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RESEARCH ARTICLE

Which mechanism for nuclear import of plasmid DNA complexed with polyethylenimine derivatives?

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Abstract

Background To investigate the nuclear import mechanism of plasmid/polyethylenimine (PEI) derivative complexes and the putative nuclear targeting of therapeutic genes by the use of oligosaccharides, we have studied the nuclear import of plasmid DNA complexed either with PEI or with lactosylated PEI (Lac–PEI) in cystic fibrosis human airway epithelial cells (∑CFTE29o- cells).

Methods and results Cells were synchronized by a double-thymidine block protocol and gene transfer efficiency was evaluated: Lac–PEI- and PEI-mediated gene transfer was greatly increased when cells have undergone mitosis during the course of transfection. However, both types of complexes were able to transfect some growth-arrested cells. When the nuclear import of plasmid/Lac–PEI or plasmid/unsubstituted PEI complexes was studied in digitonin-permeabilized cells, the nuclear uptake of both types of complexes did not follow the classic pathway of nuclear localization sequence (NLS)-containing proteins and lactose residues did not act as a nuclear localization signal.

Conclusions Our results show that for complexes made with PEI derivatives, the major route for plasmid DNA nuclear entry is a passive nuclear importation during mitosis when the nuclear membrane temporarily breaks down. However, albeit to a lesser extent as that observed in dividing cells, a plasmid DNA importation also occurs in nondividing cells by a yet unknown mechanism. Copyright © 2006 John Wiley & Sons, Ltd.

Keywords polyethylenimine; lactosylated polyethylenimine; nuclear import; nuclear pore complex; nuclear localization sequence; cell cycle

Introduction

Many extracellular and intracellular barriers to nonviral gene delivery have been identified and overcome. One of the major remaining barriers that has not been successfully overcome is the nuclear envelope [1]. Given the size of the free or complexed plasmid DNA and that of the nuclear pore complex (NPC), it is unlikely that plasmid DNA passively diffuses through the NPC. Two major routes for plasmid DNA nuclear entry have been identified [2]: an active transport through the nuclear pores or a passive nuclear importation during mitosis when the nuclear membrane temporarily breaks down. For lipofection, it has been clearly demonstrated that high levels of gene transfer are only observed in actively dividing cells [3–6]. For polyfection, results are less clear: whereas some studies suggested that the nuclear entry of complexes made with branched or linear polyethylenimine (PEI) does not

require cell division [7–10], others showed a transfection efficiency that is cell cycle dependent [5,11,12].

In order to target therapeutic genes to the nucleus of nondividing cells, bioconjugates with nuclear localization sequences (NLS) have been developed with little increase in gene expression so far [13]. An interesting alternative approach may be the use of oligosaccharides. Carbohydrate-binding proteins named lectins have been documented in the cytosol and the nucleus [14] and glycosylated proteins lacking a NLS were shown to enter the nucleus with the help of their oligosaccharides [15–17]. Moreover, a nuclear translocation of plasmid DNA/lactosylated polylysine complexes was reported that was not cell cycle dependent. It was attributed to the presence of the lactose residues on the polylysine [18].

To investigate the nuclear import mechanism of complexes made with PEI derivatives and the possible nuclear targeting of therapeutic genes by the use of oligosaccharides, we have studied the nuclear import of plasmid DNA complexed with PEI or lactosylated PEI (Lac–PEI) in cystic fibrosis human airway epithelial cells (∑CFTE29o- cells).

Materials and methods

Complex formation

Five percent of the amino groups of polyethylenimine (PEI, 25 kDa, branched polymer; Sigma, St. Louis, MO, USA) were substituted by a lactosylthiocarbamoyl residue [19] and complexes were prepared as previously described [20]. Briefly, the plasmid DNA (2.5 μg) and the designed amount of lactosylated (Lac–PEI) or unsubstituted PEI to obtain a PEI nitrogen/DNA phophorous (N/P) charge ratio of 10 (from a stock solution of PEI, 30 mM in nitrogen) were separately diluted into 25 μl of 150 mM NaCl and mixed together.

Cell synchronization and gene transfer procedure

The immortalized, human tracheal epithelial ∑CFTE29o- cells which are from a cystic fibrosis (CF) patient homozygous for the F508del *CFTR* mutation [21] were synchronized at the G_1/S boundary by using a double-thymidine block as described by Spector *et al*. [22]. Briefly, cells were incubated twice for 21 h in the presence of thymidine (2 mM), separated by a cell growing phase of 16 h. The DNA content of the cells was then studied by flow cytometry after fixation of the cells in cold ethanol and DNA staining with propidium iodide according to Spector *et al*. [23]. After the double-thymidine block, a synchronously growing population of cells was obtained; immediately after the thymidine arrest as well as upon a mitotic cycle 24 h later, cells were in majority in G_0/G_1 phase (Figure 1A). Gene transfer was studied in these synchronous cells: immediately after the release of the second thymidine block, cells were washed twice and incubated for 1 h in the presence of complexes prepared as described above. The plasmid used was the pCMV*GFP* plasmid (pGFPemd-cmv, 4.80 kb; Packard, Meriden, CT, USA) which contains the gene encoding the green fluorescent protein (GFP) under the control of the human cytomegalovirus (CMV) promoter. The number of fluorescent ∑CFTE29o- cells was determined 24 h later using a FACSort. A similar gene transfer study was performed in asynchronously growing cells and in cells arrested in S phase by leaving them in the presence of the thymidine required for the second block until flow cytometric analysis (Figure 1B).

Intracytoplasmic microinjections and GFP expression

Free plasmid DNA pCMV*GFP* (50 ng/μl in H_2O) or plasmid DNA complexed with Lac–PEI or unsubstituted PEI (N/P = 10) were diluted in a 0.5% tetramethylrhodamine isothiocyanate-dextran (155 kDa) solution. Fifty ∑CFTE29o- cells at least were then microinjected into the cytoplasm in three separate experiments according to Grosse *et al*. [20]. Twenty-four hours post-injection, cells were fixed in 3% paraformaldehyde, mounted in Vectashield solution (Vector Laboratories), and GFP-expressing cells were analyzed using an MRC-1024 confocal system (Bio-Rad, Hercules, CA, USA) mounted on a Diaphot 300 inverted microscope.

Nuclear import in digitonin-permeabilized ∑CFTE29o- cells

The ∑CFTE29o- cells were seeded on coverslips (5×10^4 cells) in a 24-well plate and permeabilized 24 h later with digitonin according to the method of Adam *et al*. [24]. The integrity of the nuclear envelope of permeabilized cells was ascertained by incubating some cells with 500 μg/ml rhodamine-labeled dextran (MW 70 000, Sigma) for 30 min at 37 °C and its exclusion from the nuclei was verified (Figure 4A). After permeabilization, cells on the inverted coverslips were incubated at 37 °C for 1 h in the presence of an import buffer (20 mM HEPES, pH 7.3; 110 mM potassium acetate; 2 mM magnesium acetate; 1 mM EGTA; 2 mM dithiothreitol; 1 μg/ml each of aprotinin, pepstatin A and leupeptin) supplemented or not with an ATP-regenerating system (0.5 mM ATP, 2.5 mM creatine phosphate, 10 U/ml creatine phosphokinase) and 50% cytoplasmic extracts (rabbit reticulocyte lysate; Promega, Madison, WI, USA). The nuclear import of a rhodamine-labeled bovine serum albumin (BSA) coupled to the SV40 karyophilic peptide (YPKKKRKVEDPRC) [17,25] or of 50 μg/ml biotinylated plasmid, either free or complexed with unsubstituted PEI or Lac–PEI, was studied. The pCMV*Luc* plasmid (pUT 650, 5.15 kb; Cayla, Toulouse, France) was used. It contains the gene encoding the firefly

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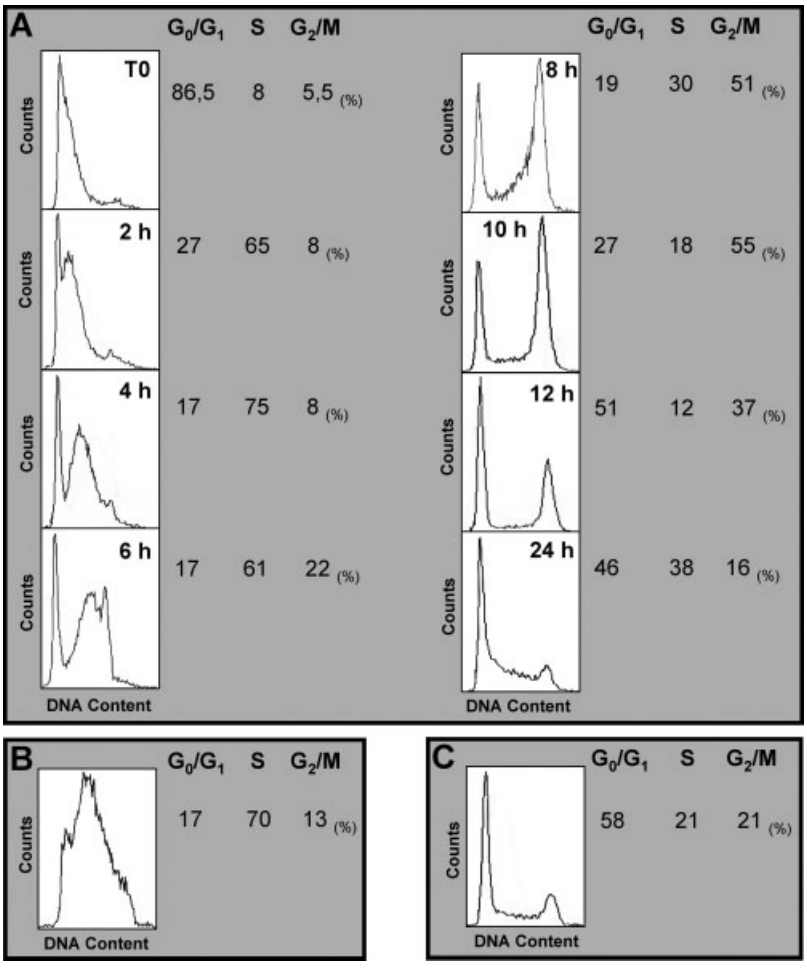

Figure 1. DNA content of synchronized (A, B) and exponentially growing (C) ∑CFTE29o- cells. (A) Synchronization of ∑CFTE29o- cells at the G1/S boundary was achieved using a double-thymidine block (T0). Cells were then released from the second thymidine block and the DNA content of the cells was analyzed by flow cytometry at different times. For each condition, the breakdown of the population according to cell cycle phase is indicated: 4 h after the block release, the majority of the cells were in S phase; after 10 h, the majority were in G_2/M phase and, after 24 h, most of the cells have undergone mitosis. (B) Cells were arrested in S phase by leaving them in the presence of the thymidine required for the second block until flow cytometric analysis. (C) An asynchronous population of cells were also analyzed

luciferase under the control of the human CMV promoter and was biotinylated using the FastTag nucleic acid labeling system (Vector Laboratories, Burlingame, CA, USA). In some experiments, the digitonin-permeabilized cells were exposed for 30 min at room temperature to 50 μg/ml wheat germ agglutinin (WGA; Calbiochem, La Jolla, CA, USA) prior to performing the nuclear import assay and 50 μg/ml WGA was also added to the complete import mixture. Cells were then washed with the import buffer, fixed in methanol/acetone for 5 min at −20 °C and incubated overnight in the presence of a goat polyclonal antibody (Ab) directed against a peptide mapping at the amino terminus of lamin A/C (Santa Cruz Biotechnology, Santa Cruz, CA, USA; dilution: 1/100) as a marker of the internal nuclear membrane. Cells were then washed and incubated for 30 min with fluorescein-conjugated donkey anti-goat Ab (Jackson ImmunoResearch, West Grove, PA, USA; dilution: 1/100). Finally, the biotinylated plasmid DNA was labeled with rhodamine-conjugated streptavidin (Molecular Probes, Eugene, OR, USA; dilution: 1/200), coverslips were washed, mounted in Vectashield solution (Vector Laboratories) and examined with an MRC-1024 confocal system (BioRad) mounted on a Diaphot 300 inverted microscope. The krypton/argon laser was tuned to produce excitation wavelengths of 488 and 568 nm. Serial sections collected at increments of 0.1-μm thick were used to define the intracellular localization of plasmid DNA. Images were obtained with a Kalman acquisition device and processed with Adobe Photoshop 7.0 software.

Statistical analysis

For the GFP expression study, data are expressed as mean ± standard error of the mean (s.e.m.) of three independent experiments. Comparisons were made using the nonparametric Mann-Whitney U-test or the Kruskal-Wallis test. Values of $p \leq 0.05$ were considered to be statistically significant.

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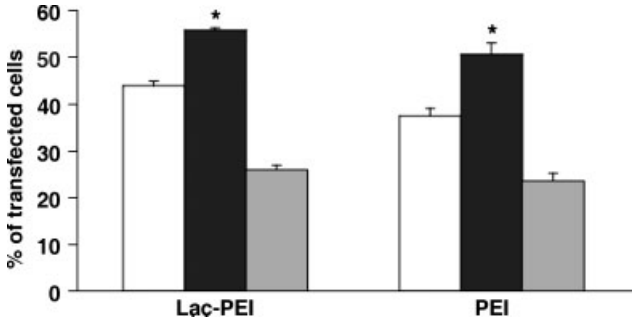

Figure 2. Influence of cell cycle on PEI derivative-mediated gene transfer efficiency in ∑CFTE29o- cells. Immediately after the release of the second thymidine block, synchronized ∑CFTE29o- cells (■) were incubated for 1 h at 37 °C in the presence of the plasmid encoding the *GFP* gene complexed with Lac–PEI or unsubstituted PEI. Twenty-four hours later, the majority of the cells have undergone mitosis (see Figure 1) and the percentage of GFP-expressing cells was determined by flow cytometry. The GFP expression was compared to that obtained with asynchronously growing cells (□) and with cells blocked in S phase (▩) by using the nonparametric Kruskal-Wallis test (*$p < 0.05$)

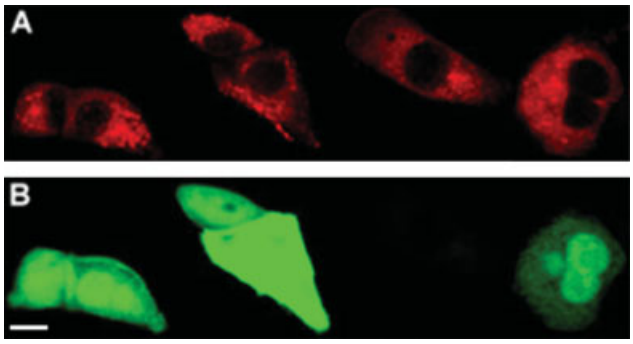

Figure 3. Influence of cell cycle on GFP expression after intracytoplasmic microinjection of plasmid/Lac–PEI complexes in synchronized ∑CFTE29o- cells. Immediately after the release of the second thymidine block, ∑CFTE29o- cells at the G_1/S boundary were microinjected into the cytoplasm with (A) a rhodamine-labeled dextran solution, to ensure the cytoplasmic localization of the microinjection and the integrity of the nuclear membrane, and (B) a plasmid encoding the *GFP* gene complexed with Lac–PEI. Twenty-four hours later, cells were fixed in 3% paraformaldehyde and analyzed by confocal microscopy: most of the GFP-expressing cells are cells that have undergone mitosis between the complex incubation and the determination of gene transfer efficiency (bar: 10 μm)

Results and discussion

Lac–PEI- and PEI-mediated gene transfer is greatly increased when cells undergo mitosis during the course of transfection

To study the influence of the cell cycle on gene transfer, we have grown three ∑CFTE29o- cell populations: synchronized cells by a double-thymidine block (Figure 1A), cells arrested in S phase (Figure 1B), and asynchronous cells (Figure 1C) (see Materials and methods for details). In these three cell populations, we have compared the gene transfer efficiency of a plasmid encoding the *GFP* gene complexed with Lac or unsubstituted PEI (Figure 2). For both types of complexes, GFP expression was greatly increased in the synchronized cells that were released from the second thymidine block at the time of complex incubation and in which most of the cells undergo mitosis between the complex incubation and the determination of gene transfer efficiency 24 h later (Figure 1A): for Lac–PEI complexes: $56 \pm 1\%$ of cells expressed GFP, as compared with $26 \pm 1\%$ of cells arrested in S phase and $44 \pm 1\%$ of asynchronous cells ($p < 0.05$, Kruskal Wallis-test; Figure 2); for unsubstituted PEI complexes: $51 \pm 2\%$ of cells that undergo mitosis expressed GFP, as compared with $24 \pm 1\%$ of growth-arrested cells and $38 \pm 2\%$ of asynchronous cells ($p < 0.05$, Kruskal-Wallis test; Figure 2). To ascertain that the differences in GFP expression observed in cells under different cell cycle conditions was not due to differences in complex cellular uptake, we studied the uptake of fluoresceinylated complexes in asynchronous cells, synchronized cells able to undergo mitosis and growth-arrested cells by flow cytometry. A similar complex uptake was observed in all conditions (data not shown).

To further verify that the nuclear entry of plasmid/Lac–or unsubstituted PEI complexes was cell cycle dependent, we microinjected free plasmid DNA encoding the *GFP* gene or plasmid DNA complexed with Lac–or unsubstituted PEI into the cytoplasm of synchronously growing cells released from a double-thymidine block. When the GFP expression was studied 24 h later, the majority of the cells had undergone mitosis (Figure 1A). As illustrated in Figure 3, 24 h after intracytoplasmic microinjection of plasmid/Lac–PEI complexes, most of the cells that expressed GFP had undergone mitosis. Among the microinjected cells that had undergone mitosis, $73 \pm 3\%$ expressed GFP, whereas $24 \pm 1\%$ of nondivided cells expressed GFP ($p < 0.05$, Mann-Whitney U-test). Similar results were obtained upon intracytoplasmic microinjection of free plasmid pCMV*GFP* or of plasmid/unsubstituted PEI complexes.

Our results show that the nuclear import of plasmid/Lac–PEI or plasmid/unsubstituted PEI complexes is greatly increased when the cells undergo mitosis during the course of gene transfer. These results together with other studies [5,11,12] suggest that for polyfection as for lipofection, the breakdown of the nuclear membrane during mitosis gives cytoplasmic plasmid DNA access to the nuclear compartment. However, for both PEI and Lac–PEI complexes, approximately 25% of growth-arrested cells were transfected. This suggests that some PEI complexes, Lac–PEI complexes or free plasmid DNA are able to cross the nuclear membrane and this is in agreement with other studies [7–10]. We therefore studied the nuclear import mechanism of plasmid DNA, either free or complexed with Lac–PEI or unsubstituted PEI, in digitonin-permeabilized cells.

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The nuclear import of free plasmid DNA, plasmid/Lac–PEI complexes or plasmid/unsubstituted PEI complexes does not follow the classic NLS-containing protein pathway

The nuclear import of a rhodamine-labeled BSA substituted with the SV40 NLS (NLS-RBSA) was studied in digitonin-permeabilized ∑CFTE29o- cells (Figure 4B). As expected, a nuclear import of NLS-RBSA was only observed in the presence of the import buffer supplemented with ATP and cytosolic extract (RRL) and was inhibited by preincubation of cells with wheat germ agglutinin (WGA) which blocks the pore by binding to the nuclear pore glycoproteins. Whatever the condition used, the import buffer alone or supplemented with ATP, RRL or both, the free biotinylated plasmid DNA or the plasmid DNA complexed with Lac–or with unsubstituted PEI accumulated at the nuclear periphery and was never observed in the nucleus of digitonin-permeabilized ∑CFTE29o- cells (Figure 1C).

The PEI derivative allows compaction of the plasmid DNA, protects it from degradation and promotes its cellular uptake and endosomal exit. To be transcribed, the plasmid DNA must enter the nucleus. It has been suggested that for some PEI derivatives, some degree of plasmid DNA/polymer dissociation occurs in the cytoplasm [26,27]. In this case, the free plasmid DNA, or most likely the plasmid DNA associated with DNA-binding proteins, polyamines or polycations present in the cytoplasm, has to cross the nuclear membrane to be transcribed. Hagstrom *et al*. have previously shown that in digitonin-permeabilized cells, double-stranded DNA is transported into the nucleus through the NPC in an energy- and temperature-dependent manner [28]. However, the nuclear uptake of DNA fragments was size-dependent: fragments larger than 2 kb remained in the cytoplasm and particularly in the perinuclear area [28]. Our results are consistent with these data. We show here that a 5 kb plasmid DNA does not passively diffuse through the nuclear pore, nor does it follow the pathway of NLS-containing proteins.

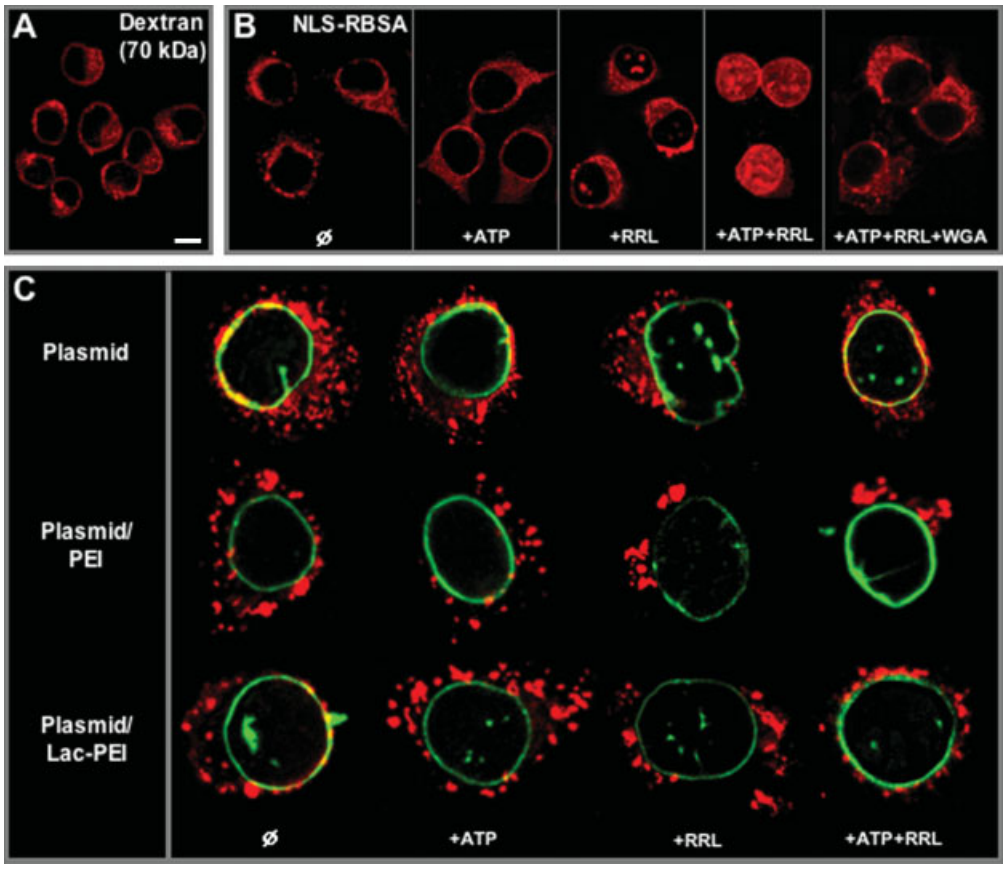

Figure 4. Nuclear import in digitonin-permeabilized ∑CFTE29o- cells. (A) Digitonin-permeabilized ∑CFTE29o- cells were incubated in the presence of rhodamine-labeled dextran and its exclusion from the nuclei shows the integrity of the nuclear membrane after digitonin treatment. (B) Digitonin-permeabilized ∑CFTE29o- cells were incubated in the presence of rhodamine-labeled bovine serum albumin substituted with the SV40 nuclear localization signal (NLS-RBSA) or (C) in the presence of biotinylated plasmid, either free or complexed with PEI or Lac–PEI, in the import buffer alone (Ø), in the import buffer supplemented with ATP and/or cytosolic extract (RRL), and in the complete import buffer supplemented with wheat germ agglutinin (WGA). Then, cells were fixed in methanol/acetone and the biotinylated plasmid was labeled with rhodamine-conjugated streptavidin and appears red. The nuclear membrane was immunolabeled with anti-lamin A/C antibodies, followed by fluorescein-labeled anti-goat antibodies and appears green, and the intranuclear localization of NLS-RBSA or of the plasmid was analyzed by confocal microscopy (bar: 10 μm)

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Although some degree of plasmid DNA/PEI derivative dissociation is likely to occur in the cytoplasm, we [20] and others [8,26,27,29] have reported that, when complexed with a PEI derivative, a plasmid enters the nucleus mostly as a complex. The maximum nuclear pore diameter is 25 nm. The approximate plasmid/25-kDa PEI complex size at N/P ratio of 6–10 is around 150 nm [30,31]. However, this complex size stands for the average of a very heterogeneous population; indeed the complex diameter is in the 30 to 700 nm range [32]. Therefore, a passage through the NPC could occur in the case of the smallest complex population. Our results show that this is not the case, or at least that, if this is the case, such a low number of complexes have entered the nucleus that they could not be detected. We conclude that most plasmid/PEI complexes do not traffic through the NPC passively and do not follow the classic pathway of NLS-containing proteins. The lactose residue was claimed to favor a nuclear translocation of plasmid DNA/lactosylated polylysine complexes [18]. Our study shows that lactose residues do not act as a nuclear localization signal. This is in agreement with our previous results demonstrating that upon intracytoplasmic microinjection, while a nuclear import was observed with serum albumin substituted with glucosyl, fucosyl, or mannosyl residues, unsubstituted serum albumin as well as serum albumin substituted with lactosyl residues were not and remained in the cytosol [15–17].

In conclusion, our study shows that, for polyfection as for lipofection, the major route for plasmid DNA nuclear entry is a passive nuclear importation during mitosis when the nuclear membrane temporarily breaks down. However, a plasmid DNA importation also occurs in nondividing cells. Whether or not the plasmid DNA is still a complex when importation occurs in nondividing cells, our results show that the nuclear importation is related neither to a passive complex importation through the NPC, nor to a classic NLS-containing protein pathway through the NPC, nor to a sugar-mediated nuclear import. Taken together, our results suggest that a small amount of plasmid DNA, either free or complexed with PEI derivative, is able to enter the nucleus by alternative routes with undeciphered mechanisms. By characterizing and understanding them, new gene transfer systems might be developed, aiming to overcome the nuclear barrier and increase transfection efficiency *in vivo*, where nonproliferating cells are often the target.

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