Plasmid DNA size does not affect the physicochemical properties of lipoplexes but modulates gene transfer efficiency

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ABSTRACT

Clinical applications of gene therapy mainly depend on the development of efficient gene transfer vectors. Large DNA molecules can only be transfected into cells by using synthetic vectors such as cationic lipids and polymers. The present investigation was therefore designed to explore the physicochemical properties of cationic lipid–DNA particles, with plasmids ranging from 900 to 52 500 bp. The colloidal stability of the lipoplexes formed by complexing lipopolyamine micelles with plasmid DNA of various lengths, depending on the charge ratio, resulted in the formation of three domains, respectively corresponding to negatively, neutrally and positively charged lipoplexes. Lipoplex morphology and structure were determined by the physicochemical characteristics of the DNA and of the cationic lipid. Thus, the lamellar spacing of the structure was determined by the cationic lipid and its spherical morphology by the DNA. The main result of this study was that the morphological and structural features of the lipopolyamine–DNA complexes did not depend on plasmid DNA length. On the other hand, their gene transfer capacity was affected by the size of plasmid DNA molecules which were sandwiched between the lipid bilayers. The most effective lipopolyamine–DNA complexes for gene transfer were those containing the shortest plasmid DNA.

INTRODUCTION

Viral vectors can mediate efficient gene transfer. However, three factors may significantly limit their clinical application: (i) viral-related immunogenicity; (ii) the potential risk associated with replication-competent virus; and (iii) the size limitation of the transgene. As an alternative, non-viral vectors, i.e. plasmid DNA, either naked or complexed with cationic molecules, have also been used for gene transfer in clinical trials. Despite encouraging results, non-viral vectors remain less efficient for transfection than viruses. A better understanding of the lipoplex and polyplex structures involved in their biological activity, cellular entry and nuclear import of plasmid DNA might improve their efficacy for transfection.

The supramolecular structure of cationic lipid–DNA lipoplexes was recently reported for some formulations. For instance, lipid bilayer covered DNA tubules were found after the addition of DNA to cationic liposomes consisting of 3-β-[N-(N',N'-dimethyllethyl)carbamoyl] cholesterol/dioleoyl phosphatidylethanolamine (DC-Chol/DOPE) (1). In contrast, the addition of linear or circular DNA to cationic liposomes of dioleoyl trimethylammonium propane/DOPE (DOTAP/DOPE) (2) and of dioctadecylammonium bromide/cholesterol (DODAB/Chol) (3) was shown to induce a topological transition from unilamellar vesicles into a higher order multilamellar structure in which DNA is sandwiched between lipid bilayers. In the latter two reports a multilamellar organization with a regular spacing of 65 Å was reported after DNA had been mixed with unilamellar vesicles of quaternary ammonium lipids, i.e. lipids bearing a single positive charge per molecule, combined with a neutral lipid or cholesterol. In a previous work, structural analysis of lipoplexes resulting from the association of plasmid DNA with a lipopolyamine, i.e. RPR120535, containing three positive charges at neutral pH showed that plasmid DNA transformed multivalent cationic lipid micelles into a supramolecular organization characterized by fully condensed DNA inside spherical particles (diameter 50 nm) resulting in a local lamellar organization with a spacing of 80 Å (4).

Despite the great efforts made to understand the physicochemical properties of the cationic lipid–DNA lipoplexes used in non-viral gene therapy (5–7), very little is known about the effect of DNA plasmid length on the ultrastructural features of these lipoplexes and its action on their gene transfer activity. However, in the near future, it will be of crucial importance to develop systems capable of transducing large DNA fragments into cells. For example, plasmovirus technology (8) and the development of regulatory systems for gene expression induced by agents like tetracycline or rapamycin are based on the transfection of large DNA molecules into cells (9,10). On
the other hand the length of human dystrophin cDNA (14 000 bp) prevents it from being introduced into currently used viral vectors, thus necessitating the use of the less efficient 6000 bp minidystrophin cDNA (11,12).

The objective of this study was to apply a previously described methodology (4), designed to provide a simple way of producing small cationic lipid–DNA lipoplexes with rigorous physicochemical characterization, to plasmid DNA of various sizes ranging from 900 to 52 500 bp. We therefore explored the correlation between the physicochemical properties of lipopolyamine–DNA complexes and transfection activity, because these structure–function relationships are of crucial importance for the future development of cationic lipid-mediated gene delivery.

MATERIALS AND METHODS

Polyanions and plasmids

Sodium hexametaphosphate was provided by Europhos (Engis, Belgium).

Plasmid construction and isolation, bacterial transformation and DNA analysis were performed as already described (13). Plasmids pXL3178 (900 bp) and pXL3032 (3700 bp) were both pCOR (plasmid with a conditional origin of replication) containing the origin of replication, the cer element from the ColE1 site-specific recombination system and the supRK selectable marker as previously described (14). Plasmid pXL3032 contained a 2600 bp luciferase expression cassette containing: (i) the pCMV promoter completed by the 5' Herpes simplex thymidine kinase untranslated region (15); (ii) the modified firefly luciferase gene (luc+) from pGL3 Basic (Promega Corp., Madison, WI); and (iii) the simian virus SV40 late polyadenylation signal, also obtained from pGL3. Plasmid pXL3326 was generated by inserting the 2600 bp BamHI–Swal luciferase expression cassette into pXL2777 (16) cut by BamHI and EcoRV. Plasmid pXL3108 (8600 bp) was a plasmid used for adenoavector vector construction and contains the adenoaviral ITR and pIX sequences (10). Plasmid pXL3141 (19 600 bp) was a derivative of pXL59 (17). Plasmid pXL3340 (19 600 bp) was linearized by cutting 3' to the poly(A) signal with NotI or SalI restriction enzyme. After 1 h incubation at 37°C, linearized plasmids were isolated by phenol/chloroform extraction followed by ethanol precipitation. Linearized plasmids were then resuspended at 100 µg DNA/ml.

HPLC analysis was performed on a Gen-Pak™ FAX anion exchange chromatography column (Waters, Saint Quentin Fallavier, France), with a DNA elution gradient of 2.5 mM NaCl/min in 25 mM Tris, pH 8, at 0.75 ml/min.

Endotoxin contaminants, co-purified with plasmid DNA, were estimated by limulus amoebocyte lysate assay (20) to comprise <0.1 EU/mg plasmid in all plasmid preparations except that of pXL3141 (endotoxin value 0.43 EU/mg plasmid).

Cationic lipid preparation, lipoplex formation and size determination

Lipopolymine RPR120535 and RPR120531 micelles (21) and the lipoplexes they form with DNA were prepared as previously described (4). Bis(guanidinium)-tren-cholesterol (BGTC)/DOPE liposomes and DNA lipoplexes were obtained as previously described (22,23).

Dynamic light scattering measurements of cationic lipid–DNA lipoplexes were performed as previously described (4). Particle size was measured using unimodal analysis with an angle of 90° and the particle size distribution using the CONTIN software.

Small angle X-ray scattering

Complexes were prepared as previously described (4). The X-ray scattering experiments were carried out using the synchrotron radiation source D3I of LURE (Orsay, France) on beamline D43. The distance between the sample and detector was 300 mm. A 2-dimensional image plate detector was used. Results are expressed in arbitrary intensity units as a function of q, where q is the momentum transfer q = 4π/λsinθ/2, θ the scattering angle and λ the X-ray wavelength. q values ranged between 0.05 and 0.30 Å⁻¹.

Cryo-transmission electron microscopy (cryo-TEM)

Cryo-TEM experiments were performed as described previously (4). Briefly, RPR120535–DNA lipoplexes were prepared at 0.5 mg DNA/ml in 20 mM NaCl, with 7 nmol RPR120535/mg DNA, corresponding to a charge ratio (+/–) of 7 and then concentrated by ultrafiltration using 300 K nanopore concentrators (Pall Filtron). RPR120535–polynucleotide complexes were prepared at 0.9 mg/ml with a charge ratio (+/–) of 6.
Cell culture and in vitro transfection
Murine NIH 3T3 fibroblast and HeLa cells (ATCC, Rockville, USA) were grown at 37°C, 5% CO₂ in Dulbecco’s modified Eagle’s medium and minimum essential medium (Gibco BRL, Cergy Pontoise, France) supplemented with penicillin and streptomycin (100 U/ml), L-glutamine (20 mM) and 10% fetal calf serum, respectively. Sodium pyruvate (1 mM) and non-essential amino acids were added to the minimum essential medium. Human aortic smooth muscle cells (AoSMC) (Biowhitaker, Walkerville, USA) were cultured in Smooth Muscle Basal Medium supplemented with 5% fetal bovine serum, human epithelial growth factor (0.5 µg/l), human fibroblast growth factor-B (2 µg/l), amphotericin B (50 µg/l), insulin (5 mg/l) and gentamicin sulfate (50 mg/l) according to the manufacturer’s indications. RPR120535–DNA lipoplexes (50 µl) containing 0.5 µg of plasmid DNA at a charge ratio (+/–) of 6 were added to 500 µl cell culture medium without serum, covering ~75 000 cells in each well of a 24-well dish. Serum (10%) was added to the medium after 3 h transfection. Transfection experiments were performed in quadruplicate. Luciferase activity was assayed 48 h later as previously described (24) using the Luciferase Assay System (Promega Corp., Madison, USA) and a Lumat LB 9501 (Berthold, Evry, France) luminometer. Luciferase activity was related to the protein concentration measured using the Pierce BCA assay (Interchim, Assnieres, France). The variable distribution of luciferase activity was found to correspond to a logarithmically normal distribution and results were given with 95% confidence limits.

RESULTS
Plasmid purification and quality control
The five different plasmids, i.e. pXL3032 (3700 bp), pXL3108 (8600 bp), pXL3340 (19 600 bp) and pXL3141 (52 500 bp) and the minicircle, contained the same luciferase expression cassette as described in Materials and Methods. Relaxed forms of the 900, 3700, 8600 and 19 600 bp plasmids were found to correspond to less than 6% of the total DNA preparation, as measured by agarose gel electrophoresis and anionic exchange chromatography. Unfortunately, the relaxed and supercoiled forms of the 52 500 bp pXL3141 could not be separated by HPLC. Therefore, it was difficult to estimate precisely the relaxed pXL3141 content in the DNA preparation. However, agarose gel electrophoresis showed that only a minor fraction of this plasmid preparation was in the relaxed conformation. RPR120535 micelles with plasmid DNA resulted in a three domain model of colloidal stability (4). These three domains, termed A, B and C (Fig. 1), were also observed for plasmid DNA lengths ranging from 900 to 19 600 bp. The zones were delimited by the concentration of RPR120535 micelles for a given plasmid DNA concentration. We defined the RPR120535–DNA charge ratio (mol positive charges/mol negative charges) as corresponding to 3/2 of the molar ratio (4). In zone A, with a charge ratio ranging from 0 to 1, lipoplexes had a mean diameter of ~180 nm. The lipoplexes in zone B, with a charge ratio of from 1 to 2, were not colloidally stable because a visible precipitate was observed. This precipitate was probably due to a charge ratio close to neutral, which favors the aggregation of lipoplexes by reducing the electrostatic repulsion between them. In zone C, with a charge ratio higher than 2, lipoplexes were found to be colloidally stable, because electrostatic repulsion was present. Note that particle size in zone C was found to be independent of plasmid length, which ranged from 900 to 19 600 bp, and that lipoplex diameters were homogeneous, at around 80 nm. It was interesting to observe that the boundaries between zones A and B and between zones B and C were independent of plasmid length in a 900–19 600 bp domain. Size distribution particle analysis revealed that, as previously shown (4), a homogeneous population of individual particles was formed in zone C with mean diameter ranging from 72 to 93 nm. Fluorescence experiments performed by exposing lipoplexes from zone C, obtained with various DNA lengths, to ethidium bromide showed that the fluorescence level was close to 0. This indicated that all DNA molecules were totally incorporated into the lipoplexes, irrespective of the DNA length. Unlike smaller size plasmid DNA, the 52 500 bp pXL3141 only exhibited, at 0.1 mg DNA/ml, a two zone colloidal stability model with a prolongation of the precipitated zone B, even at a highly positive charge ratio. A visible precipitate was indeed observed, even at a charge ratio of 20 (data not shown). However, at lower DNA concentrations, i.e. the 10 µg DNA/ml which was required for the in vitro transfection experiments, colloidal stability particles from zone C were obtained.

X-ray scattering analysis
Small angle X-ray scattering studies of RPR120535–DNA lipoplexes were performed with plasmids ranging from 900 to 52 500 bp. Figure 2A shows the X-ray scans of the 900, 8600 and 52 500 bp plasmids revealing a main peak at q = 0.0767.
As the length of supercoiled plasmid DNA did not affect particle structure, because a four-lamellar domain with 80 Å spacing was obtained in all cases, we examined the structural features of the complexes resulting from the association of RPR120535 lipopolyamine with linear double-stranded DNA fragments of 54 and 50 000 bp from λ phage or with polyphosphate as the anionic backbone. As stated above, the complexes formed with linear fragments exhibited identical diffraction patterns (82 Å spacing and four lamellae) whereas those obtained with the polyphosphate revealed a main, very narrow diffraction peak at \( d = 79 \) Å, with reflections observed at \( d/2, d/3, d/4 \) and \( d/5 \) exhibiting a lamellar organization (Fig. 2B). The effective domain size of the main diffraction peak was >1000 Å, indicating a structure including more than 12 stacked lamellae. Consequently, the molecular structure and/or the charge density of the DNA are essential for the formation of particles characterized by ordered lamellar domains with four lamellae. However, neither the size nor the topological features of the DNA molecules, i.e. whether they are supercoiled or linear, affected this formation.

We attempted to establish whether the above results were specific to RPR120535–DNA lipoplexes or could be extrapolated to lipoplexes obtained with other transfecting cationic lipids. Accordingly, small X-ray scattering was performed with lipoplexes composed of plasmids of different DNA lengths and either the lipopolyamine RPR120531 or BGTC. Interestingly, when plasmid DNA was complexed with either RPR120531 or BGTC cationic lipids, the lipid composition, but not the plasmid DNA size, was found to affect lipoplex supramolecular structure. Ordered lamellar domains with five lamellae and a spacing of 65 Å were obtained after mixing DNA plasmids composed of 900–52 500 bp with RPR120531 (data not shown), whereas ordered lamellar lipoplexes with four periodicities and a spacing of 70 Å were obtained after mixing the plasmids with BGTC/DOPE (23; data not shown).

**Electron microscopy of RPR120535 complexed with DNA and polyphosphate**

Cryo-TEM analysis was performed on complexes obtained by combining RPR120535 with plasmids of different sizes, with linear fragments of 54 or 50 000 bp or with polyphosphate. For all plasmids and for linear DNA fragments, complexes with a similar shape and a highly ordered structure were observed. They consisted of ordered microdomains ~50 nm in diameter [fig. 2G in Pitard et al. (4)]. In contrast, lipoplexes composed of RPR120535 and polyphosphate appeared as stretched fibers composed of many lamellae (Fig. 3), in agreement with the results obtained by small X-ray scattering (Fig. 2B).

**In vitro transfection**

Note that at the DNA concentration used for *in vitro* transfection experiments (10 µg DNA/ml), colloidally stable, positively charged particles were obtained with all plasmids, including the 52 500 bp plasmid. The luciferase activity was measured in transfected NIH 3T3, human AoSMC and HeLa cells with the 2900 (minicircle), 3700 (pXL3032), 8600 (pXL3108), 19 600 (pXL3340) and 52 500 bp (pXL3141) plasmids, which all contained the same luciferase expression cassette, associated with RPR120535 micelles to form cationic complexes from zone C.

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**Figure 2.** X-ray scattering analysis. (A) Small angle X-ray scattering scans of RPR120535–DNA lipoplexes (filled circle, 900 bp plasmid; open circle, 8600 bp plasmid; open square, 52 500 bp plasmid) at a cationic lipid:DNA charge ratio (+/–) of 2. (B) Small angle X-ray scattering scan of complexes between RPR120535 and polyphosphate at a charge ratio (+/–) of 6. (Inset) The logarithm of the intensity as a function of the momentum transfer (q). \( d \) and \( d/n \) with \( n = 2, 3, 4 \) and 5 indicate the first peak and higher order reflection peaks, respectively.

\( d = 2\pi q \), which corresponded to ordered domains with a spacing of 82 Å, as expressed by the formula \( d = 2\pi q \). Similar X-ray scans were obtained with the other plasmids. Although the exact structure of RPR120535–DNA lipoplexes cannot be affirmed from the X-ray scan illustrated in Figure 2A, because of the absence of the second order reflection peak due to the fairly broad first peak, in a previous work structural analysis of these lipoplexes showed a lamellar organization (4). The effective domain size given by the half-width value of the scattering peak was found to be ~300 Å, with extreme values of 260 and 320 Å for the different plasmid DNA lengths. The lipoplexes were thus shown to exhibit a four-lamellar domain independently of plasmid size, within the range 900–52 500 bp. From these results, we estimated the number of plasmid molecules contained in one particle, according to our previously published methodology (4). The estimated numbers of plasmid DNA molecules per lipoplex were 55, 17, 13, 6, 2.5 and 0.9 with plasmid sizes of 900, 2900, 3700, 8600, 19 600 and 52 500 bp, respectively.
The transfection activity of RPR120535 micelles associated with plasmid DNA of various lengths was evaluated in NIH 3T3 cells using particles containing only a single copy of the active plasmid, i.e. a plasmid containing one luciferase expression cassette. In order to transfect the cells with the same number of lipoplexes exhibiting the same physicochemical properties, the inactive plasmid pSL301, which contains no reporter gene, was used to fill the particles. Reporter gene expression in NIH 3T3 cells was shown to depend on plasmid length (Fig. 4A). With a 95% confidence interval between 4.5 and 8, luciferase activity was six times greater using the 2900 bp minicircle than it was with the 52 500 bp pXL3141. The same experiment was performed using primary cultures of human AoSMC, which are more refractory to transfection than NIH 3T3 cells. DNA size greatly affected the transfection efficiency of AoSMC cells, because, with a 95% confidence interval of between 17 and 350, the 2900 bp minicircle was shown to be 77 times more efficient than the same molarity of the 52 500 bp pXL3141 (Fig. 4B). Similar results were obtained with other cell lines, i.e. cells derived from the kidney of a male adult African green monkey (CV-1) and from the ascitic fluid of a patient with an ovarian tumor (SK-OV-3) (data not shown).

In order to study the impact of the topology of the DNA on the in vitro transfection efficiency, HeLa cells were transfected with identical mass amounts of either plasmid DNA or linearized plasmid DNA. Since the same physicochemical characteristics of lipoplexes were obtained irrespective of DNA length and topology, these conditions resulted in a total number of luciferase expression cassettes per complex which was inversely proportional to the length of the DNA molecule. Note that the same number of lipoplexes per sample were obtained in each condition. The luciferase expression was higher with plasmid DNA than with linearized plasmid DNA (Fig. 5). Similar results have been reported by Zanta et al. (25). The luciferase activities were also dependent on the size of both plasmid DNA and linearized plasmid DNA (Fig. 5). With a 95% confidence interval of between 23 and 92, lipoplexes containing 13 copies of the linearized plasmid DNA (3700 bp) were 50 times more efficient in promoting gene transfer than lipoplexes encapsulating 2.5 copies, i.e. with the same amount of lipoplexes containing five times more of the smaller plasmid, 50 times more transgene expression was detected, suggesting a 10-fold improvement of the transfer efficiency of the smaller plasmid construct.

**DISCUSSION**

Although the importance of using a plasmid DNA of minimal size for in vitro transfection activity was recently demonstrated (18), no information was given about lipoplex morphology or structure. Therefore, in vitro transfection was performed with RPR120535–DNA complexes containing a total amount of 0.5 µg DNA/well consisting of a mixture of the active plasmid and an inactive plasmid which contained no reporter gene. More precisely, 27, 35, 82, 186 and 500 ng of minicircle, pXL3032, pXL3032, pXL3340 and pXL3141, respectively, were mixed with an inactive plasmid in order to obtain a final amount of 500 ng DNA. Next, 500 ng of each DNA was complexed with the RPR120535 micelles at a charge ratio of 6 (+/–). Thus, in these conditions, lipoplexes contained one copy of the five different plasmids, i.e. minicircle, pXL3032, pXL3108, pXL3340 and pXL3141 and 16, 12, 5, 1.5 and 0 copies of the inactive plasmid, respectively. Error bars represent the 95% confidence limits. For greater clarity, luciferase activity is represented on a linear scale, even though it displayed a logarithmically normal distribution.

![Figure 3](image-url) **Figure 3.** Cryo-TEM micrograph of RPR120535 complexed with polyphosphate molecules. The lipopolyamine:polyphosphate charge ratio (+/–) was 6. The scale bar represents 100 nm.

![Figure 4](image-url) **Figure 4.** In vitro transfection of NIH 3T3 cells (A) and aortic smooth muscle cells (B). Transfections were performed with RPR120535–DNA lipoplexes containing one active plasmid, i.e. containing a luciferase expression cassette, per lipoplex. Therefore, in vitro transfection was performed with RPR120535–DNA complexes containing a total amount of 0.5 µg DNA/well consisting of a mixture of the active plasmid and an inactive plasmid which contained no reporter gene. More precisely, 27, 35, 82, 186 and 500 ng of minicircle, pXL3032, pXL3108, pXL3340 and pXL3141, respectively, were mixed with an inactive plasmid in order to obtain a final amount of 500 ng DNA. Next, 500 ng of each DNA was complexed with the RPR120535 micelles at a charge ratio of 6 (+/–). Thus, in these conditions, lipoplexes contained one copy of the five different plasmids, i.e. minicircle, pXL3032, pXL3108, pXL3340 and pXL3141 and 16, 12, 5, 1.5 and 0 copies of the inactive plasmid, respectively. Error bars represent the 95% confidence limits. For greater clarity, luciferase activity is represented on a linear scale, even though it displayed a logarithmically normal distribution.
As DNA–RPR120535 lipoplex structure and size were not modified by increasing plasmid DNA length, the number of plasmid molecules, and therefore the number of therapeutic gene expression cassettes incorporated into a single lipoplex, increased in a linear fashion as plasmid size decreased. Many studies recently focused on the development, for gene transfer, of virus-like, nanoscopic particles containing only one item of genetic information (26). Here, we found that the in vitro transfection activity of lipoplexes was proportional to the number of expression cassettes in each particle, i.e. the most active lipoplexes were those which exhibited the largest number of plasmid DNA molecules. This result was consistent with that reported by Tseng et al. (27), who recently demonstrated, on a single cell basis, that transgene expression increases with the number of intracellular plasmid copies. A large number of copies may increase the probability that a fraction of the DNA will survive intracellular degradation.

However, the number of reporter gene copies incorporated into lipoplexes cannot alone account for the differences in reporter gene expression observed with plasmids of different sizes. Thus, in the experiment illustrated in Figure 4, in which one coding cassette was inside each particle, the transfection activity of lipoplexes containing smaller plasmids was found to be greater than that of lipoplexes containing the same molarity irrespective of plasmid size. This stability only depended on the cationic lipid–DNA charge ratio (+/–). However, stable RPR120535–52 500 bp plasmid complexes from zone C could only be obtained at a low DNA concentration (10 µg DNA/ml). DNA of large size might therefore be a limitation for lipoplex-mediated gene transfer, because for clinical applications it is of crucial importance to obtain a DNA solution that is concentrated, homogeneous and colloidaly stable.

The luciferase activities measured in transfected HeLa cells (Fig. 5) were dependent on the size of the plasmid DNA, either supercoiled or linearized. Therefore, the topology of the transfected DNA molecules determines the level of gene expression in both small DNA molecules have higher transfection efficiencies than larger ones. The greater transfection efficiency of small plasmids relative to that of larger plasmids may be due to differences in a step of the transfection process which occurs after particle internalization. Behr et al. (30) also postulated that DNA is released from cationic lipid–DNA complexes through the endosomal membrane, which results in the displacement of the cationic lipid from the DNA and the subsequent release of DNA in the cytoplasm. Behr et al. (30) also postulated that DNA is released from cationic lipids in the cytoplasm through lipid mixing and competitive exchange with anionic polymers like cytoplasmic mRNA. On the basis of these reports, we suggest that DNA size may account for either the mechanism of DNA release from cationic lipids or for the intracellular migration of DNA from the cytoplasm to the nucleus, or both.

In previous studies (31,32), the authors laid special stress on the importance of the effect of cytoskeletal elements, which act as a sieve on DNA migration into the nucleus. The entry of

![Figure 5](image-url)
plasmid DNA into the nucleus required for reporter gene expression may occur due to cell division and subsequent disruption of the nuclear envelope. In particular, the highly efficient reporter gene expression obtained here with all plasmids for fast growing NIH 3T3 cells might correlate with cell division. Alternatively, DNA entry into the nucleus can also be achieved within non-dividing cells (33,34). Nuclear entry via the nuclear pores might be dependent on plasmid size. If so, this hypothesis would explain why aortic smooth muscle cell transfection is widely sensitive to plasmid size, as demonstrated by the transfection activity of the minicircle (2900 bp), which was 77 times more efficient than that of pXL3141 (52 500 bp).

A better understanding of the relative importance of both the lipid and plasmid components in lipoplex formation and ultimately in gene delivery efficiency might be useful for the design of improved DNA vectors. Further studies involving the microinjection of DNA molecules of different sizes into the intracellular elements involved in plasmid size discrimination.

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