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Review

Nucleocytoplasmic Transport of Plasmid DNA: A Perilous Journey from the Cytoplasm to the Nucleus

DELPHINE LECHARDEUR^{1,2} and GERGELY L. LUKACS¹

ABSTRACT

Nonviral vectors represent a promising approach for the safe delivery of therapeutic DNA in genetic and acquired human diseases. Before synthetic vector systems can be used for clinical applications, their limited efficacy must be addressed. At the cellular level, successful gene transfer is dependent on several additional factors including DNA uptake, release from the DNA–vector complex, and nucleocytoplasmic transport. This paper reviews the major metabolic and physical impediments that plasmid DNA vectorized by synthetic vectors encounters between the cytosol and the nucleus. Plasmid DNA that escapes the endolysosomal compartment encounters the diffusional and metabolic barriers of the cytoplasm, reducing the number of intact plasmids that reach the nuclear envelope. Nuclear translocation of DNA requires either the disassembly of the nuclear envelope during cell division or active nuclear transport via the nuclear pore complex. In the nucleus, plasmid DNA is relatively stable, but its transcription and its fate during cell division are still debated. A better understanding of the cellular and molecular basis of nonviral gene transfer during nucleocytoplasmic trafficking may provide strategies to overcome those obstacles that limit the efficiency of nonviral gene delivery. We review some of the current methods of gene transfer mediated by synthetic vectors, highlighting systems that exploit our actual knowledge of the nucleocytoplasmic transport of plasmid DNA.

INTRODUCTION

THE EVOLUTION OF MULTICELLULAR ORGANISMS depends on the relative stability of their chromosomal DNA. Multiple cellular barriers separate the nucleus from the extracellular environment and provide efficient protection against the nuclear entry of extrachromosomal and therapeutic plasmid DNA (pDNA). By hijacking a variety of cellular mechanisms, viruses have evolved strategies to bypass these barriers and ferry their genomic material into the nucleus (Anderson and Hope, 2005; Ding *et al.*, 2005). Viral gene delivery vectors, however, have severe disadvantages, such as immunogenicity and cytotoxicity (Zaiss and Muruve, 2005; Roberts *et al.*, 2006). As an alternative, nonviral or synthetic vectors have been developed with reduced immunogenicity, in addition to low cost and ease of production (Luo and Saltzman, 2000). The clinical application of nonviral vectors, however, is hampered by limited transgene expression (Wolff, 2002; Mastrobattista *et al.*, 2006).

It has been established that numerous cellular obstacles account for the low level of transgene expression by nonviral vector-mediated gene delivery (for reviews, see Lechardeur *et al.*, 2005; Medina-Kauwe *et al.*, 2005) (Fig. 1). The accessibility and specific characteristics of target tissue/organ (e.g., mucociliary clearance of the lung), as well as the rapid pDNA clearance from the extracellular milieu, can compromise gene delivery (Takakura *et al.*, 2001; Tiera *et al.*, 2006). At the cellular level the limiting membranes of organelles (e.g., cytosol, endosome, and the nucleus) constitute major obstacles to nuclear delivery of therapeutic genes (Lechardeur *et al.*, 2005). Although a new generation of synthetic delivery systems (e.g., diverse liposomal formulations, basic proteins, and polymers such as polyethylenimine) has improved the internalization of pDNA, transgene expression still remains low relative to that of viral delivery systems.

Investigations of the cellular itinerary of pDNA have highlighted some of the biochemical and physicochemical characteristics of the cytoplasm, the nuclear envelope, and the nu-

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cleosol that limit the nuclear transfer and transcription of pDNA after its escape from the endolysosomal compartment. This review summarizes some results that provide new insights into cellular obstacles encountered by pDNA during its nucleocytoplasmic trafficking.

NUCLEOCYTOPLASMIC TRANSPORT EFFICIENCY OF pDNA

On the basis of the negligible *in vitro* and *in vivo* transcriptional activity of pDNA complexed to polycations, it was postulated that complex dissociation precedes the nuclear uptake of pDNA (Zabner *et al.*, 1995). Dissociation of polycation–pDNA complexes likely occurs in the cytosol after their endosomal escape, demonstrated by reporter gene expression controlled by cytosolically expressed T7 promoter (Gao and Huang, 1993; Fasbender *et al.*, 1997a; Brisson and Huang, 1999; Subramanian *et al.*, 1999). Considering that the DNA–polyethylenimine (PEI) complex has been identified in the nucleus, dissociation may not be a prerequisite for nuclear translocation (Godbey *et al.*, 1999; Bieber *et al.*, 2002). Translocation of the complex, at least in part, occurs via the nuclear pore complex (NPC) (Hebert, 2003). These observations, collectively, argue for the exposure of pDNA to the cytosol during its nucleocytoplasmic transport and justify efforts to understand the physicochemical and biochemical properties of the cytosol.

Quantification of the nucleocytoplasmic trafficking of pDNA has been attempted by both direct and indirect methods. On the basis of expression studies of microinjected and transfected cells exposed to defined amounts of pDNA, it became evident that the number of pDNAs transcribed in the nucleus relative to that exposed to the cell is enormously reduced. It is estimated that at least 10^5 – 10^6 plasmids are required per cultured cell in the extracellular compartment to ensure the nuclear uptake and transcription of a few pDNA molecules in nonmitotic cells (Tseng *et al.*, 1997). Because the quantitative determination of pDNA copy number escaping the endosomal compartment is not feasible, only the nucleocytoplasmic pDNA transport efficiency has been measured. It was estimated that 0.1–0.001% of a cytosolically introduced thymidine kinase expression vector was transcribed in the nucleus (Capecchi, 1980). Similar results were obtained by microinjection of β -galactosidase expression vector and monitoring the translocation of radioactive or fluorescent pDNA (Dowty *et al.*, 1995; Zabner *et al.*, 1995; Pollard *et al.*, 2001). Improved quantification of nucleoplasmic transport efficiency of pDNA verified these earlier results by measuring luciferase activity in growth-arrested COS-7 cells after the injection of known amounts of reporter gene into the cytosol or the nucleus (Fig. 2). Comparison of luciferase expression of single cells indicated that only 1 of 1000–1500 cytosolic pDNA molecules is successfully transcribed in the nucleus. These results highlight the overall inefficiency of pDNA nucleocytoplasmic trafficking.

FATE OF pDNA IN THE CYTOPLASM

Restricted diffusional mobility of pDNA

On the basis of the observation that pDNA remained at the site of microinjection, it was proposed that diffusional freedom

of pDNA in the cytoplasm of myotubes is severely impeded (Dowty *et al.*, 1995), whereas oligonucleotides up to 250 bp rapidly entered the nucleus after cytoplasmic delivery (Lukacs *et al.*, 2000). Consistently, microinjection of pDNA in the proximity of the nucleus, or decreasing the expression cassette size, could significantly enhance transgene expression (Dowty *et al.*, 1995; Darquet *et al.*, 1999). According to direct microscopic observation, 1- to 6-kb double-stranded DNA entered the nuclei of digitonin-permeabilized cells or isolated *Xenopus* egg nuclei (Hagstrom *et al.*, 1997; Salman *et al.*, 2001), but was undetectable after cytoplasmic injection of pDNA into intact cells (Hagstrom *et al.*, 1997; Lechardeur *et al.*, 1997; Lukacs *et al.*, 2000). Because microinjected oligonucleotides exhibited homogeneous distribution, the molecular mass of DNA rather than specific protein–DNA interaction appeared to be responsible for the limited cytoplasmic diffusion (Leonetti *et al.*, 1991).

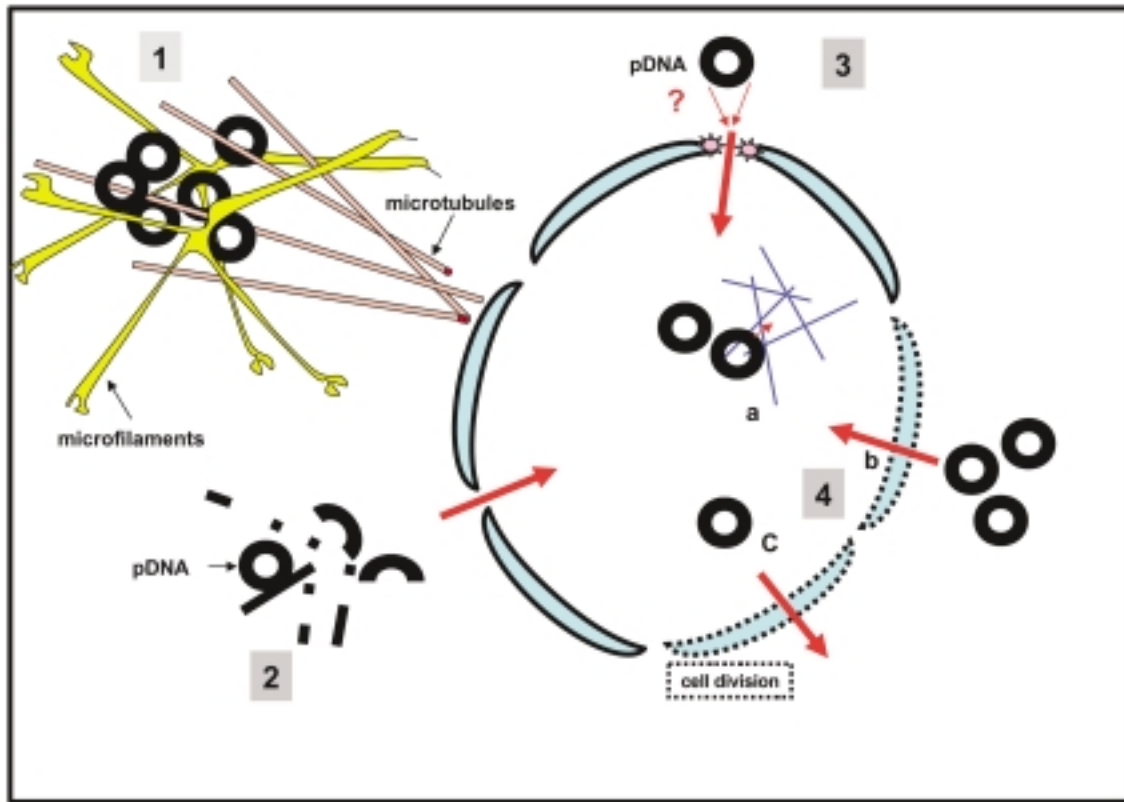
Direct determination of the diffusional mobility of fluorescein-conjugated pDNA was accomplished by fluorescence recovery after photobleaching (FRAP) (Lukacs *et al.*, 2000). Microinjected nucleic acids >2 kb were virtually immobile during the course of the measurements (a few minutes). Diffusion of 250- and 2000-bp DNA fragments was 17- and >100-fold slower, respectively, in the cytoplasm than in water (Lukacs *et al.*, 2000). Although the diffusion of smaller double-stranded DNA fragments was comparable to that of size-fractionated fluorescein-labeled Ficoll and dextran, the translational movements of larger DNAs were markedly impeded (Seksek *et al.*, 1997; Lukacs *et al.*, 2000). Presumably the mesh-like structure of the cytoskeleton accounts for the anomalous mobility of pDNA in the cytoplasm, because diffusion was significantly increased after disruption or reorganization of the actin cytoskeleton (Dauty and Verkman, 2005; Geiger *et al.*, 2006), or in cells depleted of keratin and vimentin (Shimizu *et al.*, 2005). These results demonstrate that the cytoplasm imposes a diffusional barrier to nucleocytoplasmic transport of pDNA.

Metabolic instability of pDNA

Restricted diffusion increases the residence time of pDNA in the cytoplasm, which favors the metabolic degradation of DNA. Quantitative single-cell fluorescence video image analysis in concert with *in situ* hybridization revealed that 50% of naked pDNA is eliminated in 1–2 hr from the cytoplasm of HeLa and COS-1 cells (Lechardeur *et al.*, 1999) and in about 4 hr from C2C12 cells and myotubes (Pampinella *et al.*, 2002). The fast turnover of microinjected pDNA was independent of the copy number and the conformation (linearized versus supercoiled, single versus double stranded) of pDNA and was also observed on cell cycle arrest. Similar results were obtained by monitoring pDNA turnover, using Alexa 594-labeled DNA (Shimizu *et al.*, 2005) or the polymerase chain reaction (PCR) (Pollard *et al.*, 2001). These observations, as well as potentiation of transgene expression by nuclease inhibitors (DMI-2 or aurintricarboxylic acid) (Ross *et al.*, 1998; Walther *et al.*, 2005), are in support of the conclusion that metabolic instability of pDNA may contribute to the low gene transfer efficiency of nonviral gene delivery vectors (Mirzayans *et al.*, 1992; Lechardeur *et al.*, 1999; Neves *et al.*, 1999).

A subset of nucleases plays a central role in the fragmentation of chromosomal DNA and may transiently appear in the

F2



4C

AU2

FIG. 1. Schematic representation of cellular barriers to nucleocytoplasmic traffic of pDNA. See text for details.

cytoplasm (Torriglia *et al.*, 1995). DNase I, DNase II, and NM23-H1 are thought to be released from intracellular organelles into the cytoplasm before their nuclear translocation (Wyllie *et al.*, 1980; Barry and Eastman, 1993; Polzar *et al.*, 1993). Caspase-activated DNase (CAD) and L-DNase II are ac-

tivated through proteolytic cleavage in the nucleus (Lechardeur *et al.*, 2000) or translocated on activation (Enari *et al.*, 1998; Sakahira *et al.*, 1998). Whereas intracytoplasmic injection of linear DNA increased p53-dependent apoptosis (Shimizu *et al.*, 2005), microinjected cells showed no sign of apoptosis

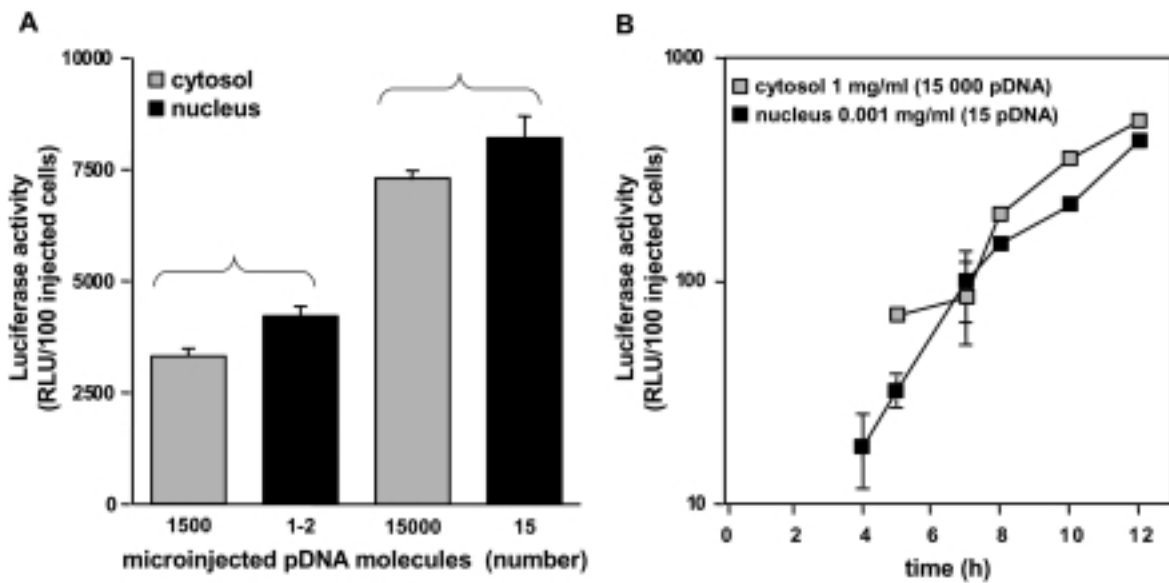


FIG. 2. Nuclear uptake efficiency of microinjected pDNA from the cytoplasm.

(Lechardeur *et al.*, 1999). The observation that isolated cytosol free of organellar contaminations displayed DNase activity (Lechardeur *et al.*, 1999; Pollard *et al.*, 2001) substantiates the role of apoptotic-independent nuclease(s) in the disposal of cytoplasmic pDNA delivered by microinjection or released during nonviral gene delivery (Fig. 1). Although the activation and inhibition profile of the cytosolic DNase show characteristics distinct from those of DNase I and DNase II (Lechardeur *et al.*, 1999), identification of the nuclease(s) responsible for the cytoplasmic pDNA degradation remains to be established.

NUCLEAR TRANSLOCATION OF pDNA

Restrictive role of the nuclear envelope

The nucleocytoplasmic trafficking of proteins and ribonucleoproteins is controlled by the nuclear pore complex (NPC), forming a channel across the nuclear envelope (Laskey, 1998). Molecules smaller than about 40 kDa diffuse passively through the NPC, whereas proteins >60 kDa are taken up by energy-dependent transport. Active nuclear accumulation requires the presence of nuclear localization sequence (NLS) in the cargo (Talcott and Moore, 1999). Docking of NLS provokes considerable conformational change in the NPC, leading to opening of the channel diameter from 9 to 40 nm (Pante and Kann, 2002). This conformational change provides a plausible explanation for the ability of the NPC to translocate substrates as large as 25–50 MDa (Harel and Forbes, 2001). The size-dependent diffusional barrier of the NPC could be visualized by the cytoplasmic microinjection of fluorescent nucleic acids: DNA fragments smaller than 250 bp (about 160 kDa) were able to diffuse into the nucleus, whereas nucleic acids larger than 250 bp were excluded from the nucleus (Leonetti *et al.*, 1991; Ludkte *et al.*, 1999; Lukacs *et al.*, 2000).

Nuclear translocation of pDNA by diffusion

The therapeutic potential of antisense oligonucleotides has prompted extensive studies of their intracellular trafficking (Leonetti *et al.*, 1991). The rapid nuclear accumulation of small DNA fragments was independent of the temperature, cytosolic ATP, and concentration of competing nonlabeled oligonucleotide (Leonetti *et al.*, 1991). Fluorescent 14- to 25-bp oligomers could be detected in the nucleus within seconds after microinjection at 22°C, suggesting that DNA fragments smaller than the cutoff of the NPC (e.g., 60-bp double-stranded oligomer has an apparent molecular mass similar to that of a 39-kDa polypeptide) have unrestricted access to the nucleosol (Clarenc *et al.*, 1993; Lukacs *et al.*, 2000). The large number of high-affinity binding sites conceivably accounts for oligonucleotide immobilization in the nucleus (Clarenc *et al.*, 1993), and was verified by FRAP measurements (Lukacs *et al.*, 2000).

The molecular mass of pDNA, depending on the size of the expression cassette, is estimated as between 2 and 10 MDa. Thus, it is unlikely that pDNA can passively enter the nucleus of postmitotic cells. Accumulation of lambda phage DNA via the NPC, however, was shown in isolated nuclei of *Xenopus* oocytes (Salman *et al.*, 2001). Whether these experimental manipulations altered the permeability barrier of the nucleus, allowing passive nuclear uptake of pDNA to occur *in vivo*, awaits further experimentations.

Active transport of pDNA by nuclear import machinery

Accumulating evidence indicates that the transport mechanisms for pDNA and polypeptides with molecular masses >60 kDa have common characteristics. The expression of microinjected reporter gene in primary myoblasts suggested that pDNA is able to enter postmitotic nuclei (Dowty *et al.*, 1995). Reporter expression was sensitive to temperature and wheat germ agglutinin (WGA), a relatively specific inhibitor of NPC-dependent active transport (Dowty *et al.*, 1995). In the same study, gold-labeled pDNA could be visualized by electron microscopy on the cytosolic and nucleosolic sides of the NPC. The WGA-sensitive, temperature- and energy-dependent translocation of pDNA strongly supports the hypothesis that plasmid molecule accumulation proceeds by active transport via the NPC (Brisson and Huang, 1999).

Association of pDNA with NLS-containing polypeptides (e.g., transcription factors) has been investigated (Dean, 1997; Dean *et al.*, 1999, 2005; Wilson *et al.*, 1999). Tethering transcription factors via specific binding sites to noncoding regions of pDNA potentiates reporter gene expression in a cell-specific manner (Vacik *et al.*, 1999; Dean *et al.*, 2005). Incorporating five tandem NF- κ B-binding motifs into pDNA increased the transfection efficiency by about 10-fold (Mesika *et al.*, 2005). Transfection was further enhanced by inducing the nuclear translocation of NF- κ B with tumor necrosis factor (TNF)- α (Mesika *et al.*, 2005). Incorporating the tetracycline operator sequence (tetO) into pDNA and coexpressing the tetracycline repressor (tetR) fused to an NLS (Vaisse, 2004) achieved comparable potentiation. These results support the notion that pDNA nuclear uptake is potentially facilitated by recruitment of the nuclear import machinery. Whether similar strategies can be used to augment the transduction efficiency of therapeutic genes *in vivo* awaits further experimentation.

Cell division and nuclear uptake of pDNA

The higher transfectability of dividing over quiescent cells appears to be compatible with the hypothesis that disassembly of the nuclear envelope enhances pDNA uptake into postmitotic nuclei (Wilke *et al.*, 1996; Mortimer *et al.*, 1999; Brunner *et al.*, 2000). The slow proliferation rate is thought to be, at least in part, responsible for the limited efficiency of lipid-mediated gene transfer of primary human airway epithelia (Fasbender *et al.*, 1997b). Blocking cell cycle progression in the G₁ phase has dramatically reduced reporter gene expression compared with that in asynchronous cell population, but has no effect on the internalization of lipoplexes (Mortimer *et al.*, 1999). A higher level of transgene expression was achieved when cells were exposed to lipoplexes before or during mitosis (Brunner *et al.*, 2000). According to other studies, cell division has little effect on transgene expression and nuclear accumulation of fluorescent pDNA (Ludkte *et al.*, 2002). These latter observations led to the proposal that during cell division, pDNA partitions between the cytoplasm and nucleus according to the respective volume of these cellular compartments. The relatively large volume of the cytoplasm compared with that of the nucleus (estimated to be 5700 and 175 μm^3 , respectively, in HeLa cells) may explain the modest effect of nuclear envelope disassembly on pDNA expression (Ludkte *et al.*, 2002).

BEHAVIOR OF pDNA IN THE NUCLEUS

Diffusion of nucleic acids of all sizes was severely restricted in the nucleus, in sharp contrast to the cytoplasm (Lukacs *et al.*, 2000; Doerr, 2005; Shimizu *et al.*, 2005). Molecular crowding cannot explain this phenomenon, because inert macromolecules up to the size of 580 kDa diffuse freely in the nuclear space (Seksek *et al.*, 1997). The limited diffusional mobility of injected DNA (250–400 bp) as well as subnuclear compartmentalization of extrachromosomal DNA (Shimizu *et al.*, 2005) indicates the presence of high-affinity binding sites for nucleic acids in the nuclear compartment.

One study confirmed and extended previous observations regarding the behavior of pDNA in the nucleus (Mearini *et al.*, 2004). First, fluorescent peptide nucleic acid (PNA)-labeled pDNA is stable in the nuclear environment (Mearini *et al.*, 2004), in line with the long persistence (hours to day) of extrachromosomal pDNA in the nucleoplasm (Gaubatz, 2000; Lukacs *et al.*, 2000; Shimizu *et al.*, 2005). Second, extraction of nuclear pDNA was resistant to detergent and high salt. The extrachromosomal DNA was preferentially associated with scaffold attachment factor-A (SAF-A), a nuclear matrix protein likely responsible for its reduced mobility, heterogeneous distribution, and exclusion from nucleoli and nuclear bodies (Gaubatz, 2000; Mearini *et al.*, 2004). This interaction was independent of plasmid sequence, the presence of functional promoter or a scaffold attachment region (SAR) in the DNA, representing the genomic attachment sites to the nuclear matrix (Baiker *et al.*, 2000; Mearini *et al.*, 2004). Because chromosomal DNA interaction with the nuclear matrix is essential for regulation of gene expression (Jenuwein *et al.*, 1997), these observations emphasize the importance of the nuclear microenvironment in nucleocytoplasmic trafficking and expression of pDNA.

pDNA behavior in the nucleus during cell division remains incompletely understood. Microinjected nuclear pDNA appears to be excluded from the nuclei of daughter cells (Ludtke *et al.*, 2002). On the other hand, the majority of pDNA labeled with PNA partitions evenly between daughter cells (Gasiorowski and Dean, 2005). According to this later study, covalently labeled pDNA with fluorescent moieties was excluded from the nuclear space in dividing cells (Gasiorowski and Dean, 2005), raising the possibility that tagged pDNA might have altered nuclear interactions and fate during mitosis. Published data have demonstrated that viral episomal DNA such as simian virus 40 (SV40) could persist in the nuclei during mitosis if nuclear matrix attachment is ensured (Baiker *et al.*, 2000). Whether nuclear pDNA behaves like chromosomal DNA or is recognized as extrachromosomal DNA during cell division needs further investigation.

STRATEGIES TO FACILITATE THE NUCLEOCYTOPLASMIC TRAFFICKING OF pDNA

Breaking the physical barriers by electroporation

Electroporation is based on the transient permeabilization of cellular membranes to a variety of water-soluble molecules including nucleic acids. Exposure of living cells to short electrical pulses induces transmembrane potential differences and opens transient pores in cellular membranes (Bigey *et al.*, 2002). Nucleofection combines chemical and electrical perturbations

to disrupt both cytoplasmic and nuclear membranes (Gresch *et al.*, 2003; Doerr, 2005) and seems to be particularly efficient for transfecting nondividing cells (Gartner *et al.*, 2006). The same principle has been adapted to deliver pDNA transcutaneously and into selected tissues (Andre and Mir, 2004). Electrical pulses not only permeabilize the membrane for DNA, but by facilitating the electrophoretic movement of pDNA into the nucleus (Satkauskas *et al.*, 2002; Zaharoff *et al.*, 2002; Andre and Mir, 2004) also exert some protection against cytosolic degradation of DNA (Capelletti *et al.*, 2002). Thus further technological improvements of electroporation may represent a promising alternative to synthetic vector delivery.

Hijacking the nuclear import machinery

Recruiting the nuclear import machinery to augment transfection efficiency requires the direct or indirect attachment of the NLS to pDNA. The NLS would promote importin- α/β binding to pDNA and subsequent nuclear translocation of the complex (for review see Cartier and Reszka, 2002; Hebert, 2003). Both classical and the nonclassical NLSs (M9 sequence of the human heterogeneous nuclear ribonucleoprotein A1) can stimulate the nuclear accumulation and transgene expression of pDNA and DNA fragments (Sebestyen *et al.*, 1998; Branden *et al.*, 1999; Ludtke *et al.*, 1999; Subramanian *et al.*, 1999; Wilson *et al.*, 1999; Zanta *et al.*, 1999).

Interaction of pDNA with a variety of cytoplasmic protein(s) harboring NLS(s) also facilitates the nuclear uptake of pDNA (Haberland and Bottger, 2005). For example, pDNA linked to a steroid derivative interacts with the glucocorticoid receptor, leading to ligand-induced nuclear targeting of the glucocorticoid receptors (Rebuffat *et al.*, 2001). Nuclear entry of pDNA was augmented by the NF- κ B DNA-binding sequence, relying on the nuclear accumulation efficiency of NF- κ B (Mesika *et al.*, 2005). This was, at least in part, accomplished by stimulating the vectorial diffusion of pDNA along microtubules by NF- κ B in a dynein-dependent manner (Mesika *et al.*, 2005). Finally, the nuclear-targeting and condensing ability of histones or high mobility group proteins, representing a subset of DNA-binding proteins with an NLS, have been tested with modest success (Hebert, 2003).

Compacting pDNA

Because cytoplasmic mobility as well as nuclear entry of nucleic acids is inversely proportional to pDNA size, it was reasonable to assume that condensing pDNA would enhance transfection efficiency. Nanoparticles are composed of specifically designed cationic molecules (e.g., polyethylenimine or organically modified neutral particles such as silica or polyethylene glycol [PEG] conjugated to polylysine), permitting the interaction of only one pDNA with a single nanocarrier (Choi *et al.*, 2003). Depending on their composition, nanoparticles can be used alone, combined with classical nonviral vectors, or delivered directly by electroporation as well as by magnetofection (Dobson, 2006). Nanoparticles can reduce pDNA to a size of 10 nm (Fink *et al.*, 2006), which is well below the 25- to 30-nm cutoff of the NPC (Pante and Kann, 2002; Liu *et al.*, 2003; Doerr, 2005; Fink *et al.*, 2006), and enhance transfection efficiency in comparison with naked DNA, particularly in slow-dividing and nondividing cells (Mastrobattista *et al.*, 2006). Microinjection of PEG-based nanoparticles increased transgene

expression by 10-fold relative to naked pDNA in postmitotic cells (Liu *et al.*, 2003). Transgene expression was WGA sensitive, suggesting that the nanoparticle was taken up via the NPC. Single-particle tracking revealed that polyethylenimine–pDNA nanocomplexes move within the cytoplasm by microtubule motor-mediated transport (Suh *et al.*, 2003). The vectorial diffusion of the particles, however, has been questioned by image correlation spectroscopy (Kulkarni *et al.*, 2005). Whether nanoparticles could improve the diffusional as well as the metabolic stability of pDNA in the cytoplasm needs to be investigated further.

CONCLUDING REMARKS

Adverse effects associated with some of the virus-based gene therapy trials have revitalized efforts to improve the efficiency of synthetic DNA delivery systems. The additive effect of extracellular and cellular hurdles, accounting for the poor transfection activity of nonviral vectors, has been largely delineated. At the cellular level the nucleocytoplasmic trafficking and expression of pDNA are impeded by both metabolic and physicochemical barriers. Thus improvements in gene transfer methods and nuclear delivery of nonviral vectors will provide important milestones to achieve safe and long-term therapeutic transgene expression, keeping in mind that sustained expression of a single-copy transgene in validated target cells would be sufficient to cure certain monogenic recessive diseases, including cystic fibrosis, the most common genetic disease in the white population (Griesenbach *et al.*, 2004).

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AU1

Preceding sentence OK as set? Or please amend.

AU2

Please explain 1, 2, 3, and 4a–c in Fig. 1? Or OK as set?