with various forms of nucleic acids, the separation of the low- and high-density fractions was performed by centrifugation at 5220*g* instead of the standard conditions (Materials and Methods). All three types of DNA formed insoluble complexes when mixed with G7 EDA at increasing dendrimer−DNA charge ratios. However, 50% of 0.1 μg/μL plasmid DNA remained in supernatant when complexed with G7 EDA dendrimer at a dendrimer−DNA charge ratio of 5 (Figure 3A). In contrast, the oligonucleotides or herring sperm DNA complexed at the same charge ratio resulted in approximately 20% of the total DNA remaining in the supernatant. While similar patterns of the precipitation curves were obtained with all three forms of DNA, aggregation of plasmid DNA proceeded at higher G7 EDA/DNA charge ratio.

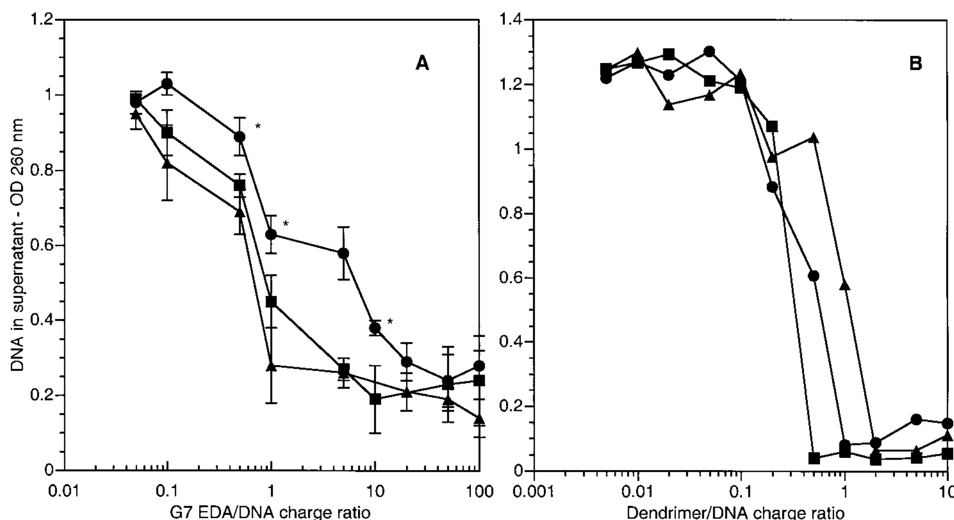

Figure 3. Formation of the aggregates with various types of nucleic acids. (A) G7 EDA complexed with 0.1 μg/μL of the different types of DNA: (■) low molecular weight, single-stranded 27 base DNA (oligo); (●) heterogeneous population of double stranded sonicated herring sperm DNA (hs DNA); and (▲) uniform population of covalently closed plasmid DNA (pGL-2). [The asterisk (*) indicates data points where $p < 0.05$ for herring sperm DNA or oligo and plasmid DNA]. (B) Plasmid DNA (pBK RSV) at 1 μg/μL complexed with EDA dendrimers. EDA dendrimers G5 (■), G7 (●), and G9 (▲).

Soluble dendrimer−plasmid DNA complexes were not observed with higher concentrations of plasmid DNA (Figure 3B). When 100 μg of pBK RSV plasmid DNA (4 kb) at a final concentration of 1 μg/μL was complexed with G5, G7, and G9 EDA in dendrimer−DNA (charge ratios ranging 0.05−1), the precipitation curves indicated that at this DNA concentration and at a dendrimer−DNA charge ratio equal to 1, all generations of dendrimers precipitated >95% of plasmid DNA. The profiles of the precipitation curves are similar, but there are subtle differences in maximum of complexation with different generations of dendrimers. Smaller dendrimers, G5 and G7 EDA, tend to form insoluble DNA complexes at lower charge ratios (∼0.5) than the larger G9 EDA dendrimer (Figure 3B). This result may suggest that the electrostatic process of complexing and aggregation of DNA may be uniquely affected by the size of the dendrimer.

To further analyze the effect of size, surface charge, and molar concentration of dendrimer on complex formation, herring sperm DNA at 0.025 μg/μL was incubated with increasing concentrations of G4, G5, G6, G7, and G9 EDA dendrimers (Figure 4). As previously observed with plasmid, at a charge ratio of 1, 95% of DNA complexed with dendrimers was found in the insoluble fractions. The efficiency of precipitation with dendrimers, defined here by the lowest molar concentration of polymer in which the maximum precipitation of DNA occurs, was proportional to the increase in size of the dendrimer. This is not unexpected given the primarily electrostatic nature of dendrimer−DNA interaction, since larger dendrimers carry higher surface charge than smaller ones (*10*). Maximum formation of insoluble complex with 0.025 μg/μL of herring sperm occurs in 0.054 μM G9 EDA, 0.215 μM G7 EDA, 0.429 μM G6 EDA, 0.57 μM G5 EDA, and 0.86 μM G4 EDA dendrimers. Consequently, calculations of the ratios between molar concentrations of G6/G7 (∼2), G7/G9 (∼4), and G6/G9 (∼8) generations reflect the inverse correlation of surface charge ratios between these dendrimers. Interestingly, the molar concentrations of larger G9, G7, and G6 EDA dendrimers most effective in the precipitation of DNA are ∼1.5 times higher than those required for neutralizating of the DNA (dendrimer−DNA charge ratio equal 1). In contrast, for the G5 and G4 EDA dendrimers, these concentrations are ∼1.3 times lower than expected for charge neutralization

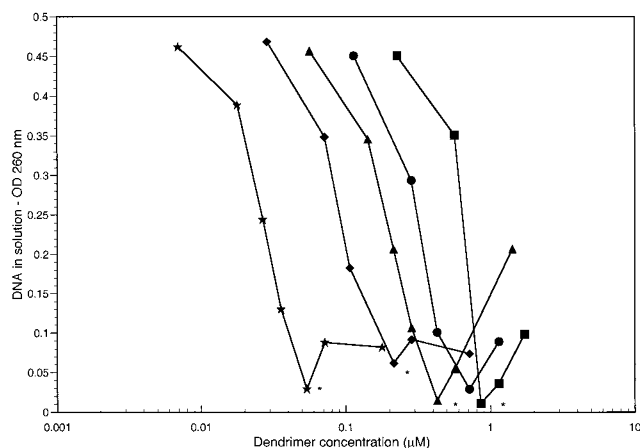

Figure 4. Effect of the size of dendrimer on the formation of aggregated dendrimer DNA complexes. Complexes were formed in a constant DNA concentration (0.025 μg/μL) with increasing concentrations of various generations of dendrimers. Data from dendrimers G4 (■), G5 (●), G6 (▲), G7 (◆), and G9 EDA (★) is described. Differences (*) between molar concentrations of dendrimers were significant with $p < 0.02$.

(Figure 4). These subtle differences in the threshold formation of an insoluble complex again suggest that the overall process of DNA aggregation is not strictly electrostatic, but also involves the size and, consequently, amount of dendrimer particles in the complex.

Efficiency of DNA Precipitation with Dendrimers and Polyamines. Spermidine and spermine are multivalent cations with charges of +3 and +4 per molecule, respectively. Both are known to induce condensation of DNA in aqueous solutions (*5*, *19*, *29*, *30*). To compare the condensation and precipitation with dendrimers, spermidine, and spermine 1 μg/μL of herring sperm DNA with radiolabeled DNA tracer was mixed with the increasing concentrations of each of the polycations. As has been reported previously, 10 mM spermine and spermidine precipitate the majority 80−90% of DNA. When the concentration reaches 100 mM, both polycations resolubilize the DNA pellet, and this transition is significantly enhanced by the presence of 25 mM NaCl (*5*). In contrast, G7 EDA at 1.12 μM (charge ratio 1) precipitates almost 90% of DNA and at 27.7 μM resolubilizes approximately

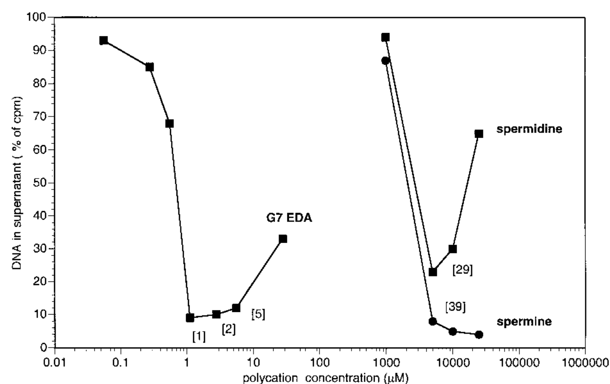

Figure 5. Comparison of DNA complex formation with G7 EDA dendrimer vs polyamines spermidine and spermine. Polycation/DNA charge ratios at the maximum of the DNA aggregation are indicated in the brackets.

30% of dendrimer–DNA complexes. Approximate polycation–DNA charge ratios of the most precipitation-effective molar concentrations of polymers are 1–5 for G7 EDA, 29 for spermidine, and above 39 for spermine (Figure 5). This indicates that spermidine and spermine require 30–40 times higher charge ratios and $(5-10) \times 10^3$ higher molar concentrations than dendrimer to induce a similar degree of DNA condensation.

Transfection Studies with Fractionated DNA–Dendrimer Complexes. To test transfection efficiency of soluble and insoluble complexes, 100 μL of mix containing 4 μg of pCMV-Luc plasmid (0.04 μg/μL) and increasing amounts of G9 EDA dendrimer were fractionated by centrifugation at 5220*g*. Two cell lines, Rat2 and D5, were transfected with either 25 μL of whole complex suspension or 25 μL of supernatant from the centrifuged samples. In all dendrimer–DNA charge ratios the majority of in vitro transfection appears to be mediated by the supernatant fractions of DNA–dendrimer complexes (Figure 6). Analysis of DNA distribution using radioactive tracer DNA, indicated that from 11% (at a charge ratio of 1) to 26% (at a charge ratio of 50) of total dendrimer–DNA complexes is in the supernatant fractions (Figure 6, insert). Although the amount of DNA in supernatant samples at charge ratios 1 and 50 differs approximately 2.5-fold, the efficiency of transfection is 5–6 orders of magnitude higher at the 20 and 50 charge ratios as compared to a charge ratio of 1. This result may suggest that the composition of the complexes is the most important factor for the efficient in vitro transfection and not simply the amount of complexed, compacted, and neutralized DNA. Transfections performed with complexes from the resuspended pellets yielded various degrees of reporter gene expression. This indicates that highly aggregated insoluble dendrimer–DNA complexes can be reconstituted to a functional form for transfection. By comparison, the efficiency of transfections performed using DNA complexed with spermidine or spermine was not better than transfections with DNA alone (data not shown).

To further analyze the nature of the most effective for transfection dendrimer–DNA complexes, G7 EDA dendrimer complexed with pCMVLuc plasmid DNA at 0.02μg/μL (charge ratio 10) was fractionated using 0 to 5% step glycerol gradient. The 100 μL samples of fractionated complexes were used in transfection of Rat2 cells. Control cells were transfected with 100 μL of regular transfection mix prepared in 2.5% glycerol solution. Transfection was augmented with 50 μM chloroquine in medium. Most efficient transfection complexes were found in the two least-dense fractions (Figure 7). Calculation of glycerol density (Sigma catalog, 1998) of these fractions enabled estimation of equilibrium density for dendrimer–DNA complexes. Lowest density fractions 1 and 2 contained complexes of density of 1.000–1.004 g/mL. Radioactive tracing of the fractionated DNA indicated almost uniform distribution across the density gradient, reflecting the polydyspersed, nonuniform nature of the dendrimer–DNA complex population (Figure 7). The approximately 30% of total DNA present in the two lowest density fractions transfeced Rat2 cells twice as effectively as unfractionated control preparations.

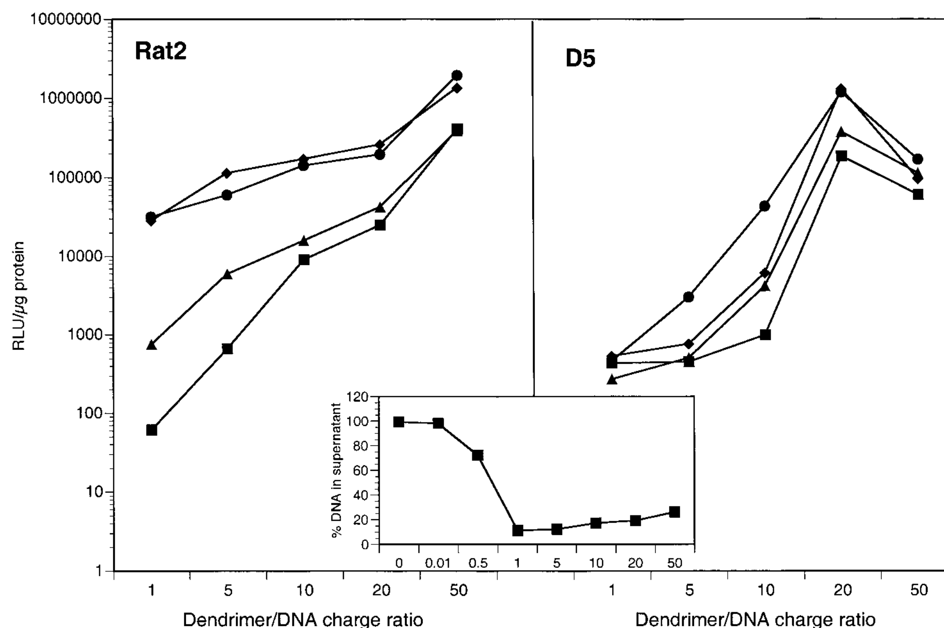

Figure 6. In vitro transfection of Rat2 and D5 cells with unfractionated dendrimer–DNA complexes vs low-density fractionated complexes. Transfection of cells with a suspension of dendrimer–DNA complexes performed either without (■) or with (●) 0.5 μM DEAE-dextran. Transfection with supernatant fractions containing soluble dendrimer–DNA complexes without (▲) and with (◆) augmentation with DEAE-dextran. Insert illustrates data obtained from radioactive tracing of fractionated G7 EDA/pCMV-Luc DNA complexes.

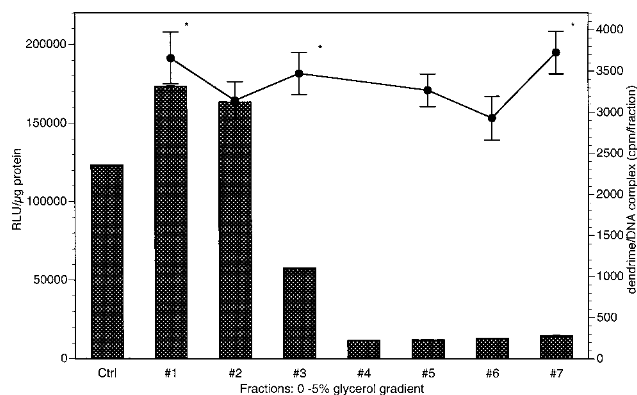

Figure 7. In vitro transfection of Rat2 cells with G7 EDA/DNA complexes. Complexes were fractionated by centrifugation on 0−5% glycerol gradient. Line (●) represents the distribution of the radioactively labeled dendrimer−DNA complexes in the gradient (right side axis). (*) $p > 0.5$ (± S.D.). The columns represent luciferase expression after transfection with unfractionated complexes vs the fractions 1−7 (left side axis).

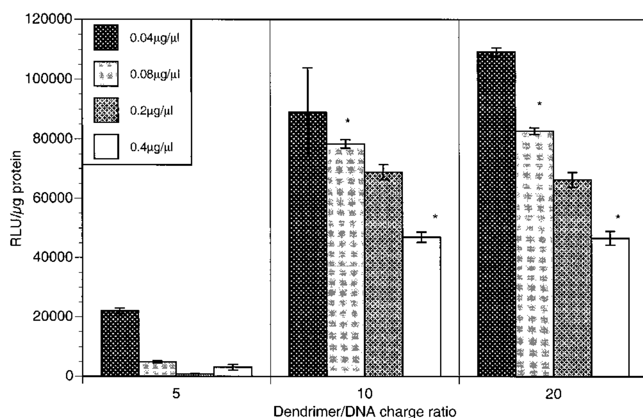

Figure 8. Effect of DNA concentration on the efficiency of in vitro transfection. COS-1 cells transfection with 1 µg of pCMV-luc plasmid DNA. DNA concentrations during complex formation with dendrimers (from 0.04 to 0.4 µg/µL) are indicated in the legend. (*) $p < 0.05$ (mean ± S.D.).

Figure 8 illustrates another approach to analyze the effect of DNA concentration-dependent compacting and aggregation on the efficiency of transfection in vitro. The G9 EDA dendrimer−DNA complexes were prepared with constant amount (1 µg) of pCMVLuc DNA at concentrations of 0.04, 0.08, 0.2, and 0.4 µg/µL and at the varying dendrimer−DNA charge ratios. The most efficient transfection of COS-1 cells occurred at the lowest DNA concentrations during complex formation, particularly in the high dendrimer−DNA charge ratios 10 and 20 (Figure 8). The precipitation experiments (Figures 2 and 3A) indicated that complexing dendrimers with DNA at lower concentrations (e.g., 0.05 µg/µL) resulted in the generation of lower density complexes which did not form precipitates. This type of complex mediated at least 2.5 times more efficient transfection of COS-1 cells than the higher density (less soluble) complexes obtained at higher DNA concentrations.

DISCUSSION

Various types of cationic molecules, including cationic lipids, polylysines, recombinant histones, and, recently, PAMAM dendrimers, have been employed as nonviral vectors for gene transfer into eukaryotic cells (*1*−*12*, *31*, *32*). These diverse materials have in common the ability to bind DNA binding via charge-based interactions, this property appearing to be necessary for transfection (*17*, *33*). The subsequent processes involved in the transfer and expression of the genetic material are not fully understood, but the compaction of DNA into condensed structures is also thought to play an important role in the cellular internalization of the genetic material. Alterations in the structure of the DNA polycation complex appear to affect the transfer and expression of the genetic material in cells and may be a critical issue in the development of nonviral gene transfer systems.

PAMAM dendrimers are an interesting new class of highly defined, spherical, cationic polymers (*10*, *14*). Recent studies have shown that these polymers can bind genetic material and facilitate gene transfer to a wide variety of cells (*11*, *12*, *15*, *26*). The characterization of the physiochemical properties of the DNA−dendrimer complex, including aggregation and precipitation are documented for the first time in this paper. We have identified that in DNA concentrations ranging 0.01−1 µg/µL, complexation with dendrimers may result in the formation of aggregates and precipitation. The primary mechanism of this process involves the electrostatic interaction between DNA and dendrimer molecules (*17*). This is illustrated in Figures 1, 3, and 4 by the generally identical shape of precipitation curves observed with dendrimers of different generations (sizes). However, the shift in the position of the curve indicates there is an influence of other parameters such as size and concentration of both dendrimers and DNA. Although all generations of PAMAM dendrimers have the same surface charge density, the interaction of smaller dendrimers G5 and G7 EDA with DNA led to formation of insoluble DNA complexes at lower charge ratios than with larger G9 EDA dendrimers. While dendrimers will form complexes with various forms of nucleic acid, including single-stranded oligonucleotides, larger fragments of salmon sperm DNA, or circular plasmid DNA, aggregation of plasmid DNA required higher dendrimer−DNA charge ratios. This indicates that average density of particles in populations of complexes formed at a specific charge ratio may differ depending on the type of DNA.

Various forms of synthetic and natural polyamines, spermidine, spermine, cobalthexamine, or polylysine interact physicochemically with DNA. Our data revealed similarities between the dendrimer mediated aggregation of DNA and that observed with other polycationic polymers (*5*, *6*, *11*, *13*, *20*, *34*). All these compounds bind various forms of DNA through charge−charge interactions, and efficiency of precipitation is dependent on the concentrations and charge ratios of both components as well as chemical structure of polycation (*5*, *26*). However, some specific differences are observed. Although the actual binding constants of large polyvalent compounds such as DNA and dendrimers are not readily determined (*5*, *8*, *35*) the high-density surface charge of the dendrimer molecules, the molar concentrations and dendrimer−DNA charge ratios that yield maximum DNA complexing are much lower for dendrimers than for other polycations. Another interesting difference is observed when comparing concentrations of DNA involved in complex formation. Spermidine and spermine seem to be most effective at forming insoluble complexes at lower DNA concentrations up to 0.01 µg/µL (*5*), while all generations of dendrimers are most effective at forming aggregates with higher DNA concentrations ranging from 0.04 to 1 µg/µL.

It is important to note that all experimental conditions tested correspond to those used for transfection in vitro and are not associated with cytotoxic effects. The excess of dendrimer provides DNA complexes with a positive net

charge. This is necessary for the efficient cellular uptake of dendrimer−DNA complexes through both energy-dependent and -independent mechanisms, as well as prolongs the survival of the transfected DNA (*12*, *17*, *25*).

In light of the precipitation studies, we have considered the link between compacting DNA and transfection in vitro. As other high molecular weight polycations, dendrimers form neutralized complexes with DNA at a charge ratio equal to 1. If compacting DNA is a crucial feature for the gene transfer as has been suggested (*1*, *2*, *9*, *32*), then one could expect that most efficient transfection with dendrimer−DNA complexes would be obtained with this conditions. Our results indicate otherwise and suggest in general that the transfection efficiency of complexes increase with an excess of cationic dendrimer (*12*). Such complexes display several unique properties in comparison to complexes formed at neutralizing concentrations of dendrimers. In particular, the appearance of the populations of low-density particles occurs only at these ratios. These low-density complexes, not yet fully characterized for structure and stoichiometry, seem to be important for achieving gene transfer since the transfection activity is contained in these soluble fractions. The complexity of polymeric polyelectrolite theories currently prevents a reliable theoretical calculation of complex stoichiometry (*5*, *34*, *35*). However, our results indicate that the conditions of complex formation (e.g., DNA concentration) define the transfection efficiency of the complex and in turn suggest that the initial complex formation may determine the final functionality of complexed DNA.

Naturally occurring polyamines are involved in numerous cellular processes (*21*) and are often used for in vitro studies of the functional properties of DNA (*18*). The correlation between condensation and aggregation of DNA and the stimulatory effect on DNA replication and transcription (*21*, *36*, *37*) have lead to the suggestion that these aggregates are fluid (*5*, *38*). Some forms of polyamine-condensed DNA are known to acquire liquid crystalline structure (*5*, *39*), and it is possible that dendrimer−DNA complexes may display properties of liquid crystals (*5*). The generation of the low-density particles at high DNA concentrations and high charge ratios closely resembles the resolubilization of DNA, which has been documented for spermidine, spermine, cobalthexamine (*5*, *29*), and other polyamines (*40*). However, the relevance of this phenomenon for gene transfer is not clear, since none of the these low molecular weight polycations is effective by itself for transfection even if they are frequently used as modifying moieties for lipid, liposome or polylysine based synthetic gene carriers (*30*−*33*).

In conclusion, the dendrimer−DNA interactions described in this study offer several insights into how these complexes form and mediate transfection. They also provide structural possibilities to enhance these polycations as carriers for cellular delivery of DNA and other genetic materials. Further characterization of the dendrimers with possible modifications of the charge and structure will clarify which properties of DNA−dendrimer complex absolutely determine its biological activity.

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