

Nucleocytoplasmic trafficking: implications for the nuclear import of plasmid DNA during gene therapy

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Key words: pore complex, nucleoporins, nuclear localization signals, karyopherin, nuclear export signals, mRNA export,

Summary

Trafficking of nuclear proteins from the site of their synthesis in the cytoplasm to the sites of function in the nucleus through pore complexes is mediated by nuclear localization signals (NLSs) on proteins to be imported into nuclei. Protein translocation from the cytoplasm to the nucleoplasm involves (i) the formation of a complex of karyopherin α with NLS-protein, (ii) subsequent binding of karyopherin β , (iii) binding of the complex to FXFG peptide repeats on nucleoporins, (iv) docking of Ran-GDP to nucleoporin and to karyopherin heterodimer by p10, (v) a number of association-dissociation reactions on nucleoporins which dock the import substrate toward the nucleoplasmic side with a concomitant GDP-GTP exchange reaction transforming Ran-GDP into Ran-GTP and catalyzed by karyopherin α , and finally (vi) dissociation from karyopherin β and release of the karyopherin α /NLS-protein by Ran-GTP to the nucleoplasm. A number of processes have been found to be regulated by nuclear import including nuclear translocation of the transcription factors NF- κ B, rNFIL-6, ISGF3, SRF, c-Fos, GR as well as human cyclins A and B1, casein kinase II, cAMP-dependent protein kinase II, protein kinase C, ERK1 and ERK2. Failure of cells to import specific proteins into nuclei can lead to carcinogenesis. For example, BRCA1 is mainly localized in the cytoplasm in breast and ovarian cancer cells whereas in normal cells the protein is nuclear. mRNA is exported through the same route as a complex with nuclear proteins possessing nuclear export signals (NES). The majority of proteins with NES are RNA-binding proteins which bind to and escort RNAs to the cytoplasm. However, other proteins with NES function in the export of proteins; CRM1, which binds to the NES sequence on other proteins and interacts with the nuclear pore complex, is an essential mediator of the NES-dependent nuclear export of proteins in eukaryotic cells. Nuclear localization and export signals (NLS and NES) are found on a number of important molecules including p53, v-Rel, the transcription factor NF-ATc, the c-Abl nonreceptor tyrosine kinase, and the fragile X syndrome mental retardation gene product; the deregulation of their normal import/export trafficking has important implications for human disease. Both nuclear import and export processes can be manipulated by conjugation of proteins with NLS or NES peptides. During gene therapy the foreign DNA needs to enter nuclei for its transcription; a pathway is proposed involving the complexation of plasmids and oligonucleotides with nascent nuclear proteins possessing NLSs as a prerequisite for their nuclear import. Covalent linkage of NLS peptides to oligonucleotides and plasmids or formation of complexes of plasmids with proteins possessing multiple NLS peptides is proposed to increase their import rates and the efficiency of gene expression. Cancer cells are predicted to import more efficiently foreign DNA into nuclei compared with terminally differentiated cells because of their increased rates of proliferation and protein import.

I. Introduction

Evolution has effectively secluded nuclear functions from cytoplasmic activities by the nuclear envelope barrier

in order to circumvent the increased organizational and regulatory problems associated with the advent of the large genomes in more complex organisms. The nuclear envelope has evolved to allow nuclear and cytoplasmic environments to be developed and to selectively keep certain regulatory proteins, such as replication factors, out, allowing cells to regulate their cell cycle and level of ploidy. This double-membrane structure effectively separates transcription of genes from translation of their mRNA into proteins and allows proteins destined to function in the nucleus to pass selectively through the lumen of the nuclear pores (reviewed by Burke, 1990; Bouliskas, 1993, 1994; Laskey et al, 1996).

The nuclear membrane prevents reinitiation of DNA replication in *Xenopus* eggs, by excluding a "licensing factor" that is essential for DNA replication; replication licensing may involve the MCM (minichromosome maintenance) complex and ORC, the origin recognition complex (Laskey et al, 1996).

The interior of the nuclear envelope is lined by a polymer of lamins intimately attached to chromatin DNA. Phosphorylation of lamins at four serines by S6 kinase II and other kinases, all of which appear to be controlled by cdc2 kinase, specifically occur as cells traverse the G2 to M checkpoint of the cell cycle; this process results in lamin depolymerization and in nuclear envelope breakdown. After completion of mitosis these processes are reversed and new nuclear envelopes are assembled around daughter cell nuclei.

Selective transport through pores creates a unique biochemical environment within the nucleus. All proteins are synthesized in the cytoplasm; the selective import of proteins through the pore complexes that straddle the inner and outer nuclear membranes is a sophisticated process dependent on energy and upon the presence of short karyophilic peptides, termed nuclear localization signals (NLS), only on nuclear proteins (Dingwall et al, 1982; Kalderon et al, 1984).

The ultimate target of gene therapy is the cell nucleus. The knowledge on nucleocytoplasmic trafficking could be used for enhancing the nuclear import of plasmids or small oligonucleotides designed to act in the nucleus. Since only a small portion (less than 1%) of the plasmid molecules that reach the cytoplasm might ultimately enter the nucleus, and only 15% of water soluble oligonucleotides which reach the cytoplasm might ultimately diffuse through pore complexes (Boutorine and Kostina, 1993), covalent linkage of NLS peptides via random-coil peptide arms to oligonucleotides or to plasmids is expected to increase their import rates and the efficiency of expression of their therapeutic gene loads. In addition, complexation of the plasmid into colloidal particles with proteins

possessing multiple NLS peptide motifs is proposed to facilitate nuclear import.

II. Morphology of the pore complexes

The nuclear envelope is often studded across both sides with transisternal "holes." These hollow cylindrical organelles spanning the two nuclear membranes are called pore complexes. Pore complexes have a width (distance from cytoplasm to nucleoplasm) of ~70 nm and a diameter of 133 nm (Hinshaw et al 1992). Their frequency greatly depends on the cell type ranging from 1 to 60 pores/mm².

Their refined model structure (Hinshaw et al, 1992) is rather complex (**Figures 1-4**). They appear as tripartite structures composed of two concave rings and a central granule. The concave rings are called pore annuli (one cytoplasmic and one nucleoplasmic), each containing eight granules arranged in an 8-fold rotational symmetry, lying on top of the pore rims. A three-dimensional electron microscopy analysis coupled with image analysis to calculate 2D and 3D maps of detergent released pore complexes revealed that this highly symmetric framework is built from many distinct and interconnected subunits arranged in such a way so as to construct a large central channel (Hinshaw et al, 1992, **Figures 1-4**).

Electron microscopy of nuclear pore complexes isolated from *Xenopus laevis* oocytes spread on a carbon-coated film has shown that each of the eight spokes seen in en face views of pore complexes is built from four morphological features: the annular, the column, the ring, and the luminal subunits (Hinshaw et al, 1992). Each spoke holds two copies of each subunit. Furthermore, an intricate network connects these subunits to one another. In addition to the large central channel, the spokes are responsible for the construction of eight peripheral channels of unknown function (Hinshaw et al, 1992). Image analysis of spokes on en face electron micrographs of pore complexes (**Figure 2a**) show that they are composed of (i) bilobed regions that form an inner annulus encircling a 42 nm diameter hole; (ii) a central region of a radius of 41 nm and (iii) an outer region of 52.5 nm. The maps obtained by Hinshaw and coworkers (1992) provide compelling evidence for a highly symmetric structure of pore complexes in accordance with previous studies (Unwin and Milligan 1982; Akey, 1989). In the oocytes of the frog *Xenopus laevis* the pore annuli have an inside diameter close to 80 nm and an outer diameter of 120 nm. The inner diameter of the pore complexes is highly constant within a certain cell type.

A total of up to 100 distinct proteins (nucleoporins) have been estimated to participate in the structure of the

Figure 1. Electron micrographs of nuclear pore complexes, (NPCs), released from the nuclear envelope by detergent. **(a)** En face views of NPCs (lower right) and rings (upper left) in the same field of view. **(b)** Image of a deep pool of stain; the two pore complexes indicated by arrows represent edge views whereas the arrowheads show oblique views of NPCs (see also the model in Figure 3); a number of en face views are also present in the same field of view. Scale bar = 500 nm. Image by courtesy of Ron Milligan and Jenny Hinshaw, The Scripps Research Institute, La Jolla, California. From Hinshaw JE, Carragher BO, Milligan RA (1992) Architecture and design of the nuclear pore complex. *Cell* 69, 1133-1141. Reproduced with kind permission from Cell Press and the authors.

Figure 2. Projection maps obtained by averaging images of each of the four structures identified in Figure 1. Regions where biological material is concentrated are darker and are enclosed by contours; regions where the negative stain is concentrated are lighter. **(a)** Average of 168 (n=168) **en face** images of detergent-released NPCs. **(b)** **Edge view** of detergent released NPCs, n=48. **(c)** **Ring views** (n=400). **(d)** **Intermediate structures** (n=23). Scale barr = 50 nm. Image by courtesy of Ron Milligan and Jenny Hinshaw, The Scripps Research Institute, La Jolla, California. From Hinshaw JE, Carragher BO, Milligan RA (1992) Architecture and design of the nuclear pore complex. *Cell* 69, 1133-1141. Reproduced with kind permission from Cell Press and the authors.

1987); thus, it is unlikely for those glycoproteins, involved in nuclear import, are integral parts of the spoke subunits (Hinshaw et al, 1992).

III. Nuclear localization signals (NLSs)

A. Historical background

The selective import of proteins that have a function in the nucleus through the pore complexes that straddle the inner and outer nuclear membranes is a sophisticated process dependent on the presence of short karyophilic peptides, termed nuclear localization signals (NLS) (reviewed by Boulikas, 1993, 1994, 1996, 1997b). A very short sequence of seven amino acids (Pro-Lys-Lys-Lys-Arg-Lys-Val or PKKKRKV), first recognized by Kalderon and coworkers (1984) in the SV40 large T antigen, is required for its normal nuclear localization.

Yoneda et al. (1988) have raised antibodies against the peptide DDDDED supposed to be present in nuclear pore or cytoplasmic receptor (transporter) protein molecules and to be involved in ionic interactions with the NLS (KKKKRK) of SV40 large T protein. Indirect immunofluorescence with these antibodies against the acidic peptide has shown punctuate staining at the nuclear rim or the nuclear surface in rat, human, bovine and murine cell lines; in addition, the antibody blocked nuclear import.

A single protein may possess more than one signals for nuclear import (Standiford and Richter, 1992). The rate of nuclear import is directly related to the number of NLS it possesses (Dworetzky et al, 1988), as was first suggested by Dingwall and coworkers (1982). A smaller number of nuclear proteins contain "bipartite" NLS hypothesized to be reconstituted by two moieties brought together by protein folding or conformational change as for example on the cytoplasmic glucocorticoid receptor by hormone binding (Welsh et al, 1986; Picard and Yamamoto, 1987).

Three approaches have been used for NLS identification: (i) gene fusion experiments between a NLS-coding DNA segment and the gene coding for puruvate kinase, β -galactosidase, or other cytoplasmic proteins; (ii) nuclear import of non-nuclear proteins conjugated to synthetic NLS peptides; (iii) site-directed mutagenesis of the NLS of a nuclear protein resulting in its cytoplasmic retention (Boulikas, 1993; Tables 1-4).

B. Rules to predict nuclear localization of an unknown protein

Several simple rules have been proposed for the prediction of the nuclear localization of a protein of an unknown function from its amino acid sequence:

(i). An NLS is defined as four arginines (R) plus lysines (K) within an hexapeptide; the presence of one or

Figure 3 Renderings of the 822-symmetrized nuclear pore complex map. En face (a), oblique (b), edge (c), and front view (d) of the 3D map. A slight ridge indicates the central plane of the 3D map and divides the assembly into two symmetrical halves. Manipulation and display of the maps were done with specific programs. Annular subunits are green, rings are yellow, and luminal subunits are blue. The remaining tan colored parts of the 3D map enclose the column subunits. Image by courtesy of Ron Milligan and Jenny Hinshaw, The Scripps Research Institute, La Jolla, California. From Hinshaw JE, Carragher BO, Milligan RA (1992) Architecture and design of the nuclear pore complex. *Cell* 69, 1133-1141. Reproduced with kind permission from Cell Press and the authors.

pore complex (Iovine et al, 1995). A pore-specific transmembrane glycoprotein gp210 (mw 204 kD) with two transmembrane domains and 13 glycosylation sites is thought to anchor the pore complex to the membrane of the nuclear envelope (Wozniak et al, 1989).

The central granule, exclusive route for import of nuclear protein, occupies the pore center. Its diameter varies from 2.5 to 35 nm and its appearance ranges from compact spherical to thin rod shaped (Akey, 1989). Fibrils, which protrude deeply into the nuclear interior forming a central ring of spokes, emanate from the central granule. The fibrils, ~3nm in diameter and extending ~200nm to the interior of the nucleus, are involved in nuclear transport: nucleoplasmin-coated gold particles associate with these tentacles (Richardson et al, 1988) and are docking sites for the karyopherin α NLS-protein complex (Rexach and Blobel, 1995). Antibodies to the O-linked glycoproteins seem to bind close to the 8-fold axis and away from the central plane of the NPC (Snow et al,

more histidines (H) in the tetrad of the karyophilic hexapeptide, often found in protein kinases that have a

Figure 4 oblique map of the pore complex (Figure 3b) superimposed over an electron micrograph of nuclear pore complexes showing the central plug of the structures. Image by courtesy of Ron Milligan and Jenny Hinshaw, The Scripps Research Institute, La Jolla, California. From Hinshaw JE, Carragher BO, Milligan RA (1992) Architecture and design of the nuclear pore complex. *Cell* 69, 1133-1141. Reproduced with kind permission from Cell Press and the authors.

cytoplasmic and a nuclear function, may specify a weak NLS whose function might be regulated by phosphorylation or may specify proteins that function in both the cytoplasm and the nucleus (Boulikas, 1996).

(ii). The K/R clusters are flanked by the α -helix breakers G and P thus placing the NLS at a helix-turn-helix or end of an α -helix. Negatively-charged amino acids (D, E) are often found at the flank of the NLS and on some occasions may interrupt the positively-charged NLS cluster.

(iii). Bulky amino acids (W, F, Y) are not present within the NLS hexapeptide.

(iv). NLS signals may not be flanked by long stretches of hydrophobic amino acids (e.g. five); a mixture of charged and hydrophobic amino acids serves as a mitochondrial targeting signal.

(v). The higher the number of NLSs the more readily a molecule is imported to the nucleus (Dworetzky et al, 1988). Even small proteins, for example histones (10-22 kDa), need to be actively imported to increase their import rates compared with the slow rate of diffusion of small molecules through pores.

(vi). Signal peptides are stronger determinants than NLSs for protein trafficking; signal peptides direct

proteins to the lumen of the endoplasmic reticulum for their secretion or insertion into cellular membranes (presence of transmembrane domains) (Boulikas, 1994).

(vii). Signals for the mitochondrial import of proteins (a mixture of hydrophobic and karyophilic amino acids) may antagonize nuclear import signals and proteins possessing both type of signals may be translocated to both mitochondria and nuclei (Beasley and Schatz, 1991; Neupert and Lill, 1995).

(viii). Strong association of a protein with large cytoplasmic structures (membrane proteins, intermediate filaments) make such proteins unavailable for import even though they possess NLS-like peptides (Boulikas, 1994).

(ix). Transcription factors and other nuclear proteins possess a great different number of putative NLS stretches; of the sixteen possible forms of putative NLS structures the most abundant types are the $\theta\theta x\theta\theta$, $\theta\theta\theta x\theta$, $\theta\theta\theta\theta$, and $\theta\theta x\theta x\theta$ where θ is R or K, together accounting for about 70% of all karyophilic clusters on transcription factors (Boulikas, 1994).

(x). A small number of nuclear proteins seem to be void of a typical karyophilic NLS; in this case either non karyophilic peptides function for their nuclear import, such molecules possess bipartite NLSs, or these NLS-less proteins depend absolutely for import on their strong complexation in the cytoplasm with a nuclear protein partner able to be imported (Boulikas, 1994); this mechanism might ensure a certain stoichiometric ratio of the two molecules in the nucleus and might be of physiological significance.

(xi) A number of proteins may be imported via other mechanisms not dependent on classical NLS (see below).

C. NLS on adenovirus proteins

The pentapeptide **KRPRP** of Adenovirus **E1a** when linked to the C-terminus of *E. coli* galactokinase, was sufficient to direct its nuclear accumulation after microinjection into Vero monkey cells (Lyons et al., 1987). The synthetic peptide **CGGLSSKRPRP** from adenovirus type 2/5 E1a crosslinked to chicken bovine **Table 1** Simple NLS

Signal oligopeptide	Protein and features
PKKKRKV	Wild-type SV40 large T protein A point mutation converting lysine-128 (double underlined) to threonine results in the retention of large T in the cytoplasm. Transfer of this peptide to the N-terminus of β -galactosidase or pyruvate kinase at the gene level and microinjection of plasmids into Vero cells showed nuclear location of chimeric proteins.
PKKKRMV	SV40 large T with a K \rightarrow M change. Site-directed mutagenesis only slightly impaired nuclear import of large T.
PKKKRKVEDP	Synthetic NLS peptide from SV40 large T antigen crosslinked to BSA or IgG mediated their nuclear localization after microinjection in <i>Xenopus</i> oocytes. The PKKGSKKA from <i>Xenopus</i> H2B was ineffective and PKTKRKV was less effective.

albumin and microinjected into HeLa cells caused nuclear localization (Chelsky et al., 1989).

Two NLS, **PPKKRMRRRIE** and **PKKKKKRP** were found on adenovirus 5 **DBP** (DNA-binding protein) which is expressed in nuclei of infected cells and is involved in virus replication and early and late gene expression. Both NLS are needed, and disruption of either site impaired nuclear localization of the 529 amino acid protein (Morin et al., 1989).

The NLS **RLPVRRRRRRVP** was determined on adenovirus **pTP1** and **pTP2** (preterminal proteins, 80 kD) between amino acid residues 362-373. The 140 kDa DNA polymerase of adenovirus when it had lost its own NLS could enter the nucleus via its interaction with pTP. This NLS, fused to the N-terminus of *E. coli* β -galactosidase, was functional in nuclear targeting (Zhao and Padmanabhan, 1988).

A "tripartite" or "doubly bipartite" NLS was found on **adenovirus DNA polymerase** (AdPol) having the sequences: signal I: **AHRARRLH** (amino acids 6-13); signal II: **PPRRRVRQQPP** (amino acids 23-33); and signal III: **PARARRRRAP** (amino acids 39-48). Signals I and II functioned interdependently as an NLS for the nuclear targeting of AdPol, for which signal III was dispensable. The combined signal II-III was more efficient NLS than signal I-II (Zhao and Padmanabhan, 1991).

IV. Nucleoporins

A number of nucleoporins (proteins of the pore complex) possess FXFG motifs and display modification of Ser/Thr by single N-acetyl-glucosamine residues; these include Nup98, p62, Nup153, and Nup214 in vertebrates and NUP1, NUP2, and NSP1 in *S. cerevisiae*. A different subset of pore complex proteins including p270, Nup214, Nup153, and Nup98 contain FXFG and GLFG repetitive peptide motifs and are able to bind specifically to NLS-containing protein models; a single motif may be a low affinity binding site and the affinity of binding could be

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CGYGPKKKRKVGG	Synthetic peptide from SV40 large T antigen conjugated to various proteins and microinjected into the cytoplasm of TC-7 cells. Specified nuclear localization up to protein sizes of 465 kD (ferritin). IgM of 970 kD and with an estimated radius of 25-40 nm was retained in the cytoplasm.
CYDDEA <u>T</u> AD <u>S</u> QH <u>ST</u> PPKKKKRKVEDPK DFESELLS	SV40 large T protein long NLS. The long NLS but not the short NLS, was able to localize the bulky IgM (970 kD) into the nucleus. Mutagenesis at the four possible sites of phosphorylation (double underlined) impaired nuclear import.
CGGPKKKKRKVGG	SV40 large T protein . This synthetic peptide crosslinked to chicken serum albumin and microinjected into HeLa cells caused nuclear localization.
PKKKIKV	A mutated (R→I) version of SV40 large T NLS . Effective NLS.
MK _{x11} <u>CRLKKLK</u> CSKEKPKCAKCLK _{x5} R _{x3} KTKR 74 N-terminal amino acid	Yeast GAL4 (99 kD). Fusions of the GAL4 gene portion encoding the 74 N-terminal amino acid with <i>E. Coli</i> β-galactosidase introduced into yeast cells specify nuclear localization.
MK _{x11} CRLKKLKCSKEKPKCA 29 N-terminal amino acid	Yeast GAL4 . Acted as an efficient nuclear localization sequence when fused to invertase but not to β-galactosidase introduced by transformation into yeast cells.
PKKARED VSRKRPR	Polyoma large T protein . Identified by fusion with puruvate kinase cDNA and microinjection of Vero African green monkey cells. Mutually independent NLS. Can exert cooperative effects.
CGYGVSRKRPRPG	Polyoma virus large T protein . This synthetic peptide crosslinked to chicken serum albumin and microinjected into HeLa cells caused nuclear localization.
APT ³ KRKG	SV40 VP1 capsid polypeptide (46 kD). NLS (N terminus) determined by infection of monkey kidney cells with a fusion construct containing the 5' terminal portion of SV40 VP1 gene and the complete cDNA sequence of poliovirus capsid VP1 replacing the VP1 gene of SV40.
AP ³ KR ³ SGVSKC (1-11)	Polyoma virus major capsid protein VP1 (11 N-terminal amino acid). Yeast expression vectors coding for 17 N-terminal amino acid of VP1 fused to β-galactosidase gave a protein that was transported to the nucleus in yeast cells. Subtractive constructs of VP1 lacking A ¹ to C ¹¹ were cytoplasmic. This, FITC-labeled, synthetic peptide crosslinked to BSA or IgG, caused nuclear import after microinjection into 3T6 cells. Replacement of K ³ with T did not.
PNKKKKRK (amino acid position 317-323)	SV40 VP2 capsid protein (39 kD). The 3' end of the SV40 VP2-VP3 genes containing this peptide when fused to poliovirus VP1 capsid protein at the gene level resulted in nuclear import of the hybrid VP1 in simian cells infected with the hybrid SV40.
EEDGPQKKKRRRL (307-318)	Polyoma virus capsid protein VP2 . A construct having truncated VP2 lacking the 307-318 peptide transfected into COS-7 cells showed cytoplasmic retention of VP2. The 307-318 peptide crosslinked to BSA or IgG specified nuclear import following their microinjection into NIH 3T6 cells.
GKKRSKA	Yeast histone H2B . This peptide specified nuclear import when fused to β-galactosidase.
KRPRP	Adenovirus E1a . This pentapeptide, when linked to the C-terminus of <i>E. coli</i> galactokinase, was sufficient to direct its nuclear accumulation after microinjection in Vero monkey cells.
CGGLSSKRPRP	Adenovirus type 2/5 E1a . This synthetic peptide crosslinked to chicken bovine albumin and microinjected into HeLa cells caused nuclear localization.
LV <u>RKKRK</u> TE ₃ SP (NLS 1) LKDKDAKKSQKQE (NLS2)	Xenopus N1 (590 amino acid). Abundant in <i>X. laevis</i> oocytes, forming complexes with histones H3, H4 via two acidic domains each containing 21 and 9 (D+E), respectively. The NLS1 is required but not sufficient for nuclear accumulation of protein N1. NLS 1 and 2 are contiguous at the C-terminus.
GNKAKRQRST	v-Rel or p59^{v-rel} the transforming protein, product of the <i>v-rel</i> oncogene of the avian reticuloendotheliosis retrovirus strain T (Rev-T). v-Rel NLS added to the normally cytoplasmic β-galactosidase directed that protein to the nucleus.
PFLDRLRRDQK PKQKRKMAR	NS1 protein of influenza A virus, that accumulates in nuclei of virus-infected cells. Determined to be an NLS by deletion mutagenesis of NS1 in recombinant SV40. The 1st NLS is conserved among all NS1 proteins of influenza A viruses.
SVTKKRKLE	Human lamin A . Dimerization of lamin A was proposed to give a complex with two NLSs that was transported more efficiently.
SASKRRRLE	Xenopus lamin A . NLS inferred from its similarity to human lamin A NLS.
TKGKRKRID	Xenopus lamin L₁ . NLS inferred from its sequence similarity to human lamin A NLS.

CVRTTKGKRKRIDV	Xenopus lamin L₁ . This synthetic peptide crosslinked to chicken bovine albumin and microinjected into HeLa cells caused nuclear localization.
CGGAMINO ACIDKRVKLD	Human c-myc oncoprotein . This synthetic peptide crosslinked to chicken bovine albumin and microinjected into HeLa cells caused nuclear localization.
PAMINO ACIDKRVKLD (M1, fully potent NLS)	Human c-myc oncoprotein . Conjugation of the M1 peptide to human serum albumin and microinjection of Vero cells gives complete nuclear accumulation. M2 gave slower and only partial nuclear localization.
RQRRNELKRSP (M2, medium potency NLS)	
SALIKKKKKMAP	Murine c-abl (IV) gene product. The p160 ^{gag/v-abl} has a cytoplasmic and plasma membrane localization, whereas the mouse type IV c-abl protein is largely nuclear.
PPKKRMRRIE PKKKKKRP	Adenovirus 5 DBP (DNA-binding protein) found in nuclei of infected cells and involved in virus replication and early and late gene expression. Both NLS are needed, and disruption of either site impaired nuclear localization of the 529 amino acid protein.
YRKCLQAGMNLEARKTKKKIKGIQQA TA (497-524 amino acid)	Rat GR , glucocorticoid receptor (795 amino acid) NLS1 determined by fusion with β-galactosidase (116 kD). NLS1 is 100% conserved between human, mouse and rat GR. Whereas the 407-615 amino acid fragment of GR specifies nuclear location, the 407-740 amino acid fragment was cytoplasmic in the absence of hormone, indicating that sequence 615-740 may inhibit the nuclear location activity. A second (NLS2) is localized in an extensive 256 amino acid C-terminal domain. NLS 2 requires hormone binding for activity.
<u>RKDRRGGRMLKHKROR</u> DDGEGRGEV GSAGDMRAMINO ACIDNLWPSPLMI <u>KRSKK</u> (amino acid 256-303)	Human ER (estrogen receptor, 595 amino acid) NLS. NLS is between the hormone-binding and DNA-binding regions; ER, in contrast with GR, lacks a second NLS. Can direct a fusion product with β-galactosidase to the nucleus.
RKFKKFNK	Rabbit PG (progesterone receptor). 100% homology in humans; F→L change in chickens. When this sequence was deleted, the receptor became cytoplasmic but could be shifted into the nucleus by addition of hormone; in this case the hormone mediated the dimerization of a mutant PG with a wild type PG molecule.
GKRKNKPK	Chicken Ets1 core NLS. Within a 77 amino acid C-terminal segment 90% homologous to Ets2. When deleted by deletion mutagenesis at the gene level the mutant Ets1 became cytoplasmic.
PLLKKIKQ	c-myb gene product; directs puruvate kinase to the nucleus.
PPQKKIKS	N-myc gene product; directs puruvate kinase to the nucleus.
PQPKKKP	p53 ; directs puruvate kinase to the nucleus.
SKRVAKRKL	c-erb-A gene product; directs puruvate kinase to the nucleus.
CGGLSSKRPRP	Adenovirus type2/5 E1a . This synthetic peptide conjugated with a bifunctional crosslinker to chicken serum albumin (CSA) and microinjected into HeLa cells directed CSA to the nucleus.
MTGSK <u>TRKHRG</u> SGA MTGSK <u>HRKH</u> PGSGA	Yeast ribosomal protein L29 . Double-stranded oligonucleotides encoding the 7 amino acid peptides (underlined) and inserted at the N-terminus of the β-galactosidase gene resulted in nuclear import.
RHRKHP KRRKHP KYRKHP KHRRHP KHKKHP RHLKHP KHRKYP KHRQHP	Mutated peptides derived from yeast L29 ribosomal protein NLS, found to be efficient NLS. The last two are less effective NLS, resulting in both nuclear and cytoplasmic location of β-galactosidase fusion protein.
PETTVV <u>RRRGR</u> SPRRRTSPRRRRSPR <u>RRRSQS</u> (One sequence, C-terminus)	Double NLS of hepatitis B virus core antigen . The two underlined arginine clusters represent distinct and independent NLS. Mutagenesis showed that the antigen fails to accumulate in the nucleus only when both NLS are simultaneously deleted or mutated.
ASKS <u>RKRKL</u>	Viral Jun , a transcription factor of the AP-1 complex. Accumulates in nuclei most rapidly during G2 and slowly during G1 and S. The cell cycle dependence of viral but not of cellular Jun is due to a C→S mutation in NLS of viral Jun. This NLS conjugated to rabbit IgG can mediate cell cycle-dependent translocation.

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GGLCSARLHRHALLAT	Human T-cell leukemia virus Tax trans-activator protein. The most basic region within the 48 N-terminal segment. Missense mutations in this domain result in its cytoplasmic retention.
DTREKKKFLKRRLLRLDE (604-620)	Mouse nuclear Mx1 protein (72 kD). Induced by interferons (among 20 other proteins) . Selectively inhibits influenza virus mRNA synthesis in the nucleus and virus multiplication. The cytoplasmic Mx2 has R→S and R→E changes in this region.
CGYGPKKKRKV (SV40 large T) CGYGDRNKKKKKE (human retinoic acid receptor) CGYGARKTKKKIK (human glucocorticoid receptor) CGYGIRKDRRGGR (human estrogen receptor) CGYGARKLKKLGN (human androgen receptor)	Synthetic peptides crosslinked to bovine serum albumin (BSA) and introduced into MCF 7 or HeLa S3 cells with viral co-internalization method using adenovirus serotype 3B induced nuclear import of BSA.
RKRQRALMLRQAR 30-42	Human XPAC (xeroderma pigmentosum group A complementing protein) involved in DNA excision repair. By site-directed mutagenesis and immunofluorescence. NLS is encoded by exon 1 which is not essential for DNA repair function.
EYLSRKGKLEL (at the N-terminus)	T-DNA -linked VirD2 endonuclease of the <i>Agrobacterium tumefaciens</i> tumor-inducing (T _i) plasmid. A fusion protein with β-galactosidase is targeted to the nucleus. The T-plasmid integrates into plant nuclear DNA; VirD2 produces a site-specific nick for T integration. VirD2 also contains a bipartite NLS at its C-terminus (see Table 2).
KKSKKKRC (95-102)	Putative core NLS of yeast TRM1 (63 kD) that encodes the tRNA modification enzyme N ² , N ² -dimethylguanosine-specific tRNA methyltransferase. Localizes at the nuclear periphery. The 70-213 amino acid segment of TRM1 causes nuclear localization of β-galactosidase fusion protein in yeast cells. Site-directed mutagenesis of the 95-102 peptide resulted in its cytoplasmic retention. TRM1 is both nuclear and mitochondrial. The 1-48 amino acid segment specifies mitochondrial import.
PQSRKKLR	Max protein; specifically interacts with c-Myc protein. Fusion of 126-151 segment of Max to chicken pyruvate kinase (PK) gene, including this putative NLS, followed by transfection of COS-1 cells and indirect immunofluorescence with anti-PK showed nuclear targeting.
QPQRYGGGRGRRW	Gag protein of human foamy retrovirus; a mutant that completely lacks this box exhibits very little nuclear localization; binds DNA and RNA in vitro.

proportional to the number of peptide motifs (see Radu et al, 1995 and the references cited therein).

The nucleoporin Nup98, containing 16 perfect and imperfect GLFG repeats and 3 FXFG repeats, is located asymmetrically at the nucleoplasmic site of the pore complex in rat cells, and, along with Nup153, is a constituent of the nuclear pore basket structure and/or nucleoplasmic ring (Radu et al, 1995). Karyopherins α/β bind cooperatively to FXFG but not GLFG repeat regions; binding of the NLS-protein/ karyopherin α/β heterodimer to FXFGs stimulated dissociation of the NLS-protein from the karyopherin α/β (Rexach and Blobel, 1995). Nup98 functions as a docking protein via its N-terminal half which contains all of the peptide repeats forming, with peptide repeats of other nucleoporins, an array of sites to mediate docking of nuclear proteins across the pore; these multiple docking sites were suggested to extend over a

distance of 250 nm from the cytoplasmically exposed fibers to the nucleoplasmic baskets (Radu et al, 1995).

Nup133 and Nup145 in *S. cerevisiae* are involved in maintaining the architecture of the nuclear envelope and the position of the pore complex in the nuclear envelope; disruption of their genes leads to clustering of pore complexes. Nup116 in yeast (Nup98 in rat, p97 in *Xenopus*) interacts with Kap95 (karyopherin β in mammals); Nup116 has a number of GLFG repeats which are required for pore function whereas the repeats in Nup49, Nup57, Nup100, and Nup145 are not. Overexpression of Nup116 blocked export of mRNA (Iovine et al, 1995).

Table 2 "Bipartite" or "split" NLS

Signal oligopeptide	Protein and features
C-terminus	Xenopus nucleoplasmin . Deletion analysis demonstrated the presence of a signal responsible for nuclear location.
AVKRPAMINO ACIDTKKAGQAKKK	Xenopus nucleoplasmin
RPAMINO ACIDTKKAGQAKKKKLD	Xenopus nucleoplasmin . Whereas these 17 amino acids had NLS activity, shorter versions of the 17 amino acid sequences were unable to locate pyruvate kinase to the nucleus.
(AVK)RPAMINO ACIDTKKAGQAKKK(KLD)	Xenopus nucleoplasmin . This 14 amino acid segment was identified as a minimal nuclear location sequence but was unable to locate pyruvate kinase to the nucleus; three more amino acids at either end (shown in parenthesis) were needed.
CGQAKKKKLD	Xenopus nucleoplasmin-derived synthetic peptide; crosslinked to chicken serum albumin and microinjected to HeLa cells specified nuclear localization. This suggests that nucleoplasmin may possess a simple NLS.
<u>KRPAMINO ACIDTKKAGQAKKK</u>	Xenopus nucleoplasmin bipartite NLS. Two clusters of basic amino acids (underlined) separated by 10 amino acid are half NLS components.
HRKYEAPRH ₆ PRKR	Yeast L3 ribosomal protein (387 amino acid) N-terminal 21 amino acid. Possible bipartite NLS. (Ribosomal proteins are transported to the nucleus to assemble with nascent rRNA). Fusion genes with β -galactosidase were used to transform yeast cells followed by fluorescence staining with b-gal antibody. The 373 amino acid of L3 fused to β -gal failed to localize to the nucleus, unless a 8 amino acid bridge containing a proline was inserted between L3 and β -gal.
NKKKRKLSRGSSQKTKGTSASAKARH RRNRSSRS (one sequence)	SV40 Vp3 structural protein . (35 amino acid C-terminus). By DEAE-dextran-mediated transfection of TC7 cells with mutated constructs.
RVTIRTVRVRPPKGGKHRK	Simian sarcoma virus v-sis gene product (p28 ^{sis}). The cellular counterpart <i>c-sis</i> gene encodes a precursor of the PDGF B-chain (platelet-derived growth factor). The NLS is 100% conserved between <i>v-sis</i> gene product and PDGF. This protein is normally transported across the ER; introduction of a charged amino acid within the hydrophobic signal peptide results in a mutant protein that is translocated into the nucleus. Pyruvate kinase-NLS fusion product is transported less efficiently than cytoplasmic <i>v-sis</i> mutant proteins to the nucleus.
KRKIEEPEPEPKKAK	Putative bipartite NLS of Xenopus laevis protein factor xnf7 . Inferred by similarity to the bipartite NLS of nucleoplasmin. During oocyte maturation xnf7 is cytoplasmic until mid-blastula—gastrula stage due to high phosphorylation. Partial dephosphorylation results in nuclear accumulation.
KKYENVVIKRS <u>PKR</u> GRPRKD	Yeast SWI5 gene product, a transcription factor. Underlined basic amino acid show similarity to bipartite NLS of Xenopus nucleoplasmin. The SWI5 gene is transcribed during S, G2 and M phases, during which the SWI5 protein remains cytoplasmic due to phosphorylation by CDC28-dependent histone H1 kinase at three serine residues two near and one (double underlined) in the NLS. Translocated at the end of anaphase/G1 due to dephosphorylation of NLS. NLS confers cell cycle-regulated nuclear import of SWI5— β -galactosidase fusion protein.
MKRKRNS 735-741 GIESIDNVMGIGILPDMTPSTEMSMRG VRISKMGVDETSSAEKIV 449-495	Bipartite NLS of influenza virus polymerase basic protein 2 (PB2). Mutational analysis of PB2 and transfection of BHK cells showed that both regions are involved in nuclear import. Deletion of 449-495 region gives perinuclear localization to the cytoplasmic side.
AHRARRLH 6-13 (BSI) PPRRRVRRQPP 23-33 (BSII) PARARRRAP 39-48 (BSIII)	"Tripartite" or "doubly bipartite" NLS of adenovirus DNA polymerase (AdPol). BSI and II functioned interdependently as an NLS for the nuclear targeting of AdPol, for which BSIII was dispensable. BSII-III was more efficient NLS than BSI-II.
KRK ₁₁ <u>KKKSKK</u> 207-226	Human poly(ADP-ribose) polymerase (116 kD). The linear distance between the two basic clusters is not crucial for NLS activity in this bipartite NLS. Lysine 222 (double underlined) is an essential NLS component. DNA binding and poly(ADP-ribosyl)ating active site are independent of NLS.
(GRKRAFHGDDPFEGEGPPDKKGD)	Herpes simplex virus ICP8 protein (infected-cell protein). This C-terminal portion of ICP8 introduced into pyruvate kinase (PK) caused nuclear targeting in transfected Vero cells. Inclusion of additional ICP8 regions to PK led to inhibition of nuclear localization.
<u>KRPREDDDGEPSEKRKRR</u> DDR	Bipartite NLS of VirD2 endonuclease of rhizogenes strains of <i>Agrobacterium tumefaciens</i> . Within the

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C-terminal 34 amino acid. Each region (underlined) independently directs β -glucuronidase to the nucleus, but both motifs are necessary for maximum efficiency. VirD2 is tightly bound to the 5' end of the single stranded DNA transfer intermediate T-strand transferred from *Agrobacterium* to the plant cell genome.

Table 3. "Nonpositive NLS" lacking clusters of arginines/lysines

Signal oligopeptide	Protein and features
<u>QLVW</u> MACNSAMINO <u>ACIDFEDLRVLSFIRG</u> TKVSPRG 327-356	Influenza virus nucleoprotein (NP). The underlined region (327-345) when fused to chimpanzee α_1 -globin at the cDNA level and microinjected into <i>Xenopus</i> oocytes specifies nuclear localization.
MN <u>KIPIK</u> DLLNPQ (NLS1 at N-terminus) VRILESWFAKNIENPYLDT (NLS2 at amino acid 141-159, part of the homeodomain)	Yeast MAT a2 repressor protein , containing a homeodomain. The two NLS are distinct, each capable of targeting β -galactosidase to the nucleus. However, deletion of NLS2 results in a2 accumulation at the pores. NLS1 and 2 may act at different steps in a localization pathway. Part of the homeodomain mediates nuclear localization in addition to DNA binding. The core pentapeptide containing proline and two other hydrophobic amino acids flanked by lysines or arginines (underlined) was suggested as one type of NLS core.
Rx7Kx ₁₅ KIPRx ₃ HFYEERLSWYSDNED 152-206 (C-terminal segment).	<i>Drosophila</i> HP1 (206 amino acids) that binds to heterochromatin and is involved in gene silencing. NLS identified by β -galactosidase/HP1 fusion proteins introduced by P-element mediated transformation into <i>Drosophila</i> embryos.
FVx7-20MxSLxYMx4MF	Adenovirus type 5 E1A internal, developmentally-regulated NLS. This NLS functions in <i>Xenopus</i> oocytes but not in somatic cells. This NLS can be utilized up to the early neurula stage.

Table 4. Nucleolar localization signals (NoLS)

Signal oligopeptide	Protein and features
MP <u>KTRRRPRRSQRKR</u> PPTP	Nucleolus localization signal in amino terminus of human p27^{si} protein (also called Rex) of T cell leukemia virus type I (HTLV-I). When this peptide is fused to N-terminus of β -galactosidase, directs it to the nucleolus. Deletion of residues 2-8 (underlined), 12-18 (double-underline) or substitution of the central RR (dotted-underlined) with TT abolish nucleolar localization. Other amino acids between positions 20-80 increase nucleolar localization efficiency.
RLP <u>VRRRRRRR</u> VP	Adenovirus pTP1 and pTP2 (preterminal proteins, 80 kD) between amino acid residues 362-373. The 140 kD DNA polymerase of adenovirus when it has lost its own NLS can enter the nucleus via its interaction with pTP. The staining was nuclear and nucleolar with some perinuclear staining as well. The NLS fused to the N-terminus of <i>E. coli</i> β -galactosidase was functional in nuclear targeting.
GR <u>KKRRQRRR</u> P	HIV (human immunodeficiency virus) Tat protein; localizes pyruvate kinase to the nucleolus. Tat is constitutively nucleolar.
R <u>KKRRQRRR</u> (AHQ) Nucleolar localization signal	Tat positive trans-activator protein of HIV-1 (human immunodeficiency virus type 1). The 3 amino acids shown in parenthesis are essential for the localization of the β -galactosidase to the nucleolus. The 9 amino acid basic region is able to localize β -gal to the nucleus but not to the nucleolus.
PAMINO ACID <u>KRVKLDQRRR</u> P	Artificial sequence from c-Myc and HIV Tat NLSs that effectively localizes pyruvate kinase to the nucleolus.
F <u>KRKHKKDISQNKRAVRR</u>	Human HSP70 (heat shock protein of 70 kD); localizes pyruvate kinase to the nucleus and nucleolus. HSP70 is physiologically cytoplasmic but with heat-shock HSP70 redistributes to the nucleoli, suggesting that the nucleolar targeting sequence is cryptic at physiological temperature and is revealed under heat-shock.
RQ <u>ARRNRRRW</u> WRERQR (35-50)	HIV-1 Rev protein (116 amino acid; nucleolar). Mutations in either of the two regions of arginine clusters severely impair nuclear localization. β -galactosidase fused to R ₄ W was targeted to the nucleus, and fused to the entire 35-50 region, was targeted to the nucleolus.
RQ <u>ARRNRRRW</u> WRERQRQ (35-51)	HIV-1 Rev protein. A fusion of this Rev peptide with β -galactosidase became nuclear but not nucleolar. The 1-59 amino acid segment of Rev fused to β -galactosidase localized entirely within the nucleolus. Whereas the NRRRRW (bold) is responsible for nuclear targeting, the RR and WRERQRQ (double underlined) specify nucleolar localization. Rev may function to export HIV structural mRNAs from the nucleus to the cytoplasm.

V. Mechanism of nuclear import and translocation across the pore complex

A. Molecular mechanisms

Transport across the pore complex is a two-step process involving binding at a site toward the periphery of the pore, docking of the molecules over the central transporter channels across the lumen of the pore complex, and release to the nucleoplasm (Akey and Goldfarb, 1989; reviewed by Nigg et al, 1991). The guided diffusion involves docking to and undocking from multiple sites across the pore (Radu et al, 1995); the movement of proteins across the pore is a stochastic process operating by means of repeated association-dissociation reactions of the NLS-protein with FXFG repeats on nucleoporins (Rexach and Blobel, 1995). Only the translocation step requires ATP (Richardson et al, 1988). The mechanism of export involves mRNA complexed with proteins containing NLSs forming a large globular mRNP (Mehlin et al, 1992); export might be specified by the presence of a large polyanion (RNA) in the complex whereas import might be specified by the NLS in the protein.

A single transport gate is located in the central domain of the transporter located within the pore complex that restricts passive diffusion; this was shown using small gold particles coated with polyethylene glycol (PEG; total particle diameter 40-70 Å) or large PEG-particles (total diameter 110-270 Å) which were microinjected into the cytoplasm or nucleoplasm of *Xenopus* oocytes; cytoplasmic injections of small gold particles showed that the particles were approximately 11 times more concentrated in the cytoplasmic half of the transporter structure whereas the particles were approximately 7 times more concentrated in the nuclear half after nuclear injections. Larger particles were less mobile and after cytoplasmic injection migrated to the surface of the pore complex, but entered the transporter less frequently (Feldherr and Akin, 1997).

Macromolecules are poor electrical charge carriers and this can be exploited to detect their movement along electrolyte-filled pores: translocating macromolecules reduce the net conductivity of the medium inside the pore; lesser values of ion conductance indicate greater macromolecular translocation (in size and/or number). This is the principle used in Coulter counter, an instrument for counting and sizing particles. The principle that ion flow is restricted during translocation of macromolecules containing nuclear targeting signals was demonstrated by Bustamante et al (1995). At least four soluble transport proteins have been identified recently: the α subunit of karyopherin involved in NLS binding (a number of other candidate NLS-binding proteins are known or might be

discovered in the future), the β subunit of karyopherin, the Ran, and p10 (Rexach and Blobel, 1995; Radu et al, 1995; Nehrbass and Blobel, 1996).

B. Components of the soluble import machinery

1. The α subunit of karyopherin

The α subunit of karyopherin, equivalent to the 60 kDa importin (Görlich et al, 1994, 1995a,b) and to SRP1 and SRP1a recognizes and binds the NLS peptide of the protein to be imported in the cytoplasm. Karyopherin α accompanies the proteins to be imported from their site of synthesis through the pores to the sites of their function in the nucleus (Görlich et al, 1995b). Two other cytosolic proteins with molecular weights of 56 and 66 kDa have been identified, along with the 66 and 90 kDa karyopherins, to form with NLS-protein a five protein complex (Imamoto et al, 1995). Karyopherins α and β cooperate to bind to FXFG but not GLFG repeats on nucleoporins (Rexach and Blobel, 1995).

Görlich and coworkers (Görlich et al, 1994, 1995b) have identified the 60 and 90 kDa **importin** subunits in both *Xenopus* and human cells corresponding to karyopherins α and β (Moroianu et al, 1996); together they constitute a cytosolic receptor for NLS binding; both subunits appear bound to the pore complex but only the larger subunit enters the nuclear interior. The 60 kDa subunit shows homology to *S. cerevisiae* **SRP1**, a pore complex protein that contributes to the maintenance of the nucleolar structure (Yano et al, 1992, 1994). Importin- α mediates nuclear protein import by binding nuclear localization signals and importin- β . A role for the α subunit of importin in RNP export has been considered (Laskey et al, 1996).

The second human homolog of yeast SRP1, hSRP1, was identified using the yeast two-hybrid system (Zervos et al, 1993) because of its interaction with a RAG-1 activator of V(D)J recombination in immunoglobulin genes (Cortes et al, 1994); the domain of RAG-1 interacting with hSRP1 was not required for recombination (Cortes et al, 1994). hSRP1 contains eight degenerate repeats of 40-45 amino acids four of which (repeats 4-7 between amino acids 245 and 437 not including the acidic stretches of the molecule) are involved in interaction with RAG-1 (Cortes et al, 1994); these repeats are known as arm motifs, have been found in other proteins, and are involved in specific protein-protein interactions (Peifer et al, 1994). For example, arm motifs participate in the interaction between the tumor suppressor adenomatous polyposis coli and β -catenin (Rubinfeld et al, 1993; Su et al, 1993). The human SRP1, interacting with

RAG-1 and perhaps also with RNA polymerases, was proposed to localize recombination and transcription near the pore complex providing the anchoring activity and assembling components essential for these processes (Cortes et al, 1994).

SRP1 interacts with the Nup1p and Nup2p nuclear pore proteins, is also implicated in the correct orientation of mitotic tubulin spindles, and has been proposed to anchor structural cytoplasmic components to pores thereby organizing proper nuclear matrix structures (Yano et al, 1994). SRP1 and importin 60 are also homologous to **Rch1**, a protein that interacts with the immunoglobulin gene recombinase RAG-1 perhaps via the NLS of RAG-1 (Cuomo et al, 1994).

2. The β subunit of karyopherin

The β subunit of karyopherin, equivalent to the 90 kDa importin (Görlich et al, 1994, 1995a,b) and to Kap95 in yeast binds to the karyopherin α /NLS-protein complex in the cytoplasm and mediates docking of the complex to nucleoporins with repetitive tetrapeptide motifs (Iovine et al, 1995; Radu et al, 1995; Weis et al, 1995; Moroianu et al, 1996). Karyopherin β enhances binding of karyopherin α to NLS-protein (Rexach and Blobel, 1995). Only the β subunit is able to bind pores and binding of the α subunit to the pore depends on karyopherin β ; karyopherin β moves only to a distance of 100 nm from its initial cytoplasmic docking site but remains associated with pores and does not appear in the nucleoplasm (Görlich et al, 1994, 1995a,b). The FXFG repeats on nucleoporins appear to stimulate the dissociation of the NLS-protein from the karyopherin α/β heterodimer (Rexach and Blobel, 1995).

3. RanGTP

Ran (in complex with p10) release the docked complex by displacing karyopherin α /NLS-protein; RanGTP and karyopherin α bind to overlapping sites on karyopherin β ; a cluster of basic residues on karyopherin β are the binding sites for RanGTP and karyopherin α (Moroianu et al, 1996). The small GTPase Ran executes the energy-dependent step of translocation across the pore complex, results in accumulation of import substrate and karyopherin α in the nucleus, and in the retention of karyopherin β in the pore complex on both sides of the nuclear pore; in the absence of Ran or energy, karyopherin α accumulates in the pore but not in the nucleoplasm in permeabilized HeLa cells (Görlich et al, 1995a,b). Ran causes the dissociation of the NLS-protein/ karyopherin α from the karyopherin β (Rexach and Blobel, 1995; Paschal and Gerace, 1995); incubation of RanGTP with karyopherin α/β heterodimer led to the dissociation of the α subunit and to the association of the β subunit with Ran; RanGDP had no effect (Rexach and Blobel, 1995).

Ran/TC4 is absolutely required for the efficient transport (Moore and Blobel, 1993; Görlich et al, 1995a,b). Ran requires the p10 protein as an active component for its efficient functioning (Nehrbass and Blobel, 1996).

The binding determinants of karyopherin β for Ran-GTP are similar to Ran BP1, a cytoplasmic Ran-GTP-binding protein, (Coutavas et al, 1993) and to similar domains on nucleoporin Nup 358 (Yokoyama et al, 1995). Displacement of Ran-GTP from karyopherin β may be a requisite for GTP hydrolysis by Ran-GAP (Floer and Blobel, 1996) and may serve to recycle karyopherin β .

4. The p10 protein

The p10 protein can associate with Ran-GDP (but not to Ran-GTP) and to karyopherin β . p10 binds to nucleoporins possessing peptide repeats (Nehrbass and Blobel, 1996). Addition of GTP to the p10/nucleoporin/Ran-GDP/karyopherin α/β complex resulted in formation of Ran-GTP causing dissociation of karyopherin α leaving the karyopherin β bound to nucleoporin (Nehrbass and Blobel, 1996). Release of karyopherin α /NLS-protein then allows the protein to be imported and karyopherin α to diffuse into the nucleus across the central plug (Görlich et al, 1995a,b).

The entrance of karyopherin α in the nucleus is consistent with the model of a shuttling nuclear import receptor (Adam et al, 1989). Dissociation of the NLS-protein from karyopherin α in the nucleoplasm might be mediated by a difference in the ionic environment between the nucleoplasm and the cytoplasm (Boulikas, 1994), by association of karyopherin α with other nuclear factors (Görlich et al, 1995b), by association of karyopherin α with shuttling proteins during their exit from the nucleus (Schmidt-Zachmann et al, 1993), or by phosphorylation of karyopherin α by a mammalian homolog of the yeast SRP1 kinase (see Radu et al, 1995; Moroianu et al, 1996).

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C. A summary on the translocation process

In summary, protein translocation from the cytoplasm to the nucleoplasm involves the following steps (Nehrbass and Blobel, 1996; Moroianu et al, 1996) (**Figure 5**):

(i). A weak complex of karyopherin α /NLS-protein is formed in the cytoplasm.

(ii). Karyopherin β interacts with karyopherin α forming a strong karyopherin β / α /NLS-protein complex. Additional proteins may participate to the formation of a larger cytoplasmic complex (Imamoto et al, 1995).

(iii). The complex binds to FXFG peptide repeats on nucleoporins at the cytoplasmic side of the pore complex via karyopherin β . The FXFG repeats may dissociate the NLS-protein from the karyopherin α / β .

(iv). p10 docks Ran-GDP to nucleoporin and to the karyopherin heterodimer.

(v). A number of association-dissociation reactions on nucleoporins dock the import substrate toward the nucleoplasmic side; this process requires the GTPase Ran and p10.

(vi). A GDP-GTP exchange reaction takes place transforming Ran-GDP into Ran-GTP catalyzed by karyopherin α which shares sequence homology with a GDP-GTP exchange factor of Ras (Görlich et al, 1994; Peifer et al, 1994). A cytosolic Ran-GTPase activating protein (Ran-GAP) in yeast has been found keeping Ran primarily in the GDP-bound form. Ran-GTP is a secondary product found locally at the pore.

(vii). Ran-GTP (but not Ran-GDP) causes dissociation of the heterodimeric α / β complex by binding to karyopherin β thus releasing the karyopherin α /NLS-protein.

(viii). A complex of karyopherin α /NLS-protein diffuses into the nucleoplasm whereas karyopherin β remains bound to the pore (because of its affinity to FXFG repeats and p10).

Shuttling proteins might contribute to coordinating nucleocytoplasmic import/export; these proteins include nucleolin and NO38 (Borer et al 1989), two hsp70-related proteins in *Xenopus* oocytes (Mandell and Feldherr 1990), the A1 pre-mRNA binding protein (Piñol-Roma and Dreyfuss 1992), Nopp 140 (Meier and Blobel, 1992), progesterone receptor (Guiochon-Mantel et al, 1991), La antigen, and several protein kinases (reviewed in Boulikas 1993, 1996). A cap-binding protein has been identified that might mediate export of RNA polymerase II transcripts (Izaurrealde et al, 1992).

In conclusion, several independent import/export pathways seem to operate in the same cell. Nonbound nucleolin is exported from nuclei and the rate of export is determined by structural domains involved in interactions in the nucleolus; a fusion construct between the cytoplasmic pyruvate kinase and the NLS of T antigen is able to shuttle between nucleus and cytoplasm; lamin B₂, a normally nuclear protein, can be converted into a shuttling protein by introducing mutations on its nuclear signal (Schmidt-Zachmann et al, 1993).

D. Import of influenza virus ribonucleoproteins

Influenza virus is unusual among RNA viruses in that it replicates in the nucleus. When infecting cells it first binds to receptors containing sialic acid and is then internalized by receptor-mediated endocytosis into late endosomes. A conformational change of peptides on hemagglutinin (HA) spikes at the lower pH of the endosome (pH 5.5) causes disruption of the endosomal membrane and release of the virus into the cytoplasm (reviewed by Bui et al, 1996). The fusogenic peptides of HA protein of influenza virus have been exploited in gene therapy for the efficient release of DNA-cationic polymer complexes from endosomes (see Fusogenic peptides in Boulikas, 1998, pages 1-172, this volume).

Since the genome is segmented, eight separate helical viral RNPs are formed containing the antisense viral RNA and numerous copies of the 56 kDa (one every 20 nucleotides); other viral proteins in the complex include the three subunits of the polymerase and the 27 kDa M1 viral matrix protein which is released from the vRNPs, presumably in the acid pH of the endosome (see Bui et al, 1996 and the references cited therein). This dissociation step is essential for nuclear import of the vRNPs; two anti-influenza virus drugs, amantadine and rimantadine, inhibit the dissociation of M1 protein from vRNPs. M1 assumes a master regulatory role for the transport of vRNPs across the cell membrane; however, the association of vRNPs with M1 inhibits their nuclear transportation across the pore complex; acidification of the cytosolic compartment caused dissociation of M1 from vRNPs and eliminated the inhibition in import.

M1 appears to prevent reimport of vRNPs into the nucleus of the infected cell and thus commits them to an assembly pathway leading to the budding of the virus particles at the cell membrane (Bui et al, 1996). When M1 was dissociated from vRNPs at late times during infection the vRNPs failed to be reimported into nuclei; cell fusion techniques, however, have shown that vRNPs which were dissociated from M1 in acid pH were import competent in the uninfected nucleus; for some unknown reason, infected nuclei, although capable of general nuclear import were no longer able to import vRNPs (Bui et al, 1996). The Hepatitis B virus that, like influenza virus, replicates in the nucleus can be reimported into the nucleus in infected cells, a fact that may explain the chronic nature of Hepatitis B infection.

VI. Regulated protein import

A number of processes have been found to be regulated by nuclear import. These include: the **NF- κ B** translocation; the import of **Dorsal** protein in dorsal but not ventral compartments of *Drosophila* embryos, a

process playing a decisive role in cell type establishment during morphogenesis; the nuclear import of the factors **rNFIL-6** and **ISGF3** after their phosphorylation in the cytoplasm; the *Xenopus* **nuclear factor 7** which is retained in the cytoplasm from fertilization through the mid-blastula stage (Li et al, 1994); and the import of a number of protein kinases including **casein kinase II**, the catalytic subunit of **cAMP-dependent protein kinase II** following stimulation of cells with cAMP, the **RSK** after its phosphorylation by MAP kinase, **protein kinase C** after stimulation of cells with the phorbol ester TPA, and the Mitogen-Activated Protein kinases (MAPK) **ERK1** and **ERK2** following stimulation of cells with growth factors or mitogens, (reviewed by Boulikas, 1995).

MAPK is activated in cytoplasm by MAPK kinase (MAPKK) in response to extracellular signals; whereas MAPK then is translocated to nucleus, MAPKK remains cytoplasmic because of a NES in the N-terminal region (residues 32-44) rich in leucine residues; the NES peptide of MAPKK, inhibited the nuclear export of ovalbumin (Fukuda et al, 1996).

The NLS of the serum response factor (**SRF**) is in close proximity to potential phosphorylation sites for the cAMP-dependent protein kinase (A-kinase) and nuclear transport of SRF proteins requires basal A-kinase activity (Gauthier-Rouviere et al, 1995).

The NLS needs to be exposed on the surface of the protein and available for binding to nuclear transporter protein molecules. The SV40 large T protein NLS is nonfunctional when it is located in a region of pyruvate kinase predicted not to be exposed to the surface (Roberts et al., 1987). In addition, nuclear proteins have been identified which are synthesized in the form of precursor molecules which remain in the cytoplasm, presumably because their NLS is hidden. Cleavage of such proteins into mature molecules by specific proteases exposes their NLS, and they are then rapidly transported to the nucleus. As an example, the p50 subunit of the transcription factor **NF- κ B** (50 kD) is synthesized in the form of a 110 kD precursor with the NLS buried in the protein; proteolytic cleavage giving the 50 kD transcription factor exposes the NLS (Henkel et al., 1992) and facilitates nuclear localization.

Nuclear translocation of protein factors, presumably by exposure of their hidden NLS or by reconstitution of a functional NLS from two remote half NLS, can be triggered by phosphorylation (Shelton and Wasserman, 1993), dephosphorylation (Moll et al., 1991; Nasmyth et al., 1990), subunit association (Levy et al., 1989), or by dissociation of an inhibitory subunit (Baeuerle and Baltimore, 1988a,b). Interferon- α regulates nuclear translocation of the transcription factor ISGF3 (Kessler et al., 1990). Thus, distant peptide regions can either "mask" the NLS or anchor the protein in the cytoplasm; examples of proteins regulated in this manner include **protein**

kinase Ca (James and Olson, 1992), human **cyclins A and B1** (Pines and Hunter, 1991), and **c-Fos** (Roux et al., 1990). In other cases, binding of hormone to a cytoplasmic protein such as **glucocorticoid receptor (GR)** induces a conformational change that exposes the NLS, or reconstitutes a functional NLS from remote half NLSs, and the protein is rapidly transported to the nucleus (Picard and Yamamoto, 1987). The ability of GRs to bind DNA is an important determinant for localization and tight binding of GR to the nucleus; mutant GRs localized to the nucleus were only weakly associated with the nuclear compartment (Sackey et al, 1996). However, the related estrogen receptor and retinoic acid receptor, contrary to the glucocorticoid receptor, are nuclear even in the absence of their receptor hormone/morphogen (Picard et al., 1990). An unusual case has been described by Lutz and collaborators (1992) where prenylation of the C-terminus of **prelamin A** by addition of a 15-carbon or 20-carbon isoprenoid, a posttranslational modification that functions in the proteolytic processing of prelamin A to lamin A in the nucleus, is also required for its nuclear import.

VII. Deregulation in nuclear import and molecular carcinogenesis

Several studies have provided a link between the deregulation in nuclear import mechanisms of specific proteins and cancer. The **BRCA1** breast cancer marker protein was found to be mainly localized in the cytoplasm in 16 of 17 breast and ovarian cancer lines and in 17 of 17 samples of cells from malignant effusions whereas in normal cells the protein was nuclear (Chen et al, 1995).

The transforming oncoprotein **v-Abl** of Abelson murine leukemia virus, a mutated form of the c-Abl nonreceptor tyrosine kinase, is a fusion protein in which portions of the retroviral Gag protein replace the N-terminal SH3 domain of c-Abl; this results in loss of phosphorylation sites in v-Abl that down-regulate its activity and render the tyrosine kinase activity of v-Abl constitutive; in addition, the viral Gag sequence provides a **myristoylation** site on v-Abl which confers a predominantly inner plasma membrane anchorage whereas c-Abl is predominantly located in the nucleus (Wong et al, 1995). Both of these properties of v-Abl, not found in c-Abl, contribute to its ability to transform cells.

VIII. RNA and protein export

A. An historical perspective

The concepts that (i) transcription occurs preferentially at the nuclear periphery near pores versus (ii) transcription occurring at any location within nuclei both have been entertained and data to support either model are available. Transcripts from the interior of nuclei have been

visualized passing through channels originating at the sites of transcription within the interior of the nucleus and emanating to the pore complex (Huang and Spector, 1991). RNA transcripts seem to be exported vectorially toward a single pore or a small subset of nuclear pores (Lawrence et al., 1989). This vectorial export of RNA might contribute to an asymmetric mRNA distribution within the cytoplasm consistent with experimental evidence (Weeks and Melton, 1987). Foci where splicing of pre-mRNA takes place are often seen associated with pore complexes via morphologically distinct channels; U3 snRNA was exclusively detected in the nucleolus and U2, U5 and U6 snRNAs were in discrete nucleoplasmic foci (Carmo-Fonseca et al, 1991).

RNA export is facilitated by proteins that shuttle between nucleus and cytoplasm. Assays based on interspecies heterokaryons and microinjection of *Xenopus* oocytes using nucleolin, mutant lamins, differing in their abilities to be incorporated into the lamina and pyruvate kinase-NLS artificial reporter protein, have shown that proteins unable to interact with large nuclear structures can be exported from nuclei; protein export was suggested not to require any export signals (Schmidt-Zachmann et al, 1993). However, recently a nuclear export sequence (NES) has been identified in proteins that are exported actively from the nucleus such as **Rev** and **PKI** (Fischer et al, 1995; Wen et al, 1995; reviewed by Gerace, 1995; Izaurralde and Mattaj, 1995). Several RNA-binding proteins have been suggested to be involved in RNA export. Export of 5SRNA requires interactions with ribosomal protein L5 or TFIIIA (Guddat et al, 1990). Export of influenza virus RNA-protein complex requires the viral protein M1 which also prevents the nuclear re-import of the viral RNA (Martin and Helenius, 1991).

Export of tRNA is a translocation process mediated by protein carrier(s) (Zaslhoff, 1983). tRNA molecules undergo a complex maturation processing involving trimming of the 5' and 3' ends, addition of three terminal CCA residues, and base modification; removal of introns (only 20% of tRNAs contain introns) is a highly regulated process occurring in association with the inner side of the nuclear envelope close to the pores (for references see Simos et al, 1996). Studies in yeast have shown that Los1 (required for pre-tRNA splicing) and Pus1 (involved in tRNA biogenesis) interact with the pore complex protein Nsp1; this involves pore proteins in the splicing of pre-tRNA (Simos et al, 1996).

Ribosomal subunits are assembled in the nucleus from imported ribosomal proteins; export of ribosomal subunits to the site of their function (cytoplasm) is energy-dependent (Khanna-Gupta and Ware 1989; Bataillé et al 1990). The nucleoside triphosphatase of nuclear envelope might be involved in the nucleocytoplasmic translocation of ribonucleoprotein (Agutter et al 1976).

The mechanism of export of mRNA deduced by microinjection of *Xenopus* oocytes is also an energy-dependent process requiring the 5' cap structure (Hamm and Mattaj, 1990; Dargemont and Kuhn, 1992). RNP particles become attached to filaments which project into the nucleoplasm and which guide the particles to the pores. The central channel can expand to permit export of large complexes such as ribosomal subunits and mRNPs and import of large nuclear proteins.

RNA export comprises (i) initial binding to the tentacles of the central plug (ii) energy-dependent translocation toward the cytoplasmic side of the pore complex (Mehlin et al, 1992). Some asymmetries between the cytoplasmic and nucleoplasmic rings have been described; for example, the cytoplasmic ring appears larger and the nucleoplasmic filaments of the pore are longer, forming a basket structure. One of the nucleoporins is attached to the nucleoplasmic side (Snow et al, 1987). These asymmetries are likely to contribute to the RNP export distinct from protein import into nuclei (Mehlin et al, 1992).

Electron microscope tomography has examined the export of large RNPs in the salivary glands of the dipteran *Chironomus*. A 75S pre-mRNA is transcribed from the Balbiani ring granules in *Chironomus tentans* and is packed into RNP ribbon particles, 30-60 nm broad and 10-15 nm thick, bent into a ring conformation; during export the particle is first oriented in a specific manner by specific recognition signals and subsequently the bent ribbon is gradually straightened and exported through the pore with the 5' end of RNA in the lead (Mehlin et al, 1992). Upon passage through pores, the proteins of the pre-mRNP dissociate; the protein composition of the particle in the nucleus and cytoplasm is different. The particle then unfolds and becomes associated with ribosomes (Mehlin et al, 1992).

Some early studies showed that no specific nuclear export signals are required. Schmidt-Zachmann et al (1993) arrived to the conclusion that a protein does not require positively acting export signals to be transported from nucleus to cytoplasm but instead, its shuttling ability is limited primarily by intranuclear interactions.

Expression of some proteins can inhibit mRNA export; the mechanism could be exerted at the splicing step rather than on the actual translocation process. Expression of NS1 protein (which is encoded by the influenza virus RNA segment 8 along with NS2 produced from the same transcript as NS1 by differential splicing) in influenza virus-infected cells induced a generalized block of mRNA export from the nucleus; NS1 mRNA, NS2 mRNA and other mRNAs were retained in the nucleus of cells expressing NS1 protein, but no effect was observed when only NS2 protein was expressed (Fortes et al, 1994).

B. Identification of NLS-independent import pathways

Proteins lacking basic type of NLS are thought to piggy-back into the nucleus in association with NLS-containing molecules. However, a new type of receptor molecule mediates nuclear import in a basic NLS-independent manner (Pollard et al, 1996; Aitchison et al, 1996; reviewed by Dingwall, 1996). This model applies to the import of hnRNPA1 molecules (Pollard et al, 1996) and to the import nuclear mRNA-binding proteins in *Saccharomyces cerevisiae* which have been isolated as a complex with Kap104 (Aitchison et al, 1996).

The cytosolic yeast karyopherin, Kap104p, acts for returning mRNA binding proteins to the nucleus after mRNA export. Indeed, Kap104p binds directly to repeat-containing nucleoporins and to the mRNA binding proteins, Nab2p and Nab4p, and functions for their nuclear import; depletion of Kap104p resulted in a rapid shift of Nab2p from the nucleus to the cytoplasm without affecting the localization of other nuclear proteins (Aitchison et al, 1996).

C. Nuclear export signals (NES) and export of mRNA: recent studies

According to Guiochon-Mantel et al (1994) the NLS of the progesterone receptor or the NLS of T antigen were shown to impart to β -galactosidase the ability to shuttle between the nucleus and the cytoplasm; microinjected proteins devoid of a nuclear localization signal were unable to exit from the nucleus. The authors thought that the nuclear import requires energy whereas the nuclear export does not and this determines whether the NLS will function as an import or export signal.

The discovery of nuclear export signals (NESs) in a number of proteins revealed the occurrence of signal-dependent transport of proteins from the nucleus to the cytoplasm. The consensus motif of the NESs is a leucine-rich, short amino-acid sequence. The NES is defined by its ability to translocate a protein from the nucleus to the cytoplasm when the two are tethered by a membrane-permeable ligand (Klemm et al, 1997). The majority of proteins with NES are RNA-binding proteins which bind to and escort RNAs to the cytoplasm; nuclear export of RNA molecules is likely to be driven by protein-based nuclear export signals (reviewed by Nakielny and Dreyfuss, 1997; Lee and Silver, 1997). Nascent pre-mRNAs associate with the abundant hnRNPs and remain associated with them throughout the time they are in the nucleus. One group of HnRNPs is strictly nuclear in interphase cells (for example hnRNP C proteins), whereas the other group, although primarily nuclear at steady state, shuttles between the nucleus and the cytoplasm via NES; NES-bearing hnRNP proteins are mediators of mRNA export (Nakielny and Dreyfuss, 1996).

Microinjection into the nucleoplasm of *Xenopus* oocytes of PEG-coated gold particles showed that these were coated with protein containing nuclear export signals (NES) suggesting that the NES is not only required for translocation, but also for migration within the nucleoplasm (Feldherr and Akin, 1997).

Nuclear trafficking of the catalytic (C) subunit of cAMP-dependent protein kinase (cAPK) is regulated by the heat-stable inhibitor (Pkl) of cAPK which contains a nuclear export signal (NES) (residues 35-49). Pkl has no obvious association with RNA. The core NES of Pkl comprises only residues 37-46, **LALKLAGLDI** and is able to trigger rapid, active net extrusion of the C-PKI complex from the nucleus.

The identification of NES have established a novel mechanism for regulation of gene expression: nuclear export of pre-mRNA can contribute to the regulation of gene expression. The processing of transcripts of TNF- β and β -globin was found to be regulated by the signal transduction pathway that includes the **Src** protein; Src seems to act on a general mechanism of splicing and/or mRNA transport. This regulation could involve RNA-binding proteins, which interact with Src (Neel et al, 1995).

D. CRM1 or exportin 1 binds NES

A protein of 110 kDa (CRM1 or exportin 1 or XPO1) was identified in *Xenopus* oocyte extracts that binds to the intact NES but not to the mutated, non-functional NES. CRM1 is an essential mediator of the NES-dependent nuclear export of proteins in eukaryotic cells (Fukuda et al, 1997). CRM1 is an evolutionarily conserved protein, shown to , originally found as an essential nuclear protein in fission yeast; *S. cerevisiae* CRM1 shows homology to importin β -like transport factors and was shown to be an essential mediator of nuclear protein export in *S. cerevisiae* (Stade et al, 1997). The cytotoxin leptomycin B which inhibits the NES-mediated transport of **Rev** protein inhibited the binding of *Xenopus* CRM1 to NES (Fukuda et al, 1997).

Overexpression of CRM1 in *Xenopus* oocytes stimulated Rev and U snRNA export from the nucleus and this process was inhibited by leptomycin B, a cytotoxin that was shown to bind to CRM1 protein; CRM1 was able to form a complex involving cooperative binding of both RanGTP and the nuclear export signal (NES) from either the **Rev** or PKI proteins implicating RanGTP in nuclear export (Stade et al, 1997; Fornerod et al, 1997). A mutation in the shuttling protein Crm1p affects not only protein export, but also mRNA export, indicating that these pathways are tightly coupled in *S. cerevisiae* (Stade et al, 1997).

Retroviruses export unspliced, intron-containing RNA to the cytoplasm of infected cells despite the fact that

intron-containing cellular RNAs cannot be exported; in HIV-1 this is accomplished by **Rev** which binds to elements in the viral RNA; in the absence of Rev, these intron-containing HIV-1 RNAs are retained in the nucleus (Zhang et al, 1996). The NES on Rev is the sequence **LQLPPLERLTL** (Wen et al, 1995). Visualization of viral transcripts using oligonucleotide probes specific for the unspliced or spliced forms of a particular HIV-1 viral RNA showed that in the absence of Rev, the unspliced HIV-1 viral RNAs were predominantly nuclear and were distributed (i) as approximately 10-20 intranuclear punctate signals of nascent transcripts and (ii) as a stable population of viral transcripts dispersed throughout the nucleoplasm excluding nucleoli (Zhang et al, 1996).

Kim and coworkers (1996) have pinpointed the NES on HTLV-1 **Rex** that fully complements HIV-1 Rev as a stretch of 17 amino acids; four leucines within the minimal region were essential for NES function; this NES peptide could serve as nuclear export signal when conjugated with bovine serum albumin.

The genome of the simpler retrovirus Mason-Pfizer monkey virus (MPMV) contains an element that serves as an autonomous nuclear export signal for intron-containing viral and cellular RNA through interaction with endogenous cellular factors; the same element is also essential for MPMV replication (Ernst et al, 1997).

E. Transportin is distinct from karyopherin β (importin)

A novel 38 amino acid transport signal was identified by Pollard and coworkers (1996) in the hnRNP A1 protein (which shuttles rapidly between the nucleus and the cytoplasm), termed M9, which confers bidirectional transport across the nuclear envelope. Furthermore, a specific M9-interacting protein, termed transportin, binds to wild-type M9. Transportin is a 90 kDa protein, distantly related to karyopherin β which also participates in mRNA export in a complex with hnRNP A1 and mRNA. Thus, it appears that there are at least two receptor-mediated nuclear protein import pathways.

Transportin mediates the nuclear import of additional hnRNP proteins, including hnRNP F. A novel transportin homolog, transportin 2, which may differ from transportin 1 in its substrate specificity has also been identified and sequenced (Siomi et al, 1997). Because transportin 1 is localized both in the cytoplasm and the nucleoplasm and a pyruvate kinase-M9 fusion, which normally localizes in the nucleus, accumulates in the cytoplasm when RNA polymerase II is inhibited, it seems that the M9 signal is a specific sensor for transcription-dependent nuclear transport. Consistent with *in vitro* data A1 dissociates from transportin 1 by RanGTP after nuclear import and becomes incorporated into hnRNP complexes, where A1 functions in pre-mRNA processing (Siomi et al, 1997).

A novel human protein, termed MIP (101 kDa) which bears significant homology to human karyopherin/importin- β , binds M9 specifically; cytoplasmic microinjection of a truncated form of MIP that retains the M9 binding site blocked the *in vivo* nuclear import of a substrate containing the M9 without affecting the import of basic NLS-bearing substrates (Fridell et al, 1997).

The shuttling **hnRNP K** protein contains also a novel shuttling domain (termed **KNS**) which has many of the characteristics of M9, in that it confers bi-directional transport across the nuclear envelope. KNS-mediated nuclear import is dependent on RNA polymerase II transcription, and a classical NLS can override this effect. Furthermore, KNS accesses a separate import pathway from either classical NLSs or M9 demonstrating the existence of a third protein import pathway into the nucleus (Michael et al, 1997).

IX. Regulated nuclear import and export

A. Proteins with NLS and NES

The transcription factor **NF-ATc** plays a key role in the activation of many early immune response genes and is regulated by subcellular localization. NF-ATc translocates from the cytoplasm to the nucleus in response to a rise in intracellular calcium. Calcineurin dephosphorylates conserved serine residues in the amino terminus of NF-AT, resulting in nuclear import (Beals et al, 1997). NF-ATc immediately returns to the cytoplasm when intracellular calcium levels fall a process mediated by a NES; glycogen synthase kinase-3 (GSK-3) phosphorylates conserved serines necessary for nuclear export and opposing Ca^{2+} /calcineurin signaling (Klemm et al, 1997; Beals et al, 1997).

The distribution of the **v-Rel** oncoprotein between the nucleus and the cytoplasm was experimentally manipulated using NLS and NES; the respective abilities of the v-Rel to localize to the nucleus in chicken embryo fibroblasts, to activate κB -dependent transcription in yeast, and to transform avian lymphoid cells were each markedly reduced by the fusion of a cis-acting NES onto v-Rel; the oncogenic properties of v-Rel were manifested only after a threshold of this protein in the nucleus was attained (Sachdev et al, 1997).

Fluorescein iodoacetamide-labeled human **p53**, injected into the cytoplasm or nuclei of 3T3 cells, was imported into or exported from nuclei within minutes. Import was inhibited by co-injection of the lectin wheat germ agglutinine (WGA). In contrast, the protein HSA conjugated with the T antigen NLS was only imported but not exported. These studies demonstrate the presence of NES on p53 in addition to import signals (NLS) and

provide new views for its implication in carcinogenesis (Middeler et al, 1997).

The subcellular localization and activity of **c-Abl** nonreceptor tyrosine kinase is regulated by cell adhesion. Upon adhesion of fibroblasts to fibronectin (an extracellular matrix protein) there is a coincident export of c-Abl from the nucleus to the cytoplasm. The cytoplasmic pool of c-Abl is reactivated within 5 min of adhesion and the activated cytoplasmic c-Abl becomes nuclear after 30 min. Thus, c-Abl can transmit integrin signals to the nucleus where it may integrate these to cell cycle signals (Lewis et al, 1996).

Fragile X syndrome, a leading cause of inherited mental retardation, is attributable to the unstable expansion of a CGG-repeat within the FMR1 (Fragile X syndrome Mental Retardation) gene; the encoded protein (**FMRP**) is a ribosome-associated RNA-binding protein that contains both NLS and NES. Immuno-gold studies provided evidence of nucleocytoplasmic shuttling of FMRP, which was localized in neuronal nucleoplasm and within nuclear pores. FMRP was highly expressed in neurons but not glia throughout the rat brain; the dendritic localization of FMRP implicated this ribosomal protein in the translation of proteins involved in dendritic structure or function that could relate to the mental retardation occurring in fragile X syndrome (Feng et al, 1997).

B. Import/export of HnRNPs

Heterogeneous nuclear ribonucleoproteins (HnRNPs) is a group of 20 different hnRNP proteins designated RNPA to RNP. Among these the C1, C2, and U molecules possess the basic NLS. However, the group A molecules do not. In spite of this, a major group of hnRNP proteins constantly shuttle between the nucleus and the cytoplasm (Michael et al, 1995; Pollard et al, 1996). HnRNPs can be divided into those that remain always nuclear and those that shuttle between the cytoplasm and the nucleus; the association of mRNA with those that possess NES and shuttle is believed to be largely responsible for mRNA export from the nucleus. hnRNP C proteins are restricted to the nucleus not because they lack an NES, but because they bear a nuclear retention sequence (**NRS**) that is capable of overriding NESs (Nakielny and Dreyfuss, 1996). The NRS in hnRNP C1 is a stretch of 78 amino acids; it was proposed that removal of NRS-containing hnRNP proteins from pre-mRNA is an essential step for mRNA export (Nakielny and Dreyfuss, 1996).

The total number of hnRNPA1 and hnRNPA2 molecules in each HeLa cell nucleus is in the order of 70-90 millions; during mitosis these proteins are released into the cytoplasm and are reimported after biogenesis of the new nuclear envelopes around the daughter cell nuclei. The import rate for these molecules could be 500 molecules per pore per minute assuming one hour for re-

accumulation of the hnRNPA molecules in the nucleus (Dingwall, 1996). HnRNPA1 is one of a set of hnRNP proteins that do not possess an NLS. A stretch of 38 amino acids in A1, which interacts with a human protein called transportin, is both necessary and sufficient for nuclear import. Yeast Kap104 seems to be the analog of transportin and both display a region with homology to a domain in importin- β (Görlich and Mattaj, 1996) which might interact with similar domain in nucleoporins to mediate docking of the transportin-hnRNPA1 or Kap104-protein complex through the pore (Aitchison et al, 1996).

X. Import and export of U snRNPs

In contrast to the concept of export of mRNPs there are cases where RNA-protein complexes are imported into nuclei. Small nuclear ribonucleoprotein particles (snRNPs) in particular U1 snRNPs, are assembled in the cytoplasm and are then imported into nuclei to facilitate splicing. Import of snRNPs and proteins may involve distinct pathways (Fischer et al 1991; Michaud and Goldfarb, 1992). The import of U1 snRNPs requires a trimethyl-G cap structure as well as protein binding to the Sm domain of U1 snRNA (Hamm et al, 1990; Fischer and Lüthmann, 1990).

Recently a role of the yeast importin- α (SRP1p) in nuclear export of capped U snRNAs has been unraveled in a remarkable series of events. Approximately 30% of SRP1p were found in a nuclear complex with the *Saccharomyces cerevisiae* nuclear cap-binding protein complex (CBC) which promotes nuclear export of capped U snRNAs and shuttles between nucleus and cytoplasm. Xenopus CBC is associated with importin- α in the nucleus and CBC might shuttle while bound to importin- α . Binding of importin- β in the cytoplasm, a binding which displaces the RNA from the CBC-importin- α complex, and the commitment of CBC for nuclear reentry trigger and promote the release of capped U snRNAs into the cytoplasm (Görlich et al, 1996).

XI. Observations on nucleocytoplasmic trafficking pertaining to plasmid import

A model was proposed (Boulikas, 1997a) for the import of plasmid DNA by taking into consideration the following observations or ideas:

(i) DNA microinjected into *Xenopus* eggs or naked DNA incubated with frog oocyte extracts is spontaneously condensed into nucleosomes and chromatin and is then assembled into nuclei *in vitro* by the formation of a double nuclear envelope around the condensed DNA; the egg or egg extracts contain all the essential components for this process (Newport, 1987). We expect plasmid DNA to be complexed with a number of DNA-binding proteins in the cytoplasm and after nuclear import to be converted into

chromatin structures; attachment to the nuclear matrix is a prerequisite for transcription and replication.

(ii) RNA is exported from nuclei in the form of a complex with proteins (Mehlin et al, 1992); ribosomal subunits are preformed in the nucleus from imported ribosomal proteins and are then exported as large RNA-protein complexes. Some U snRNPs are imported into nuclei (Hamm et al, 1990). We expect expulsion of plasmid DNA through pore complexes to the cytoplasm after its nuclear import to be negligible.

(iii) Condensation of plasmid DNA with histones increases several-fold the efficiency of expression of foreign genes (Wagner et al, 1991; Fritz et al, 1996).

(iv) Complexation of the DNA with HMG proteins shortens significantly the time required for gene expression after transfection (Kaneda et al, 1989). According to this procedure Sendai virus was used to fuse DNA-loaded ganglioside liposomes with protein-containing membrane vesicles purified from red blood cells; cointroduction of HMG-1 protein showed rapid uptake of plasmids by nuclei; replacement of HMG-1 by BSA resulted in localization of the grains of the in situ hybridization in the cytoplasm after 6 h reaching the nucleus only after about 24h (Kaneda et al, 1989).

(v) Plasmid DNA condensation with polylysine also enhances transfection of cell cultures; however, polylysine (18-24 kDa), microinjected into the cytoplasm of *Tetrahymena*, remained cytoplasmic; polylysine (5-9 kDa) was evenly distributed between the cytoplasm and the micro- and macronuclei of *Tetrahymena* by diffusion following microinjection; thus, large polylysine molecules cannot be imported into nuclei (White et al, 1989). Polylysine-plasmid complexes are proposed to be uncomplexed in the cytoplasm followed by binding of nascent nuclear proteins before plasmid import can take place. Plasmid complexation with polylysine may only help internalization through the cell membrane but not nuclear import.

(vi) Oligonucleotides tagged with NLS target the nucleus more efficiently than free oligos (Seibel et al, 1995); the same oligos tagged with mitochondrial signals enter mitochondria (Seibel et al, 1995).

(vii) *Agrobacterium tumefaciens* elicits tumors on plant hosts by transporting a single-stranded (ss) copy of transferred DNA (T-DNA) portion of Ti (tumor-inducing) plasmid which enters infected plant cells and integrates into plant nuclear DNA (direct repeats define the T-DNA ends on Ti plasmid). Transfer begins when the VirD2 endonuclease produces a site-specific nick. Two *Agrobacterium* proteins, VirD2 and VirE2 containing NLS associate directly with T plasmid and mediate its nuclear import. VirE2 alone, which has been shown to actively transport ssDNA into the plant cell nucleus, packages ssDNA into semi-rigid, hollow cylindrical filaments with a telephone cord-like coiled structure as was shown by

scanning transmission electron microscopy (STEM); these complexes were proposed to be actively imported through pore complexes (Citovsky et al, 1997). This is a clear example of plasmid import mediated by plasmid-associated proteins possessing NLS.

(viii) Binding NLSs from SV40 T antigen to luciferase plasmid DNA promoted transgene expression following injection of DNA-NLS complexes into the cytoplasm of zebra fish eggs; NLS peptides, but not nuclear-import-deficient peptides, mediated import of DNA from the cytoplasm into embryo nuclei, under conditions in which naked DNA was not imported. Thus, use of NLS may reduce the need for elevated DNA copy numbers in some gene transfer applications (Collas et al, 1996; Collas and Alestrom, 1997).

(ix) Intact, protein-free SV40 DNA was localized to the nucleus after it was cytoplasmically injected into cells in a process which was inhibited (i) by wheat germ agglutinin (ii) by an anti-nucleoporin antibody which block the nuclear pore complex and (iii) by energy depletion. During this process the DNA accumulated at the nuclear periphery before its import and, as opposed to protein import, DNA import required transcription; furthermore, imported DNA colocalized with the SC-35 splicing complex antigen, suggesting localization to areas of transcription or message processing. The SV40 origin of replication and the early and late promoters supported import, whereas bacterial sequences alone and other SV40-derived sequences did not (Dean, 1997).

(x) Fusion of liposomes with the cell membrane will release the encapsulated DNA into the cytoplasm; this mechanism is rather rare and liposomes seem to be internalized by receptor mediated endocytosis if appropriate ligands are exposed on its surface, by poration, especially when cationic lipids are present, or via phagocytosis ending into endosomes and lysosomes (Martin and Boulikas, 1997). Cationic lipids destabilize the biological membranes (both cytoplasmic and lysosomal) and mediate rapid delivery of plasmid to the cytoplasm (reviewed by Boulikas, 1998 page 1-172, this volume). Lysis of the liposome in the endosome or caveolae will release DNA inside vesicles; fusion of the liposome with the endosome or caveolae membranes will release the DNA into the cytoplasm; the presence of fusogenic peptides on the liposome will promote lysis of the endosomal membrane and release of the liposome-plasmid complex into the cytoplasm.

(xi) Viruses have evolved different mechanisms for entry into cells (and into nuclei). For example, after entry into the cytoplasm the adenoviral particle is attached to the cytoplasmic side of pore complexes and the DNA is released to the interior of pore annuli entering the nucleoplasm. These highly ordered processes are accompanied by losses or protease degradation of specific proteins on the viral particles; a viral protease, L3/p23,

located inside the capsid at 10 copies per virion, plays a key role in the stepwise dismantling and in the weakening of the capsid structure culminating with the release of the adenovirus DNA by degrading of the viral capsid protein VI (Greber et al, 1996).

(xii) Spliced mRNAs are exported from nuclei via interaction with RNA-binding proteins (mainly from the HnRNP family) which contain nuclear export signals. Since these interactions are specific we expect the export of plasmid DNA, once it is imported to the nucleus, to be negligible.

(xiii) Fluorescently labeled oligonucleotides, after delivery using DOTAP liposomes, entered the cell using an endocytic pathway and redistributed from punctate cytoplasmic regions into the nucleus; nuclear uptake took place only with positively charged complexes; DOTAP increased over 100 fold the antisense activity of a specific anti-luciferase oligonucleotide (Zelphati and Szoka, 1997). The nuclear membrane was found to pose a barrier against nuclear import of oligonucleotides which accumulated in the perinuclear area; although DOSPA/DOPE liposomes could deliver ODNs into the cytosol, these liposomes were unable to mediate nuclear import of ODNs; on the contrary oligonucleotide-DDAB/DOPE complexes with a net positive charge were released from vesicles into the cytoplasm and mediated nuclear import of the oligos (Lappalainen et al, 1997). Labeled oligonucleotides delivered to animals by tail vein injection in complexes with DC-Chol:DOPE liposomes were localized primarily to phagocytic vacuoles of Kupffer cells at 24 h post-injection; nuclear delivery of oligonucleotide *in vivo* was not observed (Litzinger et al, 1996).

XII. A model for the nuclear import of plasmid DNA

Taking into account these observations we have proposed a plausible model for the nuclear import of plasmid DNA after its cytoplasmic localization (Boulikas, 1997a; **Figure 5**). Plasmid is complexed with nuclear proteins in the cytoplasm as a prerequisite for its import. The binding efficiency and the type of proteins that are complexed in the cytoplasm with the plasmid DNA are matters of speculation. A number of studies support the model that nascent cytoplasmic proteins containing nuclear localization signals are complexed with the transfected DNA and mediate its nuclear import. What type of nascent nuclear proteins might be responsible for mediating plasmid translocation into nuclei *in vivo*? Certainly histones are abundantly synthesized in dividing cells and histone H1 has been shown to display an affinity for supercoiled over relaxed DNA plasmids (Singer and Singer, 1976). A number of transcription factors (TFs) and other nuclear proteins are synthesized *de novo* in actively proliferating cells (but at lower rates in terminally

differentiated cells); these proteins could bind to the plasmid, especially to promoters and enhancers in a sequence-specific manner, and mediate the import of the plasmid-TF complex.

Emerging concepts in plasmid import

J-P Behr PNAS

A novel quantitative method to study plasmid transport from the cytoplasm to the nucleus was used by James and Giorgio (2000) following cationic lipoplex transfection of a Cy3-labeled pGreenLantern plasmid to CV1 cells that are less effective in translocating plasmid to the nucleus than HeLa cells; the average Cy3-pGL-positive HeLa cell contained approximately 2470 plasmid copies.

James MB, Giorgio TD (2000) Nuclear-associated plasmid, but not cell-associated plasmid, is correlated with transgene expression in cultured mammalian cells. **Mol Ther** 1, 339-346.

XIII. Perspectives

Antisense and triplex-forming oligonucleotides, in their single- or double-stranded form, as well as RNA or DNA oligonucleotides have been extensively used in targeting nuclear DNA. The chemistry for covalent coupling of oligonucleotides to peptides has been established. Linkage of oligonucleotides to NLS peptides or to mitochondrial import peptides resulted in nuclear or mitochondrial targeting, respectively (Seibel et al, 1995). Oligo-nucleotides may enter nuclei after their crosscomplexation with nuclear proteins in the cytoplasm. Studies with fluorescent-labeled single-stranded oligonucleotides show binding to RPA *in vitro* (Costas Koumenis, Stanford, Personal communication). RPA is the main single-stranded DNA-binding activity present in mammalian cells.

Understanding the rules that govern trafficking through the pore complex is instructive to our comprehension of plasmid uptake by nuclei during somatic gene transfer and for developing strategies to overcome obstacles for foreign gene expression by enhancing the nuclear import.

Because of their increase rates of proliferation and protein import, cancer cells are expected to be more susceptible to nuclear import of plasmid and to uptake transfected plasmid at higher rates compared with terminally differentiated cells. However, cancer cells especially solid tumors of epithelial origin (lung, colon, head & neck, brain tumors) do not readily internalize particles such as liposomes (Martin and Boulikas, 1997); the step of translocation across the cell membrane, and not the step of nuclear import, is expected to be the rate limiting step in the overall gene transfer procedure in these cancer cells.

Figure 5. A model for the import of proteins into nuclei. The pore complex is shown with its octagonal symmetry. **Step 1.** A complex of the plasmid DNA with one or more proteins possessing NLS (NLS-protein) is formed in the cytoplasm; NLS proteins might include histones, HMGs, transcription factors, or other DNA-binding proteins after their de novo synthesis on polyribosomes; the NLS-protein then binds to karyopherins α/β . **2.** The complex is docked by binding to multiple sites on nucleoporins (structural proteins of the pore complex). **3.** The p10 and Ran-GTP dissociate karyopherin α /NLS-protein-plasmid complex which is expelled to the nucleoplasm resulting in plasmid DNA nuclear import. Adapted from Boulikas T (1997a) Nuclear localization signal peptides for the import of plasmid DNA in gene therapy. *Int J Oncol* 10, 301-309. Reproduced with kind permission from the International Journal of Oncology.

Cancer is a disease of the control of the cell cycle and cell signaling involving mutations in a number of oncogenes and tumor suppressor genes (Spandidos, 1985). Our prediction that tumor cells will import plasmid-

protein complexes across the nuclear envelope more efficiently than nondividing cells provides a basis for the preferential targeting of cancer cells and might have important implications in human gene therapy.

Acknowledgments

Special thanks to Emile Zuckerkandl for stimulating discussions.

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