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MINIREVIEW

Nuclear Import and Export of Viruses and Virus Genomes

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Many viruses replicate in the nucleus of their animal and plant host cells. Nuclear import, export, and nucleo-cytoplasmic shuttling play a central role in their replication cycle. Although the trafficking of individual virus proteins into and out of the nucleus has been well studied for some virus systems, the nuclear transport of larger entities such as viral genomes and capsids has only recently become a subject of molecular analysis. In this review, the general concepts emerging are discussed and a survey is provided of current information on both plant and animal viruses. Summarizing the main findings in this emerging field, it is evident that most viruses that enter or exit the nucleus take advantage of the cell's nuclear import and export machinery. With a few exceptions, viruses seem to cross the nuclear envelope through the nuclear pore complexes, making use of cellular nuclear import and export signals, receptors, and transport factors. In many cases, they capitalize on subtle control systems such as phosphorylation that regulate traffic of cellular components into and out of the nucleus. The large size of viral capsids and their composition (they contain large RNA and DNA molecules for which there are few precedents in normal nuclear transport) make the processes unique and complicated. Prior capsid disassembly (or deformation) is required before entry of viral genomes and accessory proteins can occur through nuclear pores. Capsids of different virus families display diverse uncoating programs which culminate in genome transfer through the nuclear pores.

INTRODUCTION

Viruses that replicate in the nucleus derive several advantages from processes occurring in this compartment. Some make use of cellular DNA and RNA polymerases for replication and transcription; others take advantage of the RNA-splicing machinery for RNA processing. A few exploit the cellular chromatin for integration and regulated expression. Given the economy imposed by a limited genome size, the nucleus is clearly an ideal location for replication and transcription of viral DNA and processing of viral RNA.

The price paid for these conveniences is additional obstacles during entry into and exit from the host cell. Not only do viruses have to deliver their genomes and accessory proteins across the plasma membrane or the endosomal membrane into the cytosol, they must target their genome to the nucleus and overcome the barrier of the nuclear envelope. They have to find routes of transport from the sites of penetration to the nucleus, circumventing the tight regulation and strict size limits imposed on nuclear transport. Furthermore, since DNA is not among the macromolecules generally transported into and out of the nucleus, DNA viruses must modify existing mechanisms.

In this review, we describe emerging insights into the mechanisms by which animal and plant viruses move through the nuclear membrane during entry and exit from the cell. Although the information is for the most part still incomplete, it is clear that the mechanisms and strategies differ considerably among virus families. Some have nucleocapsids small enough to pass through the nuclear pores, some have capsids that undergo uncoating in the endosome, some bud across the nuclear membrane, and others rely on dissociation of the nuclear membrane during cell division.

During viral replication many types of nuclear transport events can involving viral components can occur. There is (1) nuclear import of the incoming viral genome; (2) import of viral structural proteins that have regulatory and other functions in the nucleus; (3) export of transcribed mRNAs for translation in the cytosol; (3) export of full-length viral genomes for assembly in the cytosol; (4) import of structural and nonstructural viral proteins after translation in the cytosol, and (5) export of assembled viral capsids and nucleoproteins from the nucleus. In thinking about virus infection

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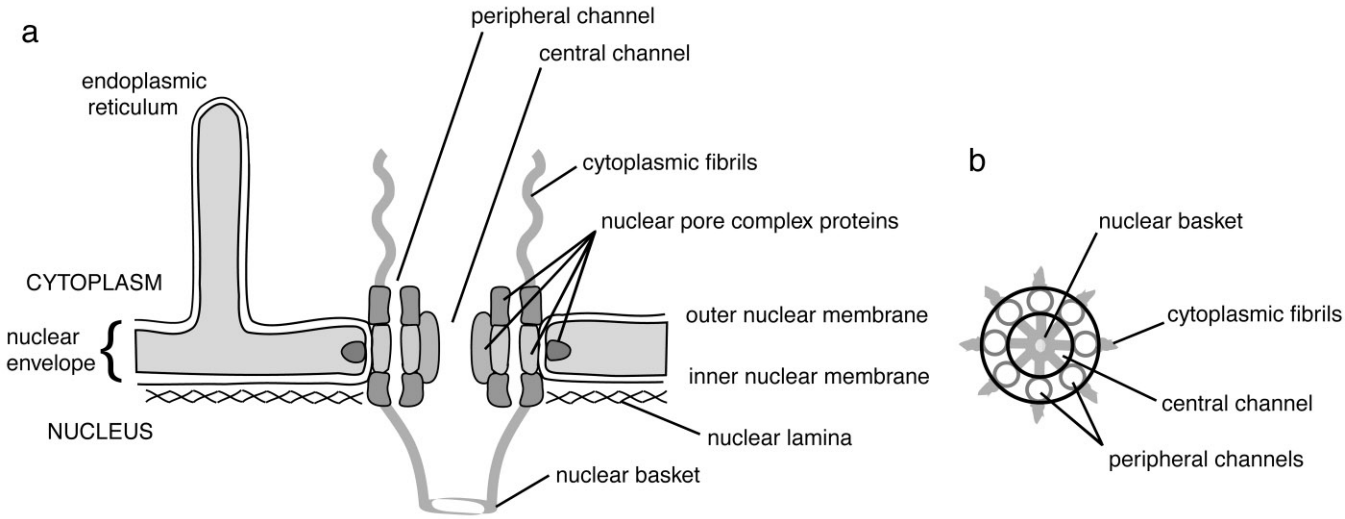


FIG. 1. The nuclear pore complex. A typical nuclear pore complex is shown (a) in a cross-section view to show the interaction of the NPC components with the nuclear envelope and (b) from above to show the relative orientation of the central channel to the eight peripheral channels. The nuclear pore complex spans the outer and inner nuclear membranes (ONM and INM) with some proteins contained between. This space is continuous with the lumen of the ER. The pore has fibrils extending toward the cytoplasm and a basket-like structure facing the nucleoplasm.

and the nucleus it is important to distinguish between these different processes. The same viral components may be under different stages of the replication cycle and be part of multiple transfer events in and out of the nucleus, each of which may utilize different mechanisms and different signals.

NUCLEAR TRANSPORT IN THE CELL

The nuclear envelope and pore complexes

Viruses make use of the cellular machinery in all stages of their life cycle. Transport into and out of the nucleus is no exception. Therefore it is essential to review some general aspects of nuclear pore structure and nuclear transport. Since only some of the features pertinent to viruses will be mentioned here, the reader should consult recent reviews on the topic for more details (Corbett and Silver, 1997; Görlich, 1997; Görlich and Mattaj, 1996; Nakielny and Dreyfuss, 1997; Nakielny *et al.,* 1997, Doye and Hurt, 1995, 1997; Goldberg and Allen, 1995; Panté and Aebi, 1993).

The nucleus and the cytosol are separated by a double membrane, the nuclear envelope. Both membranes of the nuclear envelope are part of the endoplasmic reticulum (ER), and the space between them is part of the ER lumen. Transport between the cytosol and the nucleoplasm occurs through protein-lined aqueous channels called nuclear pore complexes (NPC). These can be thought of as sites of connection or fusion between the inner and the outer membranes, stabilized by a protein complex.

A diagrammatic representation of a nuclear pore and its relationship to the nuclear envelope is shown in Fig. 1. The proteins that make up the NPC, called nucleoporins, have a combined molecular mass of about 125 MDa (Rout and Wente, 1994). A number of them contain multiple copies of short degenerate repeat sequences such as FXFG, GLFG, or FG. Some are modified by O-linked *N*-acetylglucosamine residues (Fabre and Hurt, 1994), which explains why translocation across the NPC is blocked by the lectin wheat germ agglutinin.

The maximal diameter of particles that can pass through the pore by facilitated transport is about 23 nm (Dworetzky and Feldherr, 1984). Transport is dependent on specific targeting signals, on a set of cytosolic factors, and on guanosine 5′-triphosphate (GTP). RNA is generally transported in the form of ribonucleoproteins (RNPs), whereas DNA during normal cell life is not transported through the NPC.

Although signal-mediated, the process of nuclear transport is fundamentally different from protein transport into organelles such as the ER and mitochondria. First, it does not involve direct passage of macromolecules through a membrane, but rather through a large membrane pore equivalent to the channel formed after fusion between two membranes. Second, unlike proteins that enter through membranes, proteins that pass in and out of the nucleus do not need to be unfolded. Not only are they transported in a native folded form, but often as oligomeric complexes that can contain both protein and nucleic acid molecules.

Nuclear transport signals

Facilitated transport into and out of the nucleus is determined by specific sequence motifs that constitute localiza-

tion signals. For import, these are called nuclear localization sequences (NLSs), and for export, nuclear export signals (NESs). They are, as a rule, transferable, i.e., they can confer nuclear transport competence to proteins otherwise restricted to the cytoplasm or the nucleus.

The first NLS to be identified was a short stretch of basic amino acids (PKKKRKV) in simian virus 40 (SV40) large T-antigen (Kalderon *et al.,* 1984a,b). Similar sequences were subsequently found in numerous viral and cellular proteins with karyophilic properties. Other versions of this motif display a bipartite sequence, e.g., in nucleoplasmin (KRPAATKKAGQAKKK) (Robbins *et al.,* 1991). We will refer to these import signals as "classical" NLSs, as they were the first to be discovered.

Recently, entirely new targeting sequences have emerged from studies on heterogeneous nuclear RNPs (hnRNPs). For hnRNP A1 the signal is present in a glycine-rich segment of 40 amino acids (termed M9) (Siomi and Dreyfuss, 1995) and for hnRNP K the signal is an unrelated 40-amino-acid signal (termed KNS) (Michael *et al.,* 1997). The uracil-rich small nuclear (U sn) RNPs have a unique signal that is composed of the Sm core protein, a component of the U snRNP, and the trimethylguanosine (m_3G) cap (Fischer *et al.,* 1991, 1993; Hamm *et al.,* 1990).

Two types of NESs have been identified on proteins. First, the nuclear import sequences in hnRNP A1 and K also function as NESs (Michael *et al.,* 1995, 1997). Second, sequences rich in leucine residues act as NESs in the HIV-1 Rev molecule (LPPLERLTL) (Bogerd *et al.,* 1995; Fischer *et al.,* 1995) and in the catalytic domain of cAMP-dependent protein kinase inhibitor, PKI, (LALKLAGLDI) (Wen *et al.,* 1995). These leucine-based NESs may be widely used because they are present in a variety of exported proteins (Fritz and Green, 1996). Signals for RNA export are generally found on the monomethylated (m^7G) cap structures (Dargemont and Kuhn, 1992; Hamm and Mattaj, 1990).

Pathways for nuclear import

There are at least four independent import pathways for proteins, all of which rely on interaction with soluble receptors called karyopherins or importins (for reviews see (Corbett and Silver, 1997; Görlich and Mattaj, 1996; Nigg, 1997). The current state of knowledge of this rapidly growing family of import receptors is shown in Table 1. The importin α/β complex recognizes NLSs of the classical type, i.e., those typified by SV40 T-antigen and nucleoplasmin. Overall, the growing family of related importin α molecules, combined with their multiple roles in binding, allows considerable flexibility and specialization during nuclear import of proteins containing a classical NLS (Görlich *et al.,* 1996; Malik *et al.,* 1997).

An alternative pathway for nuclear import is mediated by an importin β-related molecule, termed transportin (Pollard *et al.,* 1996). The transportins mediate import of hnRNPs that contain a nonclassical NLS. Independent import pathways are also mediated by related, but distinct, importin β-like molecules. These pathways are used to import ribosomal subunits and mRNA-binding proteins, but the NLSs responsible are so far unknown (Pemberton *et al.,* 1997; Rosenblum *et al.,* 1997; Rout *et al.,* 1997; Schlenstedt *et al.,* 1997).

After receptor binding to the karyopherin proteins in the cytoplasm, attachment occurs to the fibrils extending from the NPC. The receptor–NLS substrate interacts specifically with FXFG, GLFG, or FG repeats on the nucleoporins. Actual translocation through the central channel of the pore is mediated by a complex that includes a small GTP-binding protein, Ran (Moore and Blobel, 1993), and an accessory molecule, termed p10 or NTF2 (Moore and Blobel, 1994; Paschal and Gerace, 1995).

Pathways for nuclear export

The mechanisms of export from the nucleus must account for a wide variety of substrates. A range of RNA species (mRNA, tRNA, rRNA, and snRNAs) and many different proteins are exported. RNA and protein export have been the focus of several reviews (Nakielny and Dreyfuss, 1997; Nakielny *et al.,* 1997; Nigg, 1997). The current state of knowledge of nuclear export receptors is shown in Table 1.

The first export receptor identified was a nuclear protein termed RIP (also known as Rab) (Bogerd *et al.,* 1995; Fritz *et al.,* 1995). However, recent evidence suggests that the RIP receptors may play a role in docking to the NPC, but may not be bona fide nuclear export receptors (Stutz *et al.,* 1997; Ullman *et al.,* 1997). A family of importin β-related molecules has recently been identified and shown to directly mediate the export of proteins containing leucine-based NESs (Neville *et al.,* 1997). These exportins probably mediate a wide variety of protein export from the nucleus and may account for the recycling of the importin α nuclear import receptor, to complete the cycle of import/export (Kutay, *et al.,* 1997).

For the alternative, hnRNP-based export pathway, it is likely that transportin, the receptor for nuclear import, also mediates export. hnRNPs are commonly found as part of the pre-mRNA splicing complex, and the majority of mRNA export is likely to be mediated via the transportins. However, the export of some RNAs does not appear to occur via a protein-mediated pathway. U snRNAs rely on CBP 20, part of the nuclear cap binding complex (CBC) for export. As with import, the different export pathways converge on the nuclear pore and export is dependent on Ran-like GTPases, with the possible exception of tRNA (Richards *et al.,* 1997).

Regulation of nuclear transport

Phosphorylation is the best understood regulatory mechanism for nuclear transport. The modulation of import signal itself by phosphorylation has been demonstrated for the SV40 T NLS (see Jans and Hübner, 1996, for a review). Alternatively, phosphorylation may regulate nuclear import by causing cytoplasmic retention (Görner, *et al.,* 1998; Rao *et al.,* 1997; Chow *et al.,* 1997).

Phosphorylation and methylation play also a regulatory role in nuclear export. Protein kinase C activation inhibits the rate of mRNA transport from isolated nuclei (Schröder *et al.,* 1988), most likely due to phosphorylation of NPC proteins. The export of several other proteins is known to be affected by phosphorylation (Beals *et al.,* 1997; Yang *et al.,* 1997). Many hnRNPs, and other RNA-binding proteins, are dimethylated by a unique arginine methyltransferase (Henry and Silver, 1996; Liu and Dreyfuss, 1995) which is likely provide a mechanism for regulation.

NUCLEAR IMPORT OF VIRAL GENOMES

The virus families known to replicate in the nucleus are listed in Table 2. The majority are DNA viruses, and most of them nonenveloped. The likely reason for the rather low number of RNA viruses is that the nucleus has less to offer an RNA virus. It cannot supply enzymes for genome replication because it lacks RNA-dependent RNA polymerases. Furthermore, the gene-regulation machinery present in the nucleus is essentially useless for a typical RNA virus. However, once the viral mRNAs are transcribed, the splicing machinery can be used by RNA as well as DNA viruses. This "service" is, in fact, exploited by the few RNA viruses that replicate in the nucleus.

Typically, incoming viruses enter the nucleus from the cytosolic compartment (see Fig. 2). The early steps in virus entry include a membrane penetration that results in the delivery of nucleocapsids and accessory proteins into the cytosol of the host cell. Thus, whether originally trapped inside a viral envelope or not, the delivered complexes are membrane-free and ready to transfer the genome to the nucleus.

For the nuclear import step, viruses make use of two quite different overall strategies:

(A) They can wait in the cytosol until the cell undergoes mitosis. With the nuclear envelope temporarily disassembled, the viral genome can associate itself with the cellular chromatin and enter a newly assembling nucleus. Used by most retroviruses, this strategy restricts infection to dividing cells.

(B) Alternatively (and more commonly), they can deliver the viral genome through the envelope of the interphase nucleus. This allows infection of nondividing cells and provides a way to infect terminally differentiated cells. For this strategy to work, the viruses must have mechanisms for transporting the genomes to the nucleus and delivering the infectious genome through the nuclear membrane.

While in all well-studied cases, passage into the nucleus occurs through the NPCs, it may be premature to entirely dismiss the possibility that some viruses may bypass the nuclear pores and enter directly through one or both of the nuclear membranes. Few viruses and capsids are small enough to pass through the NPCs in modified form. In most cases, the particles must undergo partial or full disassembly before the genome enters.

Nuclear entry of RNA viruses

Lentiviruses use multiple modes of nuclear targeting

Lacking a mechanism for entry into the nucleus of interphase cells, most retroviruses rely, as mentioned above, on disassembly of the nuclear envelope during mitosis. However, the lentiviruses including human immunodeficiency virus type 1 (HIV-1) (Cullen, 1992) constitute an exception. They have evolved sophisticated mechanisms that ensure efficient transport of their genome in to the nucleus of nondividing cells (Lewis and Emerman, 1994). This allows them not only to infect terminally differentiated cells, but determines many of their pathogenic properties.

Entry of HIV-1 begins at the plasma membrane where the incoming viruses bind to CD4 and other surface receptors (see Doms and Peipert, 1997). These interactions trigger pH-independent fusion of the viral envelope with the plasma membrane. Once inside the cytosol, the viral RNA (trapped inside a capsid complex) is reverse-transcribed into a linear double-stranded DNA. The resulting DNA-containing particle, the viral preintegration complex, is imported into the nucleus generally within 4 h of infection.

The molecular dimensions of the preintegration complex are not known, but it sediments at a rate between 160S and 400S (Stevenson, 1996). In addition to the DNA, it contains the viral core protein (NC), the reverse transcriptase (RT), the p6 protein, the viral integrase (IN), the Vpr protein, and copies of the viral matrix protein (MA) (Stevenson, 1996). The particle is likely to be too large to enter through NPCs without disassembly or remodeling.

Several studies have shown that import is mediated by Vpr, a viral accessory protein that binds to the p6 domain of Gag (Heinzinger *et al.,* 1994). Since Vpr-mediated import is not blocked by peptides corresponding to the SV40 T-antigen NLS, it is unlikely to involve classical NLSs. Mutational analysis of Vpr has identified N-termi-

TABLE 1

Family name	Specific proteins	Homologues	Molecule(s) transported, e.g.	Signal
		IMPORT		
Importin α (Görlich *et al.,* 1994)	(a) Rch 1 (Cuomo *et al.,* 1994)	hSRP1 (Weis, *et al.,* 1995)	SV40 T-antigen nucleoplasmin	"Classical" NLS basic basic bipartite
	(b) NPI-1 (O'Neill and Palese, 1995)	p54/56 (Adam and Adam, 1994)		
	(c) Qip 1 (Miyamoto *et al.,* 1997)	Karyopherin α (Moroianu, *et al.,* 1995)		
	(d) hSRP1γ (Nachury *et al.,* 1998)	OHO31 (pendulin) (Torok *et al.,* 1995)		
	ySRP1/Kap60 (Enenkel *et al.,* 1995)	At IMPα (Smith *et al.,* 1997)		
		NBP70 (Stochaj *et al.,* 1991)		
		PTAC58 (Imamoto *et al.,* 1995b)		
Importin β (Görlich *et al.,* 1995)		p97 (Adam and Adam, 1994)		
		Karyopherin β/Kap95 (Radu *et al.,*) 1995		
		RSL1 (Koepp *et al.,* 1996)		
		PTAC97 (Imamoto *et al.,* 1995a)		
Transportin (Nakielny *et al.,* 1996)	(a) Transportin1 (Siomi *et al.,* 1997)	Karyopherin β2 (Bonifici *et al.,* 1997)	hnRNP A1, F	"Nonclassical" NLS/NES (glycine-rich), e.g., 40-aa M9 domain
	(b) Transportin2 (Siomi *et al.,* 1997)	Kap 104 (Aitchison *et al.,* 1996)		
		MIP (Fridell *et al.,* 1997)		
			hnRNP K	"Nonclassical" 40-aa domain
	Mtr10 (Pemberton *et al.,* 1997)		mRNA-binding proteins	
	Karyopherin β3 (Yaseen and Blobel, 1997)	Yrb 4 (Schlenstedt *et al.,* 1997)	Ribosomal proteins	Novel basic NLS
	Sxm 1 (Rosenblum *et al.,* 1997)		Ribosomal proteins	
	Importin β (Palacios *et al.,* 1997)		U snRNAs	m_3G cap Sm core protein
Exportin 1	CRM1 (Fornerod *et al.,* 1997; Ossareh-Nazari *et al.,* 1997)	Xpo1 (Stade *et al.,* 1997)	HIV-1 Rev PKI	Leucine-based NES
	Pse1 (Seedorf and Silver, 1997)			
Exportin 2	CAS (Kutay *et al.,* 1997)		Importin α	
	Kap123 (Seedorf and Silver, 1997)			
	Sxm1 (Seedorf and Silver, 1997)			
	hRIP (Fritz *et al.,* 1995)	Rab (Bogerd *et al.,* 1995)	HIV-1 Rev	Leucine-based NES
Transportin	Transportin1 (Siomi *et al.,* 1997)		mRNA	"Nonclassical" NES/NLS
CBC (Izaurralde *et al.,* 1992)	CBP20 (Izaurralde *et al.,* 1995)		U snRNA mRNA	m^7G cap
	Exportin t (Kutay *et al.,* 1988)		tRNA	
			rRNA	NES on protein?

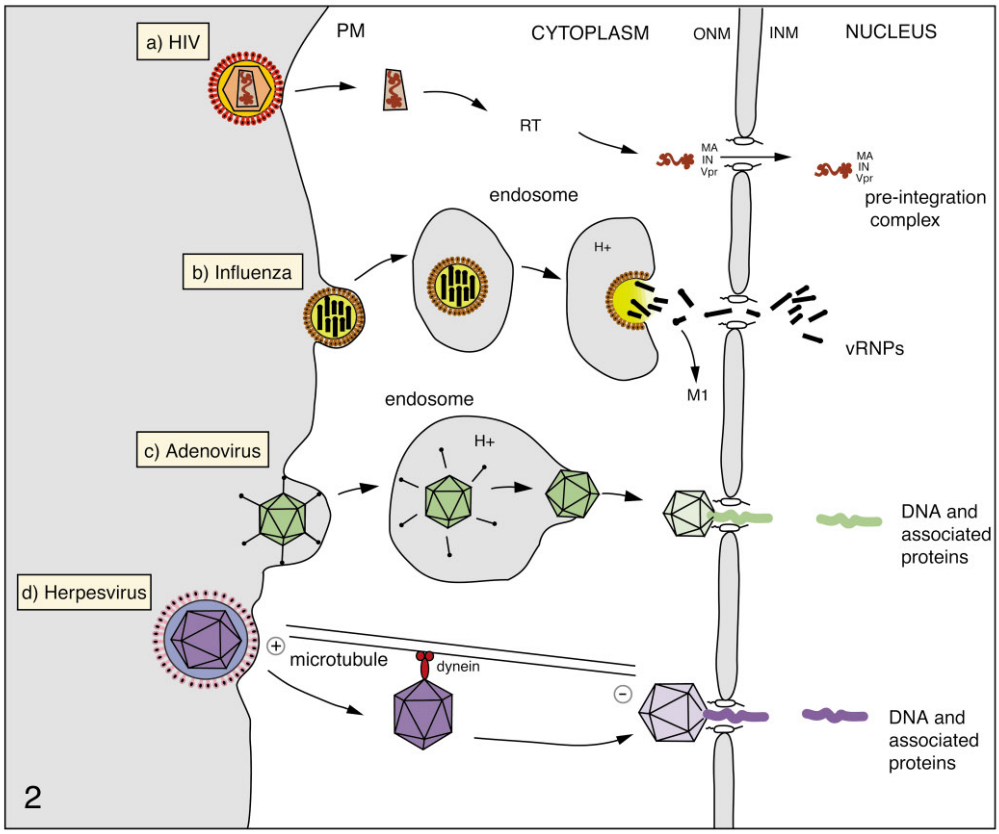
TABLE 2

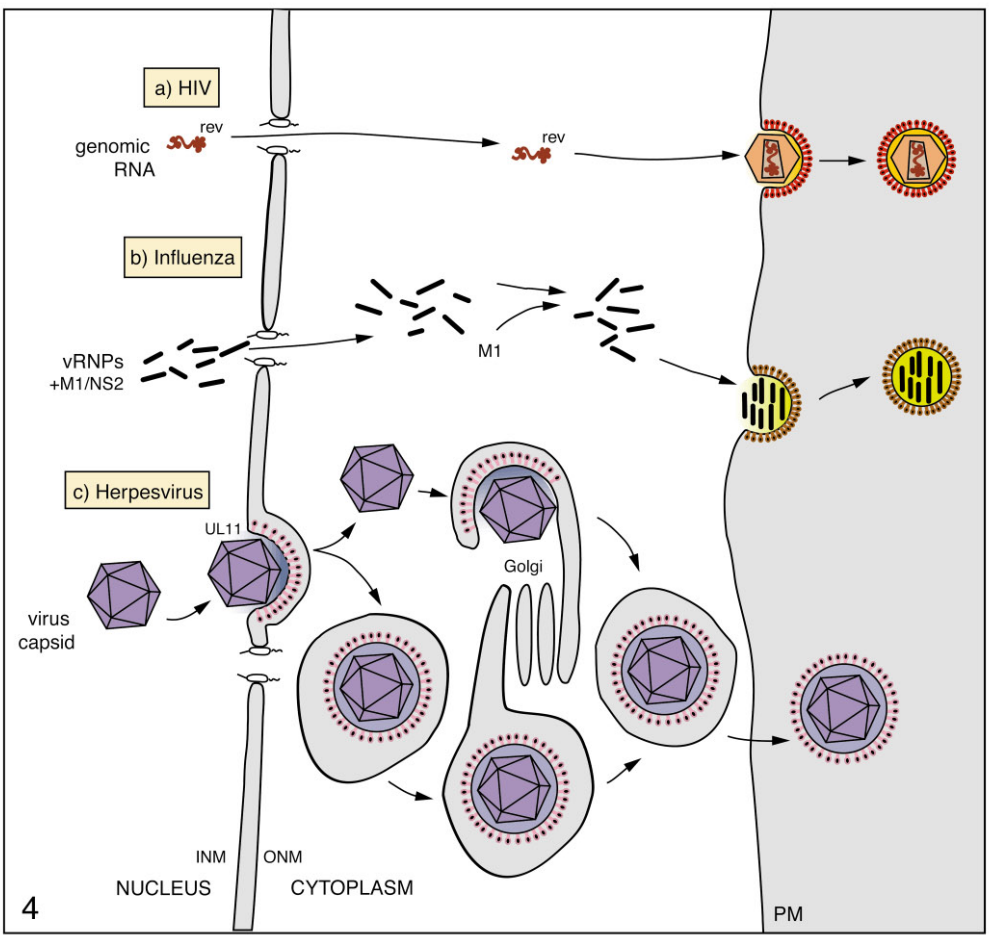
Virus family	Selected example(s)			Mechanism of import	Mechanism of export
Retroviridae		Animal	RNA (RT)		
(a) Oncovirus	(a) Simian retrovirus type 1			(a) Breakdown of NE during mitosis	(a) Translocation of viral RNA through the NPC
(b) Lentivirus	(b) Human immunodeficiency virus type 1			(b) Translocation of RNP through the NPC	(b) Translocation of viral RNA through the NPC
Orthomyxoviridae	Influenza virus	Animal	ss RNA (−)	Translocation of RNP through the NPC	Translocation of RNP through the NPC
Bornaviridae	Borna disease virus	Animal	ss RNA (−)	Translocation of RNP through the NPC?	Unknown
Rhabdoviridae	Lettuce necrotic yellows virus	Plant	ss RNA (−)	Unknown	Budding of capsid through the INM
Hepadnaviridae	Hepatitis B virus	Animal	ds DNA (RT)	Docking to the NPC and translocation of DNA	Translocation of RNA through the NPC
Caulimoviridae	Cauliflower mosaic virus	Plant	ds DNA (RT)	Unknown	Translocation of RNA through the NPC?
Geminiviridae	Bean dwarf mosaic virus Squash leaf curl virus	Plant	ss DNA	Translocation of virus through the NPC?	Translocation of virus through the NPC?
Parvoviridae	Minute virus of mice ParvovirusB19	Animal	ss DNA	Translocation of virus through the NPC?	Translocation of virus through the NPC?
Papovaviridae	Papovavirus	Animal	ds DNA	Budding of capsid through the NE?	Nuclear lysis upon cell death
	Simian virus 40			Transport of virus through the NPC	Budding of virus through membrane?
Adenoviridae	Adenovirus 2	Animal	ds DNA	Docking to the NPC and translocation of DNA	Nuclear lysis upon cell death
Herpesviridae	Herpes simplex virus Cytomegalovirus	Animal	ds DNA	Docking to the NPC and translocation of DNA	Budding of capsid through the INM
Polydnaviridae	Ichnovirus	Animal	ds DNA	Unknown	Envelopment of capsid in the nucleus
Iridoviridae	Frog virus 3	Animal	ds DNA	Unknown	Export of DNA?
Baculoviridae	*Autographa californica* multiple nuclear polyhedrosis virus	Animal	ds DNA	Docking to the NPC and translocation of DNA	Budding through the NE Envelopment of capsid in the nucleus

nal α-helices as key regions for promoting the nuclear transport (Di Marzio *et al.,* 1995; Mahalingam *et al.,* 1997, 1995). Interestingly, visualization of expressed Vpr often shows a nuclear rim distribution, rather than an intranuclear localization raising the possibility that Vpr acts at the nuclear membrane (Mahalingam *et al.,* 1997, 1995;

FIG. 2. Nuclear import of virus genomes. (a) Human immunodeficiency virus (HIV) binds to the plasma membrane (PM) and the core is internalized into the cytoplasm. The genome is then converted to a complimentary DNA form by reverse transcriptase (RT). The matrix protein (MA), integrase (IN), and/or the Vpr protein mediate import across the pore and into the nucleus. (b) Influenza virus binds to the PM and is internalized into endosomes by receptor-mediated endocytosis. The viral envelope fuses with the endosomal membrane in the low pH environment of the late endosome and the matrix protein and the eight RNA segments are released into the cytoplasm. The individual segments are then imported across the pore and into the nucleus. (c) Adenovirus binds to the PM and is internalized into endosomes by receptor-mediated endocytosis. The low pH environment of the late endosome induces an adenovirus-mediated rupturing of the endosomal membrane that releases the fiberless capsid into the cytosol. The capsid then docks to the cytoplasmic face of the pore and the DNA is translocated into the nucleus. (d) Herpesvirus binds to the PM and is internalized into the cytoplasm. The capsid attaches to cytoplasmic microtubules and is transported toward the nucleus using dynein as a motor. The capsid then detaches and docks to the cytoplasmic face of the pore and the DNA is translocated into the nucleus. ONM, outer nuclear membrane; INM, inner nuclear membrane.

FIG. 4. Nuclear export of viruses. (a) The RNA of human immunodeficiency virus (HIV) interacts with the Rev protein in the nucleus, which mediates the export of the RNA across the pore and into the cytoplasm. The RNA is then encapsidated in the cytoplasm and the virus buds across the plasma membrane (PM). (b) The export of the eight influenza genome segments is initiated by the presence of M1 in the nucleus. The segments are exported across the pore and into the cytoplasm, where M1 also coordinates virus assembly and virus budding across the PM to release virus from the cell. (c) Herpesvirus capsids also assemble in the nucleus and bud through the inner nuclear membrane (INM). Two mechanisms for further transport of the virus are then possible. The virus may fuse though the outer nuclear envelope (ONM) to release a naked capsid into the cytoplasm. This capsid then becomes enwrapped by membranes of the Golgi apparatus to form a cytoplasmic virus within a vesicle. Alternatively, the virus buds across the outer nuclear membrane to form a double-membraned virus in the cytoplasm. The outer of these two membranes then fuses with the Golgi apparatus to allow late modifications of the viral glycoproteins. As with the former pathway, the outcome of trafficking is the presence of an enveloped virus within a cytoplasmic vesicle. In both cases the vesicle then fuses with the PM to release an enveloped virus from the cell.


a) HIV
PM
CYTOPLASM
ONM
INM
NUCLEUS
RT
MA
IN
Vpr
MA
IN
Vpr
pre-integration
complex
endosome
H+
b) Influenza
vRNPs
M1
endosome
H+
c) Adenovirus
DNA and
associated
proteins
d) Herpesvirus
microtubule
dynein
DNA and
associated
proteins
2


a) HIV
genomic
RNA
rev
rev
b) Influenza
vRNPs
+M1/NS2
M1
c) Herpesvirus
UL11
Golgi
virus
capsid
INM
ONM
NUCLEUS
CYTOPLASM
PM
4

Vodicka *et al.,* 1998). It has been speculated that the α-helical N-terminus of Vpr would interact with the NPC or importins via "coiled-coil"-type protein–protein interactions rather than via an NLS (Bukrinsky and Haffar, 1997; Emerman, 1996).

It has recently been shown that as part of the viral preintegration complex, Vpr associates both with importin α and the NPC, i.e., it may take the place of importin β in the importin α/β–nucleoporin docking process (Vodicka *et al.,* 1998). During HIV-1 infection of macrophages, an importin α-Vpr–nucleoporin complex may be formed. Alternatively, Vpr may bind to importin α and increase its affinity for other NLS-containing, viral proteins, facilitating the nuclear import of the preintegration complex (Popov *et al.,* 1998).

There is also evidence that nuclear import occurs by other mechanisms. Peptides corresponding to the SV40 T-antigen NLS have been shown to inhibit infection, suggesting a role for a classical NLS (Gulizia *et al.,* 1994). There is evidence that the MA protein, the most N-terminal of the proteins derived from the viral Gag gene, serves as a key factor in nuclear targeting (Bukrinsky *et al.,* 1993; von Schwedler *et al.,* 1994). It has an NLS-like sequence close to the N-terminus, which allows it to interact with importins (Gallay *et al.,* 1996). Tyrosine phosphorylation of the MA may be necessary for expression of the karyophilic properties of this sequence (Camaur *et al.,* 1997; Gallay *et al.,* 1995a,b).

However, the importance of the MA-mediated pathway is difficult to judge at present. The studies showing a role for MA were all performed with Vpr-negative virus lines (Bukrinsky *et al.,* 1993; von Schwedler *et al.,* 1994). Moreover, it has been shown that the phosphorylation pattern of MA varies between strains (Bukrinskya *et al.,* 1996), and mutation of the "critical" tyrosine does not have any effect on infection (Freed *et al.,* 1997). In fact, elimination of the entire N-terminal basic domain of MA has no effect on HIV replication, and fusion proteins or peptide conjugates containing the MA NLS do not confer nuclear-import competence to cytosolic proteins (Fouchier *et al.,* 1997). Clearly, more studies are needed to clarify this confusing area.

Recently, a third mechanism has been proposed involving an independent contribution of the integrase (Gallay *et al.,* 1997). A bipartite NLS in this molecule has been found to interact directly with importin α mediating nuclear import of the preintegration complex. That IN plays a role in nuclear targeting is reinforced by the recent finding that nuclear import of the Ty1 retrotransposon in yeast is mediated by an NLS present on the Ty1 IN (Kenna *et al.,* 1998; Moore *et al.,* 1998). It is possible that the karyophilic properties ascribed to MA are actually mediated via its interaction with IN.

Nuclear import of influenza virus vRNPs occurs following pH-dependent uncoating

Influenza viruses are enveloped animal viruses with a segmented, negative-sense RNA genome. They have seven or eight distinct RNA molecules individually packaged into complexes that contain, in addition to the RNA, nucleoprotein NP and a polymerase complex (PA, PB1, and PB2). With one copy per 20 nucleotides, the NP constitutes the major protein component of the viral ribonucleoprotein (vRNP). NP forms a proteinaceous core around which the RNA is wrapped in a helical fashion, reminiscent of the DNA in a nucleosome. In this helical structure, the RNA is exposed and is sensitive to RNase.

The available evidence shows that incoming vRNPs enter the nucleus in intact form through the NPCs (Kemler *et al.,* 1994; Martin and Helenius, 1991b). The NP and the polymerase accompany the RNA into the nucleus, where they are required for transcription and replication. The length of the individual vRNPs varies from 20 to 80 nm depending on the size of the RNA. However, the diameter of the rod-shaped particles is constant and is small enough (10–20 nm) to allow active passage of vRNPs if they move lengthwise through the pore. However, it cannot be excluded that the vRNPs undergo conformational alterations during passage.

Two distinct low-pH-induced changes, affecting different structural proteins, the hemagglutinin (HA) and the matrix or membrane protein (M1), are required for successful entry of influenza virus into the cell and the nucleus. After binding to the cell surface, the particles are endocytosed by coated vesicles (Matlin *et al.,* 1982). The acid-induced conformational change in HA that allows delivery of the virus into the cytoplasm is well understood (see Carr and Kim, 1994; Hernandez *et al.,* 1996 for reviews). The vRNPs are thus delivered to the cytosolic compartment from which they rapidly and efficiently move into the nucleus via the NPCs (Martin and Helenius, 1991b).

Studies with amantadine, an anti-influenza A drug, have been instrumental in revealing the second, acid-induced phenomenon. The inhibitor was found to block the dissociation of incoming nucleocapsids in the cytosol and to inhibit the transport of vRNPs into the nucleus (Bukrinskaya *et al.,* 1982; Martin and Helenius, 1991a). The inhibitory action of amantadine has long been known to involve the M2 protein, a small viral transmembrane protein present in a few copies in the viral membrane (Zebedee and Lamb, 1988). More recently it was found that M2 serves as an acid-activated transmembrane channel for protons (Pinto *et al.,* 1992; Sugrue and Hay, 1991). Martin and Helenius (1991a) proposed that the M2 channel is needed to expose the nucleocapsid inside the incoming virus to low pH in the

endosomes and thus allow dissociation of the vRNPs from each other and from M1.

Further studies have provided support for this model. Acidification has been shown to induce an irreversible change in M1 resulting in its dissociation from vRNPs (Bui *et al.,* 1996). M1 has emerged as a central regulator of vRNP transport into and out of the nucleus (for review see Whittaker *et al.,* 1996b). Moreover, in some virus strains, there is direct evidence that acidification *in vitro* causes dissociation of nucleocapsid components (Zhirnov, 1992).

How are the vRNPs targeted at the nuclear pore? Do they have NLSs and do they make use of importins? All four components of the vRNPs carry some type of nuclear targeting sequence because they undergo transport to the nucleus after synthesis in infected cells. Although the three polymerase subunits have been shown to contain classical NLSs (Akkina *et al.,* 1987; Jones *et al.,* 1986; Nieto *et al.,* 1994), the karyophilic signal(s) of NP remains ill-defined.

Following expression, NP has been shown to accumulate in the nucleus of *Xenopus* oocytes and tissue culture cells (Davey *et al.,* 1985; O'Neill and Palese, 1995; Wang *et al.,* 1997). In HeLa cells it interacts with two homologs of importin α, NPI-1 and NPI-3 (O'Neill and Palese, 1995; Wang *et al.,* 1997). When added to permeabilized cells, purified NP is transported into the nucleus by importins α and β, Ran, and p10 (O'Neill *et al.,* 1995). It can also mediate the import of RNA with which it is complexed, implying that the NLS does not overlap the RNA interaction domain. Nuclear import of expressed NP has recently been shown to depend on basic residues close its N-terminus (Neumann *et al.,* 1997; Wang *et al.,* 1997). This basic NLS may be of a novel type, as it appears not to recognize the standard NLS-binding site on NPI-1, but instead recognizes a distinct domain at the C-terminus (P. Palese, personal communication), which is also recognized by the NLS of the cellular Stat-1 transcription factor (Seikimoto *et al.,* 1997). A second signal has also been identified for nuclear accumulation of NP in *Xenopus* oocytes. The signal bears no relationship to any previously identified NLS (Davey *et al.,* 1985) and its role in mammalian cells is unclear. Unfortunately, the nature of the *in vitro*-synthesized NP–RNA complexes so far used leaves open the mechanism by which authentic vRNPs are targeted *in vivo.* One cannot exclude the possibility that the assembled vRNPs enter by a mechanism distinct from that used by NP.

It is possible that influenza virus makes use of its segmented genome to facilitate nuclear entry. However, such a conclusion may be premature, as there are other segmented RNA viruses, such as bunya- and arenaviruses, that do not replicate in the nucleus. In addition, Borna disease virus (BDV) is an RNA virus that replicates in the nucleus and does not have a segmented genome (Briese *et al.,* 1992). Little is currently known about BDV nuclear transport, but infection does not appear to be sensitive to amantadine (Cubitt and de la Torre, 1997; Hallensleben *et al.,* 1997). However, it is apparent that the large RNA molecules, packaged in helical RNP structures, can be channeled into the nucleus.

Nuclear entry of DNA viruses

DNA viruses employ a variety of entry strategies (see Greber, 1998, for a recent review). We will discuss these mechanisms in some detail, starting with adenovirus, which binds to the NPC before releasing its DNA. Other viruses, such as herpes-, baculo-, and hepadnaviruses, are less well understood. Despite different routes through the cytosol, they all seem to have a similar docking and disassembly mechanisms at the NPC. This is in contrast to papovaviruses, which probably undergo extensive disassembly prior to their arrival at the NPC.

Adenoviruses follow a strict disassembly program

Adenoviruses are large nonenveloped DNA viruses (70–100 nm in diameter) with an outer protein shell composed of 12 pentonal complexes and 240 hexon subunits. Each penton complex contains a base and a projecting fiber. Overall, the virus contains eleven different types of viral polypeptides.

Adenoviruses 2 and 5 bind to cells via two receptors: the fiber receptor has recently been identified as a glycoprotein of the immunoglobulin family called CAR for Coxsackie Adenovirus Receptor (Bergelson *et al.,* 1997). The second receptor, the fibronectin-binding integrin (integrin $\alpha v\beta 5$), binds to the penton base protein (Wickham *et al.,* 1993). The virus enters by receptor-mediated endocytosis and the particle is delivered in fiber-free form to the cytosol by a low-pH-dependent membrane lysis event that ruptures the endosomal membrane (Chardonnnet and Dales, 1970; Greber *et al.,* 1994; Pastan *et al.,* 1986). The lytic event is caused by the viral penton protein and the adenovirus–integrin complex (Wickham *et al.,* 1994).

Having reached the cytosol, the virus undergoes a series of further disassembly events. It loses proteins IIIa, IX, and XX that stabilize the facets in the capsid wall (Greber *et al.,* 1993). Protein VI, which bridges the viral DNA and the capsid walls, is degraded by the L3 protease, a protease contained within the virion (Cotten and Weber, 1995; Greber *et al.,* 1996). It is a cysteine protease and is activated by exposure of the incoming virus to the reducing environment of the cell.

Transport of the viral capsid toward the nucleus appears to be microtubule-mediated judging by the observation that many of the viruses appear to bind to microtubules (Dales and Chardonnet, 1973; Luftig and Weihing, 1975). The entry of DNA-associated protein VII into

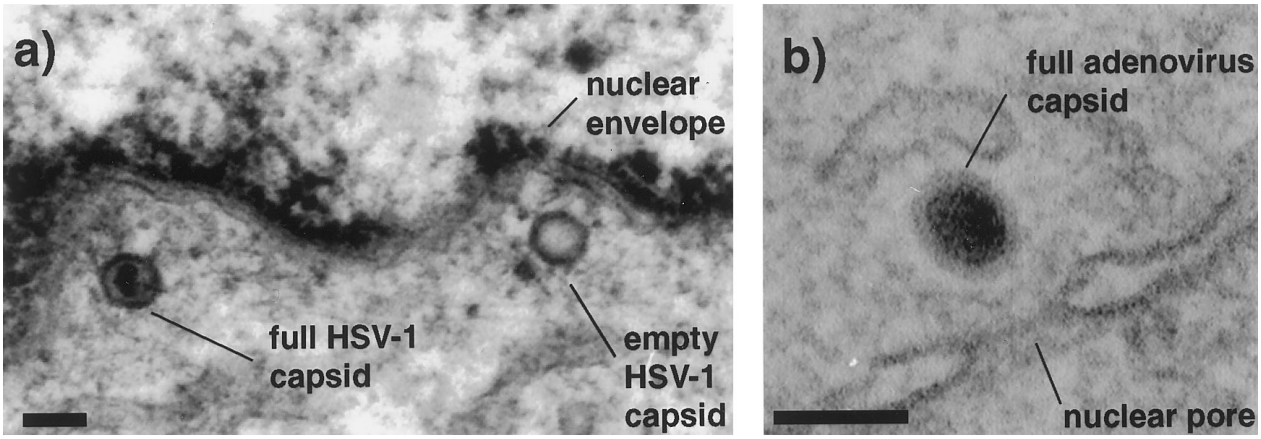


FIG. 3. Binding of herpesvirus and adenovirus to the NPC. Ultrathin Epon sections of cells infected at high multiplicity with (a) herpes simplex virus type-1 (HSV-1) and (b) adenovirus type 4. HSV-1 capsids are bound to the nuclear envelope and may contain the electron-dense DNA core (full capsids) or may be DNA-free (empty capsids). Adenovirus, however, is seen bound only to the nuclear envelope when it contains DNA. For both viruses binding appears to occur to the filament components of the NPC, as the caspid is found a short distance away from the nuclear envelope. (a) This figure was kindly provided by Melanie Ebersold and Beate Sodeik, Yale University School of Medicine (New Haven, CT). (b) This figure was kindly provided by Paul Webster, Yale University School of Medicine (New Haven, CT) and Urs Greber, University of Zurich (Switzerland). Bars, 100 nm.

the nucleus, which serves as an indicator of virus disassembly, is in fact abolished after drug-induced microtubule depolymerization (U. Greber, personal communication).

That adenovirus capsids bind to the cytoplasmic face of the NPCs has been known for a long time (Morgan *et al.,* 1969). Within an hour of entry into the cell, the majority of virus particles can be seen associated with NPCs. Docking to the NPC is followed by transfer of the DNA into the nucleus, a process that depends on metabolic energy (Chardonnnet and Dales, 1972). During nuclear import, the viral DNA and the DNA-condensing viral proteins including protein VII are imported through the NPC (Greber *et al.,* 1997). A small fraction of the hexon protein is also observed to enter the nucleus, but this transfer may occur independently of the DNA import.

The receptors and ligands involved in docking of the adenovirions to the NPC and the mechanism of final capsid dissociation and DNA transfer into the nucleus remain unclear. The structural proteins of the virus have the necessary signals for nuclear import, because during virus synthesis they are all transferred from the cytosol to the nucleus. Whether these same signals are used for targeting and import of the incoming virus particle remains to be tested.

Recent studies using drugs that deplete the ER of calcium have shown that the release of adenovirus DNA into the nucleus requires that the NPCs are open (U. Greber, personal communication). Calcium depletion causes a reversible closure of the nuclear pores and inhibits active transport of proteins and diffusion of small dextran molecules (Greber and Gerace, 1995; Perez-Terzic *et al.,* 1997).

No empty capsids are visible at the nuclear pores, nor are virus particles seen inside the nucleus, strongly suggesting that the capsids disassemble after or during the release of DNA (U. Greber and P. Webster, personal communication). Pretreatment of the virus with inhibitors of the L3 protease, such as the alkylating agent *N*-ethylmaleamide, block uncoating of the virus at the nuclear membrane (Greber *et al.,* 1996).

Herpesviruses make use of a dynein motor and enter through NPCs

Herpesviruses are large enveloped DNA viruses. Inside the envelope is an icosahedral capsid with a diameter of 120 nm, surrounded by an amorphous protein layer, the tegument. The particles typically contain over 30 structural proteins.

Herpes simplex virus type 1 (HSV-1), the most widely studied of the Herpesviridae, fuses its envelope with the plasma membrane whereby the capsid and tegument proteins are released into the cytosol (Roizman and Sears, 1996). Most of the tegument proteins dissociate from the capsid (Sodeik *et al.,* 1997). The capsid thereafter associates with microtubules (Kristensson *et al.,* 1986; Sodeik *et al.,* 1997). This association is mediated by dynein, a minus-end-directed motor protein, which transports the capsid along microtubules to the microtubule organizing center (MTOC), located adjacent to the nucleus (Sodeik *et al.,* 1997). While microtubule-mediated transport is not absolutely necessary for infection in tissue culture cells, it probably plays an important role in the infection of neurons in which the distance from the site of penetration to the nucleus may be large. It is not yet known which of the viral proteins serves as the

receptor for dynein or for adapter proteins such as dynactin.

As with adenoviruses, albeit more slowly, the herpesvirus capsids accumulate at the nuclear envelope and associate with the NPCs (Granzow *et al.,* 1997; Sodeik *et al.,* 1997) (see Fig. 3). Electron microscopy suggests that attachment occurs via the fibrils that emerge from the pore into the cytosol. Since capsids purified from virus particles bind directly to nuclear pores in isolated nuclei, they must contain signals for nuclear pore attachment. Such *in vitro* binding is sensitive to anti-NPC antibodies and wheat germ agglutinin (P. Ojala, personal communication).

At the pore, the virus releases its DNA, which is translocated into the nucleus leaving an "empty" capsid docked onto the NPC. The events during the actual translocation of the DNA are unclear, but electron microscopy analysis of virus capsids treated with guanidine–HCl *in vitro* showed DNA extruded from discrete sites that coincided with the capsid vertices (Newcomb and Brown, 1994). A transient alteration in the capsid pentons at the vertices may allow local escape of DNA after binding of the capsid to the NPC. Optimally, only one vertice would open—that facing the NPC.

During infection of cells with a mutant of HSV-1, *ts*B7, capsids bind to the NPC but fail to release the DNA (Batterson *et al.,* 1983). The viral components modified in the *ts*B7 mutant have not been categorically identified, but it is likely that a mutation in the large tegument protein, VP1/2, is at least partly responsible for the block in nuclear import of the viral DNA (Batterson *et al.,* 1983).

Baculoviruses probably use actin to get to the NPCs

The baculovirus family comprises a collection of large, enveloped DNA viruses that infect invertebrates. It is divided into two genera; the nuclear polyhedrosis viruses (NPVs) and the granuloviruses (GVs) (Blissard and Rohrman, 1990; Murphy *et al.,* 1995). All have a rod-shaped nucleocapsid, 250–300 $\times$ 30–60 nm, with a distinctive "nipple" structure at the apical end. Both generas of baculoviruses can produce two types of virion, either intracellular (in occlusion bodies; ODVs) or extracellular (budded viruses; BVs).

During cell entry, the BV form enters by endocytosis and the nucleocapsid is released into the cytosol (Blissard, 1996; Miller, 1996). Release of virus from the endosome induces actin polymerization, with incoming capsids localized to one end of polymerized actin tails (Charlton and Volkman, 1993), which may require a nucleocapsid-associated actin-binding protein (Lanier *et al.,* 1996). Treatment of cells with drugs that inhibit actin filament formation delays early gene expression (Lanier and Volkman, 1998). Therefore, it is likely that the capsids are transported through the cytosol by actin polymerization behind the nucleocapsid, which allows them to rapidly reach the nucleus, which possibly is similar to how vaccinia virus egresses from infected cells (Cudmore *et al.,* 1995).

Apparently intact NPV nucleocapsids have been seen inside the nucleoplasm (Granados, 1978; Granados and Lawler, 1981; Hirumi *et al.,* 1975; Knudson and Harrap, 1976) but little is known about their transit into the nucleus. However, electron microscopy has shown that nucleocapsids of GV dock at the NPCs (Raghow and Grace, 1974; Summers, 1971). They bind perpendicularly to the pore, possibly oriented by the nipple structure on the capsid. Some of the NPC-associated capsids contain DNA, whereas others are empty, suggesting that the capsids of NPV remain cytosolic after DNA release. Overall, the mechanism of baculovirus entry into the nucleus remains unclear.

Hepadnavirus capsids: Phosphorylation-dependent association with NPCs

Hepadnaviruses are enveloped viruses with a partially double-stranded DNA genome. The icosahedral capsids have a diameter of 30–34 nm and contain either 180 or 240 copies of a single core protein. In addition to the DNA, the capsid houses a reverse transcriptase/polymerase covalently bound to the DNA, as well as heat shock protein 90 (Hsp 90) and protein kinase C captured from the infected cell cytosol (Kann *et al.,* 1995; Nassal and Schaller, 1993).

The lack of a convenient tissue culture system has made it difficult to study hepadnavirus–cell interactions. Thus, it is still unclear how and where the fusion event occurs that delivers the core into the cytosol (Ganem, 1996; Rigg and Schaller, 1992). Since the DNA is repaired and transcribed in the nucleus, it is thought that the capsid binds to an NPC and delivers the DNA–polymerase complex through the pore. It has been found, consistent with this model, that DNA–polymerase complexes devoid of other viral components are transported to the nucleus after microinjection into cells (Kann *et al.,* 1997).

Recent studies using woodchuck hepatitis virus capsids generated in *Escherichia coli* have shown that phosphorylation of the core protein by the trapped protein kinase C may be essential for nuclear binding of the capsids (Kann *et al.,* submitted for publication). The core proteins of mature capsids are, in fact, phosphorylated at one or more sites close to the C-terminus. This is a basic region of the sequence thought to be located inside the capsid and to interact with viral RNA during assembly and also to contain several putative NLSs.

Only phosphorylated capsids bind to nuclear pores when added to digitonin-permeabilized cells. Binding is dependent on the presence of importins α and β and is

inhibited by peptides corresponding to known NLS sequences and to two sequences from the core protein C-terminal domain (Kann *et al.,* submitted for publication). It is also inhibited by antibodies to NPCs and by wheat germ agglutinin. The phosphorylated capsids thus seem to associate with NPCs through the classical pathway for import of karyophilic proteins. It is possible that phosphorylation by the trapped protein kinase C exposes the C-terminal peptides and the nuclear localization signals on the surface of the icosahedral particle. This may be the reason why the kinase is part of the final virus particle.

Hepatitis B viruses are unusual because not only are the incoming viruses transported to the nucleus, but also the newly assembled progeny viruses. Newly assembled capsid particles in the cytosol can either bud through the membranes of the early secretory pathway and exit the cell as enveloped Dane particles or they can be targeted for the nucleus for amplification of the infection (Ganem, 1996). In other words, unlike with influenza virus (see below), there is no block against reinfection of the nucleus by progeny capsids that have not left the cell. This reinfection process is frequent early in infection and is thought to allow maintenance of infection.

Papovaviruses translocate through the NPC as an uncoated nucleoprotein complex

Papovaviruses, of which the best known are simian virus 40 (SV40) and polyoma virus, are nonenveloped and have an icosahedral capsid structure, approximately 50 nm in diameter. Virions of SV40 and polyoma contain three proteins, VP1, VP2, and VP3, which form the icosahedral capsid shell.

Incoming SV40 binds to major histocompatibility complex (MHC) class I molecules that are concentrated in specialized surface invaginations of the plasma membrane called caveolae (Anderson *et al.,* 1996; Stang *et al.,* 1997). The particles are then internalized in small vesicles and transported to other compartments of the cell, most frequently the ER (Kartenbeck *et al.,* 1989; Maul *et al.,* 1978). Since transport of macromolecules from the plasma membrane to the ER is a relatively rare event in mammalian cells, the transport of SV40 is of considerable interest. It raises several questions. Is it using a pathway that exists under normal physiological conditions? Does it really utilize caveolin-coated vesicles? Is this mechanism in any way part of the process of productive infection?

That SV40 virions may be delivered to the cytosol before entering the nucleus is suggested by experiments using microinjection of intact SV40 virions into the cytoplasm of cells (Clever *et al.,* 1991; Yamada and Kasamatsu, 1993). The DNA of these viruses was imported into the nucleus and the cells were infected. As the maximal pore size is 23 nm, it is difficult to see how translocation through the NPC takes place without either uncoating or a major remodeling of the capsid.

It is likely that the microinjected SV40 was uncoated, because structural studies show that the NLS of the capsid proteins are buried in the interior of the intact capsid (Liddington *et al.,* 1991). *In vitro* reconstitution experiments have shown addition of VP3 (but not of VP1 or VP2) to SV40 DNA allowed efficient translocation of the DNA through the NPC within 2 h of injection (Nakanishi *et al.,* 1996). Although protein-free SV40 DNA can be imported into the nucleus after microinjection, it is an inefficient and probably random process (Dean, 1997).

Like SV40, polyoma virus particles added to cells in culture were taken up by small endocytic vesicles at the plasma membrane (Cole, 1996; Mackay and Consiligi, 1976). These are thought to then fuse with the outer nuclear membrane and thus release the capsid into the nucleus (Griffith *et al.,* 1988). Intact virus particles have, in fact, been observed in the nucleus (Mackay and Consiligi, 1976; Mattern *et al.,* 1966). However, fusion of a pinocytic vesicle with the outer nuclear membrane would deliver the capsid only into the lumen. The suggested nuclear entry process needs to be reexamined with current cell biological techniques, to better understand the various steps.

NUCLEAR EXPORT OF VIRAL GENOMES

The barrier of the nuclear pore is a major factor also during exit of viruses from the nucleus. In some cases the barrier is overcome by lysis of the nucleus along with the cell. This occurs late in infection when the nucleus is filled with assembled virus particles and the cell is in late stages of apoptosis. It is most likely the mechanism used by nonenveloped DNA viruses such as adeno-, papova-, and parvoviruses, which mature inside the nucleus.

The morphogenesis of enveloped viruses by budding from a cellular membrane requires a more orderly and less destructive mechanism of capsid exit from the nucleus. The membrane systems of the cell need to remain intact and membrane transport must be functional.

The ways in which newly assembled capsids and nucleoproteins leave the nucleus are poorly understood. However, there are clearly several different strategies depending on the size of the transported particles. Exit via the NPC is used by retroviruses and influenza virus, which have relatively small RNP genomes. The main assembly process occurs in the cytosolic compartment, usually in contact with membranes at the budding sites. Larger capsids that cannot pass through the nuclear pore may bud across the nuclear envelope (herpesviruses and baculoviruses). In some cases, they may acquire an envelope inside the nucleus. For polydnaviruses, this has been suggested to occur by *de novo*

membrane formation in the nucleus. For occluded baculoviruses it may occur by invagination of the nuclear envelope. Finally, the plant geminivirus (small nonenveloped DNA viruses) express a specialized "movement protein" for transit into and out of the nucleus. The exit and morphogenesis of some of these viruses (HIV, influenza virus, and herpesviruses) is outlined in Fig. 4.

Nuclear exit of RNA virus genomes

Retroviruses have *cis*- or *trans*-acting nuclear export factors

Retroviruses produce three classes of mRNA in the nucleus: completely spliced, unspliced, or partially spliced. The unspliced RNA also functions as genomic RNA and must be exported to the cytoplasm prior to virus assembly. This presents a problem as there is no established cellular pathway for nuclear export of unspliced RNAs. The retroviruses have nuclear export factors that are part of their genome. As reviewed recently (Cullen, 1992; Hope, 1997; Nakielny *et al.,* 1997), these are either *cis*-acting elements (for oncoviruses) or separate genes that act *in trans* (for lentiviruses).

The nuclear export factor for HIV-1 (a lentivirus) is encoded by one of the early viral genes, termed Rev. Early in infection, completely spliced RNAs, including splice variants that transcribe Rev, are exported from the nucleus. Rev is synthesized along with the other early proteins, Tat and Nef. It enters the nucleus using a classical NLS and interacts with the viral RNA via the highly structured Rev-response element (RRE) (Cullen, 1992). When Rev reaches a sufficient level, nuclear export of incompletely and unspliced RNAs is initiated.

Rev mediates export directly (Fischer *et al.,* 1994) because it contains a leucine-based NES as part of the effector domain of the protein (Fischer *et al.,* 1995). This NES has recently been shown to interact with the exportin family of nuclear export receptors (see above) (Fornerod *et al.,* 1997). Complex formation is inhibited by the drug leptomycin B (Wolff *et al.,* 1997), which specifically targets the interaction of the Rev NES with the CRM1 exportin (Fornerod *et al.,* 1997).

An additional cellular protein, the eukaryotic initiation factor 5A (eIF-5A) has also been implicated in Rev-dependent nuclear export. eIF-5A binds specifically to the RRE (Ruhl *et al.,* 1993) and eIF-5A mutants block Rev *trans*-activation and Rev-mediated nuclear export (Bevec *et al.,* 1996). It is possible that eIF-5A acts as a cofactor for nuclear export, somehow linking the Rev NES to the nuclear export receptors.

Export of the HIV genome occurs via the NPC (Fischer *et al.,* 1994; Malim and Cullen, 1991). Virus assembly takes place at the plasma membrane following specific interactions of the RNA with the Gag precursor and the membrane (Stevenson, 1996).

The oncoviruses also use unspliced RNAs to translate structural proteins. However, they do not have Rev-like *trans*-acting factors. Instead, they appear to have circumvented the problem of exporting unspliced RNAs by encoding *cis*-acting elements that interact with the cellular RNA-export machinery (Pasquinelli *et al.,* 1997; Saavedra *et al.,* 1997). These so-called constitutive transport elements (CTEs) have been found in Mason–Pfizer monkey virus (MPMV) and simian retrovirus type-1 (SRV-1). When moved into the HIV-1 genome, the CTEs allow replication of Rev- and RRE-deficient mutants (Bray *et al.,* 1994; Zolotukhin *et al.,* 1994). The cellular protein adenosine 5′-triphosphate-dependent RNA helicase A binds the CTE from SRV-1 (Tang *et al.,* 1997). Helicase A shuttles from the nucleus to the cytoplasm in cells transiently expressing CTE-containing RNA, accounting for the nuclear export of oncovirus genomes. Whether the helicase acts as a nuclear export receptor or whether it stabilizes the structure of the unspliced RNA or releases the RNA from the spliceosome remains to be determined.

Similar *cis*-acting elements are known to occur in the avian retroviruses, where avian cell-specific CTEs confer nuclear export competence to the unspliced genomic RNA (Ogert *et al.,* 1996; Simpson *et al.,* 1997). In principle, therefore, all oncoviruses encode their own CTEs for nuclear export.

Export of hepatitis B virus RNA has features common to oncoviruses and lentiviruses

During hepadnavirus replication, progeny viral RNA is transcribed in the nucleus from covalently closed circular (ccc) DNA. Since these transcripts are not spliced, hepadnaviruses face the problem of having to induce the export of unspliced genomic RNA. As with oncoviruses, a *cis*-acting posttranscriptional regulatory element (PRE) is used (Huang and Yen, 1995, 1994). The nuclear export of the HBV therefore seems to share features with both classes of retrovirus. This is suggested by the report that export of the hepatitis B virus (HBV) genome is blocked by peptides corresponding to the HIV-1 Rev NES (Roth and Dobbelstein, 1997). It is currently unclear to what extent HBV export mechanisms are similar to those of retroviruses.

Influenza vRNP export is controlled by M1

The eight subgenomic influenza virus RNAs are packaged into individual viral RNP complexes in the nucleus (Whittaker *et al.,* 1996b). They are small enough to be transported through the NPC. The exodus begins a few hours after infection, when the viral matrix protein (M1) begins to be synthesized (Martin and Helenius, 1991a). Although the vRNPs are thought to be capable of nuclear exit in the absence of M1, the process is slow (Neumann *et al.,* 1997; Whittaker *et al.,* submitted for publication).

The actual role that M1 plays in vRNP export is still uncertain. However, when vRNPs are trapped in the nucleus by H7 treatment, nuclear M1 is clearly needed to induce vRNP export (Bui *et al.,* submitted for publication). In addition M1 may allow the completion of vRNP assembly, it may contain its own NES, it may mediate the exposure of export signals on the vRNPs, or it may release the vRNPs from nucleoplasmic structures. Its effect on vRNP transfer may also be connected to its capacity to inhibit reimport of exported vRNPs (Whittaker *et al.,* 1996a).

A temperature-sensitive M1 mutant, *ts*51, has yielded valuable information on the role of M1 in nuclear export. It is hyperphosphorylated at the nonpermissive temperature (Whittaker *et al.,* 1995) and differs from the wild type in that it accumulates in the nucleus (Rey and Nayak, 1992). This M1 is able to release the vRNPs from the nucleus. The vRNPs reach the cytosol, but appear to bind very low amounts of M1. These results imply that the role of M1 in the export of vRNPs is to release the particles from the nucleus, but it does not have to form a stable complex with the vRNPs once export has occurred.

As well as acting as a trigger for vRNP nuclear export, M1 plays an important role in the overall regulation of nuclear transport. Late in infection, M1 associates with both the plasma membrane and the vRNPs and prevents cytoplasmic vRNPs from reentering the nucleus (Whittaker *et al.,* 1996a). In heterokaryons consisting of infected and uninfected cells, vRNPs do not enter the uninfected nucleus late in infection. If M1 is prevented from interacting with the cytosolic vRNPs, either by acidifying the cell or by use of the *ts*51 mutant at the nonpermissive temperature, vRNPs can then enter the uninfected nucleus (Bui *et al.,* 1996; Whittaker *et al.,* 1996a). Thus M1 is a major modulator of influenza vRNP nuclear transport.

It is not unlikely that influenza vRNPs have more than one mechanism for nuclear exit. The NS2 protein, in a complex with M1, is thought to be involved in nuclear export because it contains a functional NES, which interacts with the RIP/Rab family of nucleoporins and can functionally replace the effector domain of HIV-1 Rev (O'Neill *et al.,* 1998). It is presently unclear what similarities exist between Rev and NS2. Although NS2 appears to contain an effector domain that can function like Rev, it is not known to bind RNA, and so any role in exporting unspliced RNAs is likely to be mediated through M1.

As well as export being mediated by viral proteins, it is also possible that the influenza vRNPs alone can exit the nucleus. When the NP is expressed in cells it can redistribute to the cytoplasm after initial concentration in the nucleus (Neumann *et al.,* 1997). In fact, it can shuttle between the nucleus and the cytosol (Whittaker *et al.,* 1996a), suggesting that NP may have NESs as well as NLSs.

Nuclear exit of DNA viruses

Herpesviruses and plant rhabdoviruses bud through the nuclear envelope

Herpesvirus capsids are assembled in the nucleus (Roizman and Sears, 1996) and due to their large size (120 nm), transport through the NPC is not physically possible. To exit the nucleus, HSV-1 binds to the inner nuclear membrane and buds into the lumen, see Fig. 5 (Baines and Roizman, 1992; Granzow *et al.,* 1997; Roizman and Sears, 1996). The UL11 gene may be a key factor in this initial budding event, as capsids from mutant viruses bind normally to the inner nuclear membrane but are found in fewer numbers in the lumen. UL11 encodes a myristoylated protein that binds to the inner nuclear membrane and the virus envelope, but not to virus capsids (Baines *et al.,* 1995).

Once initial budding at the inner nuclear membrane has occurred, the subsequent exit pathway of herpesviruses through the cytoplasm is uncertain. Results obtained principally through morphological analysis by electron microscopy suggest two possible models for virus exit (see Fig. 4c). In the first model, capsids would transit directly into the ER, follow the constitutive cellular secretory pathway through the Golgi apparatus, and so reach the cell surface (Harson and Grose, 1995; Johnson and Spear, 1982). Alternatively, capsids become deenveloped at the outer nuclear membrane and accumulate in an unenveloped form in the cytoplasm (Gershon *et al.,* 1994; Granzow *et al.,* 1997; Stackpole, 1969). When HSV-1 mutant *ts*Prot.A is analyzed, double-membraned viruses are seen adjacent to the cytoplasmic face of the nuclear envelope, possibly attached to the outer membrane by a stalk (Church and Wilson, 1997) (Fig. 5b). Within 60 min, large numbers of naked cytoplasmic *ts*Prot.A capsids are then seen, indicating that the newly enveloped capsids are rapidly deenveloped upon leaving the nucleus. The capsids then somehow become wrapped by the membranes of the Golgi apparatus (Granzow *et al.,* 1997) (Fig. 5c) where final glycosylation takes place. This wrapping mechanism shows similarities to the second wrapping step during vaccinia virus morphogenesis (Schmelz *et al.,* 1994; Sodeik *et al.,* 1993).

Two additional pieces of data support the notion that a second envelopment occurs in the Golgi apparatus. Browne *et al.* (1996) used a mutant HSV-1 in which the essential glycoprotein, gH, contained a KKXX ER retrieval signal. This recombinant gH was synthesized, but trapped in the ER. The progeny mutant virus produced did not contain gH, and infectivity was decreased. These results are consistent with virus maturation at a point in

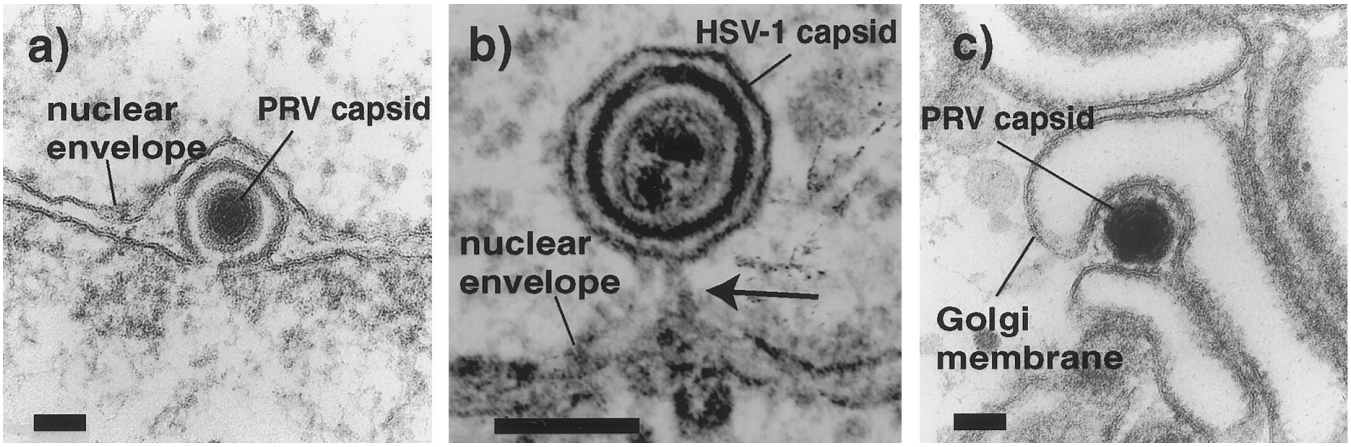


FIG. 5. Envelopment of herpesvirus during exit. Ultrathin Epon sections of cells infected with pseudorabies virus (PRV). (a) PRV capsids bud across the inner nuclear membrane and into the space between the inner and outer nuclear membrane (ONM). (b) Enveloped HSV-1 capsid exiting the ONM. Arrow denotes a "stalk" attaching connecting the virus and the ONM. (c) Naked PRV capsids in the Golgi area of the cytoplasm are wrapped by membranes of tubular vesicles; (a) and (c) were kindly provided by Harald Granzow, Federal Research Centre for Virus Diseases of Animals (Insel Reims, Germany); (b) was kindly provided by Duncan Wilson, Albert Einstein School of Medicine (New York). Bars, 100 nm. Reproduced by permission, American Society for Microbiology.

the secretory pathway downstream of the ER. Using a very different experimental approach, van Genderen *et al.* (1994) showed that the lipid composition of the extracellular HSV envelope contains lipids enriched in Golgi membranes. This also indicates that the final envelope of HSV is derived from the Golgi apparatus. However, it is possible that different herpesviruses acquire envelopes at different intracellular locations.

Rhabdoviruses are enveloped negative-sense RNA viruses, occurring in animals and plants. Whereas replication in animals is cytoplasmic, some plant rhabdoviruses replicate in the nucleus (Francki and Randles, 1979; Matthews, 1991). Electron microscopy analysis of potato yellow dwarf virus (PYDV) (Chiu *et al.,* 1970), bean yellow mosaic virus (Kitajima *et al.,* 1969), and sonchus yellow net virus (SYNV) (van Beek *et al.,* 1985) shows virus cores assembled in the nucleus. The cores have sizes of approximately 75 × 250 nm, i.e., too large to be translocated through the NPC. As with herpesviruses, the cores of PYDV and another plant rhabdovirus, maize mosaic virus, bud through the inner nuclear membrane and accumulate in the lumen (Chiu *et al.,* 1970; McDaniel *et al.,* 1985). The virus envelope then seems to fuse with the outer nuclear membrane, releasing the core components into the cytosol (van Beek *et al.,* 1985). The further trafficking steps leading to egress from the cell are not known.

Polydnaviruses apparently assemble a membrane inside the nucleus

The polydnaviruses are unusual in many ways. Not only do they have a segmented double-stranded DNA genome comprising 15–30 DNA molecules, but they have a symbiotic relationship with species of a parasitic wasp (order Hymenoptera) (Fleming, 1992; Summers and Dib-Hajj, 1995). The viruses occur in two forms, ichnoviruses and bracoviruses. Members of the genus *Ichnovirus* appear to have two membrane envelopes. The inner is thought to be assembled *de novo* in the nucleus and the outer is acquired during budding from the plasma membrane (Norton and Vinson, 1983). The second genus, *Bracovirus,* has a single unit membrane, again assembled *de novo* within the nucleus (Stoltz and Vinson, 1979); they are thought to exit the cell after lysis.

A significant and possibly unique feature of the polydnaviruses is the synthesis of membrane within the nucleus. The production and composition of this membrane is unknown and to date has been characterized by electron microscopy only as an electron-dense structure surrounding the capsid. This form of envelopment is not consistent with current models of eukaryotic lipid biosynthesis or membrane assembly. No other examples of *de novo* membrane assembly within the nucleus are currently known.

Baculoviruses either bud outward through the nuclear membrane or invaginate inward

Assembly of baculovirus capsids occurs in the nucleus (Blissard and Rohrman, 1990), resulting in the formation of nucleocapsids with dimensions of 30–60 × 250–300 nm (Miller, 1996). The large size means that exit from the nucleus via the NPC is not possible. Instead, exit occurs by budding and envelopment. At early stages of infection, from 12 to 24 h, progeny nucleocapsids bud through the inner nuclear membrane, with envelopment apparently initiated by interactions of the "nipple" end of

the nucleocapsid with the inner nuclear membrane (Adams *et al.,* 1977). This route of exit may show features common to the herpesvirus and plant rhabdovirus budding pathway described earlier. Further trafficking events for baculovirus maturation are unclear, but capsids are apparently deenveloped in the cytoplasm and a final envelopment of the nucleocapsids occurs at the plasma membrane (Miller, 1996) to produce the mature enveloped virus (the budded virus or BV) (Hirumi *et al.,* 1975; Raghow and Grace, 1974).

A second group of baculovirus nucleocapsids (the occlusion-derived virus or ODV) are produced late in infection, from 24 h. They are seen to be enveloped within the nucleus (Knudson and Harrap, 1976; Stoltz *et al.,* 1973). Nuclear envelopment was originally thought to occur with a membrane assembled *de novo,* as predicted for the polydnaviruses. However, electron microscopy analysis of baculovirus-infected insect cells has shown regions of the inner nuclear membrane extending inward (Summers and Arnott, 1969). Double-membraned invaginations of the nuclear envelope have been characterized in many cell types (Fricker *et al.,* 1997), but invaginations of the inner nuclear membrane alone have been documented only in insect cells (Parke and de Boni, 1992). It is possible that such invaginations envelop the baculovirus capsid, forming what are termed "baculovirus induced intranuclear microvesicles" (Hong *et al.,* 1994).

Evidence for nuclear envelopment of ODV cores is threefold. First, lipid-like microvesicles are seen to pinch off from the inner nuclear membrane and are subsequently seen surrounding the capsid within the nucleus (Braunagel *et al.,* 1996). Second, lipid analysis of viral envelopes shows ODV envelopes have a similar lipid profiles to isolated nuclei, whereas BVs do not (Braunagel and Summers, 1994). Third, green fluorescent protein (GFP)-tagged virus envelope proteins ODV-E66 and ODV-E25 are first seen in association with the nuclear envelope and as infection progresses, they move to localized regions within the nucleus (Hong *et al.,* 1997). The enveloped viruses within the nucleus are subsequently covered by polyhedron protein to form large aggregates of nuclear viruses, termed polyhedra. Their maturation includes the addition of a protein and carbohydrate "envelope" around the periphery. Upon death of the host and cell lysis, the polyhedra are released into the environment.

Geminiviruses have a movement protein for nuclear shuttling

Geminiviruses are small plant viruses, which, because of their DNA genome, replicate in the nucleus. The capsids are geminate, or twinned, consisting of two incomplete icosahedra, both approximately 25–30 nm each (Murphy *et al.,* 1995). Because of the rigid cell wall, plant viruses have unique problems, as release of viruses into the extracellular space and direct viral entry into cells are, for example, not possible. Therefore specialized movement proteins (MPs) allow infection (Deom *et al.,* 1992). They are implicated in local cell–cell movement and long-distance transport through the plant vascular system.

Geminiviruses have developed two MPs, one of which is likely to mediate shuttling of the virus into and out of the nucleus, while the other is responsible for intracellular transport from one cell to the next (Sanderfoot and Lazarowitz, 1996). Nuclear transport has been studied in bean dwarf mosaic geminivirus (BDMV), which has an MP, BR1, that acts as a nucleo-cytoplasmic movement protein (Pascal *et al.,* 1994). BR1 is responsible for both nuclear import and export. It contains two classical NLSs responsible for nuclear import (Sanderfoot *et al.,* 1996), but the signals for export have not been identified. Therefore, it is unclear precisely how the switch between import and export occurs. The second MP, BL1, which facilitates viral movement through plasmadesmata and across cell boundaries (Noueiry *et al.,* 1994), may provide directionality by redirecting BR1 from the nucleus to the cell periphery (Sanderfoot and Lazarowitz, 1995).

ANALOGIES TO TRANSPORT OF OTHER NUCLEOPROTEIN PARTICLES

Translocation of viral core structures may have analogies to other systems of nucleic acid transport through the NPC. Transport of a specific premessenger RNP, the Balbiani ring granule in the salivary glands of *Chironomus tentans,* has been studied extensively. Nuclear export of the 10–15 $\times$ 30–60 nm particles occurs only after the ring-like structure of the RNP is straightened into an extended ribbon and is oriented with the 5′ end in the lead (Mehlin *et al.,* 1992, 1995). It has recently been shown that a specific protein, hrp36, accompanies the RNP out of the nucleus (Visa *et al.,* 1996a). hrp36 is homologous to the human hnRNP A1 and contains a similar glycine-rich M9-like domain, but its role in nuclear export has not been shown definitively. In addition a second protein, CBP20, is also involved in the nuclear export of *C. tentans* RNPs (Visa *et al.,* 1996b). CBP20 binds to the caps of RNAs in the nucleus and associates with the pre-mRNA during transcription. It remains attached to the 5′ end of the RNP during translocation through the nuclear pore.

A similar system may be present also in plants, where interaction of *Agrobacterium* with plant cells results in crown-gall tumor formation. The *Agrobacterium* single-stranded DNA intermediate T-strand is composed of a complex (12.6 nm $\times$ 3.6 μm) with a single VirD2 molecule at its 5′ end and multiple VirE2 molecules along its

length (reviewed in (Citovsky *et al.,* 1997; Citovsky and Zambryski, 1993)). Both VirD2 and VirE2 contain NLSs (Citovsky *et al.,* 1992; Howard *et al.,* 1992), and it is thought that the DNA docks to the NPC via the VirD2 molecule, at which point transport is initiated. The multiple VirE2 molecules then mediate the translocation of the long DNA through the NPC (Zupan *et al.,* 1996).

CONCLUDING REMARKS

Today, many steps in virus replication are well understood at the molecular level. Virus binding, penetration, assembly, and budding are currently under intensive study in many systems. Nuclear transport steps during virus entry and exit have, however, remained virtually uninvestigated. These steps in the replication cycle are no longer intractable experimentally. The cell biology of nuclear transport of cellular macromolecules has progressed rapidly in recent years, providing extensive new concepts and tools. The structural organization of the NPC is now increasingly clear, specific targeting signals and receptors have been identified, and a variety of *in vitro* and *in situ* assays have been developed. It is now seems possible to analyze the various steps that lead to virus targeting into and out of the nucleus.

A major challenge will be that different virus families use different strategies. The transport through the nuclear membrane is, moreover, often coupled to complex disassembly steps necessary to release the genome and accessory proteins in a transport-competent form. The nature of these steps relies on complex interactions between cellular and viral molecules that may be distinct between viruses.

As with other general processes such as membrane fusion, endocytosis, the molecular biology of nucleic acid, and the immune response, viruses have the potential as model systems to lead the way to new insights otherwise difficult to discover. Examples already exist in this field: SV40 T-antigen and HIV-1 Rev have already played central roles in the identification of nuclear targeting signals and cellular receptors for nuclear protein export, and vesicular stomatitis virus matrix protein has shown novel features of Ran-dependent nuclear transport (Her *et al.,* 1997).

Furthermore, viruses are increasingly used as vectors for delivery of heterologous genes into cells. An understanding of the intracellular trafficking of the virus genome is critical to the development and improvement of new gene therapies. Finally, viruses remain important pathogens for humans, livestock, and crops and an understanding of the nuclear transport pathways offers opportunities for new antiviral strategies.

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