REVIEW

Utilization of synthetic peptides containing nuclear localization signals for nonviral gene transfer systems

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The ability of nonviral gene delivery systems to overcome extracellular and intracellular barriers is a critical issue for future clinical applications. In recent years, several efforts were focused on the elucidation of the gene transfer mechanisms and on the development of multicomponent systems in order to improve both targeted gene delivery and transfection efficiency. The transport of the therapeutic DNA from the cytoplasm into the nucleus is an inefficient process and is considered as the major limiting step in nondividing cells. One of the strategies to improve nuclear uptake of DNA is

taking advantage of the cellular nuclear import machinery. Synthetic peptides containing a nuclear localization signal (NLS) are bound to the DNA so that the resulting DNA–NLS complex can be recognized as a nuclear import substrate by specific intracellular receptor proteins. In this review, we critically summarize recent studies applying this approach with a particular focus on NLS-sequence specificity. Implications of the observed results are also discussed in regards to future developments of this technology.

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Introduction

The goal of gene therapy is either the replacement of a defective human gene or the introduction of a new gene encoding a therapeutic protein. To be successful, this requires both delivery of the therapeutic DNA into human cells and its subsequent expression. It is widely recognized that the transfer of exogenous DNA into somatic cells is a complex process involving the passage through numerous biological barriers and is particularly complicated in the case of *in vivo* applications. Those barriers include the extracellular matrix, the cell membrane, the intracellular environment and also the nuclear envelope.

To overcome these barriers, the development of nonviral gene transfer systems tends towards multicomponent structures which may contain (poly)cationic elements for DNA condensation, lipidic compounds for enhanced protection and affinity to cell membranes and additional ligands for cell targeting and intracellular processing. Because of their structural and functional properties they are described as artificial virus-like particles and will be termed herein as heteroplexes in analogy to lipopolyplexes.¹ It is claimed that heteroplexes can circumvent the current limiting factors of viral vectors such as immunogenicity, size limitations of the transgene, potential mutation to replication-competent recombinant viruses and the expensive production. However, nonviral gene transfer systems still suffer from a relatively low transfection efficiency compared with viral vectors, hindering their broad clinical application.

Gene delivery can also be achieved by physical methods such as *in vivo* electroporation,^{2,3} increase of hydrodynamic pressure,^{4,5} particle gun^{6,7} and jet injection.⁸ The latter has recently been applied to tumors *in vivo*.⁹

The transfection mechanism of the targeted cell can be described as a sequence of several transport and dismantling steps of the heteroplex and are depicted in Table 1. The initial step is the binding of the heteroplex to the cell surface and its subsequent internalization. For this purpose, ligands or antibodies specific to cell membrane receptors can be displayed on the heteroplex surface to enhance cell type-specific targeting and cell entry via receptor-mediated endocytosis. However, one cannot exclude the possibility of direct entry into the cell by transient permeabilization of the cell membrane, especially when heteroplexes containing liposomes or when amphiphilic peptides are used.^{10,11}

The next step concerns the intracellular transport of the heteroplex up to the nucleus. The endocytotic pathway potentially offers vesicular transport to the perinuclear region. However, this requires proper sorting of the endosome, a process depending on physico-chemical properties of the endocytosed material.¹² Different strategies are used to allow the release of the vector into the cytoplasm, including the application of endosomolytic peptides or lipids^{11,13} and polyethyleneimines acting as a protonic sponge and inducing osmotic disruption of the endosome.^{14,15}

Once in the cytoplasm, the heteroplex is exposed to soluble and insoluble factors, which may lead to cytoplasmic entrapment and subsequent degradation. It is also expected that intracellular transport and degradation processes which interfere with the gene transfer mechanism will depend on the cell physiology and therefore

Table 1 Gene transfer as a result of transport and dismantling steps of a heteroplex^a

Gene transfer mechanism	Limiting factors	Possible enhancing factors
<p>Heteroplex</p> <p>Cytoplasm</p> <p>Nucleus</p>	Cell membrane (1)	Cell surface receptors (2)
		Endocytosis (3)
	Recycling endosomes? (4)	
		Vesicular transport (5)
	Fusion with lysosomes and degradation (6)	
		pH-driven escape from the lysosome (7)
	Cytoplasmic entrapment (8)	
		Nuclear import machinery (9)

^aLeft, schematic representation at the cellular level. Factors limiting the transfection efficiency (middle) and mechanisms which could potentially enhance targeting and efficiency (right) of the heteroplex are indicated.

also on the specific cell type and its microenvironment. The last step is finally the transport into the nucleus and the release of the DNA,¹⁶ in order to permit transcription of the therapeutic gene. One approach is to avoid nuclear import altogether by developing a cytoplasmic expression system based on the T7 RNA polymerase.^{17,18} Another strategy is to take advantage of the nucleocytoplasmic transport machinery. The general proof of this concept is provided by the fact that some viruses, among them DNA viruses, as part of their life cycle transport their genome through the nuclear pore complex (NPC), probably by interaction with the cellular nuclear import machinery.^{19,20} However, this crucial translocation step of the viral DNA is not completely understood, so that it cannot be transposed directly to nonviral gene transfer systems. Nevertheless, numerous efforts were made to modify plasmid DNA so that it can be recognized by cellular factors as a nuclear import substrate. Modifications include additional cloning of specific DNA sequences recognized by transcription factors,²¹ the attachment of glycosylated moieties²² and synthetic pep-

tides acting as nuclear localization signals (NLS). Over the past few years, tremendous advances have been realized elucidating the nuclear transport mechanism of macromolecules. It resulted in the identification of a wide range of potential NLS sequences and possibilities for regulation, which may be applied to the drug delivery systems.²³ The aim of this review is a critical analysis of recent publications concerning the use of synthetic peptides carrying NLS sequences for nonviral gene transfer.

Nuclear import of proteins

The nucleus in eukaryotic cells is separated from the cytoplasm by the nuclear envelope, consisting of two chemically distinct membranes, the inner and outer membranes, which are spaced by the perinuclear cisterna. The outer membrane is continuous to the endoplasmic reticulum. Transport of macromolecules between the cytoplasm and the nucleus occurs through nuclear pore complexes (NPC) spanning the nuclear envelope. The NPC is an exceptionally large complex of about 125 MDa and is

composed of 50–100 different proteins, termed nucleoporins.²⁴ Molecules smaller than 9 nm in diameter or 60 kDa can diffuse passively through the central channel of the NPC. Larger molecules comprising of proteins and RNA–protein complexes are transported actively by nuclear transport receptors through the NPC, thereby the NPC is capable of dilating up to 10–25 nm. Physiological factors can induce structural changes of the NPC modifying its permeability. Indeed, cytoplasmic ATP concentrations can modulate size and shape of the NPC. Furthermore, calcium depletion from the nuclear envelope cisterna inhibits passive diffusion and NLS-mediated transport through the NPC, probably by inducing conformational changes.^{25,26}

The broad variety of import substrates led the cell to develop a wide range of specific nuclear import pathways with corresponding regulation mechanisms, from which the importin β -pathway is one of the best characterized.^{27–29} Import substrates bind either directly to importin β or through the adapter protein importin α . The latter situation is found for cargoes bearing a ‘classical’, arginine/lysine-rich NLS, such as for the SV40 T large antigen or nucleoplasmin and is shown schematically in Figure 1. The cargo/importin complex docks through the association of other factors to cytoplasmic filaments emanating from nucleoporins and translocates through the central channel by a still unknown mechanism. The central channel constitutes a hydrophobic meshwork composed of phenylalanine-rich repeats from specific nucleoporins.³⁰ Finally, the complex dissociates by an energy-consuming reaction and transport factors are recycled back to the cytosol and are thus available for a next import cycle. One important factor is the small GTPase Ran, which allows association of the cargo–importin complex in its GDP-form, whereas its GTP-form dissociates the complex. The GDP- and GTP-form are maintained respectively by the Ran GTPase activating protein 1 (RanGAP 1) in the cytoplasm and the nucleotide exchange factor RanGEF in the nucleoplasm. Therefore, directionality of the transport is dictated by the unequal distribution of RanGAP 1 and RanGEF between the cytoplasm and the nucleus.

Several steps of the transport process are potentially targets for regulation mechanisms, including: (1) Accessibility or masking of NLS and availability of import factors; (2) regulation of the NLS affinity to its import receptor, eg by phosphorylation; (3) existence of cytoplasmic or nucleoplasmic retention signals; (4) regulation of NPC permeability; (5) possible regulation of the cargo affinity to the hydrophobic central channel.

The nuclear envelope as a barrier for nonviral gene transfer

Early studies indicated that the nuclear envelope represents a major barrier for nonviral gene transfer. Microinjection of naked DNA into the nucleus of cultured mammalian cells resulted in 50–100% transfection, whereas no transfection was observed following cytoplasmic injection.³¹ These results were later confirmed by microinjection studies using fluorescent and biotin-labeled plasmid DNA. No DNA could be detected in the nucleus following microinjection into the cytoplasm as compared with direct nuclear injection.³² Additional microinjection experiments into living cells demonstrated

Figure 1 ‘Classical’ nuclear import pathway of proteins mediated by importin α/β . (1) Trimeric complex formation between the import substrate, importin α and importin β . (2) Docking of the trimeric complex to the NPC. (3) Translocation through the central channel. (4) Dissociation of the complex and release of the import substrate into the nucleoplasm. A, The GTP-form of Ran is hydrolysed by RanGAP 1 to its GDP-form, which mediates the formation of the trimeric complex. B, The exchange of GDP with GTP on Ran is mediated by RanGEF and leads to the dissociation of the complex. Cargo-NLS, protein containing a NLS (import substrate); α , importin α ; β , importin β ; NPC, nuclear pore complex; RAN, small GTPase Ran; GEF, nucleotide exchange factor RanGEF; GAP, GTPase Ran activating protein RanGAP1.

that the nuclear import of naked DNA was size-dependent and was only detectable with linear DNA fragments which were smaller than 1.5 kb.^{33,34} Characterization of cells transfected with lipopolyamine–plasmid DNA complexes by transmission electron microscopy indicated that only one of 100 plasmid DNA molecules present in the cytoplasm reached the nucleus.³⁵ Furthermore, it was shown that co-transfection of cells with lipoplexes and a vaccinia virus expressing T7 RNA-polymerase permitting expression of the plasmid DNA in the cytoplasm leads to a transfection rate of almost 100%, as compared with 10% using a control expression system with a plasmid lacking the T7-promotor.³⁶ In addition, several groups provided experimental evidence that cells undergoing mitosis were far more readily transfected than cell-cycle arrested or quiet cells,^{36–40} suggesting that the dissociation of the nuclear membrane during mitosis greatly facilitates the transfection.

Taken together, these studies demonstrate that the transport of plasmid DNA through the NPC is an inefficient and rare event limiting the efficiency of nonviral gene transfer systems tremendously.

Studies on nuclear protein import showed that col-

loidal gold particles of a diameter of up to 170 Å and coated with NLS sequences from a karyophilic protein accumulate in the nucleus.⁴¹ Similarly, cytoplasmic reporter proteins such as β -galactosidase or pyruvate kinase fused to NLS are transported into the nucleus by a nuclear import pathway specific to the NLS sequence.²⁵ These observations lead to the utilization of karyophilic DNA-binding proteins in order to facilitate nuclear accumulation of plasmid DNA. Histone or high mobility group proteins were complexed to plasmid DNA and used in a calcium-based transfection system⁴² or in conjunction with cationic liposomes.^{43,44} However, it remains questionable whether these systems were effectively improved by the specific karyophilic property of the proteins. Indeed, NLS sequences seem to overlap with DNA-binding domains of the protein and thus may be impaired in their nuclear import capacity. Furthermore, fusion of an additional NLS to the C-terminal domain of human histone 1° protein did not lead to a significant increase of the transfection efficiency.⁴³

NLS sequences used for nonviral gene transfer

Synthetic NLS peptides were attached to the DNA and combined with conventional transfection methods in order to facilitate delivery of the modified DNA into the cell. Recent studies describing the application of synthetic peptides in nonviral gene transfer systems are summarized in Table 2. The various approaches mainly differ in the chosen NLS sequence, the method used for the attachment of the NLS to the DNA and the transfection system employed.

The majority of the studies involved the well-characterized SV40 T large antigen NLS-sequence ¹²⁶PKKKRKV¹³² (NLS^T, Table 2). In a few studies, the NLS^T was used together with its natural flanking regions (NL-NLS, P101, Table 2) containing a serine phosphorylation site which is recognized by the protein kinase CK2. Phosphorylation of this site is known to enhance binding of the NLS to its receptor importin α/β and consequently its rate of nuclear import.⁴⁵ Another NLS sequence is the M9, a cellular NLS from the heterogeneous nuclear ribonucleoprotein (hnRNP) A1 which is a major nuclear pre-mRNA binding protein.⁴⁶ M9 is a 'nonclassical' NLS rich in glycine and aromatic residues (²⁶⁸NQSSNFGPMKGG NFGGRSSGPYGGGGQYFAKPRNQGGY³⁰⁵) recognized by the importin β -like shuttle protein transportin 1.⁴⁷ Furthermore, a NLS sequence derived from the HIV-1 viral protein (Vpr) was also used in transfection experiments. Vpr is implicated in nuclear import of the HIV-1 pre-integration complex and harbors a C-terminal, 'nonclassical' NLS (Vpr52-93, ⁵²DTWTGVEALIRILQQLFIHFRIGCR HSRIGIIQQRTRNGA⁹³). The originality of this NLS relies on its ability to mediate nuclear import of a reporter protein independent of the importin α/β or transportin pathway, probably through direct interaction with the NPC and without the requirement of energy.⁴⁸

Finally, an adenoviral peptide sequence from the 20 N-terminal residues of the Ad3 fiber protein was used to enhance the transfection of human cells. This peptide (¹AKRRLSTSFNPVYPYEDS²⁰) contains a hydrophobic domain (FNPVYPY), a NPXY domain capable of receptor-mediated endocytosis and a NLS (KRRLSTSF),

which is able to carry a reporter protein efficiently into the nucleus.⁴⁹

The NLS can be covalently attached to the DNA. In this case, care has to be taken to avoid binding of the NLS within the expression cassette blocking subsequent transcription of the transgene. Noncovalent attachment of the NLS is performed through electrostatic interactions with the negatively charged DNA. This can be achieved either by taking advantage of cationic residues within the NLS itself or by fusion or covalent binding of a cationic moiety (poly/oligolysine, histone 1-DNA-binding domain, scrambled NLS^T) to the NLS. A particularly elegant method is the use of a peptide nucleic acid (PNA) sequence, which functions as a sequence-specific DNA-binding domain. This allows control of both the number of attached molecules and their binding site on the DNA. In a similar approach an oligonucleotide (ODN) sequence was used instead of the PNA. In this case, the ODN was covalently attached to the target DNA sequence using photo-active psoralen (Pso-ODN).

For the transfection of cells *in vitro* the preformed peptide-DNA complexes were combined with cationic polymers or lipids, such as polylysine, polyethylenimine, lipofectAMINE, lipofectin and other cationic lipids. In some studies the NLS-DNA complexes were delivered into the cytoplasm by direct microinjection. In other studies, the NLS-containing peptide could also mediate the cell entrance without the need of additional transfection agents. A possible explanation is that NLS-containing peptides bind and condensate the DNA-forming aggregates which are taken up by the cells. Except for a few studies,^{50–52} the incorporation of a NLS-containing peptide significantly increased the transfection efficiency of the particular transfection system used.

Evaluation of NLS sequence specificity in transfection systems

In principle, the NLS sequence specificity for plasmid accumulation in the nucleus and concomitant enhanced transgene expression should be demonstrated by using appropriate control sequences lacking the capacity to facilitate nuclear import. The studies showing enhanced transfection efficiency with NLS-containing peptides were therefore screened for data demonstrating the sequence-specific action of the NLS in the transfection process and an overview is given in Table 2. Sequence specificity was clearly demonstrated in two studies where the NLS was bound to a linear DNA fragment containing the expression cassette.^{34,53} An impressive piece of work involved the use of a 3.4-kb linear fragment containing the luciferase reporter gene and capped with an ODN hairpin structure. One end of the fragment harbored a covalently attached NLS^T and transfection was performed in combination with EXGen500 (linear polyethylenimine). This DNA-NLS^T construct led to a 150-fold higher gene expression compared with a construct carrying a mutated NLS sequence.⁵³ It was also shown that a circular plasmid DNA covalently attached to a NLS-containing peptide retained its transfection capacity. However, following transfection with this construct the NLS peptide did not significantly increase the reporter gene expression.^{50,51,54}

Several studies using noncovalently bound NLS-containing peptides did not provide any data as controls

Table 2 Utilization of synthetic NLS-sequences in nonviral gene transfer systems

NLS	DNA	Attachment of the NLS to DNA	c/nc ^a	Transfectionmethod/ reporter gene	NLS-specific increased transgene expression ^b	Conclusion	Ref.
NL-NLS ^T /mNL- NLS ^T /mNLNLS ^T	linear 900 bp linear 3.4 kbp pDNA	Streptavidin- Biotinylated DNA Oligonucleotide- NLS-cap Pso-ODN triple helix	nc c c	Microinjection/GFP EXGen500/luciferase	Yes Yes (150-fold)	NL-NLS ^T increases transfection efficiency of 900bp linear DNA Covalently bound NLS increases transfection efficiency of linear DNA No effect of the NLS on transfection	34 53 50
NLS ^T	pDNA	CPI	c	Cationic lipid/ β -galactosidase	ND	No effect of the NLS on transfection Sequence-specific effect of covalently NLS on transfection was not demonstrated	50,51 54
NLS ^T /mNLNLS ^T NLS ^T /mNLNLS ^T or D- NLS ^T	pDNA pDNA	lissamine CPI	c c	Cationic lipid/ β -galactosidase Lipofectin or microinjection/luciferase	No No	No effect of the NLS on transfection Sequence-specific effect of covalently NLS on transfection was not demonstrated	73
NLS ^T /revNLS ^T	pDNA	PNA	nc	25 kDa PEI/ β -galactosidase and luciferase	ND	Sequence-specific effect of the NLS on transfection was not demonstrated	52
NLS ^T	pDNA	Histone 1 DNA- binding domain	nc	Cationic lipid/luciferase/ β -galactosidase	ND	No effect of the NLS on transfection	57
P101/P101T (NLS ^T /mNLS ^T) P101/P101T (NLS ^T /mNLS ^T)	pDNA pDNA	110 kDa Polylysine 110 kDa Polylysine	nc nc	Transferrin-polylysine/ β -galactosidase Calcium phosphate or lipofectAMINE/ β -galactosidase	~1.3-fold increase No	Little effect of the NLS compared with the mNLS on transfection Maximum transfection efficiency was not increased with P101 compared with P101T	58
P101/P101T (NLS ^T /mNLS ^T) M9/scM9	pDNA pDNA	110 kDa Polylysine scrambled NLS ^T	nc nc	HGM fusion protein/ β -galactosidase LipofectAMINE/ β -galactosidase	~2-fold increase Yes (~10-fold)	Slight effect of the NLS compared with the mNLS on transfection non covalently bound M9 enhances transfection efficiency compared with scM9 about 10-fold	59 60
NLS ^T /revNLS ^T	pDNA	direct binding to DNA	nc	Microinjection into zebrafish eggs/luciferase	Yes	microinjected NLS/DNA-complexes leads to transfection. No transfection using revNLS	55,56
Vpr52-93	pDNA	direct binding to DNA	nc	Transfection with DNA/peptide complexes/luciferase	ND	Sequence-specific effect of the NLS on transfection was not demonstrated	15
Ad3 sequence (Peptide I)	pDNA	20 N-terminal lysine residues	nc	Transfection with DNA/peptide complexes/luciferase	ND	Sequence-specific effect of the NLS on transfection was not demonstrated	74
NLS ^T (MPG)	pDNA	direct binding to DNA	nc	Transfection with DNA/peptide complexes/luciferase	ND	Sequence-specific effect of the NLS on transfection was not demonstrated	75
NLS ^T (l oligomer4)	pDNA	branched lysine residues	nc	Transfection with DNA/peptide complexes/luciferase, β -galactosidase	ND	Sequence-specific effect of the NLS on transfection was not demonstrated	76

^aCovalent or non-covalent binding of the NLS to the DNA.

^bIncrease of maximum transgene expression after transfection using the NLS compared with a control sequence known to be deficient in nuclear import NLS, nuclear localization signal. NLS^T, NLS of simian virus 40 T large antigen; the prefix 'm' and 'rev' denote respectively a mutated and a reversed form of the NLS which are deficient on facilitated nuclear import; NL-NLS^T, NLS^T plus flanking regions containing a phosphorylation site known to enhance the nuclear import rate; GFP, green fluorescent protein; EXGen500, linear 22 kDa polyethylenimine; pDNA, plasmid DNA; Pso-ODN, psoralen modified oligonucleotide; ND, no data provided; D-NLS, D-stereoisomer of NLS; CPI, cyclopropylpyrrolidone; PNA, peptide nucleic acid; PEI, polyethylenimine; P101, NLS plus flanking region; P101T, mutated form of P101; HGM, a fusion protein containing the α -melanocyte stimulating hormone fused to the transcription factor GAL4 DNA-binding domain; M9, NLS from the heterogeneous nuclear ribonucleoprotein (hnRNP) A1; scM9, scrambled sequence of M9; peptide I, 20 N-terminal residues of adenovirus 3 fiber protein harboring both a NLS (residues 2-5) and a ligand for receptor-mediated endocytosis; MPG, bifunctional peptide containing NLS^T and a hydrophobic fusion sequence from HIV-1 gp41; L oligomer4, lysine-based branched multidomain peptide containing 8 NLS^T.

using NLS-scrambled or mutated sequences as controls (indicated as ND in Table 2).

In other studies, plasmid DNA was noncovalently bound to a peptide containing a NLS^T without additional DNA-binding sequence. As a control for NLS-sequence specificity, NLS^T was replaced by a reversed NLS^T (revNLS^T). The resulting NLS^T-DNA complexes were micro-injected into Zebrafish eggs and transfection activity studied. Expression of the luciferase reporter gene was only detected in combination with the NLS^T, but not with the revNLS^T-control sequence.^{55,56} In a number of studies, the P101-sequence (enhanced NLS^T) was coupled to polylysine and combined with different transfection agents including polyfection, calcium-phosphate, lipofection and a chimeric protein.^{57–59} Minimal increase of β -galactosidase reporter gene expression (1.3–2-fold) was observed at a lysine to nucleotide charge ratio of 0.4 as compared with a mutated peptide (P101T). However, when the charge ratio was increased from 0.4 to 4.0 the expression level was overall significantly higher, but under these conditions incorporation of either P101 or P101T had no additional effect. These studies indicate that the charge ratio, which affects the complex structure, has a large influence on the transfection efficiency while the incorporation of the NLS peptide did not significantly improve reporter gene expression.

The only work showing a significant improvement of mammalian cell transfection mediated by a specific NLS peptide noncovalently attached to plasmid DNA, involved the M9 sequence.⁶⁰ In this study, M9 was fused to a scrambled NLS^T for DNA-binding and combined with lipofectAMINE for transfection. In this case, M9 induced a 10-fold increase of β -galactosidase reporter gene expression compared with a scrambled M9 sequence (Table 2).

Model systems to investigate nuclear import of DNA–NLS complexes

Several model experimental systems have been developed to investigate the nuclear import of DNA–NLS complexes and are summarized in Table 3. The ability of the DNA–NLS complex to physically interact with import factors has been tested in quantitative *in vitro*-binding assays using recombinant proteins. The variation of both the number and sequence of bound peptides, provided information regarding the affinity and specificity of the interaction. Translocation through the NPC has been investigated in partially permeabilized cells and in isolated nuclei. These systems require nuclei having an intact nuclear envelope and functional NPCs. The exact nuclear import mechanism can be further characterized using appropriate factors known to participate in specific import pathways or by manipulating the transport assay conditions. Nuclear import in living cells has been investigated by microinjection of DNA–NLS complexes into the cytoplasm. Factors known to interfere or to compete with the nuclear import process can be co-injected and the resulting effect may provide basic information on the nuclear import pathway. However, co-injection of additional factors may be limited if they interact directly with the DNA–NLS complexes. Furthermore, microinjection is an invasive method which induces considerable stress on the investigated cell. Consequently, the natural

behavior of the cell may be modified which makes the interpretation of such experiments difficult.

Evaluation of the NLS-facilitated nuclear import of DNA

Similar to the evaluation of the transfection experiments above, we analyzed the data published on nuclear import of DNA–NLS complexes with regard to the NLS-sequence specificity (Table 3). The *in vitro*-binding assays demonstrated that recombinant importins are able to interact with the NLS^T in the NLS-plasmid complex, regardless of covalent or noncovalent binding of the NLS^T to the DNA. The interaction was shown to be NLS-sequence specific as demonstrated by comparison with a NLS^T-control sequence.^{50,51,57–59} The experiments using permeabilized cells or isolated nuclei showed that DNA complexes carrying the NLS^T accumulated in the nuclei to a higher extent than DNA complexes containing NLS-control sequences. These results were obtained both with linear and plasmid DNA, independent of how the NLS^T was attached to the DNA (see Table 2). The nuclear import of DNA was shown to be an active process and was inhibited at 4°C or after energy depletion. In some studies, the passage of the complex through the NPC was demonstrated by specific blocking experiments using lectin wheat germ agglutinin (WGA).^{54–56} Finally, a competitive inhibition experiment using NLS^T-streptavidin suggested that nuclear import of NLS^T covalently attached to plasmid DNA occurred through the importin α -pathway.⁵⁴

An impressive study involving NLS P101, cross-linked to polylysine,^{57–59} investigated NLS sequence specificity. In this study, P101 was compared with its mutated form (P101T) in combination with various transfection methods. Transfection efficiency was correlated to the charge ratio of lysine to nucleotide in the polylysine–DNA complex, to physical parameters (morphology and size) of the resulting DNA complexes and also to their capacity to interact with the nuclear import machinery.

It is interesting to note that with a lysine to nucleotide charge ratio of 0.4 the binding of the complex to recombinant importins was increased 10-fold in a NLS sequence specific manner. In addition, an approximate 1.7-fold NLS sequence specific enhancement of nuclear accumulation was observed in permeabilized cells.⁵⁸ However, as discussed previously, at this charge ratio only a slight NLS sequence specific increase in the reporter gene expression (1.3–2-fold) was observed in the transfection experiments (see Table 2).

At a charge ratio of 4.0, reporter gene expression was increased considerably over the one obtained with the charge ratio of 0.4, but there was no difference between the complexes with the peptide P101 and P101T. The binding of the complexes to recombinant importins was also increased (2.3-fold) at this charge ratio compared with the binding capacity at ratio 0.4, but was also non-specific to the used NLS. Surprisingly the nuclear accumulation was the same for both NLS and similar to the maximum level obtained at charge ratio 0.4.

These results indicate that overall the NLS used had little effect on the transfection efficiency. This also emphasizes the difficulty in correlating the interaction of the complexes to the nuclear import machinery with the transfection efficiency. Indeed, complexes formed with a

Table 3 *In vitro* models to investigate the NLS-facilitated nuclear import of DNA

Ref.	50,51	57–59 ^c	34	55,56	54	60
NLS (NLS ^T /mNLS ^T)	NLS ^T /mNLS ^T	P101/P101T	NLS ^T /mNLS ^T or D-NLS ^T linear 70bp-3kb	M9/scM9 pDNA	pDNA CPI	pDNA scrambled NLS ^T
DNA	pDNA	NLS ^T /revNLS ^T pDNA	Streptavidin-Biotinylated DNA	direct binding to DNA		
NLS-DNA binding	lissamine	110 kDa Polylysine	nc	nc	c	nc
c/nc	c	nc	nc	nc	c	nc
<i>In vitro-binding assays: interaction of complexes with recombinant importins</i>						
Method	GST-importin α binding assay	ELISA-based importin α/β -binding assay	—	—	—	—
NLS-enhanced binding ^a	yes	yes for Ly/Nu = 0.4; no for Ly/Nu = 4 ^c	—	—	—	—
<i>Permeabilized cells or isolated nuclei: transport through the nuclear pore complex of nuclei with an intact nuclear envelope</i>						
Method	—	mechanical cell perforation	digitonin-permeabilization	isolated sea urchin pronuclei	digitonin-permeabilization	digitonin-permeabilization
NLS enhanced import ^b	—	yes for Ly/Nu = 0.4; no for Ly/Nu = 4 ^c ; active transport	yes for a 510bp fragment; active transport	yes active transport through NPC	yes active transport through NPC; inhibited by NLS-streptavidin	ND transport of M9/DNA-complexes through NPC
<i>Cytoplasmic microinjection: Studying nuclear import in living cells</i>						
Cell type	CV-1	—	HeLa	Zebrafish eggs	HeLa	—
Detection of the complex	Rhodamine labeled peptide	—	Texas Red-labeled DNA	PCR analysis of injected DNA after nuclear extraction	Rhodamine labeled DNA	—
NLS enhanced import ^b	weak diffusion to the nucleus; no detectable nuclear localization	—	yes for a 900bp fragment	yes no signal after co-injection with BSA-NLS	no detectable nuclear localization	—

^aIncrease of importin binding using the NLS compared with a control sequence known to be deficient in nuclear import.

^bIncrease of nuclear accumulation using the NLS compared with a control sequence known to be deficient in nuclear import.

^cIn the study of Chan 2001,⁵⁹ NLS-polylysine was added to HGM-precomplexed DNA. Data were provided for Ly/Nu = 0.4 only. Abbreviations, if not indicated, are the same as in Table 1. GST, glutathione S-transferase; Ly/Nu, lysine to nucleotide ratio indicating the charge ratio of the DNA-peptide complex; FITC, fluorescein-5-isothiocyanate. BSA, bovine serum albumin.

charge ratio of 2.0 containing P101 bind to recombinant importins almost as efficiently as complexes with a charge ratio of 4.0, although the interaction is about seven-fold higher than with the P101T. However, at the charge ratio of 2.0 the transfection is almost abolished. This was attributed to a change in the morphology and size of the complexes, at a charge ratio of 2.0. Complexes were characterized by electron and atomic force microscopy as 40–80 nm toroids at charge ratio 0.4, collapsed beads at charge ratio 4.0 and 150–800 nm large irregular aggregates at charge ratio 2.0. This suggests that physico-chemical properties of the complexes influence the transfection efficiency to a significantly greater extent than the incorporated NLS sequence. In addition, although increased transgene expression was observed with P101 as compared with P101T regardless of the transfection method employed, P101 used in combination with calcium phosphate is still less efficient than P101T in combination with lipofection. These observations indicate that the charge ratio of the complex and the chosen transfection system readily determine the maximum transfection level and are able to surpass the effect of the NLS.

Finally, it should be noted that the DNA complexes were not combined with the transfection system for the binding studies, the nuclear accumulation experiments and the microscopy. In order to correlate the nuclear import results and physical parameters of the complex with the transfection efficiency observed for the complex in combination with transfection systems, one has to assume that the complex released into the cytoplasm is identical to the complex before combination with the transfection system. Additional studies will be necessary to determine the exact physico-chemical properties of the complexes released into the cytoplasm which may provide insights into the properties required for efficient nuclear import and expression.

WGA blocking experiments performed with the M9 sequence and permeabilized cells demonstrated that DNA–M9 complexes accumulate in the nuclei and translocate through NPCs.⁶⁰ However, no corresponding data were presented using the scrambled M9 sequence as control, as used in the transfection experiments (see Table 2). Therefore, it remains questionable whether the observed M9 sequence-specific increase of reporter gene expression is indeed due to an enhanced M9-mediated nuclear uptake of the DNA.

The microinjection experiments showed that a linear 900 bp DNA fragment accumulates in the nucleus to a higher extent when noncovalently attached to the NL–NLS^T (enhanced NLS^T) than with the mNL–NLS^T-control sequence.³⁴ Nuclear localization was also observed after microinjection of plasmid DNA complexes containing noncovalently bound NLS^T into Zebrafish eggs. In this case, nuclear accumulation could be blocked by co-injection of BSA–NLS, suggesting that the nuclear import of DNA–NLS^T complexes occurs through the importin α -pathway.^{55,56} Finally, microinjection of plasmid DNA covalently bound to NLS^T was performed in CV-1 and HeLa cells, however, no nuclear localization of the injected DNA could be detected.^{50,51,54}

Possible nonspecific functions of the NLS during transfection

The rationale to incorporate NLS sequences into the transfection systems was to facilitate the transport of

DNA from the cytoplasm into the nucleus via the nuclear import machinery in a NLS sequence-specific manner. As mentioned previously, not all studies included experiments using a NLS-control peptide to demonstrate sequence specificity. In these cases, one cannot exclude the possibility that the incorporated peptide may enhance the transfection efficiency through a mechanism other than the nuclear import machinery.

It is likely that the basic residues of the NLS interact directly with the DNA contributing to its condensation and increasing the total charge of the particular transfection system.

Recent studies suggest that cell binding and internalization of DNA complexes is mediated by heparan sulfate proteoglycans (HSPGs).^{61–63} These molecules are displayed on the cell surface and bind to positively charged DNA complexes through nonspecific electrostatic interactions. The expression of HSPG depends on many factors including the cell type and cell differentiation. HSPG is also found intracellularly and especially in the nucleus.⁶⁴ Furthermore, proteoglycans harboring different types of glycosaminoglycans (GAGs) are major components of the extracellular matrix in many organs. Interaction of lipoplexes and polyplexes with GAGs was demonstrated *in vitro*⁶⁵ and may lead to entrapment or dissociation of the DNA complex inside the organ. Coating of the polyplex with an anionic lipid prevented the inhibition of polyfection *in vitro* by hyaluronic acid.⁶⁶ Taken together, it is possible that NLS-containing peptides incorporated in heteroplexes interact with proteoglycans. This interaction may enhance cell entry of the heteroplex and possibly interferes with other steps of the gene transfer mechanism.

The anionic residues of NLS-containing peptide could also interact with the polycations typically used for DNA condensation or the hydrophobic residues with lipid components. The NLS could thereby influence the physical properties of the heteroplex such as particle size, DNA condensation rate, surface charge distribution and intracellular stability. These effects could be sequence-specific and could influence the transfection efficiency of the complex. Therefore, it is important to design studies that permit the detection of nuclear import selectively.

So far only four different NLS sequences have been used in combination with gene transfer systems and most of the analyzed studies involve NLS^T which has a high content of lysine and arginine residues, which increases the cationic residues:DNA ratio within the complex. It will be important to include NLS sequences with different physico-chemical properties, eg bipartite NLS consisting of two basic clusters separated by a linker region. The bipartite NLS bind importin receptor at multiple sites,⁶⁷ offering greater sequence diversity.⁶⁸ Furthermore, a broad variety of other types of NLS sequences have been identified with different corresponding receptors and potentially different nuclear import pathways that may be of interest for gene transfer systems.

Conclusions regarding the application of synthetic NLS-containing peptides for gene transfer

In view of the NLS sequence specificity to facilitate nuclear import for efficient transfection, the following conclusions can be drawn from the data presented to

date. (1) *In vitro*, NLS^T bound to plasmid DNA is able to interact with its importin receptor and carries DNA through the NPC into the nucleus, presumably via the importin pathway. (2) The NLS^T was shown to enhance nuclear uptake of linear DNA in contrast to plasmid DNA in mammalian cells. Note, naked linear DNA itself accumulates in nuclei in a size-dependent manner, whereas to date nuclear import of plasmid DNA has not been directly observed. (3) In transfection experiments, NLS^T clearly enhances reporter gene expression when linear DNA is used. M9 is able to enhance transfection efficiency, however the mechanism involved remains to be determined.

In summary, the concept of facilitated nuclear import of DNA through specific NLS sequences, which results in an improved nonviral gene transfer system, was only demonstrated conclusively for linear DNA. The applicability of this approach to plasmid DNA requires further investigation and is complicated by the fact that the translocation mechanism of plasmid DNA into the nuclei of living cells is still not understood.

Additional comments and future prospects

The need of standardization

The studies presented differ widely in several critical aspects including the cell types, transfection systems used and also in the experimental model systems and approaches employed to investigate the role of the NLS-containing peptide during transfection. A detailed analysis and meaningful comparison of the results obtained in order to derive clear conclusions describing the current state of the art, remain thus a difficult task. Therefore, certain standardization or a detailed description of factors known to influence transfection efficiency is believed to be of great value for future studies. These may include at least the following points: (1) analysis and assessment of parameters describing the purity of DNA; (2) storage and handling of synthetic peptides; (3) detailed procedure of DNA complex formation; (4) methods for the physico-chemical characterization of DNA complexes and their validation; and (5) general definition of what should be valuable control experiments, particularly regarding peptide sequence specificity. In addition to these specific considerations, one may also emphasize the following general points, which are particularly critical for the utilization of gene transfer technologies: (1) Standard cell culture conditions for a given cell type or definition of experimental criteria in order to describe physiological parameters such as growth rate and confluency. Information on the origin and the number of passages of the considered cell line, as well as frequencies of mycoplasma decontamination may also be of interest. (2) Accurate statistical analysis of experimental data. Precise information about the statistical method and the corresponding preliminary validation test would be helpful if presented in a clear manner.

The effect of size and conformation of the DNA on nuclear import

Transport into the nucleus, via the NPC, occurs for a broad variety of macromolecules including proteins and nucleic acids. For both, size-dependence seems to be a common theme. Indeed, passive diffusion through the

NPC is observed for proteins smaller than 60 kDa and ODN or small DNA fragments up to 200 bp.^{34,69} With increasing size, diffusion becomes weaker, but nuclear accumulation can be actively enhanced by taking advantage of the NLS-dependent nuclear import machinery. This means that the cell's machinery actually accelerates the transport process of molecules which can be classified as 'permissive' for nuclear uptake.

Extensive microinjection studies using linear DNA fragments showed that size is a major factor limiting cytoplasmic mobility⁷⁰ and transport into the nucleus,³⁴ which may lead to cytoplasmic entrapment and subsequently to degradation. However, it remains unclear whether size is also a significant factor limiting intracellular transport processes of plasmid DNA and particularly of DNA complexes used in transfection systems. Circular and supercoiled DNA is believed to be the predominant form of transfection-active plasmid DNA. Their hydrodynamic diameter is therefore highly reduced as compared with linear DNA, especially when additional condensing agents are used. Furthermore, endocytosed DNA complexes can be localized in the perinuclear region after a few hours following transfection,^{32,71} suggesting that a high number of plasmid DNA molecules are closely localized to the nuclear envelope. Finally, molecules commonly used for transfection are smaller than 10 kb. In comparison, a DNA virus such as the herpes simplex virus 1 is able to transport its 152-kb genome through the NPC.²⁰

One may therefore hypothesize that additional factors beyond a simple size constraint hinder nuclear accumulation of plasmid DNA. Plasmid DNA may belong to a class of 'non-permissive' molecules for nuclear uptake, against which the cell has developed mechanisms for exclusion from the nucleus. Potential mechanisms may include cytoplasmic retention, down-regulation of NPC permeability and rapid nuclear export.

Understanding the behavior of DNA complexes inside the cell

To date, little is known about the transport process of plasmid DNA or DNA complexes into the nucleus. Since large aggregates are supposedly excluded from the nucleus, single DNA molecules or perhaps small DNA complexes must be released at some point during transfection. It is unknown whether plasmid DNA enters the nucleus in a circular and condensed form and whether cellular factors are involved in the translocation process.

Cellular factors have to be identified which physically interact with DNA and thereby hinder nuclear transfer either directly or by inducing additional cellular mechanisms. Based on this knowledge, specific modifications of the DNA could produce molecules 'permissive' for nuclear localization and with an increased translocation rate through the NPC.

The current belief is that DNA complexes utilize the endocytotic pathway for cell entry and intracellular traffic and subsequently gain entry into the nucleus during mitosis. However, the sensitivity of most experimental methods only allows tracking of relatively large DNA complexes. Therefore, one cannot exclude the existence of other pathways, which may effectively lead to transfection. A recent study demonstrates that although micro-injected plasmid DNA accumulated in the nucleus primarily in cells which underwent mitosis, a number of

DNA molecules were able to translocate into the nucleus in non-mitotic cells.⁴⁰

Rational design of heteroplexes with *in vivo* application

Synthetic elements derived from the elucidation of the gene transfer mechanism must be assembled in an appropriate way so that each component retains its expected activity within the heteroplex. Furthermore, the physico-chemical properties of heteroplexes also depend on the production procedure and determine the intracellular fate. Hence, the ability to build heteroplexes with defined physico-chemical properties is a critical issue in the design of multicomponent transfection systems.

Another important aspect concerns the applicability of the heteroplex *in vivo*. As mentioned earlier, the heteroplex is subjected to numerous extracellular factors depending on the administration route. In the case of intravenous delivery, the heteroplex may interact with blood elements including albumin, opsonins and the complement system, altering its resistance to degradation. A previous study based on 2D-PAGE analysis demonstrated that interaction of liposomes with plasma proteins could be altered upon specific surface modifications and also influenced subsequent organ distribution *in vivo*.⁷² Therefore, the cell entry mechanism and intracellular fate may vary significantly to that expected from cell culture experiments. Consequently, optimization of a heteroplex only makes sense if the cell entry mechanism *in vivo* and the properties of the internalized material are well characterized and reproducible in *in vitro* model systems or in cell culture.

To conclude, the successful design of novel heteroplexes suitable for *in vivo* gene therapy will require a better understanding of both extra- and intracellular processes and synergistic efforts with polyelectrolyte chemists and colloid scientists.

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