

REVIEW

Nuclear entry of nonviral vectors

DA Dean¹, DD Strong² and WE Zimmer³

¹Division of Pulmonary and Critical Care Medicine, Feinberg School of Medicine, Northwestern University, Chicago, IL, USA;

²VA Loma Linda Health Care System, Loma Linda, CA, USA; and ³Department of Medical Pharmacology and Toxicology, College of Medicine, Texas A&M University, College Station, TX, USA

Nonviral gene delivery is limited to a large extent by multiple extracellular and intracellular barriers. One of the major barriers, especially in nondividing cells, is the nuclear envelope. Once in the cytoplasm, plasmids must make their way into the nucleus in order to be expressed. Numerous studies have demonstrated that transfections work best in dividing populations of cells in which the nuclear envelope disassembles during mitosis, thus largely eliminating the barrier. However, since many of the cells that are targets for gene therapy do not actively undergo cell division during the

gene transfer process, the mechanisms of nuclear transport of plasmids in nondividing cells are of critical importance. In this review, we summarize recent studies designed to elucidate the mechanisms of plasmid nuclear import in nondividing cells and discuss approaches to either exploit or circumvent these processes to increase the efficiency of gene transfer and therapy.

Gene Therapy (2005) 12, 881–890. doi:10.1038/sj.gt.3302534; Published online 28 April 2005

Keywords: nuclear import; nuclear envelope; plasmid; nuclear localization signal; nonviral gene transfer

Introduction

The success of nonviral gene therapy has been largely limited by inefficient gene delivery due to the presence of multiple extracellular and intracellular barriers to gene transfer. Perhaps, the major barrier that has not been successfully overcome is the nuclear envelope. Although the molecular mechanisms of the nuclear import and export of proteins, mRNAs, snRNPs, and ribosomes have been studied in detail over the past 20 years, the mechanisms of plasmid nuclear import have received much less attention. It is clear that unless plasmids can enter the nucleus, they cannot be transcribed. Thus, just as entry into the cytoplasm across the plasma or endosomal membrane is a prerequisite for gene transfer, transport across the nuclear membrane is equally vital.

Nuclear envelope as a barrier to gene transfer

In 1980, Mario Capecchi demonstrated that when pBR322-based plasmids were injected into the nuclei of mouse fibroblasts, expression could be detected in about 50–100% of cells, but when the same number of plasmids were injected into the cytoplasm, no expression was detected in any of the 1000 injected cells.¹ Similar microinjection experiments by Graessman *et al*² showed that when between 1000 and 3000 copies of a plasmid were delivered to the cytoplasm, the level of gene

expression detected was less than 3% of that found when the DNA was microinjected into the nucleus. Zabner *et al*³ also found that in *Xenopus* oocytes, the same trend was true: whereas DNA microinjected into the nucleus produced robust gene expression, cytoplasmic injection resulted in very little expression. Other experiments in a number of mammalian cell types have confirmed these findings.^{4–7}

Implicit in all of these experiments, and the conclusion that the nuclear envelope is a barrier to gene transfer, is the assumption that the cells studied were nondividing. Indeed, it is known that when cells undergo mitosis, the nuclear envelope breaks down and the permeability barrier to the nucleus is lost. Thus, if plasmids (or viral genomes) are present in the cytoplasm, they can enter the 'nucleus' when the envelope is disrupted during this stage of the cell cycle. For example, it has long been known that in contrast to lentiviruses such as HIV, the reverse-transcribed genomes of oncoretroviruses cannot enter the nucleus to integrate and express unless the cells go through mitosis.^{8–10} Similarly, it is well appreciated that nondividing or growth-arrested cells cannot be easily transfected by almost any method. By contrast, cells undergoing mitosis are much more receptive to transfection. In one study using primary human airway epithelial cells, nondividing cells, as measured by lack of BrdU incorporation, were only 10% as likely to express gene product as their dividing counterparts.¹¹ More recent studies using synchronized cells have shown that cells transfected with various lipoplex formulations, polyethyleneimine (PEI), or polylysine, expressed between 50- and 3000-fold more gene product when transfected in the G2 or G2–M stage as compared to those transfected in G1.^{12,13}

Correspondence: Professor DA Dean, Division of Pulmonary and Critical Care Medicine, Feinberg School of Medicine, Northwestern University, 240 E Huron Ave, McGaw 2336, Chicago, IL 60611, USA
Published online 28 April 2005

Regardless of whether the cells undergo mitosis or not, the levels of DNA that reach the nucleus are low at best. It has been estimated that following lipoplex-mediated transfection, between 2000 and 10 000 plasmids are delivered per cell.^{14–16} Using quantitative PCR, Southern blot analysis, or electron microscopy, between 1 and 10% of the plasmids were found in the nuclei of the cells at up to 24–36 h following DNA addition.^{6,17} In another study in which plasmids were fluorescently labeled randomly throughout the DNA and quantified in cells by fluorescence activated cell sorting, the numbers were much higher, with between 30 and 60% of the intracellular DNA being in the nuclear compartment, depending on the cell type used.¹⁶ This suggested that different cells have differing capacities for DNA nuclear localization. However, in this study, the modification of the DNA with fluorophores every 22 bp may have resulted in plasmids that are not completely representative of native DNA, and thus these numbers may be an overestimation.^{18,19} Regardless, these results demonstrate that a significant proportion of the DNA that enters the cytoplasm never arrives in the nucleus. In studies from our lab, increasing concentrations of expression plasmids were microinjected into the cytoplasm or nucleus and 24 h later, after the synchronized cells had divided, the levels of gene expression were quantified. We found that it took 30–100 times more plasmid injected into the cytoplasm compared to the nucleus to give equivalent levels of gene expression, suggesting that even in dividing cells, the amount of DNA that gets to the nucleus is low.⁷ Similar studies from Jon Wolff's group later confirmed these findings and illustrate that nuclear import of DNA is a relatively inefficient process, even when the nuclear envelope breaks down.¹⁹

One major reason that the majority of cytoplasmic plasmids fail to reach the nucleus is the presence of cytoplasmic nucleases that act to degrade the 'free' DNA. Clearly, following endocytosis, a significant percentage of internalized DNA is targeted to the lysosomal compartment, where it will be degraded. However, once DNA is freed into the cytoplasm, it is still subject to degradation prior to nuclear entry. Studies from Lechardeur *et al*²⁰ showed that single- and double-stranded plasmids are degraded in the cytoplasm of HeLa and COS cells with an apparent half-life of between 50 and 90 min. Similarly, Pollard and co-workers²¹ demonstrated that this nuclease activity was calcium-dependent and degraded DNA in cells with a half-life of less than 2 h. By contrast, several other studies have not detected such high rates of degradation, although it is clear that some degree of DNA disappearance is occurring.^{22,23} For example, several studies from Todd Giorgio's group have developed mechanistic models for nonviral gene delivery and their experimental findings suggest that cytoplasmic DNA may be degraded at rates between 30 and 1400 molecules per min, depending on the total amount of input DNA in the cell.^{15,16,24} While these rates seem high, they would translate to a cytoplasmic half-life of roughly 5 h. Regardless of the exact rate of degradation, it is clear that there is a competition between DNA degradation in the cytoplasm and efficient nuclear import; if the DNA persists too long in the cytoplasm, there will be less of it around to enter the nucleus and lead to gene expression.

Intracellular trafficking of 'naked' DNA

The function of the cationic lipid and polymer components of transfection complexes is to condense the DNA, protect it from degradation, and to promote association with the plasma membrane so the DNA can interact with and enter the cell. Following endocytosis, in order for the DNA to enter the nucleus, it must escape the endosome and become 'freed' into the cytoplasm. One question surrounding this and the nuclear entry of the DNA is whether the DNA dissociates from the lipoplex or polyplex complexes prior to or after nuclear entry. Experiments have been performed that support both possibilities, but the preponderance of data suggests that following lipoplex-mediated transfections, the DNA that drives gene expression in the nucleus is likely free of lipid. Zabner *et al*³ demonstrated that when liposome-complexed plasmids were injected directly into the cytoplasm or nucleus at the same lipid:DNA ratios that gave optimal transfection efficiency, no gene expression could be detected. By contrast, certain polyplex complexes, such as PEI, may remain complexed with the DNA after transport into the nucleus: when PEI-DNA complexes were microinjected into the cytoplasm or nucleus, gene expression was detected in either case.^{25–27}

Regardless of whether the complexes dissociate in the cytoplasm or nucleus, it is unlikely that the DNA remains 'free' for very long. The cytoplasm and nucleus are filled with numerous DNA-binding proteins, polyamines, and other polycations that will complex with the released DNA. These associations will likely neutralize the charge on the DNA, condense the DNA, and thereby reduce the size of the plasmid to possibly aid in nuclear transport. They may also prevent or promote cytoplasmic degradation of the DNA. Further, some of these proteins may actually mediate the specific nuclear import of the plasmids.

Plasmid nuclear entry in nondividing cells

Wolff and co-workers first demonstrated that plasmids can be transported into the nuclei of nondividing cells via the nuclear pore complex (NPC).²⁸ All macromolecular traffic that enters or exits the intact nucleus occurs through the NPC.²⁹ They showed that when microinjected into the cytoplasm of cultured myotubes, plasmids localized to the nucleus, based on detection of reporter gene expression, in a dose- and energy-dependent process. Further, coinjection of agents that block transport through the NPC (either wheat germ agglutinin or an antibody against NPC components) also blocked plasmid nuclear entry and gene expression. Using a similar microinjection approach, but using *in situ* hybridization to detect the injected DNA directly, our lab later confirmed that plasmids enter the nucleus via the NPC, but do so in a sequence-specific manner.³⁰ When the 5243 bp SV40 genome was microinjected into the cytoplasm of a variety of growth-arrested cell types, it localized to the nucleus within 6–8 h in the absence of cell division. Import was inhibited by wheat germ agglutinin, energy-depletion, and antibodies against the NPC. However, in attempts to compete for import using a second plasmid type, it was found that import was sequence specific. Whereas the SV40 genome localized to

the nucleus in the absence of cell division, plasmids lacking SV40 sequences, such as pBR322, pUC19, or pGL3-basic, remained in the cytoplasm. This was most striking when cells were coinjected with SV40 DNA and one of these other plasmids; the SV40 *in situ* signal was nuclear, but the other plasmid's signal was completely cytoplasmic. By cloning various sequences from the SV40 genome into pUC19 or pBR322, it was found that the SV40 enhancer was all that was necessary for this DNA nuclear import.^{7,30} This sequence and other sequences supporting DNA nuclear import were termed DNA nuclear targeting sequences, or 'DTS'.

DNA nuclear targeting sequences (DTSs)

The DNA sequence from the SV40 genome that supported nuclear import of an otherwise cytoplasmically localized plasmid contained the 72 bp enhancer repeat.^{7,30} When as little as one copy of this enhancer was cloned into pBR322, the resulting plasmid localized to the nuclei of microinjected, nondividing cells with the same kinetics as the full-length SV40 genome with import first being detected within 40–60 min of injection (Figure 1). Such sequence-specific DNA nuclear import has been observed in all mammalian cell types tested to date, including primary cells and cell lines from mice, rats, chickens, hamsters, monkeys, and humans.^{7,30–33} In support of these findings, Greassman *et al*² demonstrated that the 72 bp SV40 enhancer lead to increased transcription of a herpes TK promoter-driven gene in actively dividing cells, compared to plasmids lacking the enhancer, confirming the role of the sequence as an enhancer. However, when he microinjected the plasmids into the nucleus or cytoplasm and followed expression, he found that the enhancer-containing plasmid was more efficient at stimulating gene expression when the DNA was microinjected into the cytoplasm than when it was delivered to the nucleus, suggesting that the classical 'enhancer' activity was not the only function of this sequence. More recent work from other labs has supported these findings.^{34,35}

Based on the sequence required for DNA nuclear import, a model was developed to account for the sequence-specificity (Figure 2). The SV40 enhancer contains binding sites for a number of ubiquitously expressed, general transcription factors, such as AP1, AP2, AP3, NF- κ B, Oct1, TEF-1, etc.³⁶ Normally, combinations of these transcription factors would bind to the SV40 enhancer when the genome is in the nucleus to

regulate gene expression during the infectious cycle. However, since transcription factors, like other proteins, are translated in the cytoplasm, it is possible that they could bind to their binding sites on the SV40 enhancer-containing plasmid to create a protein–DNA complex. Further, since transcription factors function in the nucleus, they contain nuclear localization signals (NLSs) within their amino-acid sequences to direct nuclear import via interactions with the importin machinery and the NPC. Normally these transcription factors would be translated in the cytoplasm, transported into the nucleus using their NLSs and the importin proteins, and then bind to their target sites on various promoters and enhancers within the nucleus. However, if they first bind to a cytoplasmic SV40 DTS-containing plasmid, the plasmid could become coated at one or more sites with a number of proteins that harbor NLSs, which could in turn bind to the importins. This large multiprotein–DNA complex could then be carried into the nucleus by the classic signal-mediated NPC pathways. Thus, the SV40 DTS can be seen as a scaffold for transcription factors and their bound importin family members, which results in nuclear import of the entire protein–DNA complex. Similar mechanisms for nuclear import of the reverse-transcribed HIV preintegration complex, and other viral genomes have been suggested.^{10,37} However, the main difference here is that the SV40 DTS functions to promote plasmid nuclear import using only endogenous cellular proteins in the absence of any viral component.

Based on this model for plasmid nuclear import, it is possible that any eukaryotic promoter, enhancer, insulator, or regulatory sequence could act as a DTS. However, this is not the case. A number of strong viral and cellular promoters have been tested for their ability to promote nuclear import of plasmids following transfection or cytoplasmic microinjection, and to date, most sequences have no such activity. For example, the CMV immediate early promoter/enhancer, the herpes TK promoter, and the RSV LTR promoter, all of which are as robust for transcription as the SV40 early promoter/enhancer and contain multiple binding sites for a number of similar transcription factors, have no DTS activity.^{2,7,32} Further, the presence of multiple transcription factor binding sites is not sufficient to create nuclear-localizing plasmids. How then is the SV40 DTS active for import but many of these other sequences are not?

First, not all transcription factors can mediate or participate in DNA nuclear import. In order for a transcription factor to act as an adapter between the DTS scaffold and the importin machinery, it must have functionally and spatially distinct NLS and DNA-binding domains. Sp1, for example, contains an NLS that is buried within the zinc-finger DNA-binding domain.³⁸ Under normal circumstances, the NLS is recognized by the importins, Sp1 is transported into the nucleus, and then it binds to DNA. However, if it were to bind to the SV40 promoter/enhancer (which it does) in the cytoplasm, the NLS would not be accessible to the importins. In an elegant set of studies, Chan and Jans^{39,40} demonstrated that although the GAL4 transcription factor from yeast can bind to the GAL4 UAS when present on a plasmid and can interact with high affinity with importin α , it cannot do both at once. Thus, contrary to initial hopes, GAL4 did not enhance nuclear import of plasmids or gene expression. By contrast, the NLS of

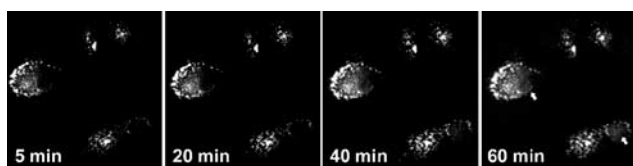


Figure 1 Plasmid nuclear import in nondividing cells. Plasmids were labeled using a Cy5-PNA clamp and microinjected into the cytoplasm of TC7 cells as previously described.^{7,43} The DNA can be seen to begin to enter and accumulate in the nucleus as early as 40 min following cytoplasmic injection. If the cells are allowed to incubate for longer times, up to 100% of the PNA-labeled DNA is detected within the nucleus. Arrows indicate nuclei in two injected cells.

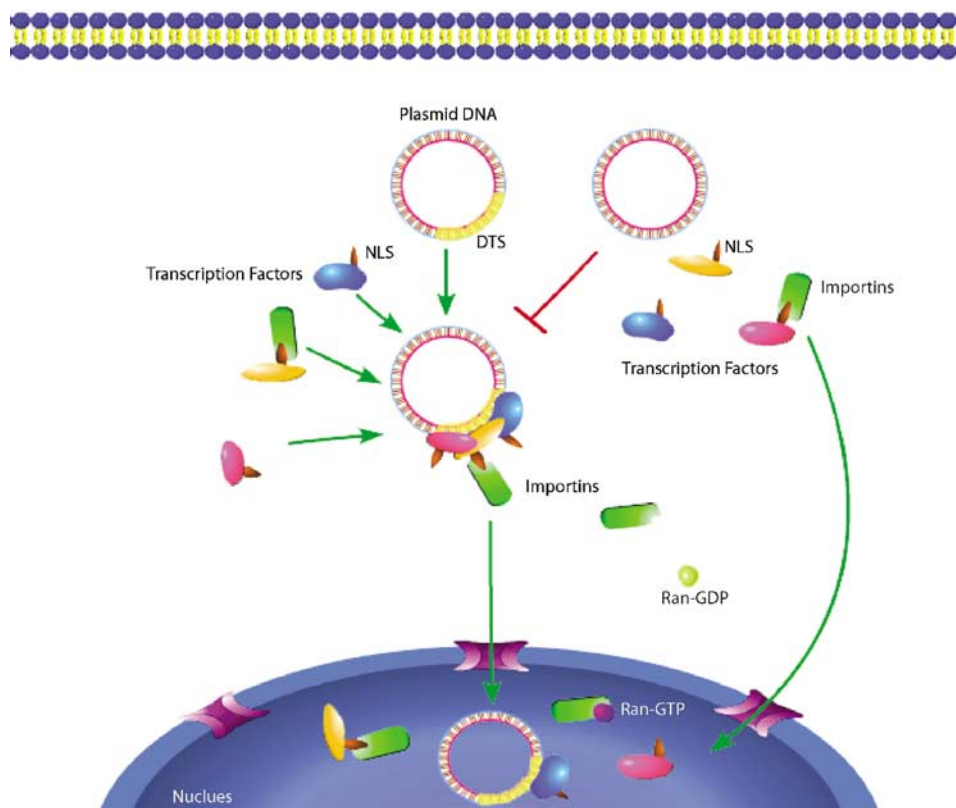


Figure 2 Model for sequence-specific DNA nuclear import. Once in the cytoplasm, plasmids containing a DTS (depicted in yellow) can interact with newly synthesized transcription factors to form protein–DNA complexes. Since transcription factors contain NLSs for their nuclear localization, the DTS-containing DNA can become coated at distinct sites with one or more NLS, which can then interact with the importins to mediate nuclear import of the entire complex. By contrast, plasmids lacking a DTS cannot form the appropriate DNA–protein complexes for nuclear import.

NF- κ B is far removed from the DNA-binding domain of the protein and could be involved in importin recognition and nuclear import of the DNA complex.^{41,42}

The presence of the SV40 DTS on a plasmid has been shown to increase nuclear import of the DNA in nondividing cells by directly following the DNA using fluorescently-labeled, triplex-forming peptide nucleic acid (PNA) clamps and by *in situ* hybridization.^{7,44} In both cases, plasmids with the SV40 DTS localized to the nuclei of nondividing or synchronized cells prior to cell division, whereas plasmids lacking the sequence remained in the cytoplasm. The DTS import activity has also been followed in synchronized cells that were microinjected with GFP-expressing plasmids by quantifying gene expression.⁷ Since the CMV immediate early promoter does not mediate plasmid nuclear import, it can be used to drive expression of a reporter gene in a nuclear import assay. Matching CMV promoter driven GFP plasmids containing or lacking the SV40 DTS downstream of the GFP gene were microinjected into either the cytoplasm or nuclei of synchronized cells. Differences in GFP expression levels following nuclear injection reflected differences in transcription, whereas differences in expression following cytoplasmic injection reflect DNA nuclear import activity. When equal numbers of plasmids were injected into the nucleus, they gave similar levels of GFP expression, suggesting that the plasmids are transcriptionally equivalent. Further, injection of as few as 1–3 plasmids per nucleus

produced GFP⁺ cells at both 4 and 8 h postinjection.⁷ When as many as 1000 copies of the DTS-lacking plasmid were injected into the cytoplasm of cells that divided at 14 h postinjection, no gene expression was detected prior to cell division. By contrast, when anywhere between 10 and 1000 copies of the SV40 DTS-containing plasmid were injected into the cytoplasm of these cells, gene expression could be detected as early as 2–4 h following microinjection. However, comparing the efficiency of nuclear *versus* cytoplasmic injection, even with a DTS, it took 20 times more DNA injected into the cytoplasm, compared to the nucleus, to yield equivalent gene expression.⁷

Several other DNA sequences have been proposed to act as DTSs. Mesika *et al*⁴² cloned five repetitive NF- κ B binding sites into a luciferase-expressing plasmid and found that gene expression was increased 12-fold compared to the same plasmid lacking these binding sites when transfected into cells. This increased gene expression was enhanced by the addition of TNF- α , an NF- κ B activator. When the plasmids were fluorescently labeled using rhodamine-labeled PNA clamps and transfected into cells using dendrimers, the presence of the NF- κ B binding sites increased nuclear localization as detected by confocal microscopy, and addition of TNF- α increased nuclear localization even further. Thus, the authors concluded that these NF- κ B binding sites may act to increase DNA nuclear import. Although many of the described experiments used plasmids that also

contained the SV40 early promoter and enhancer, suggesting that the import detected could be due to a combination of NF- κ B and SV40 DTS activities, the stimulation of import and expression by the NF- κ B binding sites suggests that these sequences do have DTS activity. Indeed, more recent studies for the same group have shown that NF- κ B binding sites alone on a plasmid can support nuclear import that is dependent on wild type NF- κ B p50 binding, confirming this model of DNA nuclear import.⁴³

Two other studies have developed matched DNA binding site–transcription factor pairs that act as DTSs. In one case, the OriP sequence from the Epstein–Barr virus (EBV) was cloned into a plasmid and shown to increase gene expression by six- to seven-fold following cytoplasmic microinjection in EBNA-1 expressing cells, when corrected for transcriptional differences between the plasmids and compared to plasmids lacking the OriP sequence.⁴⁵ EBNA-1 is an EBV protein that binds to the OriP sequence and contains an NLS. The authors interpreted these experiments to suggest that the EBNA-1 protein bound to the OriP containing plasmids and aided nuclear import resulting in increased gene expression. A more recent paper has used combinations of the tet operator and a modified tetracycline repressor containing an NLS (tetO and TetR-NLS, respectively) to create a system in which DNA nuclear localization and subsequent gene expression was increased.⁴⁶ When two copies of the tetO were cloned into a plasmid and then transfected into cells expressing TetR-NLS, gene expression increased almost 20-fold in growth-arrested cells, and nuclear localization increased by four-fold. This increased nuclear localization and expression was dependent on both the presence of the tetO sequences and the TetR-NLS protein. Further, the authors compared the efficacy of the tet pair to plasmids bound to a PNA-NLS conjugate and found that the PNA-NLS showed no specific effects on nuclear localization or gene expression, suggesting that the spatial localization of the NLS with respect to the DNA may be important for importin interaction and nuclear import activity.

Cell-specific DNA nuclear import

Apart from the SV40 DTS and the other specific examples just discussed, several DTSs have been identified that act in cell-specific manners. Based on the model proposed for DTS action, it was reasoned that there may exist other DNA sequences that could act with cell-specificity based on their ability to bind to cell-specific transcription factors. Indeed, two such sequences have been shown to have DNA nuclear import activity: the smooth muscle gamma actin (SMGA) promoter and the flk-1 promoter.^{32,47} The SMGA gene is expressed in smooth muscle cells and is regulated at the transcriptional level by at least two factors, serum response factor (SRF) and Nkx3.^{48–50} When portions of this promoter were placed into a pBR322-based plasmid and microinjected into the cytoplasm of smooth muscle cells, the DNA localized to the nucleus in the absence of cell division.³² By contrast, when injected into other cell types, such as endothelial cells, epithelial cells, or fibroblasts, the plasmids remained in the cytoplasm. Both SRF and Nkx3 play roles in the nuclear import of

the DNA. When SRF was expressed in CV1 cells, which normally do not express this transcription factor and support no nuclear import of the SMGA constructs, nuclear import of the SMGA DTS plasmid was detected.³² Additionally, when binding sites for either SRF or Nkx3 were mutated within the SMGA promoter, nuclear import was abolished. This smooth muscle-specific DTS also increased gene expression in transfected growth-arrested smooth muscle cells, but not in nondividing epithelial cells, suggesting that nuclear import of plasmids can be restricted to specific cell types.

The flk-1 promoter also acts as a cell-specific DTS that is functional only in endothelial cells. The flk-1 promoter drives expression of a VEGF receptor and is restricted largely to endothelial cells.⁵¹ When several different endothelial cell-specific promoters were cloned into the CMV promoter-driven GFP nuclear import reporter plasmid and tested for their ability to direct GFP expression in nondividing cells by cytoplasmic microinjection, the flk-1 promoter showed nuclear import activity, whereas three other promoters did not.^{47,52} Further, *in situ* hybridization confirmed that the flk-1 promoter caused nuclear import of the DNA in microinjected cells.

Unfortunately, there is no way to predict which sequences will act as import sequences and which will not. Thus, to identify DNA sequences with nuclear import activity, a brute force approach has been taken in which multiple cell-specific promoters are cloned into appropriate vectors and tested for import activity by microinjection or transfection assays. Hopefully as more sequences are identified, common features will emerge, allowing for a more directed approach for DTS identification.

Protein factor requirements for DNA nuclear import

In order to better characterize the mechanisms of DNA nuclear import, several groups have used digitonin-permeabilized cells. This system has been extensively utilized to characterize the mechanisms of signal-mediated protein nuclear import and export.⁵³ Using fluorescently labeled linear fragments of DNA, Hagstrom demonstrated that nuclear import could be detected in permeabilized cells and that import was saturable and inhibited by agents that block the NPC or deplete energy. However, import was not inhibited by excess NLS-containing proteins, suggesting that DNA utilized a pathway distinct from that of NLS-containing proteins. In support of this, it was found that these linear DNA fragments were imported into the nuclei without addition of exogenous cytoplasmic extracts, suggesting that the importin machinery was not required. Further, they demonstrated that DNA fragments larger than 1 kb were excluded from the nuclei, while those smaller than 1 kb readily entered. This is in contrast to what has been seen in intact cells, where plasmids up to 15 kb are transported into the nucleus in the absence of cell division.^{7,28,30} However, in another study from the same lab, when NLS peptides were fused to the DNA, larger DNA fragments and even plasmids could be imported into the nuclei of the permeabilized cells, but this time in a manner that was absolutely dependent on cytoplasmic

extracts.¹⁸ These results suggest that NLS-modified DNA uses the NLS-dependent nuclear import machinery for import whereas the NLS-free linear DNA did not. Interestingly, one aspect to both of these studies that may limit their relevance to the mechanisms used in intact cells is the fact that in both cases, when the substrates were microinjected into the cytoplasm of intact cells, neither the fluorescently labeled linear DNA fragments nor the NLS peptide-modified DNA was able to localize to the nucleus.

Another study using the same permeabilized cell system obtained different results that more closely resembled the results found in intact cells.⁴⁴ In this report, Wilson and co-workers used plasmids that were fluorescently labeled at distinct sites with fluorescently labeled PNA clamps.^{23,44} When added to the permeabilized cells, the DNA began to localize to the nuclei within 1 h and was abundantly imported by 4 h. As seen by Wolff's group, import was inhibited by WGA, energy-depletion, and antibodies against the NPC. However, unlike the Wolff group, import of intact plasmids between 4 and 14 kbp was detected and required cytoplasmic and nuclear extracts. The dependence of cell extracts suggested that the importin machinery was necessary for import. When recombinant importin α , importin β , and RAN were added to the DNA, no nuclear import was observed, but when nuclear extracts were also provided, DNA nuclear import was detected. Since the importins cannot bind DNA directly, the nuclear extracts likely provided transcription factors and other DNA-binding proteins to act as adapters between the importins and the DNA. Moreover, when a labeled plasmid lacking the SV40 DTS was added to the permeabilized cells in the presence of either cell extracts or importin α , β , RAN, and nuclear extracts, no nuclear import was observed. These results support the model for sequence-specific DNA nuclear import in which transcription factors are needed to bridge the DTS scaffold to the import machinery.

More recent experiments have studied the nuclear import of several DNA-peptide or DNA-polymer conjugates and found that plasmids can indeed be imported into the nuclei of permeabilized cells and that import is energy-, time-, and NPC-dependent.^{13,54} In one study, it was even demonstrated that when a peptide containing and RGD motif was linked to the DNA via an oligolysine peptide, nuclear import could be detected in permeabilized cells leading to expression of a reporter gene.⁵⁴ Finally, several other studies using isolated nuclei from either cardiac myocytes or coconut syncytia demonstrated that plasmids could be imported through the NPC in a manner that was dependent on cytoplasmic and nuclear extracts, as well as the presence of the SV40 DTS.^{55–57} Moreover, these studies also demonstrated that the isolated nuclei could not only import the DNA but also export expressed mRNA leading to reporter gene expression.

Approaches to increase DNA nuclear import

Most of the work aimed at characterizing the mechanisms of plasmid nuclear uptake has been done largely for the purpose of trying to increase nuclear localization of DNA to increase gene delivery and expression. To this

end, almost all of the focus on improving DNA nuclear import has been on the use of NLS-containing peptides or proteins as agents to drive the DNA into the nucleus (Figure 3). Such proteins and peptides have been complexed to DNA by electrostatic interactions,^{58–68} random covalent attachment to the DNA,^{18,69–72} and by site-specific attachment to the DNA using PNAs or unique chemistries.^{73–79} However, success has been varied: some papers have reported that inclusion of NLS-containing proteins or peptides increases gene transfer and expression, while others have found no such enhancement. A recent review by Cartier and Reszka⁸⁰ has summarized the field beautifully and draws a similar conclusion.

However, despite the lack of consensus on the positive effects of inclusion of proteins or NLS-peptides to increase nuclear trafficking and gene expression of plasmids, several recent studies are worth mentioning, if only for their novel approaches to nuclear targeting. First, as discussed above, almost all attempts to increase plasmid nuclear targeting have relied on using classical NLS sequences. In contrast, one recent study has covalently linked the importin β -binding (IBB) domain from importin α to plasmids and then transfected complexes into cells.⁸¹ During transport of classic NLS-containing proteins, the NLS binds to importin α , which in turn binds to importin β through its IBB, and the NLS-importin α/β complex is transported into the nucleus.²⁹ Scherman and co-workers ingeniously reasoned that by binding the IBB to the DNA, only one intermolecular interaction would need to form (eg, IBB-importin β) in order for the complexed plasmid to be imported into the nucleus. Unfortunately, although improved transfection efficiency was observed using the IBB peptide, studies in permeabilized cells indicated that the increased expression was not due to increased nuclear import, but rather to the physicochemical properties of the IBB-DNA complexes.⁸¹

Another unique approach to increase nuclear import of plasmids has exploited the glucocorticoid receptor (GR).^{82,83} Under normal circumstances, GR resides in a multiprotein complex in the cytoplasm with its NLS masked. Upon ligand binding, the NLS is exposed and the ligand-bound GR translocates into the nucleus. Rebuffat *et al* attached the GR ligand dexamethasone to plasmids by either a direct linkage via a psoralen linker or using a PNA clamp. In both cases, the dexamethasone reagents were able to interact with GR and induced nuclear localization of the receptor. When steroid-complexed DNA was transfected into nondividing cells expressing GR, gene transfer and expression was 20- to 40-fold greater than that seen with unmodified DNA. Thus, inclusion of a classic NLS-peptide is not the only approach to improve nuclear trafficking and subsequent gene expression.

In vivo DNA nuclear import

Despite the advances in characterizing the mechanisms of DNA nuclear import in cultured and transfected cells, there has been relatively little success transforming the information gathered into better methods for DNA delivery *in vivo*. Several papers have demonstrated that complexation of DNA with NLS peptides can lead to

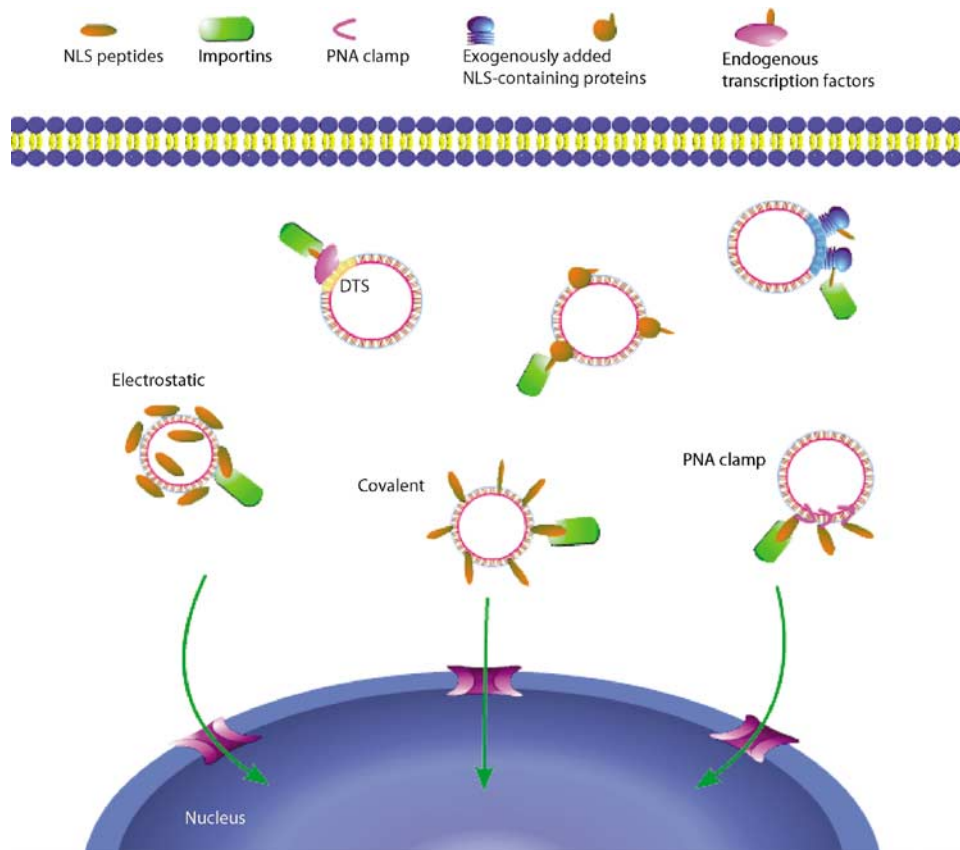


Figure 3 Various approaches to increase nuclear localization of transfected plasmids. A number of methods have been proposed and tested to increase the nuclear import of plasmids following transfection. Most rely on the addition of NLS-peptides or NLS-containing proteins to the DNA, by either electrostatic, covalent, or PNA clamps, to increase nuclear import. Other approaches have used general or cell-specific DTSs (in yellow), or synthetic DTS/NLS-protein pairs (in blue).

enhanced nuclear uptake and gene expression in zebrafish and shrimp embryos, but apart from these isolated reports, most studies aimed at increasing DNA nuclear import by addition of NLS-containing proteins or novel polymers have remained restricted to cultured cell studies.^{58,59,84} The only successes in adult animals reported to date have focused on the inclusion of DNA nuclear targeting sequences to increase gene transfer and expression *in vivo*. Incorporation of one or two copies of the SV40 DTS into plasmids downstream of the reporter gene resulted in a 20-fold increase in gene expression in murine skeletal muscle following naked DNA injection and electroporation.^{35,85} When DNA was delivered without electroporation, similar increases in gene expression were detected. Although the intracellular localization of the DNA was not studied in these reports, the authors proposed that the increases were due to the nuclear import activity of the SV40 DTS and not classical enhancer activity. More definitive proof for the *in vivo* function of the SV40 DTS as a nuclear import sequence came from studies by Young *et al*³³ on electroporation-mediated gene transfer to the rat mesenteric vasculature. In this study, inclusion of the SV40 DTS downstream of CMV-driven reporter genes resulted in 40- to 200-fold increased gene expression in intact arteries and veins. When the location of the transferred DNA was determined by *in situ* hybridization, it was found that plasmids lacking the SV40 sequence were present

throughout the tissue at 8 h but were cleared from the tissue within 24 h (based on a lack of detectable signal), but plasmids containing the SV40 DTS continued to be detected at 24 and 48 h post-transfer. Moreover, plasmids with an SV40 DTS localized to nuclei of cells in the tissue even as early as 8 h and by 48 h, the only detectable plasmid DNA was in the nuclei.

Unlike the situation seen in cultured cells where the dependence on the DTS appeared almost absolute, a number of studies have shown that in many tissues, especially skeletal muscle, robust gene transfer and expression can be obtained using plasmids lacking any nuclear import sequence. Additionally, using highly effective transfection reagents, certain cell types have not shown an absolute dependence on the presence of a DTS for gene transfer and expression. It is likely that when the cytoplasm becomes filled with large concentrations of plasmids, at least some of the plasmids can randomly make their way to the nuclear envelope and be imported into the nucleus independent of any DTS. Indeed, when linear DNA is brought close enough to the nuclear envelope using laser tweezers, it is pulled in.⁸⁶ Further, it has been shown that when DTS-lacking plasmids are delivered to the cytoplasm of a mouse myotube *in vivo*, no gene expression is observed until 1 000 000 plasmids are injected, suggesting that mass action could account for the DTS-independent nuclear localization.⁸⁷

Conclusions

It is clear that unless plasmids enter the nucleus, no gene expression, integration, or replication of any vector DNA can take place. Although the nuclear envelope breaks down during mitosis thus eliminating this barrier to gene transfer, most of the cells that are targets for gene therapy are either slowly dividing or do not divide at all. Thus, we must understand these mechanisms of plasmid nuclear import if we are to develop ways to improve intracellular trafficking. Despite the great focus on development of better transfection reagents that increase cell entry or the improvement of viral, nonviral, and synthetic promoters to increase transcription, relatively little success has been made at developing techniques and reagents to increase nuclear DNA transport. Hopefully, the elucidation of the basic mechanisms of plasmid nuclear import will change this and lead to more effective nonviral gene therapy approaches.

Acknowledgements

We thank Rui Zhou and Joshua Z Gasiorowski for providing figures and all the members of our labs for intriguing discussions and critical reading of the manuscript. Work in the authors labs was supported in parts by grants HL59956 (DAD), HL71643 (DAD), and CA95608 (WEZ) from the NIH, and by The National Medical Test Bed and the US Department of the Army (Cooperative Agreement Number DAMD17-97-2-7016). The content of the information does not necessarily reflect the position or policy of the government or the NMTB. DDS is supported by the Office of Research and Development, Medical Research Service, Department of Veteran Affairs.

References

- 1 Capecchi MR. High efficiency transformation by direct microinjection of DNA into cultured mammalian cells. *Cell* 1980; **22**: 479–488.
- 2 Graessman M *et al*. Helper activity for gene expression, a novel function of the SV40 enhancer. *Nucleic Acids Res* 1989; **17**: 6603–6612.
- 3 Zabner J *et al*. Cellular and molecular barriers to gene transfer by a cationic lipid. *J Biol Chem* 1995; **270**: 18997–19007.
- 4 Mirzayans R, Remy AA, Malcom PC. Differential expression and stability of foreign genes introduced into human fibroblasts by nuclear versus cytoplasmic microinjection. *Mutat Res* 1992; **281**: 115–122.
- 5 Thornburn AM, Alberts AS. Efficient expression of miniprep plasmid DNA after needle micro-injection into somatic cells. *Biotechniques* 1993; **14**: 356–358.
- 6 Labat-Moleur F *et al*. An electron microscopy study into the mechanism of gene transfer with lipopolyamines. *Gene Therapy* 1996; **3**: 1010–1017.
- 7 Dean DA, Dean BS, Muller S, Smith LC. Sequence requirements for plasmid nuclear entry. *Exp Cell Res* 1999; **253**: 713–722.
- 8 Lewis PF, Emerman M. Passage through mitosis is required for oncoretroviruses but not for the human immunodeficiency virus. *J Virol* 1994; **68**: 510–516.
- 9 Miller DG, Adam MA, Miller AD. Gene transfer by retrovirus vectors occurs only in cells that are actively replicating at the time of infection. *Mol Cell Biol* 1990; **10**: 4239–4242.
- 10 Dvorin JD, Malim MH. Intracellular trafficking of HIV-1 cores: journey to the center of the cell. *Curr Top Microbiol Immunol* 2003; **281**: 179–208.
- 11 Fasbender A, Zabner J, Zeiher BG, Welsh MJ. A low rate of cell proliferation and reduced DNA uptake limit cationic lipid-mediated gene transfer to primary cultures of ciliated human airway epithelia. *Gene Therapy* 1997; **4**: 1173–1180.
- 12 Brunner S *et al*. Cell cycle dependence of gene transfer by lipoplex, polyplex and recombinant adenovirus. *Gene Therapy* 2000; **7**: 401–407.
- 13 Escriou V *et al*. Critical assessment of the nuclear import of plasmid during cationic lipid-mediated gene transfer. *J Gene Med* 2001; **3**: 179–187.
- 14 Coonrod A, Li FQ, Horwitz M. On the mechanism of DNA transfection: efficient gene transfer without viruses. *Gene Therapy* 1997; **4**: 1313–1321.
- 15 Tseng W, Haselton F, Giorgio T. Transfection by cationic liposomes using simultaneous single cell measurements of plasmid delivery and transgene expression. *J Biol Chem* 1997; **272**: 25641–25647.
- 16 James MB, Giorgio TD. Nuclear-associated plasmid, but not cell-associated plasmid, is correlated with transgene expression in cultured mammalian cells. *Mol Ther* 2000; **1**: 339–346.
- 17 Tachibana R, Harashima H, Shinohara Y, Kiwada H. Quantitative studies on the nuclear transport of plasmid DNA and gene expression employing nonviral vectors. *Adv Drug Deliv Rev* 2001; **52**: 219–226.
- 18 Sebestyén MG *et al*. DNA vector chemistry: the covalent attachment of signal peptides to plasmid DNA. *Nat Biotechnol* 1998; **16**: 80–85.
- 19 Ludtke JJ, Sebestyén MG, Wolff JA. The effect of cell division on the cellular dynamics of microinjected DNA and dextran. *Mol Ther* 2002; **5**: 579–588.
- 20 Lechardeur D *et al*. Metabolic instability of plasmid DNA in the cytosol: a potential barrier to gene transfer. *Gene Therapy* 1999; **6**: 482–497.
- 21 Pollard H *et al*. Ca²⁺-sensitive cytosolic nucleases prevent efficient delivery to the nucleus of injected plasmids. *J Gene Med* 2001; **3**: 153–164.
- 22 Escriou V *et al*. Cationic lipid-mediated gene transfer: analysis of cellular uptake and nuclear import of plasmid DNA. *Cell Biol Toxicol* 1998; **14**: 95–104.
- 23 Zelphati O, Liang X, Hobart P, Felgner PL. Gene chemistry: functionally and conformationally intact fluorescent plasmid DNA. *Hum Gene Ther* 1999; **10**: 15–24.
- 24 Banks GA, Roselli RJ, Chen R, Giorgio TD. A model for the analysis of nonviral gene therapy. *Gene Therapy* 2003; **10**: 1766–1775.
- 25 Pollard H *et al*. Polyethylenimine but not cationic lipids promotes transgene delivery to the nucleus in mammalian cells. *J Biol Chem* 1998; **273**: 7507–7511.
- 26 Ogris M *et al*. PEGylated DNA/transferrin-PEI complexes: reduced interaction with blood components, extended circulation in blood and potential for systemic gene delivery [In Process Citation]. *Gene Therapy* 1999; **6**: 595–605.
- 27 Carlisle RC *et al*. Adenovirus hexon protein enhances nuclear delivery and increases transgene expression of polyethylenimine/plasmid DNA vectors. *Mol Ther* 2001; **4**: 473–483.
- 28 Dowty ME *et al*. Plasmid DNA entry into postmitotic nuclei of primary rat myotubes. *Proc Natl Acad Sci USA* 1995; **92**: 4572–4576.
- 29 Gasiorowski JZ, Dean DA. Mechanisms of nuclear transport and interventions. *Adv Drug Deliv Rev* 2003; **55**: 703–716.
- 30 Dean DA. Import of plasmid DNA into the nucleus is sequence specific. *Exp Cell Res* 1997; **230**: 293–302.
- 31 Dean BS, Byrd Jr JN, Dean DA. Nuclear targeting of plasmid DNA in human corneal cells. *Cur Eye Res* 1999; **19**: 66–75.

- 32 Vacik J, Dean BS, Zimmer WE, Dean DA. Cell-specific nuclear import of plasmid DNA. *Gene Therapy* 1999; **6**: 1006–1014.
- 33 Young JL, Benoit JN, Dean DA. Effect of a DNA nuclear targeting sequence on gene transfer and expression of plasmids in the intact vasculature. *Gene Therapy* 2003; **10**: 1465–1470.
- 34 Mir B, Piedrahita JA. Nuclear localization signal and cell synchrony enhance gene targeting efficiency in primary fetal fibroblasts. *Nucleic Acids Res* 2004; **32**: e25.
- 35 Blomberg P et al. Electroporation in combination with a plasmid vector containing SV40 enhancer elements results in increased and persistent gene expression in mouse muscle. *Biochem Biophys Res Commun* 2002; **298**: 505–510.
- 36 Wildeman AG. Regulation of SV40 early gene expression. *Biochem Cell Biol* 1988; **66**: 567–577.
- 37 Whittaker GR. Virus nuclear import. *Adv Drug Deliv Rev* 2003; **55**: 733–747.
- 38 Kuwahara J, Azumano M, Takeda T. Nuclear localization of transcription factor Sp1. *Nucleic Acids Symp Ser* 1999; **42**: 293–294.
- 39 Chan CK, Jans DA. Synergy of importin alpha recognition and DNA binding by the yeast transcriptional activator GAL4. *FEBS Lett* 1999; **462**: 221–224.
- 40 Chan CK, Hubner S, Hu W, Jans DA. Mutual exclusivity of DNA binding and nuclear localization signal recognition by the yeast transcription factor GAL4: implications for nonviral DNA delivery. *Gene Therapy* 1998; **5**: 1204–1212.
- 41 Müller CW et al. Structure of the NF- κ B p50 homodimer bound to DNA. *Nature* 1995; **373**: 311–317.
- 42 Mesika A, Grigoreva I, Zohar M, Reich Z. A regulated, NF κ B-assisted import of plasmid DNA into mammalian cell nuclei. *Mol Ther* 2001; **3**: 653–657.
- 43 Mesika A et al. Enhanced intracellular mobility and nuclear accumulation of DNA plasmids associated with a karyophilic protein. *Hum Gene Ther* **16**: 200–208.
- 44 Wilson GL, Dean BS, Wang G, Dean DA. Nuclear import of plasmid DNA in digitonin-permeabilized cells requires both cytoplasmic factors and specific DNA sequences. *J Biol Chem* 1999; **274**: 22025–22032.
- 45 Langle-Rouault F et al. Up to 100-fold increase of apparent gene expression in the presence of Epstein–Barr virus oriP sequences and EBNA1: implications of the nuclear import of plasmids. *J Virol* 1998; **72**: 6181–6185.
- 46 Vaysse L et al. Development of a self-assembling nuclear targeting vector system based on the tetracycline repressor protein. *J Biol Chem* 2004; **279**: 5555–5564.
- 47 Dean DA. Nucleocytoplasmic trafficking. In: Mahato RI (ed). *Pharmaceutical Perspectives of Nucleic Acid-based Therapeutics*. Harwood Academic Publishers: London, 2002, pp 229–260.
- 48 Carson JA, Fillmore RA, Schwartz RJ, Zimmer WE. The smooth muscle gamma-actin gene promoter is a molecular target for the mouse bagpipe homologue, mNkx3-1, and serum response factor. *J Biol Chem* 2000; **275**: 39061–39072.
- 49 Browning CL et al. The developmentally regulated expression of serum response factor plays a key role in the control of smooth muscle-specific genes. *Dev Biol* 1998; **194**: 18–37.
- 50 Kovacs AM, Zimmer WE. Cell specific transcription of the smooth muscle γ -actin gene requires both positive and negative acting cis-elements. *Gene Exp* 1998; **7**: 115–129.
- 51 Patterson C et al. Cloning and functional analysis of the promoter for KDR/flk-1, a receptor for vascular endothelial growth factor. *J Biol Chem* 1995; **270**: 23111–23118.
- 52 Young JL, Byrd JN, Wyatt CR, Dean DA. Endothelial cell-specific plasmid nuclear import. *Mol Biol Cell* 1999; **10S**: 443a.
- 53 Adam SA, Marr RS, Gerace L. Nuclear protein import in permeabilized mammalian cells requires soluble cytoplasmic factors. *J Cell Biol* 1990; **111**: 807–816.
- 54 Colin M et al. The nuclear pore complex is involved in nuclear transfer of plasmid DNA condensed with an oligolysine-RGD peptide containing nuclear localisation properties. *Gene Therapy* 2001; **8**: 1643–1653.
- 55 Bustamante JO et al. Dendrimer-assisted patch-clamp sizing of nuclear pores. *Pflugers Arch* 2000; **439**: 829–837.
- 56 Bustamante JO et al. Calcium, ATP and nuclear pore channel gating. *Pflugers Arch* 2000; **439**: 433–444.
- 57 Bustamante JO. Nuclear pore ion channel behavior in live syncytial nuclei. *Pflugers Arch* 2002; **444**: 286–290.
- 58 Collas P, Alestrom P. Rapid targeting of plasmid DNA to zebrafish embryo nuclei by the nuclear localization signal of SV40 T antigen. *Mol Mar Biol Biotechnol* 1997; **6**: 48–58.
- 59 Collas P, Husebye H, Alestrom P. The nuclear localization sequence of the SV40T antigen promotes transgene uptake and expression in zebrafish embryo nuclei. *Transgenic Res* 1996; **5**: 451–458.
- 60 Collas P, Alestrom P. Nuclear localization signal of SV40 T antigen directs import of plasmid DNA into sea urchin male pronuclei *in vitro*. *Mol Reprod Dev* 1996; **45**: 431–438.
- 61 Aronsohn AI, Hughes JA. Nuclear localization signal peptides enhance cationic liposome-mediated gene therapy. *J Drug Target* 1998; **5**: 163–169.
- 62 Chan CK, Jans DA. Enhancement of polylysine-mediated transfection by nuclear localization sequences: polylysine does not function as a nuclear localization sequence. *Hum Gene Ther* 1999; **10**: 1695–1702.
- 63 Subramanian A, Ranganathan P, Diamond SL. Nuclear targeting peptide scaffolds for lipofection of nondividing mammalian cells. *Nat Biotechnol* 1999; **17**: 873–877.
- 64 Kaneda Y, Iwai K, Uchida T. Increased expression of DNA cointroduced with nuclear protein in adult rat liver. *Science* 1989; **243**: 375–378.
- 65 Fritz JD, Herweijer H, Zhang G, Wolff JA. Gene transfer into mammalian cells using histone-condensed plasmid DNA. *Hum Gene Ther* 1996; **7**: 1395–1404.
- 66 Bottger M et al. Acid nuclear extracts as mediators of gene transfer and expression. *Biochim Biophys Acta* 1998; **1395**: 78–87.
- 67 Hagstrom JE et al. Complexes of non-cationic liposomes and histone H1 mediate efficient transfection of DNA without encapsulation. *Biochim Biophys Acta* 1996; **1284**: 47–55.
- 68 Chan CK, Jans DA. Enhancement of MSH receptor- and GAL4-mediated gene transfer by switching the nuclear import pathway. *Gene Therapy* 2001; **8**: 166–171.
- 69 Hagstrom JE et al. Nuclear import of DNA in digitonin-permeabilized cells. *J Cell Sci* 1997; **110**: 2323–2331.
- 70 Ludtke JJ, Zhang G, Sebestyen MG, Wolff JA. A nuclear localization signal can enhance both the nuclear transport and expression of 1 kb DNA. *J Cell Sci* 1999; **112**: 2033–2041.
- 71 Ciolina C et al. Coupling of nuclear localization signals to plasmid DNA and specific interaction of the conjugates with importin alpha. *Bioconjug Chem* 1999; **10**: 49–55.
- 72 van der Aa MA et al. An NLS peptide covalently linked to linear DNA does not enhance transfection efficiency of cationic polymer based gene delivery systems. *J Gene Med* 2005; **7**: 208–217.
- 73 Zanta MA, Belguise-Valladier P, Behr JP. Gene delivery: a single nuclear localization signal peptide is sufficient to carry DNA to the cell nucleus. *Proc Natl Acad Sci USA* 1999; **96**: 91–96.
- 74 Branden LJ, Mohamed AJ, Smith CI. A peptide nucleic acid-nuclear localization signal fusion that mediates nuclear transport of DNA. *Nat Biotechnol* 1999; **17**: 784–787.
- 75 Neves C, Byk G, Scherman D, Wils P. Coupling of a targeting peptide to plasmid DNA by covalent triple helix formation. *FEBS Lett* 1999; **453**: 41–45.
- 76 Zelfhati O et al. PNA-dependent gene chemistry: stable coupling of peptides and oligonucleotides to plasmid DNA. *Biotechniques* 2000; **28**: 304–316.

- 77 Branden LJ, Christensson B, Smith CI. *In vivo* nuclear delivery of oligonucleotides via hybridizing bifunctional peptides. *Gene Therapy* 2001; **8**: 84–87.
- 78 Liang KW, Hoffman EP, Huang L. Targeted delivery of plasmid DNA to myogenic cells via transferrin-conjugated peptide nucleic acid. *Mol Ther* 2000; **1**: 236–243.
- 79 Morris MC *et al*. Combination of a new generation of PNAs with a peptide-based carrier enables efficient targeting of cell cycle progression. *Gene Therapy* 2004; **11**: 757–764.
- 80 Cartier R, Reszka R. Utilization of synthetic peptides containing nuclear localization signals for nonviral gene transfer systems. *Gene Therapy* 2002; **9**: 157–167.
- 81 Carriere M, Escriou V, Savarin A, Scherman D. Coupling of importin beta binding peptide on plasmid DNA: transfection efficiency is increased by modification of lipoplex's physico-chemical properties. *BMC Biotechnol* 2003; **3**: 14.
- 82 Rebuffat A *et al*. Selective enhancement of gene transfer by steroid-mediated gene delivery. *Nat Biotechnol* 2001; **19**: 1155–1161.
- 83 Rebuffat AG *et al*. Gene delivery by a steroid-peptide nucleic acid conjugate. *FASEB J* 2002; **16**: 1426–1428.
- 84 Arenal A *et al*. The SV40T antigen nuclear localization sequence enhances nuclear import of vector DNA in embryos of a crustacean (*Litopenaeus schmitti*). *Gene* 2004; **337**: 71–77.
- 85 Li S *et al*. Muscle-specific enhancement of gene expression by incorporation of the SV40 enhancer in the expression plasmid. *Gene Therapy* 2001; **8**: 494–497.
- 86 Salman H *et al*. Kinetics and mechanism of DNA uptake into the cell nucleus. *Proc Natl Acad Sci USA* 2001; **98**: 7247–7252.
- 87 Utvik JK, Nja A, Gundersen K. DNA injection into single cells of intact mice. *Hum Gene Ther* 1999; **10**: 291–300.