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# Emerging significance of plasmid DNA nuclear import in gene therapy

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**Abstract**

The signal-mediated import of plasmid DNA (pDNA) into nondividing mammalian cell nuclei is one of the key biological obstacles to nonviral therapeutic pDNA delivery. Overcoming this barrier to pDNA transfer is thus an important fundamental objective in gene therapy. Here, we outline the rationale behind current and future strategies for signal-mediated pDNA nuclear import. Results obtained from studies of the nuclear delivery of pDNA coupled to experimentally defined nuclear localisation signal (NLS) peptides, in conjunction with detergent-permeabilised reconstitution cell assays, direct intracellular microinjection, cell-based transfection, and a limited number of in vivo experiments are discussed.


*Keywords:* Plasmid DNA (pDNA); Nuclear import; Cyto-nucleoplasmic transport; Gene therapy; Nonviral gene transfer; Nuclear localising signals; Peptide nucleic acid (PNA); Nuclear localising signals (NLS)

**Contents**



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## 1. Introduction

There has been a substantial interest recently in the nuclear delivery of deoxyribonucleic acid (DNA) in gene therapy strategies such as the now well-established gene augmentation, and more recently for site-specific gene repair (reviewed in Refs. [1,2]). Non-viral DNA delivery, involving the use of naked DNA, cationic polymer and lipid-based carriers is an appealing way of delivering therapeutic nucleic acids [3,4]. However, their use is limited, at present, by their comparatively low efficiency. In comparison to many of the other key obstacles to gene transfer, the intracellular transport and fate of exogenously added nucleic acids is poorly understood. Converging evidence from a number of different experimental approaches demonstrates that delivery of therapeutic plasmid DNA to the nonproliferating cell nucleus is an inefficient process and is limiting for exogenously added transgene activity.

An early report showed 50–100% of mouse embryonic fibroblast thymidine kinase-negative ($LMTK^-$) cells expressed kinase activity when the *Herpes simplex* virus thymidine kinase (HSV-TK) gene was injected directly into the nucleus, compared with $<0.01\%$ when plasmid was injected into the cytoplasm [5]. Zabner et al. [6] demonstrated the presence of plasmid in the cytoplasm of $\sim 98\%$ of African green monkey kidney fibroblast-like Cos-1 cells transfected with a cytoplasmic transcribing *Vaccinia* virus system compared to the 10% seen in cells transfected with plasmid containing the cyto-megalovirus (CMV) promoter system. These and other findings are consistent with experimental data showing that in dividing eukaryotic cells the end of prophase and the beginning of prometaphase is characterized by the disassembly of the nuclear envelope (NE), the double-membrane delineating the nucleus from cytoplasm [7–9]. The NE would, therefore, not be expected to be an obstacle to delivery of pDNA to mitotic cell nuclei. In contrast however, the intact NE excludes large macromolecules from the nucleus (following cellular uptake/internalisation).

Translocation of macromolecules across the NE is via pores that serve as size-exclusion barriers, with small molecules entering the nucleus by passive diffusion while larger macromolecules only enter via highly regulated active processes. Small oligonucleotides (ODNs), 18–28 bp in size, have been shown to rapidly and preferentially accumulate into the nucleus after transfection [10,11] and the size limit for passive diffusion of ODNs was found to be between 200 and 310 bp [12]. This is consistent with the notion that smaller ODNs may readily pass through nuclear pores (not withstanding active sequence-specific cytoplasmic retention/degradation), which have a diffusion limit of approximately 40 000 Da [13], whereas much larger plasmids are excluded. Moreover, nucleic acids lack a nuclear targeting component and plasmids are therefore unlikely to traverse the pores as free molecules. This then led many workers in the field to consider the tagging of targeting moieties to therapeutic nucleic acid molecules in order to circumvent the nuclear entry bottleneck.

In theory, the use of nuclear targeting motifs to direct the passage of nucleic acids across the NE into the nucleus should be simple. A well-defined nuclear targeting signal is conjugated to the DNA of interest, and the DNA–signal conjugate is then used for cell transfection or microinjected directly into the cytoplasm (Fig. 1). Nuclear delivery of the DNA–NLS conjugate is then evaluated by assaying for reporter gene activity after transfection, assessing the intracellular localization of fluorescent tagged DNA–NLS conjugates after microinjection and/or import into viable nuclei of detergent-permeabilised cells. In practice, over the last few years however, this approach has produced ambiguous outcomes. Here, we review recent work on nuclear localizing signal (NLS)-mediated DNA nuclear import. We begin by briefly describing macromolecular transport from the cytoplasm into the nucleus (hereafter referred to as cyto-nucleoplasmic transport) and refer readers to the primary literature in this area, which has been extensively reviewed [14–16]. Examples of experimentally defined nuclear targeting motifs and their conjugation to nucleic acids will then be considered. We then summarize the status of the field of signal-mediated DNA nuclear import and close with a discussion on alternative strategies for harnessing current knowledge on cyto-nucleoplasmic transport to facilitate efficient DNA nuclear delivery.

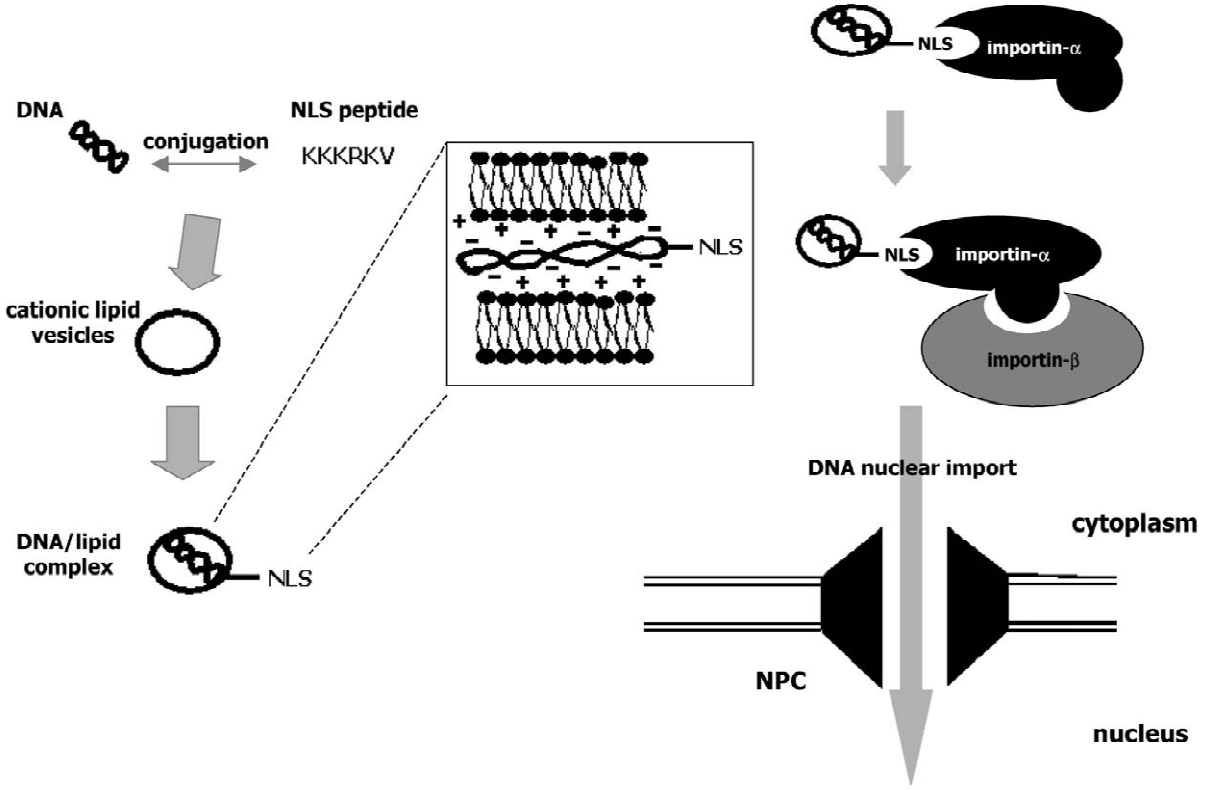


Fig. 1. Schematic representation of the nuclear localising peptide-conjugated DNA nuclear import mechanism. A well-defined nuclear targeting peptide signal sequence is conjugated to the DNA. Intracellular delivery of the DNA–NLS conjugate is mediated by formation of lipoplexes via the electrostatic interaction of negatively charged DNA molecules with cationic lipid molecules. In the cytoplasm, the importin-α transport receptor binds the DNA–NLS conjugate and together with importin-β mediates interaction with the nuclear pore complex to translocate the import complex into the nucleus. NLS, nuclear localizing signal.

## 2. Nuclear pore complex and cyto-nucleoplasmic transport

In the past few years, major advances have been made in the field of macromolecular cyto-nucleoplasmic transport. It is now established that transport proceeds through specialised NE spanning channels called nuclear pore complexes (NPCs) (reviewed in Refs. [17,18]). These cylindrical supramolecular structures, each composed of a central cylinder anchored in the NE by a surrounding spoke–ring complex [19,20], are about 130 nm in diameter and 70 nm thick, and perforate the NE. The mammalian NPC is composed of ~50 unique proteins (approximately eight copies of each per NPC), known collectively as nucleoporins (Nups) (reviewed in Ref. [21]). Filamentous attachments that presumably interact with incoming or outgoing receptor complexes extend to 50–100 nm at both the cytoplasmic and nucleoplasmic faces [22,23]. Passive diffusion of metabolites and small macromolecules across a centrally located channel is rapid but highly inefficient for proteins of ≥20–40 kDa [24]. The diameter of the channel can expand from ~10 to ~25 nm to translocate cargo macromolecules that are as large as several Mega-Daltons (close to 40 nm in diameter) [25–27]. NPCs are very stable structures that turn over very slowly in the entire duration of interphase. They are organized in an elastic network and are immobile in the plane of the nuclear membrane. Typically, a mammalian cell nucleus possesses about 2000 NPCs at a density of 4 NPCs/

$\mu m^2$ [28]. Each NPC has been estimated to carry out a maximum rate of $\sim 10^3$ transport events per second [29].

Selective nuclear import of macromolecules larger than 40 kDa is mediated by nuclear localising signals (NLSs). In the best characterised import pathway, the NLS is recognised by a heterodimeric protein complex of importin-α (also known as karyopherins-α, Kap-α) and importin-β (Kap-β). Importin-α then interacts directly with the cargo NLS, whereas importin-β docks the complex to the NPC by specifically binding to a subset of hydrophobic phenylalanine–glycine-rich repeats (FG repeats, standard IUPAC single-letter amino acid code) repeat containing NPC proteins (nucleoporins, or nups) [30–32]. Directionality of translocation across the NPC is subsequently determined by the nucleotide-bound state of the small guanine nucleotide triphosphatase (GTPase) Ran and is energy dependent (reviewed in Ref. [33]). Although the precise mechanism by which the karyopherins–cargo complex translocates across the pore is controversial, recent evidence suggests that NPC passage involves a partitioning of the entire complex into a hydrophobic phase formed by the FG-rich clusters. Differential partitioning across this permeability barrier is thought to facilitate NPC passage [34].

The rate of flux across the NPC of DNA has been shown to be significantly slower than that of NLS-bearing proteins. Fluorescent-tagged NLS-conjugated

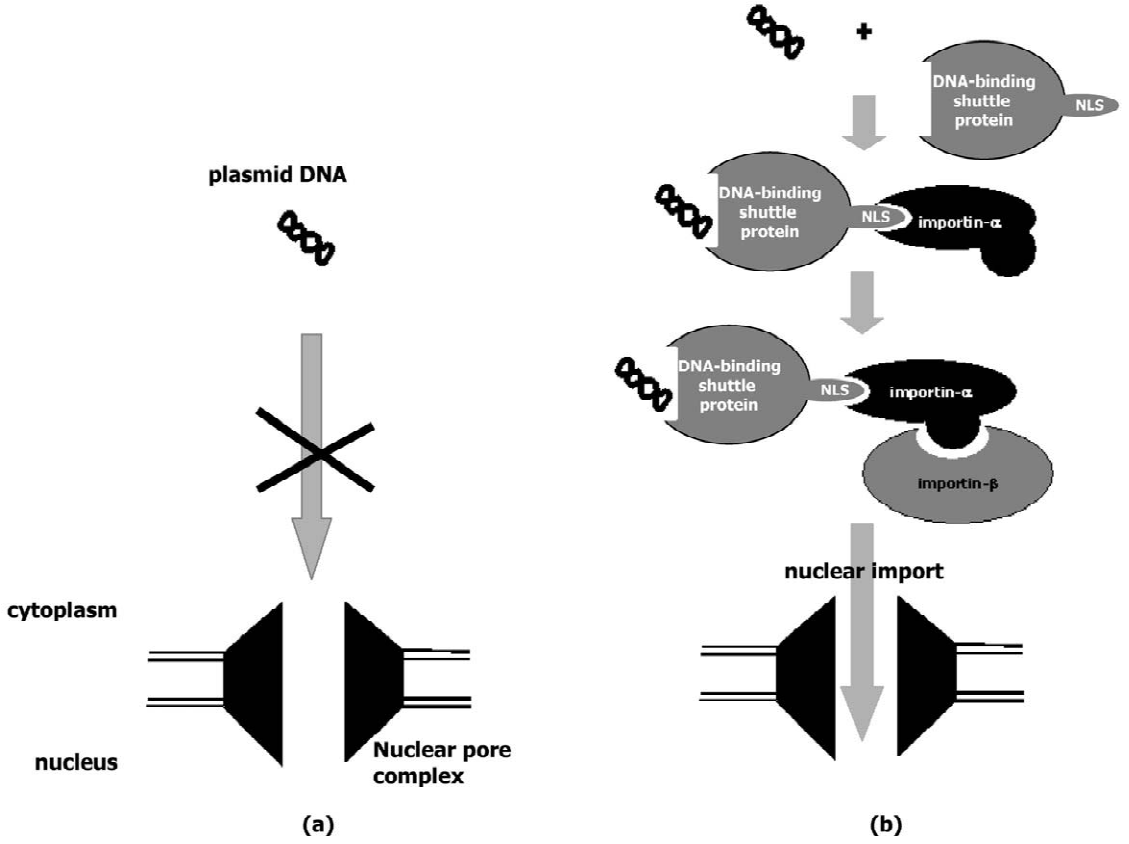


Fig. 2. Plasmid DNA nuclear import. The ‘piggyback’ model for plasmid DNA nuclear import predicts that because comparatively large plasmid DNA molecules lack nuclear targeting capability, they are unlikely to traverse the nuclear pore complexes as free molecules and are, therefore, excluded from the nucleus (a). For nuclear import to occur, the plasmid DNA cargo molecule binds to the adapter importin-α via soluble DNA-binding, NLS-possessing karyophilic shuttle proteins and is translocated to the nucleus using the nuclear import machinery (b).

BSA transport substrate is detected in the digitonin-permeabilised cell nucleus within 5 min, and is completely relocalised to the nucleus by 30 min [34]. In intact baby hamster kidney (BHK) fibroblastic cells, using computational and experimental approaches, the steady state flux rate of GTPase Ran, a key NLS-possessing nucleocytoplasmic transport regulator, was calculated to be 520 molecules per nucleus per second [36]. In contrast, when both fluorescent labelled or native pDNA are microinjected into the cytoplasm of intact cells, DNA is only detected (by confocal microscopy and in situ hybridisation, respectively) after 6–8 h. Fluorescent pDNA is observed within digitonin-permeabilised cell nuclei in 90 min and is maximal by 4 h [35,37]. These data suggest the import of the pDNA into the nucleus is approximately two orders of magnitude slower than that of NLS-bearing proteins. One explanation for the reduced import of pDNA is that DNA needs to first access NLS-bearing DNA-binding 'shuttle proteins' before being fortuitously piggybacked across the NPC into the nucleus (Fig. 2). This is consistent with the idea that the binding of DNA and shuttle protein, together with the movement of the DNA–protein complex within the cytoskeleton and the accessing of the protein import machinery will be rate-limiting for pDNA nuclear import [35,38].

In summary, nucleic acids lack NPC translocation-promoting elements and even when highly condensed or compacted (typically 30–100 nm diameter) are unlikely to passively diffuse across the NPC [39,40]. NPC passage of potential therapeutic DNA thus requires endowment of an indirect nuclear targeting capability.

## 3. Diversity of nuclear localizing signals

In the wake of Blobel's groundbreaking *Signal Hypothesis* [41], extensive data describing mechanisms by which proteins are directed to many distinct subcellular addresses have been accumulated. These include numerous reports detailing the nuclear transport of macromolecules and leading to the identification of a wide range of potential NLS sequences. NLS motifs are non-cleaved signal sequences within a transport substrate and at present do not fit a tight consensus. The best described NLS, the monopartite motif, resembles that of the SV40 large tumour antigen (SV40 Tag, $P^{126}$KKKRKV) and consists of a cluster of basic residues preceded by a helix-breaking proline residue [42,43]. A variation on this basic theme is the bipartite NLS, which is made up of two clusters of basic residues separated by 9–12 residues typified by the NLS of the *Xenopus* phosphoprotein nucleoplasmin (KRPAATKKAGQAKKKK$^{170}$) [44]. Both these localizing sequences use importin-α as their cognate receptor (reviewed in Ref. [45]). A striking deviation from the basic NLSs are ones only recognised by importin-β as exemplified by the arginine-rich sequence($^{35}$RQARRNRRRRWRERQRQ$^{51}$) from human immunodeficiency virus type 1 (HIV-1) Rev protein [46]. Non-classical target sequences include the M9 sequence from heterogeneous nuclear ribonucleoprotein A1 (hnRNP A1) protein. The M9 motif has been mapped to a ~38 amino acid glycine-rich sequence located between residues 268 and 305 of the 320 amino acid hnRNP A1 protein [47]. Specific recognition and passage across the NPC of the M9 sequence requires an endogenous carrier protein, transportin [48].

Finally, it is noteworthy that not all experimentally known NLSs comply with the above 'rules' and indeed a number of non-nuclear proteins appear to possess NLS-like sequences [49]. The definition of an NLS motif as a determinant of NPC-mediated nuclear import is therefore operational and not based on its primary structure [50]. For example, the Tat–NLS (GRKKRRQRRRAP$^{59}$) from the accessory protein of HIV-1 and similar so-called protein-transduction-domains (PTD) are capable of targeting heterologous cargo proteins in an importin-α/β-independent fashion [51]. Interestingly, glycosylated proteins lacking classical NLS motifs have also been shown to enter the nucleus in an energy-independent and O-linked *N*-acetylglucosaminyl residue-dependent manner. In these experiments, it was demonstrated that BSA substituted with β-di-*N*-acetylchitobioside (GlcNAc β-4GlcNAc) is transported from the cytosol to the nucleus in a sugar-dependent manner [52,53]. The aforementioned NLS motifs and many other experimentally determined sequences have recently been collated in a comprehensive database available at: http://cubic.bioc.columbia.edu/predictNLS [49].

## 4. Conjugation of nuclear localising signals to DNA

Conventional chemistries to synthesize peptide and nucleic acids simultaneously are not compatible, so varieties of noncovalent and covalent approaches have been used to associate DNA to NLS peptides. In early studies, workers simply mixed the reactants to form ionic complexes between negatively charged DNA and the purified NLS peptides. These complexes were then used to transfect cells using conventional protocols. Significant enhancement of transgene expression was achieved following direct microinjection of the preformed DNA/NLS–peptide conjugates into the cytoplasm or transfection of amphibian and mammalian cells [54–56]. Further studies also showed increased transgene transfection efficiency when the NLS motif was fused or covalently bound to a cationic moiety such as poly/oligolysine or the histone 1–DNA-binding domain [57,58]. However, even with appropriate controls (utilising reversed or mutant NLS peptides), DNA/NLS–peptide complex experiments should be interpreted with some caution as the effect of the basic NLS peptide (and/or any other linked positively charged moieties) on the DNA–cationic lipid/polymer interactions is poorly understood.

An alternative to associating NLS peptides to DNA electrostatically is to couple the peptides directly by chemical conjugation to the DNA. This covalent approach also involves the separate preparation of the NLS peptide and the DNA with subsequent formation coupling of the two highly purified moieties to produce a conjugate with the desired properties [59]. Crosslinking agents such as cyclo-propapyrroloindole [60] and 4-(*N*-maleimidomethyl)cyclohexane-1-carboxylic acid *N*-hydroxysuccinimide ester (SMCC) [61], together with photoactive *p*-azido-tetrafluoro-benzyl–NLS peptide conjugates have all been used successfully to associate NLSs to DNA [62]. In addition, streptavidin-conjugated NLSs have also been successfully coupled to biotinylated DNA [12]. Major drawbacks to the above covalent coupling include reduction in transcriptional activity resulting from chemical modifications in the transcription cassette. In addition, sequence-specific conjugation is not possible because the covalent adducts resulting from the reactions are randomly positioned on the nucleic acid.

With this in mind, we and others [35,63] (and reviewed in Ref. [64]) have recently used peptide nucleic acids (PNA) to link molecules to DNA in a sequence-specific manner. PNAs are synthetic homologues of nucleic acids with the sugar backbone replaced by an uncharged mimic of repeating 2-aminoethyl-glycine units. This pseudo-peptide backbone provides biological stability [65] and access to a variety of chemical modifications (reviewed in Ref. [66]), making PNA an ideal linker molecule. The preparation of PNA–peptide hybrids is achieved by the coupling of thiol-derivatized PNA with maleimido-peptides. A discrete PNA target sequence far away from the transcriptional site is chosen presumably making it possible to hybridise the PNA chimeric peptide to DNA in a site-specific manner [67]. Awasthi and Nielsen [68] have recently introduced a semi-automatic protocol for parallel synthesis of PNA–peptide hybrids using a *t*-butoxycarbonyl (Boc)-protection strategy. The method dispenses with the requirement for separate synthesis and purification of the reactant PNA and NLS moieties. Furthermore, subsequent purification of the product, the PNA–NLS hybrid, is unnecessary which minimises the unavoidable loss of material seen in conventional coupling reactions. The potential for high reproducibility and ease of scale-up that this method offers, will hopefully become apparent in the near future [68].

## 5. Signal-mediated DNA nuclear import: status of the field

### *5.1. DNA nuclear translocation and transfection studies*

Pioneering studies established the standard modus operandi for studying NLS-mediated macromolecular NPC-passage using synthetically conjugated or fused NLS peptides to fluorescent-tagged macromolecules of interest. Substrate accumulation was then fluorescently monitored in digitonin-permeabilised mammalian cell nuclei using confocal laser scanning microscopy (CLSM). This led to the unravelling of

the molecular mechanism of translocation through the NPC, including the identification of soluble transport factors such as the karyopherins [69]. This cell-free nuclear reconstitution experimental system has also led to insights into the complexities of DNA nuclear translocation across the NE by allowing for the study of NPC translocation events in isolation from other possible confounding factors such as the plasma–membrane (cellular entry), cytoplasmic processing and the cytoskeleton. Thus DNA-associated NLS peptides have been shown to accumulate in the nucleus in an energy-dependent, NPC-binding wheat-germ agglutinin (WGA)-inhibitable, and saturable manner [54,55,60].

The ability of NLS–peptides to mediate delivery of biologically active pDNA has been tested indirectly by monitoring the reporter gene activity following direct introduction into the cell via microinjection and/or transfection of the DNA–NLS conjugate into the cells. To date, a limited number of studies have demonstrated bona fide sequence-specific enhancement of reporter gene activity in cells following microinjection or transfection with NLS conjugated reporter gene DNA (Fig. 1). Ludtke et al. [12] showed that microinjection of a construct consisting of a biotinylated 900-bp green fluorescent protein (GFP) expression vector with streptavidin conjugated to a 39 amino acid peptide (H-CK-KKSSSDDEATADSQHSTPPKKKRKVEDPKDFP - SELLS) containing a functional SV40 large T antigen NLS resulted in a four-fold increase in GFP expression compared to that seen with constructs coupled to mutant NLS [12]. This was followed by a second report in which a single SV40 Tag NLS peptide (PKKKRKVEDPYC) was attached, via an ester/maleimide bifunctional linker (SMCC), to a capped 3.3-kbp CMV luciferase linear DNA fragment (CMVLuc–NLS). A 150-fold increase in luciferase activity was demonstrated in a number of cell types transfected with this conjugate compared with construct carrying a mutated NLS sequence. Interestingly, the CMVLuc–NLS produced a 1000-fold increase in dividing HeLa and 3T3 cells compared to the modest 10–30-fold increase seen in nondividing macrophage and dorsal root ganglion neurons [61]. Further demonstration of enhanced nuclear import and transfection efficiency of NLS-conjugated DNA was shown using large oligonucleotides and *lacZ* or enhanced green fluorescent protein (EGFP) reporter constructs hybridised to PNA–SV40 Tag NLS. An increase of up to eight-fold was observed in both the nuclear translocation of fluorescence-marked oligonucleotides, and the efficacy of polyethylenimine (PEI)-mediated plasmid transfection of Cos-7 cells compared to that seen in constructs lacking the PNA–NLS hybrid. Although control transfections with constructs hybridised to PNA-mutant NLS hybrids were not carried out, no nuclear translocation of the fluorescence-marked ODNs hybridised to PNA-inverted SV40 Tag NLS (VKRKKKP) was detected [70].

Taken together, observations from the few well-controlled studies are consistent with the notion that both pDNA nuclear translocation and transfection efficiency can be improved by rendering DNA transport-competent by conjugation with NLS(s).

### 5.2. Ex vivo and in vivo studies

The data discussed in the previous section provide insight into how transfection efficiency is dependent on NPC passage of transgenes in nondividing mammalian cells. However, few examples of successful use of NLS-conjugated DNA to enhance nuclear delivery ex vivo and in vivo have been reported. Branden et al. [71], using a sense Cy-5 fluorochrome-labeled 15-mer ODN hybridised to PNA–SV40 Tag NLS, showed active nuclear translocation of the PNA–NLS/ODN complex following PEI-mediated transfection in different mouse organs compared to that seen for the control, a Cy-3 fluorochrome-labeled 15-mer ODN antisense for the PNA sequence. In addition, although the data were not shown, no detectable transfection was seen when they used PNA lacking the NLS moiety hybridised to the ODN. Schirmbeck et al. [72] conjugated NLS peptide (PKKKRKVEDPYC), via a sulpho-MBS bifunctional crosslinker, to closed linear plasmid DNA. This DNA fragment contained the minimal immunogenetically defined gene expression (MIDGE) vector for hepatitis B surface antigen (HbsAg) required to stimulate an antibody response to HbsAg after gene gun immunisation of BALB/c mice. Compared to the identical expression vector

not conjugated to the NLS peptide, the HbsAg plasmid–NLS conjugate enhanced by 15-fold, both the priming of antibody responses to HbsAg after intramuscular injection and transfection efficiency in vitro. The enhanced immune response observed following treatment with import-competent NLS conjugated plasmid was not, however, compared to a control import-deficient conjugated plasmid.

Intriguingly, in a recent study of asialoglycoprotein receptor ligand asialofetuin (ASF) liposome-mediated human $\alpha_1$-antitrypsin (hAAT) gene expression in an in vivo mouse model, coupling of NLS peptide to the cationic lipid enhanced transfection efficiency and long-term duration of expression. In these experiments, NLS peptides were covalently conjugated to ASF targeted and non-targeted liposome/transgene complex via the helper lipid, dipalmitoylphosphatidylethanolamine (DPPE). In vivo transfection efficiency of the transgene, containing the complete genomic sequence of the hAAT gene under control of the endogenous promoter, was evaluated by measuring plasma levels of hAAT following intravenous injection and in the presence of partial haptectomy. The NLS peptide-conjugated ASF-targeted liposomes showed a greater transfection efficacy and longer duration of expression ($>12$ months) compared to non-targeted NLS-conjugated liposomes which was attributed to increased NLS-mediated liposome/DNA complex nuclear penetration [73].

In conclusion, relatively few studies have taken the field of NLS-mediated nuclear import into the in vivo setting. Furthermore, the necessary rigorous controls have sometimes not been incorporated. We, therefore find it difficult to conclude that there is as yet overwhelming evidence for the success of this approach in vivo. Finally, it may turn out that different organs and tissues will have variable susceptibility to these approaches in vivo.

## 6. Pitfalls and alternative approaches of optimising DNA nuclear import

There are a number of potential pitfalls in the use of NLS peptides to enhance DNA nuclear import. Firstly, all the aforementioned approaches involve the use of chemically synthesized proteinaceous material which pose a theoretical possibility of triggering the host immune surveillance, a case of an 'artificial virus' mimicking adverse viral properties. Even humanised NLS peptides might not be able to escape this fate as most common conjugation chemistries necessitate the addition of non-native cysteine residues. Secondly, and even though some recent evidence has demonstrated the sequence-specific interaction of the 'classic' SV40 TAg NLS, either tethered to DNA or cationic polymers, with recombinant importins, much still remains to be learned.

Using solution binding assays, Wils and co-workers [62,74] showed that specific recognition of DNA–NLS conjugates by importin-α depended on the number of peptides conjugated to the DNA but concluded that the effect of DNA size and topology was not well enough understood. More recent experiments by Chan and Jans [57,58] have used similar assays to study the accessibility of the NLS–polylysine conjugate to importin-α/β in studies aimed at investigating the basis of enhanced of polylysine-mediated transfection in rat hepatoma HTC cells. Interestingly, in this study at high polymer–DNA ratios even the mutant NLS–polymer/DNA complex exhibited significant non-specific importin binding comparable to that seen in the absence of DNA. Upon conjugation of signal peptide sequences to DNA, either alone or associated with cationic polymer/lipid, it is reasonable to expect the relatively large negatively charged DNA molecule to interact significantly with the positively charged NLS amino acid residues. In so doing, the anionic DNA could theoretically shield the NLS peptide and, therefore, hamper optimal binding to the importins or perturb interaction with the import machinery. High-resolution crystal structures of importin-α bound to both SV40 and the Myc NLS peptides have recently been reported and show that these similar but distinct NLSs bind to the same site on the importin-α. The NLSs bind in an extended conformation with equivalent amino acid residues each docking into identical pockets of the importin-α binding cleft [75–77]. This cleft on the importin-α combines hydrophobic and electrostatic elements and includes strategically placed negatively charged glutamate and aspartate residues at the edges that promote affinity and prevent indiscriminate interaction with hydrophobic

peptides [75]. The overwhelmingly large negative charge contributed by cationic polymer/lipids in transfection experiments using NLS–DNA conjugate/lipid complexes has the potential to impose severe conformational constraints on the NLS–peptide sequence thereby perturbing optimal binding and subsequent signal-mediated nuclear import of the NLS–DNA conjugate. Further insight into DNA bound NLS peptide behaviour and the mechanism of its access to the nuclear import machinery should, therefore, help in designing optimum nuclear targeting moieties.

Recently, alternative approaches to circumvent the NE barrier to DNA nuclear import by harnessing the import pathway have been reported. These are based on the use of endogenous or engineered DNA-binding karyophilic proteins to both bind/condense the therapeutic DNA and facilitate its signal-mediated nuclear import (Fig. 2). Condensation and nuclear import of DNA has been demonstrated using genetically engineered human fusion proteins of DNA-binding domains and NLS amino acid residues. Wolf and co-workers [78,79] have designed a recombinant histone (NLS–H1) containing both the SV40 large T antigen nuclear localization signal and the carboxy-terminal domain of human histone H1(0). NLS–H1 fusion protein–plasmid DNA complexes were shown to mediate reporter gene transfer into cultured cells with similar efficiencies to plasmid DNA–cationic lipid (lipofectin) complexes. In addition, NIH-3T3 or COS-7 cells transfected with NLS–H1-plasmid DNA–lipofectin complexes expressed 20 times more luciferase or had 2.5 times more β-galactosidase-positive cells than those transfected with plasmid DNA–lipofectin complexes. An alternative strategy is the inclusion of nucleotide sequences into the DNA, which have affinity for nuclear transport-mediating cellular proteins, such as transcription factors. A 72-bp repeat in the SV40 enhancer, previously shown to have nuclear import function in vitro increased CMV promoter-driven gene expression by as much as 20-fold in murine tibialis muscle in vivo [80,81]. More recently Mesika et al. [82] demonstrated a 12-fold increase in nuclear delivery and an increase in gene expression using a DNA vector that contained repetitive binding sites for the inducible transcription factor NFκB. Furthermore, this strategy can be extended to design cell-specific nuclear targeting vectors. Studies from our laboratories have shown that plasmid DNA containing the chicken smooth muscle γ-actin (SMGA) promoter is selectively transported into the nuclei of differentiated smooth muscle cells due at least in part to the serum response (transcription) factor (SRF). The SRF, when expressed in non-smooth muscle cells, was able to mediate nuclear targeting by SMGA promoter-containing DNA [83].

## 7. Conclusion

Several examples exist in the recent literature of the use of signal-mediated DNA nuclear import, which relies on association with, and/or conjugation of, experimentally determined NLS peptides. As discussed above, most studies so far have used the SV40 TAg NLS although other motifs, including hnRNP A1 protein M9 NLS and the (HIV-1) Rev protein NLS have also been used. There is, however, a paucity of data on NLS-mediated enhancement of DNA nuclear import in vivo, with only two studies reported to date. As previously mentioned, a major weakness in a majority of studies reporting DNA–NLS conjugate functionality is the lack of appropriate experimental controls. In a timely review of NLS peptides used for nonviral gene transfer, Cartier and Reszka [84] recently underscored the need for rigorous standards to demonstrate NLS–DNA conjugate functionality. Chief among these requirements was the need for inclusion of control scrambled or mutant NLS sequence peptide–DNA conjugates in all experiments. Nevertheless, the methods and results summarized in this review illustrate the rapid progress in the understanding of a key stage in the intracellular fate of exogenously added DNA. The efficient nonviral delivery of therapeutic DNA into the non-dividing cell nucleus should benefit from this effort.

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