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Expert Review

The Nuclear Pore Complex: The Gateway to Successful Nonviral Gene Delivery

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Abstract. One of the limiting steps in the efficiency of nonviral gene delivery is transport of genetic material across the nuclear membrane. Trafficking of nuclear proteins from the cytoplasm into the nucleus occurs via the nuclear pore complex and is mediated by nuclear localization signals and their nuclear receptors. Several strategies employing this transport mechanism have been designed and explored to improve nonviral gene delivery. In this article, we review the mechanism of nuclear import through the nuclear pore complex and the strategies used to facilitate nuclear import of exogenous DNA and improve gene expression.

KEY WORDS: karyopherins; nonviral gene delivery; nuclear localization signal; nuclear pore complex.

INTRODUCTION

The purpose of gene therapy is to correct for dysfunctional or missing genes by delivering therapeutic genes into the nucleus of cells. This requires an efficient and safe gene delivery system. Much progress has been made in the development of safe nonviral gene delivery systems, yet they remain less efficient than virus-based gene delivery systems (1). The reason for this can be found in a combination of inefficient intracellular trafficking and, in particular, intranuclear delivery of DNA with nonviral vectors. Before the exogenous DNA can be expressed, it needs to pass several barriers, which are schematically depicted in Fig. 1. The barriers include the cell membrane (1) or, in case of internalization of the gene complexes via endocytosis, the endosomal membrane (2), the cytoplasm (3), and the nuclear envelope (NE) (4). Lack of capacity to cross the NE seems to be one of the main reasons for the low transfection efficiencies observed with nonviral vectors. Reaching the nucleus is accomplished more easily in proliferating cells than in quiescent cells as during cell division the NE is temporarily broken down. On completion of mitosis, components of the NE are used to reassemble a new NE in each daughter cell (2). In addition, both the rate of nuclear uptake and the functional size of the transport channels are greater in proliferating cells (3,4). In quiescent, nondividing cells, the NE acts as a selective barrier around the nucleus that impedes nuclear import of pDNA and nonviral gene delivery systems. Feldherr described in 1962 that nucleocytoplasmic transport of colloidal gold particles occurs through specialized pores in the NE, the so-called nuclear pore complexes (NPCs) (5). In addition, Bonner showed that protein migration into the nuclei is restricted to small proteins and nuclear proteins (6,7). Both large and cytoplasmic proteins were excluded from the nuclei. This controlled transport through the NPC in the NE is now recognized as an important mechanism for regulating gene expression (8). Nucleocytoplasmic transport is mediated by nuclear receptors from the karyopherin β family, like importin β and transportin.

Here, we will review transport processes across the NPC. The working mechanism of the NPC at the molecular level will be summarized and the most important factors limiting the efficient import of exogenous DNA are discussed. Finally, an overview will be given of strategies used to allow efficient uptake of DNA into the nucleus with the aim to improve the efficiency of nonviral vectors.

Nuclear Pore Complex

The NE is composed of an outer membrane (ONM), which is continuous with the endoplasmic reticulum, an inner

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ABBREVIATIONS: CAS, cellular apoptosis susceptibility gene; CF, cytoplasmic filament; CR, cytoplasmic ring; GEF, GDP–GTP exchange factor; GR, glucocorticoid receptor; HEAT, histidine, glutamic acid, alanine, and threonine; hnRNP, heterogeneous ribonucleoprotein; IBB, importin β binding domain; NE, nuclear envelope; NES, nuclear export signal; NLS, nuclear localization signal; NPC, nuclear pore complex; PEG, polyethylene glycol; PEI, polyethylenimine; PLL, poly-L-lysine; PNA, peptide nucleic acid; POM, pore membrane proteins; SMGD, steroid-mediated gene delivery; Vpr, viral protein R.

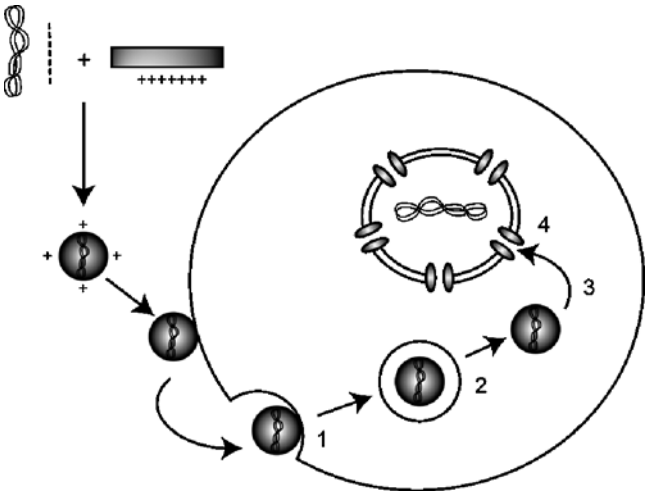

Fig. 1. A schematic picture of the intracellular barriers a nonviral gene delivery system has to cross. A positively charged particle is formed from negatively charged DNA and an excess of a positively charged carrier. The resulting positively charged particle can bind to the cellular membrane and is taken up via endocytosis (1), inside the cell it has to escape from the endocytic vesicle (2), travel toward the nucleus (3) and across the nuclear envelope (4).

membrane (INM), which lies within the nucleus, and the pore membrane. The latter is formed by fusion of the inner and outer membranes (9). The resulting channel forms the connection between cytoplasm and nucleoplasm and is called the nuclear pore complex (NPC) (Fig. 2). The number of NPC per nuclear membrane is dynamic and depends on the cell type and its metabolic activity. A proliferating human cell is estimated to have 3000–5000 NPCs (10). Winey *et al.* showed that in the yeast *Saccharomyces cerevisiae* the number of NPCs per cell varies between 65 and 182 (11). Membrane proteins localized in this channel are called pore membrane proteins (POMs), which likely play a role in NPC assembly by initiating the formation of the pore membrane domain, stabilizing it, and serving as a membrane anchor site for the NPC. All transport mechanisms into and out of the nucleus, both active and passive, occur through a cylindrical tubular element in this NPC (12). Its importance is underlined by the observation that the NPC seems to be conserved in all eukaryotes (13–15).

The NPC is made up of approximately 50 different nucleoporins, which occur in multiples of eight (16,17). Nup107–Nup160 (Nup160, Nup133, Nup107, Nup96, Nup85, and Sec13) are the main building blocks of the NPC (18). Both stationary and mobile nucleoporins reside in the NPC, indicating that the NPC is a dynamic rather than a static structure (19). Nucleoporins are characterized by the presence of FG dipeptide (Phe-Gly) repeat motifs. These so-called FG nucleoporins are present throughout the entire NPC and are strategically positioned to interact with cytosolic receptor proteins called karyopherins (see the next section) (20,21). These karyopherins are thought to play a role in the diffusion of ions and small molecules and to participate in the import of inner nuclear membrane proteins and/or the maintenance of NE electrical conductance (22).

The NPC has a tripartite architecture. It consists of a central framework and a cytoplasmic and nuclear ring moiety (Fig. 2). The central framework is formed by eight spokes (S) and spans the NE (23). From the cytoplasmic ring (CR) eight ~50-nm-long, kinky, cytoplasmic filaments (CF) emerge (24). The nuclear ring (NR) consists of a basketlike structure that is formed by eight ~150-nm-long nuclear filaments (NFs), which are joined at the distal end by a 30- to 50-nm-diameter ring (25). The central pore has a length of ~90 nm, is narrowest (45–50 nm in diameter) at the level of the NPC's midplane, and widens to ~70 nm toward its cytoplasmic and nuclear periphery. The total mass of the NPC has been estimated to be 90–120 MDa (26).

Nucleocytoplasmic Transport

Transport through the NPC is selective and energy dependent (27). Ions and small proteins (<40 kDa) with a diameter smaller than 9 nm enter the nucleus passively, whereas larger molecules with a maximum diameter of 39 nm require active transport (28,29). Kinetic studies have indicated that a single NPC can accommodate a mass flow of up to 80 million daltons per second, which equals 1000 translocation events per second per NPC, including both passive and active transport (30,31).

The exact mechanism for translocation of proteins through the NPC remains to be elucidated. Nevertheless, different models for translocation through the NPC have been proposed such as the affinity gradient model (32), Brownian affinity-gate model (16,33), and the selective phase model (30). In the affinity gradient model, it is proposed that transport through the nuclear pore is mediated by sequential interactions with nucleoporins. Transport is directed to the nucleus by increasing affinity of the transport receptor karyopherin β for the subsequent nucleoporins. However, this model does not correlate with the fast translocation kinetics through the NPC of 1000 events per second (30).

In the Brownian affinity-gate model, which was suggested by Rout *et al.*, transport through the NPC occurs through diffusion (16). Targeting toward the NPC by a

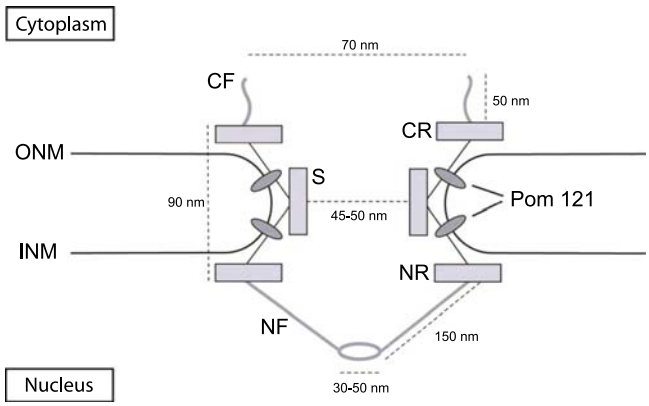

Fig. 2. The nuclear pore complex (NPC). The NPC is formed in the channel between the cytoplasm and nucleus where the inner (INM) and outer membrane (ONM) are fused. Pore membrane proteins, like Pom 121, play a role in NPC assembly and stabilization. The NPC consists of eight spokes (S), connected to flanking rings (CR and NR), from which the cytoplasmic (CF) and nuclear filaments (NF) emerge. The nuclear filaments are joined at the distal end to form a basketlike structure.

transport receptor increases the probability that a macromolecule can translocate through the NPC. Interaction of transport receptors with nucleoporins increases the accumulation of macromolecules adjacent to the channel entrance, thereby increasing the local concentration. Because the CFs of the NPC are dispensable for nuclear import, binding has to occur more proximal to the central channel (34). This model coincides with the rapid transport through the NPC. However, it does not explain the transport of large ribonucleoprotein particles that require structural changes in the central channel during translocation (35).

The third model, the selective phase model, was put forward by Ribbeck and Görlich (36). Here the nucleoporins that line the central channel interact with each other through weak hydrophobic interactions and thereby form a meshwork that fills the central channel and allows only passage of macromolecules with a maximum diameter of 39 nm. However, immunolocalization of nucleoporins by electron microscopy places nucleoporins, containing a sequence motif of phenylalanine–glycine (FG) repeats, mainly at the cytoplasmic and nuclear periphery of the NPC rather than in the central channel (37,38).

None of the three mechanisms discussed above completely describes nucleocytoplasmic transport as measured experimentally, but a combination of the positive aspects of these models might give a more mechanistic transport model as was suggested by Fahrenkrog and Aebi (39). In all three models, signal-bearing cargo is targeted toward the nucleus. Therefore, a longer dwell time near the NPC and a higher probability to diffuse through the central channel occurs. In addition, interactions with nucleoporins enable signal-bearing cargoes to cross the NPC even in the presence of a meshwork, whereas this barrier repels nontargeted cargoes.

Recently, Kubitscheck *et al.* showed with single-molecule far-field fluorescence that the dwell time at the nuclear pore complex of the transport receptor transportin-1 is reduced upon binding to a specific transport substrate, suggesting that translocation is accelerated for loaded receptor molecules (40). Taken together, these results indicate the importance of nuclear targeting for efficient nuclear import.

Karyopherins

Active transport of macromolecules into the nucleus involves several steps and starts in the cytoplasm by complex formation of the macromolecule and transport receptor. Transport receptors are mainly members of the karyopherin β family that mediate nuclear transport of specific cargoes. The different members of the karyopherin β family share similar molecular weights (90–150 kDa) and isoelectric points (4.0–5.0) and contain multiple tandem helical repeats (histidine, glutamic acid, alanine, and threonine) termed HEAT repeats (41). They have different functions in both nuclear import and export (42) (Table I). Karyopherin β1 (importin β) mediates nuclear import of positively charged nuclear localization signals (NLSs), known as classical NLSs, via karyopherin α (importin α). Karyopherin β2 (transportin) mediates nuclear import of a set of mRNA binding proteins (43) and karyopherin β3 and β4 of a set of ribosomal proteins (44). In addition, nuclear export is also mediated by members of the karyopherin β family, namely, CRM1 and cellular apoptosis susceptibility gene (CAS) (45–47).

In nonviral gene delivery, nuclear uptake is mainly pursued via targeting to importin β or transportin. Figure 3 shows a schematic overview of nuclear transport mediated by importin β. This route has similarities to the transportin import/export cycle as will be highlighted below. Importin β mediates nuclear import of macromolecules after binding its cargo directly or indirectly, via an adaptor protein (48). Adaptor proteins characterized so far are importin α, which recognizes classical NLSs (49,50), snurportin (51), RanBP7 (52), and XRIP α (53). Importin β has distinct domains for binding to the adaptor protein, Ran and two nucleoporin FG-repeat binding sites (54). The crystal structure of importin β bound to a fragment of importin α shows that 13 of the 19 HEAT repeats are involved in importin α binding (55). Importin α and snurportin 1 interact with importin β via an importin β binding (IBB) domain, which is an arginine-rich domain of 41 residues (56). The N-terminal IBB domain of importin α serves a dual role: importin β binding and autoinhibition. When importin α is not bound to importin β the autoinhibitory sequence within the N-terminal domain interacts with the NLS-binding pocket (57). However, the binding is not that strong, because NLS can still bind to importin α in the absence of importin β albeit with a significantly lower affinity. In addition to the flexible N-terminal IBB domain, importin α is composed of a highly structured domain composed of ten tandem armadillo (ARM) repeats, which contains two NLS-binding sites (ARM repeats 2–4 and 7–9). In contrast to importin β, several isoforms of importin α exist in the cell, which can be grouped into three subfamilies (58). The

Table I. Members of the Karyopherin β Familiy Involved in Nuclear Transport Pathways Discussed in this Review

Transport direction	Mammalian cells	Cargo	Reference
Import	Karyopherin β1/Importin β	Many cargoes, mainly with basic NLSs via importin α, UsnRNPs via snurportin	(48)
	Karyopherin β2/Transportin	HnRNPA1, histones, ribosomal proteins	(43)
	Karyopherin β3	Ribosomal proteins	(44)
	Karyopherin β4	Ribosomal proteins	(44)
	Importin 7	HIV RTC, glucocorticoid receptor, ribosomal proteins	(136)
Export	Crm1	Leucine-rich NES cargoes	(46)
	CAS	Importin α	(47,50)

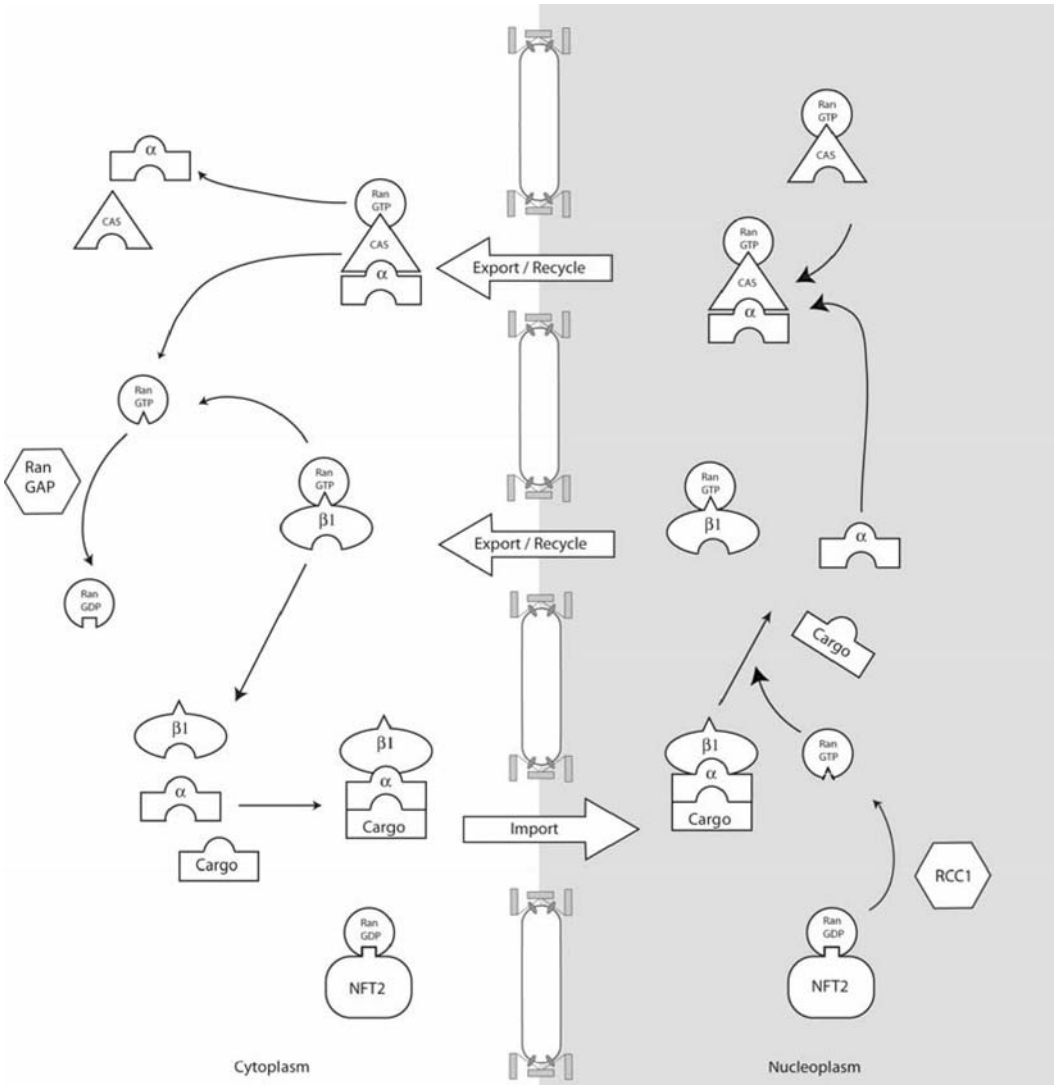

Fig. 3. Model for nuclear import and export pathway of importin β and α. The NLS-containing cargo binds in the cytoplasm to the importin β/α heterodimer, which then mediates transport into the nucleus [adapted from Moroianu (139)]. Inside the nucleus, RanGTP binding to importin β results in release of importin α and the cargo. Importin β is shuttled back into the cytoplasm by RanGTP and importin α by CAS/RanGTP.

different importin α proteins can interact with the same substrates, but with a different affinity (59). The importin β/importin α/NLS–cargo complex binds to docking sites such as the phenylalanine ring of the FG repeat in nucleoporins at the periphery of the NPC via hydrophobic residues on their surface, which triggers translocation through the NPC. Inside the nucleus, the complex is dissociated after binding of the small nuclear GTPase Ran-GTP to importin β, as is depicted in Fig. 3. Ran is transported into the nucleus by NTF2 in its GDP-bound form (60,61). NTF2 also mediates transport through the NPC through interaction with the FG repeat in nucleoporins (62). Inside the nucleus, Ran-GDP is converted into Ran-GTP by the GDP–GTP exchange factor (GEF) RCC1, which is a nuclear, chromatin-associated, protein (63). RanGTP binds to importin β and actively displaces importin α (54). After binding of Ran-GTP, importin β is shuttled back to the cytoplasm together with Ran-GTP, which is then converted into Ran-GDP by cytoplasmic Ran GTPase-activating protein (GAP). Dissociation of Ran-GDP leaves the transport receptor free to bind a new cargo and transport it into the nucleus. Importin α is shuttled back into the cytoplasm by CAS, a karyopherin β family member (50). CAS binds karyopherin α in the presence of Ran-GTP and exports it to the cytoplasmic side of the NPC.

Recently, the role of importin β in other cellular processes was described (64,65). Importin β facilitates not only nuclear import, but is also a potential motor adaptor for movement along microtubules and transduces damage signals from axons of injured neurons back to the cell body. In addition, importin β1 plays an inhibitory role on the assembly of the mitotic spindle, centrosome dynamics, nuclear membrane formation, and nuclear pore assembly (65). In the latter two the ratio of importin β1 to RanGTP is critical (66).

Transportin mediates transport of the heterogeneous nuclear ribonucleoprotein A1 (hnRNP A1) into the nucleus. Transportin recognizes and binds a nonclassical NLS se-

quence directly without an adaptor protein (as is discussed in the next paragraph). Transport through the NPC of transportin and its cargo is the same as for importin β. Binding of Ran-GTP inside the nucleus mediates release of the cargo and transport to the cytoplasm of transportin.

Nuclear Localization Signals

For active transport of proteins into the nucleus a nuclear localization signal (NLS), which can interact with the nuclear transport system and thereby initiate nuclear import, is required (67–69). Different classes of NLS sequences exist (70), which are divided into classical and nonclassical sequences. The classical NLS sequences are characterized by a stretch of basic, charged amino acids. Different examples of classical NLS sequences exist, like monopartite NLS sequences, which have one cluster of basic amino acids, and bipartite NLSs, which contain two clusters of basic amino acid residues separated by 10–12 neutral residues (69,71). These NLSs interact with the importin α/importin β heterodimer to facilitate nuclear import of their protein cargo (72). Some other NLSs can be recognized by importin β alone (70). These sequences resemble the IBB domain from importin α. Cingolani *et al.* identified a second cargo binding site on importin β, distinct from the IBB domain binding site, which is able to bind an NLS peptide directly without the interference of importin α or other adaptor molecules (73).

The nonclassical NLS lacks the stretch of basic amino acids and binds to transportin instead of importin β (43). Within the hnRNP A1 a 38 amino acid domain, termed M9, has been identified to be responsible for localization of hnRNP A1 into the nucleus (74,75). In contrast to classical NLS sequences that exclusively mediate nuclear import, the M9 NLS sequences also contain a nuclear export signal (NES). NESs are largely hydrophobic leucine-rich sequences that have been identified in several proteins (70,76).

Identification of NLS sequences experimentally is limited, and theoretical generalization for NLSs [hexapeptides with at least four basic residues and neither acidic nor bulky residues (77)] matches only a few nuclear and many nonnuclear sequences. To be able to identify NLSs more easily and more specifically, Cokol *et al.* designed a database for *de novo* prediction of NLS motifs (78). Through iterated *in silico* mutagenesis, they extended a set of 91 experimentally verified NLS motifs to a set of 214 potential NLS sequences.

Nuclear Entry of Plasmid DNA in Nondividing Cells

Delivery of plasmid DNA into intact nuclei involves transport through the NPC. Some groups showed nuclear uptake of plasmid DNA. Dowty *et al.* observed nuclear localization of plasmid DNA in postmitotic nuclei of primary rat myotubes (79) and Escriou *et al.* demonstrated uptake of plasmid DNA into the nuclei of CV1 cells (80). However, this transport is inefficient. Plasmid DNA microinjected into the nucleus results in high expression levels, whereas plasmid DNA injected into the cytoplasm is only poorly expressed. Pollard *et al.* observed that only 0.1% of naked DNA or 1% of polymer-complexed DNA reached the nucleus of COS-7 cells following microinjection into the cytoplasm (81). In addition, the rate of nuclear uptake of plasmid DNA, even when containing an NLS peptide, has been shown to be much slower than that of NLS-bearing proteins (82). DNA microinjected into the cytoplasm of cells reached the nucleus in 6–8 h. In digitonin-permeabilized cells nuclear uptake occurred after 90 min (83,84).

Both the size of the DNA and the sequence proved to be important for nuclear uptake. DNA with a size up to 1 kb can enter the nucleus rather efficiently after coupling of an NLS peptide via active transport involving the NPC (85,86). Important for the size of the DNA is also the structure of the DNA. Supercoiled DNA has a smaller diameter than open circular DNA and is transcriptionally more active (87,88). Moreover, a region of simian virus (SV) 40 DNA is sufficient for promoting nuclear entry of exogenous plasmid DNA (83,89,90). The sequence contains an origin of replication, portions of two promoters, and the SV40 enhancer. An important feature of this sequence is that it contains binding sites for transcription factors. Binding to these transcription factors can facilitate nuclear uptake of the DNA. Greassman *et al.* demonstrated that the 72-bp SV40 enhancer sequence was already sufficient to increase gene expression (91). After electroporation of blood vessels with a plasmid containing the SV40 enhancer sequence, gene expression was 10-fold higher than with a control plasmid, showing its functionality *in vivo* (92). This increase was caused by more efficient and faster transport into the nucleus and by the transcriptional enhancer function of the sequence. In addition, other sequences were reported to enhance nuclear uptake of plasmid DNA (93), some of them in a cell-specific manner by binding to cell-specific transcription factors [reviewed by Dean *et al.* (84)].

Some cationic polymers, such as polyethylenimine (PEI), have been described to be able to transfect nondividing cells *in vivo* and/or enter the nucleus together with DNA, however, to a lesser extent than viral vectors (94). The mechanism that leads to this translocation and subsequent transfection under those conditions is not well understood so far.

NLS-MEDIATED NUCLEAR TARGETING OF DNA

To facilitate nuclear uptake of exogenous DNA, peptides resembling NLS sequences are used to target the DNA toward the nucleus and more preferably into the nucleus. Such NLS peptides can be coupled to the negatively charged DNA based on the predominantly positive charge that is characteristic of classical NLS peptides. Alternatively, the NLS peptides can be covalently coupled to either the condensing agent of the nonviral gene delivery system (in most cases cationic polymers such as PEI) or directly to the phosphate backbone of the DNA. These different strategies (schematically depicted in Fig. 4) yield different outcomes in terms of transfection efficiency as will be discussed in the next section.

Noncovalent and Sequence-Independent Association of NLS Peptides to DNA

The most commonly used NLS peptide is the SV40 large T-antigen-derived NLS (PKKKRKV), the first identified

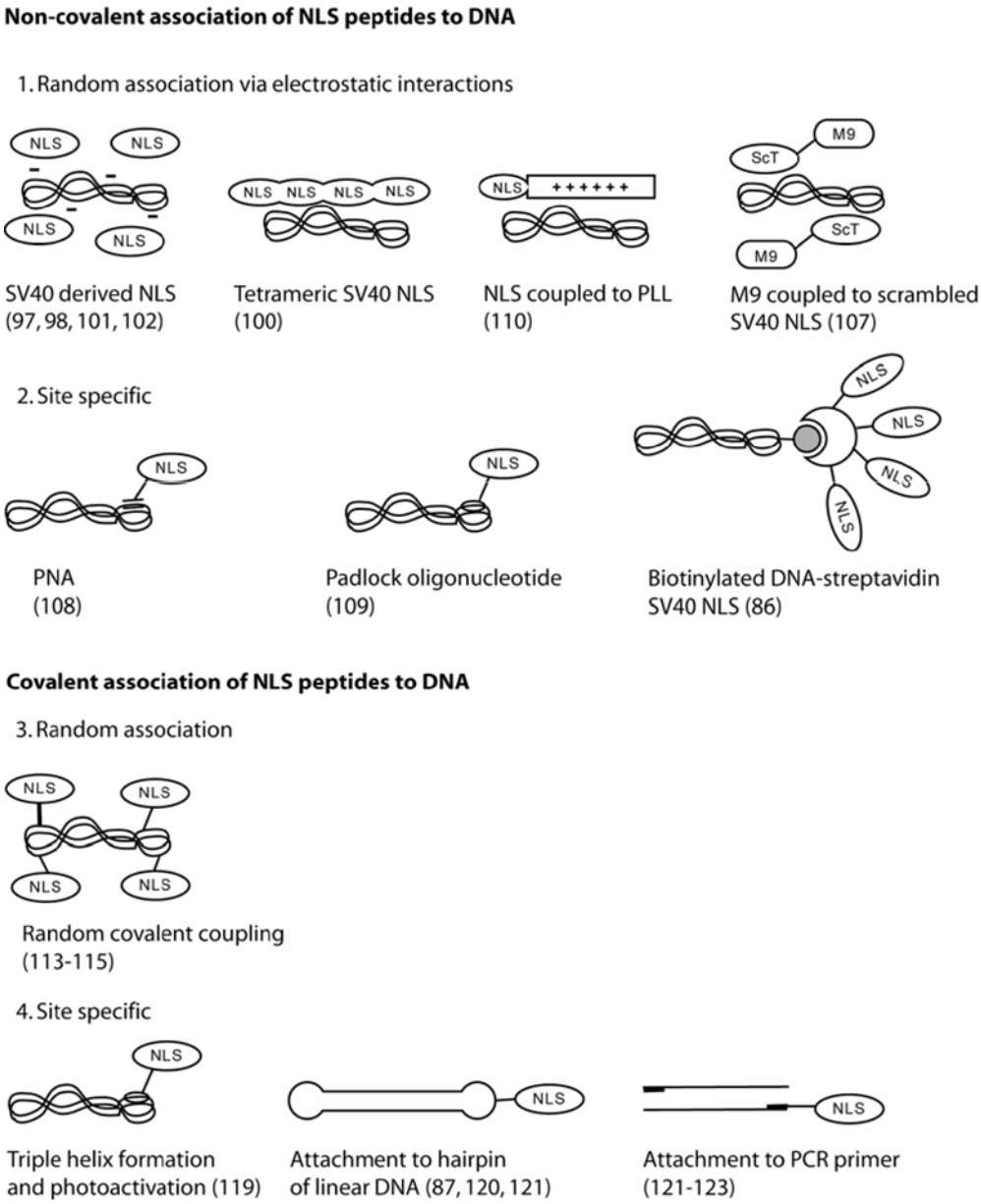

Fig. 4. Different strategies used to facilitate nuclear uptake of DNA and thereby enhance transfection efficiency.

NLS sequence (69) (Fig. 4.1). This cationic peptide binds DNA via electrostatic interactions. It can facilitate nuclear uptake of nonnuclear proteins with a molecular weight up to 465 kDa (95). Collas and Alestrom associated SV40-derived NLS peptides (CGGPKKKRKVG-NH2) via ionic interactions to luciferase-encoding plasmid DNA and microinjected the DNA–peptide complex into the cytosol of zebrafish embryos (96). The NLS peptide enhanced the expression of luciferase and facilitated nuclear uptake of plasmid DNA in isolated nuclei incubated in a cell-free extract (97,98). Efficient condensation of the plasmid DNA is important for cellular uptake, intracellular trafficking, and nuclear uptake. Condensation of DNA can be achieved by peptides with eight or more positively charged amino acids (99). Therefore, Ritter *et al.* designed a 4.4-kDa peptide consisting of four identical repeats of the SV40 NLS peptide with intervening glycine residues as spacers (100). This multimeric NLS peptide construct was able to condense plasmid DNA into small complexes and increase both transgene expression and nuclear uptake of the plasmid DNA in various cell lines, such as 16HBE14o, HeLa S6, and COS-7, when compared to a nuclear transport-deficient mutant sequence of the NLS. Especially, the early onset of gene expression was remarkable in comparison to different cationic polymers, like poly-L-lysine (PLL) and PEI. Plasmid DNA was detected in the nucleus already within 2 h, indicating that the multimeric NLS peptide construct can facilitate nuclear import. Addition of the SV40 derived NLS peptide to DNA could also enhance transfection efficiency of liposomes, as was shown by Aronsohn and Hughes (101) and Keller *et al.* (102). The SV40-derived NLS peptide was added to plasmid DNA by electrostatic interactions before complexation with the liposomes. In both studies, the NLS peptide enhanced transgene expression. Another strategy is to use an NLS coupled to a

DNA-binding and condensing peptide. Colin *et al.* used an oligolysine peptide, consisting of 16 lysine residues, coupled to an integrin-targeting tripeptide motif consisting of Arg-Gly-Asp (RGD) (103). RGD peptides have been found to stimulate cell adhesion and are widely employed to target to alpha-V/beta-3 integrins on the cell membrane. When the RGD peptide was coupled to oligo-L-lysine, luciferase expression increased 10-fold compared to the oligo-L-lysine alone or oligo-L-lysine coupled to RGE, indicating that the increase was specific to the RGD sequence. The increase in gene expression was caused by improved nuclear transfer of the plasmid DNA through the NPC of 56 FHTE8o cells (104). A similar construct, which also contains an integrin-targeting sequence, is able to transfect nondividing cells both *in vivo* and *in vitro* (105,106). In these studies, a peptide that targets an extracellular receptor also mediates nuclear uptake of its cargo, providing a complete nuclear delivery system.

Subramanian *et al.* used the nonclassical NLS peptide M9 to enhance transfection efficiency of lipofectin in confluent endothelial cells (107). However, this peptide does not contain many ionic residues and, therefore, a scrambled sequence of the SV40 T-antigen consensus NLS was added to improve DNA binding. Addition of the M9 peptide fused to the scrambled NLS sequence increased marker gene expression 63-fold.

Noncovalent and Sequence-Specific Association of NLS Peptides to DNA

The studies described above made use of electrostatic interactions to bind NLS peptides to DNA (Fig. 4.2). This does not allow control of the DNA-binding site of the NLS peptide, which could interfere with the transcription domain of the DNA. Using peptide nucleic acid (PNA) Branden *et al.* bound the NLS peptide in a sequence-specific manner to the plasmid DNA (108). With this technique, they were able to bind the NLS peptide to the plasmid DNA in a region not involved in gene expression. They observed a 5- to 8-fold increase in reporter gene expression after transfection of NIH/3T3 cells with PEI-complexed DNA compared to plain plasmid DNA. However, they did not show that this increase was specific for the NLS peptide, nor did they show nuclear import of the DNA. Roulon *et al*. described coupling of NLS peptides to a specific site in the plasmid DNA using a triple helix-forming padlock oligonucleotide; however, no enhancement of transgene expression was observed in HeLa cells when using cationic liposomes or polymers as transfection agents (109). Ludtke *et al.* used NLS-streptavidin to interact with biotinylated DNA (86). Addition of the NLS peptide increased both nuclear uptake and expression of DNA in a size-dependent manner after microinjection into the cytosol of HeLa cells.

NLS Peptides Coupled to Carrier Components

In addition to using the NLS peptide as a carrier to condense DNA, the peptide can also be attached to the DNA-condensing agent of the nonviral gene delivery carrier. Chan *et al*. observed a moderate increase in gene expression after covalent attachment of the SV40 large tumor antigen to PLL (molecular weight 110 kDa) using the Bioproducts transferrinfection kit in HTC cells (110). This increase in transfection efficiency could be attributed to the ability of the construct to bind to importin α/β heterodimer, which PLL itself cannot do.

A newly discovered nuclear targeting sequence is mellitin, a component of bee sting venom, which is often used in gene or drug delivery because of its membrane-destabilizing properties (111). When covalently conjugated to PEI (25 kDa) it also increases nuclear uptake of complexed DNA (112). Microinjection of melittin PEI/DNA complexes into the cytoplasm of HeLa cells resulted in 4-fold higher gene expression than after microinjection of PEI/DNA complexes alone. Coinjection with wheat germ agglutinin (WGA), an inhibitor of the NPC, abolished this effect of melittin, indicating the involvement of the NPC in gene delivery.

Covalent and Sequence-Independent Association of NLS Peptides to DNA

The disadvantage of noncovalent binding of an NLS peptide to DNA is that dissociation of the complex can occur during intracellular trafficking. To prevent and/or control dissociation, the peptide can be coupled covalently to DNA (Fig. 4.3). Sebestyen *et al.* covalently linked between 25 and 100 NLS peptides per kilobase pair DNA via a cyclopropapyrroloindole linker (113). When more than 40 NLS peptides per kilobase pair were attached, the plasmid DNA was transported into the nucleus of digitonin-permeabilized HeLa cells. The number of peptides attached influenced the rate and the extent of nuclear import of the plasmid DNA. Interestingly, coupling of NLS peptides did not enhance the nuclear uptake of plasmid DNA after microinjection into the cytoplasm, which may indicate sequestering of the DNA in the cytoplasm. In addition, other studies showed importin α binding after coupling of an NLS peptide to plasmid DNA, but observed no or only a minor increase in gene expression. Ciolina *et al.* covalently associated NLS peptides to plasmid DNA by photoactivation (114). They observed binding of their conjugates to importin α when 10 NLS peptides were attached to the DNA, but they detected no DNA in the nucleus after microinjection into the cytoplasm of NIH/3T3 cells. Nagasaki *et al*. randomly coupled the SV40 large T-antigen NLS peptide to plasmid DNA via a diazocoupling, which resulted in increase in binding to importin α (115). A polyethylene glycol (PEG) spacer (molecular weight 3400) was used to increase the distance between the cationic NLS peptide and the anionic DNA. Both the number of peptides attached and the length of the spacer were important for enhanced binding to importin α and gene expression. Coupling of 5.1 NLS peptides via a 3400-Da PEG spacer resulted in a 4-fold increase in gene expression in COS-1 cells. However, no nuclear uptake of the plasmid DNA was observed after microinjection into the cytoplasm of COS-7 cells. Carrière *et al*. coupled the importin β binding peptide (IBB) covalently to plasmid DNA at different peptide/DNA ratios (116). Despite binding to cytoplasmic receptors, no increase in transfection was detected in HeLa cells. Coupling of higher ratios of peptide via photoactivation showed decrease in gene expression. Noncovalently bound IBB peptide was also able to mediate the binding of plasmid DNA

to importin β. Therefore, they evaluated the effect of IBB peptide addition, without covalent coupling to the DNA, on transfection efficiency of DNA/cationic lipid complexes. Addition of the peptide increased transfection 20-fold.

Taken together, these studies indicate that covalent coupling of NLS peptides to plasmid DNA did not markedly enhance nuclear uptake or increase reporter gene expression. The reason for the poor nuclear localization could be the poor diffusion through the cytoplasm, the fast turnover of the DNA in the cytoplasm, or the large size of the DNA (86,117,118). Moreover, the lack of increase in transfection efficiency could be caused by transcriptional inactivation after covalent attachment of ligands to DNA.

Covalent and Sequence-Specific Association of NLS Peptides to DNA

To prevent inhibition of reporter gene expression by covalent attachment of peptides to nonspecific sites on the plasmid DNA, several groups coupled the NLS peptide to a specific location in the plasmid DNA (Fig. 4.4). Neves *et al.* coupled a targeting peptide to plasmid DNA (7257 bp) by covalent triple-helix formation and photoactivation. This resulted in functional conjugates that could bind to importin α. However, attachment of the NLS peptide did not increase transgene expression in NIH/3T3 cells (119). Maybe the lack of enhanced transfection in these studies is caused by the large size of plasmid DNA, which makes it hard to cross the NPC (86).

Following this line of thought, Zanta *et al.* coupled one SV40-derived NLS peptide to linearized plasmid DNA (87). The 3.3-kb linearized DNA was capped at the ends with a hairpin to prevent degradation by cellular exonucleases. The SV40-derived NLS peptide was attached to the hairpin located at the 3′ end of the linear DNA construct. Zanta *et al.* observed a 10- to 1000-fold increase in gene expression when compared to linear DNA without the NLS peptide. The enhancement factor depended on the cell type used. They did not directly assess the nuclear uptake of the linear DNA constructs. The promising results with single NLS peptides attached to end-capped linear DNA has stimulated other groups, including ours, to do the same. Tanimoto *et al.* covalently coupled the SV40-derived NLS peptide to the hairpin located at the 3′ end of the linear DNA or to both sides (120). However, this did not result in increase in transfection efficiency of COS-7 cells. They observed that introduction of a modified base in the hairpin of the linear DNA construct decreased transgene expression after microinjection into the cytoplasm or the nucleus, indicating that transcription activity or stability in the nucleus may be reduced by this modification. Our group has followed the same approach as Zanta *et al.* with the exception that the SV40 NLS was attached to the 5′ end rather than the 3′ end of the linear DNA construct (121). No increase in transfection efficiency was observed in our hands. Nor did we observe nuclear uptake of the linear DNA constructs in digitonin-permeablized OVCAR-3 cells (Fig. 5). The NLS peptide did mediate nuclear uptake of BSA (Fig. 5A), but was not capable to transport linear DNA into the nucleus (Fig. 5C). The discrepancy between results from the several approaches and various research groups indicates that the outcome of transfection experiments may be dependent on differences in experimental setup that at first sight may look trivial.

An easy method to synthesize large amounts of linear DNA, containing only eukaryotic regulatory elements, antigen-encoding DNA, and a polyadenylation sequence, is PCR (121–123). We synthesized PCR constructs using primers with phosphorothioate modifications in the backbone to stabilize the constructs in the cytoplasm. One of these primers contained the SV40-derived NLS peptide. Unfortunately, only a minor effect of the NLS peptide on gene expression in COS-7 cells was detected when compared to PCR-generated constructs without peptide and no increase was observed when compared to plasmid DNA. The minor increase in gene expression after attachment of the NLS peptide could also be attributed to protection of the DNA toward exonuclease digestion because the constructs were not stable in the presence of exonucleases (121).

Association of Plasmid DNA with Viral Proteins

Viral vectors are very efficient in nuclear delivery of exogenous DNA, and this could be exploited to improve the nuclear uptake and thereby the transfection efficiency of nonviral gene delivery systems. Viral proteins or peptides that mediate nuclear import may be coupled to cationic polymers to enhance their transfection efficiency. Carlisle *et al.* covalently coupled the adenovirus hexon protein to PEI

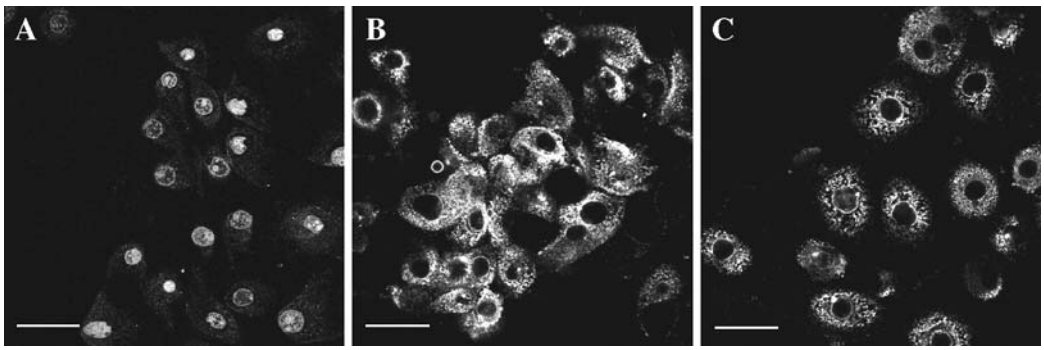

Fig. 5. Assay of nuclear import of BSA–Texas Red with SV40-derived NLS peptide (A) and fluorescent LDNA (B) and NLS-LDNA (C) in digitonin-permeabilized OVCAR-3 cells. Fluorescent microscopy pictures are shown. The scale bar represents 40 μm.

(800 kDa) (112). Activity of the conjugate was compared to PEI and PEI linked to albumin. Transgene expression was 10-fold higher after transfection of HepG2 cells with the adenovirus hexon–PEI complexes compared to PEI–DNA. This was not caused by an increase in cellular uptake due to the presence of the hexon protein. Microinjection experiments showed that the constructs mediate gene transfer via the NPC, even though the hexon protein does not contain a clear NLS sequence.

Moreover, proteins with nuclear localization and DNA condensation properties can be used for gene delivery. The viral protein R (Vpr) of human immunodeficiency virus type 1 plays a significant role in the viral life cycle by facilitating nuclear import of the preintegration complex in nondividing cells. Vpr is also able to interact with DNA, and Kichler *et al.* showed that the C-terminal domain of Vpr (residues 52 to 96) is able to condense plasmid DNA and transfect specific cells with efficiencies comparable to PEI and up 10- to 1000-fold higher than PLL (124). Mechanistic studies showed that the domain [Vpr5-(55–82)] that adopts an α-helix conformation mediates gene transfer by DNA condensation and membrane-destabilizing capabilities (125).

Association of Plasmid DNA with Karyophilic Proteins

Complexation of exogenous DNA with karyophilic proteins is also a commonly used method for nuclear delivery. Several karyophilic proteins have been used to facilitate nuclear uptake of DNA, like importin β (126), histones (127), nuclear factor-κB (NFκB) (128,129), and nuclear steroid receptors (130). Importin β plays an essential role in nuclear import and, therefore, association of plasmid DNA to importin β could enhance nuclear uptake of exogenous DNA. Nagasaki *et al.* coupled importin β via streptavidin to biotinylated plasmid DNA (126). A PEG spacer was introduced to increase nuclear uptake. Conjugation resulted in a 2.6-fold increase in the number of NIH/3T3 cells expressing the reporter gene. However, biotinylation of the plasmid DNA reduced gene expression as previously described (131).

Histones are nuclear proteins that electrostatically bind to DNA. Recently, it was shown that histones H1, H2A, H3, and H4 not only translocate into the nucleus, but also cross the cell membrane directly and not via endocytosis (132). In addition, the histones were able to mediate cell penetration of covalently attached bovine serum albumin molecules, indicating their potential as carriers for the delivery of macromolecules into living cells. Several groups have described the beneficial effect of histones H1 and H2A on gene transfection (127,133–135). In general, gene expression was comparable or better than conventional gene delivery systems, like liposome-based systems.

NFκB is a transcription factor that plays an important role in carcinogenesis. Mesika *et al.* observed that the NLS-carrying NFκB proteins p50 and p65 not only mediated nuclear uptake of plasmid DNA, but also its migration through the cytoplasm along microtubules toward the nucleus of HeLa cells (128). However, the majority of plasmid DNA molecules failed to reach the NE and enter the nucleus. This could be caused by formation of aggregates inside the cytoplasm, which greatly impairs transport through the cytoplasm even if it occurs via the microtubuli.

Rebuffat *et al.* developed a strategy called steroid-mediated gene delivery (SMGD), which uses steroid receptors that are present in the cytosol as shuttles to facilitate the uptake of exogenous DNA into the nucleus (130). They used glucocorticoid receptors (GRs) as a model system to test the principle of SMGD. The GR is cytoplasmic without hormone and localizes to the nucleus after hormone binding. The GR contains two NLS sequences, one that is similar to the SV40 NLS and one that is poorly defined, and one NES sequence (136). Both importin α/importin β and importin 7 mediate nuclear import of GR. Rebuffat *et al.* synthesized a bifunctional steroid derivative (DR9NP), which consisted of a steroid hormone to bind to the GR, a spacer, and a DNA-binding domain. The linker was covalently attached to plasmid DNA via UV irradiation. Both nuclear uptake and gene expression were enhanced in CV-1 cells. To prevent inhibition of gene expression after cross-linking the linker within the biologically important regions of the plasmid DNA, Rebuffat *et al.* developed a coupling method via PNA (137). The advantage of this method is its selective targeting to cells that express a specific nuclear receptor.

CONCLUSIONS

The success of nonviral gene delivery will be mainly determined by its ability to transfect nondividing cells. It is clear that overcoming the nuclear envelope in nondividing cells is still a major bottleneck for efficient gene therapy. Several strategies to improve nuclear import and thereby gene expression have been followed, but until now only with moderate success. Several reasons can be given for the somewhat disappointing outcomes. First, despite extensive research, the mechanisms underlying the NLS-mediated import of pDNA are still poorly understood. Second, only the endpoint of transfection, i.e., reporter gene expression, is often used to assess the efficiency of nuclear import by NLS peptides. However, as the transfection efficiency is dependent on many factors it is often difficult to determine the role of the NLS peptide. The absence of increase in transfection efficiency does not automatically mean that the NLS peptide did not work. For example, covalent conjugation of NLS peptides to DNA may lead to very efficient nuclear import of the NLS–DNA conjugates, but at the same time, may block transcription of the reporter gene. Third, differences in experimental setup and cell lines used make comparative evaluations of the role of NLS peptides on transfection efficiency very difficult, if not impossible. A standardized assay to evaluate the nuclear import of NLS–DNA constructs directly is therefore highly desired.

It is clear that an NLS peptide, covalently or non-covalently coupled to DNA, alone does not improve transfection efficiency sufficiently. Reasons for this could be an interaction of the peptide with the DNA or aggregation of the DNA with cellular structures in the cytoplasm. However, nuclear import remains extremely important for efficient gene delivery. To achieve this, a closer look is required at NPC functionality and the requirements for nuclear transport mechanisms used by nuclear receptors and viruses with regard to size, charge, and exposure of NLS sequences. A nuclear targeting sequence will be an important part of future gene delivery systems. These systems will have to combine

efficient cellular uptake and intracellular trafficking properties with nuclear delivery to result in an efficient nonviral gene delivery system (138).

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