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Review

How viruses access the nucleus ☆

Sarah Cohen [1], Shelly Au [1], Nelly Panté *

Department of Zoology, University of British Columbia, 6270 University Boulevard, Vancouver, British Columbia, Canada V6T 1Z4

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ABSTRACT

Many viruses depend on nuclear proteins for replication. Therefore, their viral genome must enter the nucleus of the host cell. In this review we briefly summarize the principles of nucleocytoplasmic transport, and then describe the diverse strategies used by viruses to deliver their genomes into the host nucleus. Some of the emerging mechanisms include: (1) nuclear entry during mitosis, when the nuclear envelope is disassembled, (2) viral genome release in the cytoplasm followed by entry of the genome through the nuclear pore complex (NPC), (3) capsid docking at the cytoplasmic side of the NPC, followed by genome release, (4) nuclear entry of intact capsids through the NPC, followed by genome release, and (5) nuclear entry via virus-induced disruption of the nuclear envelope. Which mechanism a particular virus uses depends on the size and structure of the virus, as well as the cellular cues used by the virus to trigger capsid disassembly and genome release. This article is part of a Special Issue entitled: Regulation of Signaling and Cellular Fate through Modulation of Nuclear Protein Import.

1. Introduction

In order to establish a productive infection, viruses must overcome multiple barriers within the host cell. These barriers include the plasma membrane and underlying cell cortex, an extremely dense cytoplasm through which molecular traffic is highly restricted (reviewed in [1]) and any other membranes that must be crossed in order to access the sites of viral replication or assembly. How different viruses accomplish these feats depends to a large degree on the size and structure of the virus. Viruses consist of an RNA or DNA genome surrounded by either multiple copies of capsid proteins (non-enveloped viruses) or both capsid proteins and a lipid membrane (enveloped viruses). The size of animal viruses ranges from approximately 25 nm to over 300 nm. The key features of the viruses discussed in this review are summarized in Table 1.

Viruses first attach to the host cell through interactions between viral membrane proteins (enveloped viruses) or three-dimensional structures on the capsid (non-enveloped viruses) and cell surface receptors; viruses are then internalized either by direct fusion of the viral envelope with the plasma membrane, or via one of the cell's many endocytic pathways (reviewed in [2–4]). If entry is by endocytosis, then the virus escapes from the endocytic compartment to the cytosol. The escape strategy depends on the type of virus. For enveloped viruses, this involves fusion of the viral envelope with endosomal membranes. For non-enveloped viruses the endosomal escape process is less well understood, but can involve lysis of the endosomal membrane employing lytic peptides [3]. The released viral capsid or nucleoprotein complex then traverses the cytoplasm, often by associating with cellular motor proteins which traffic along various cytoskeleton components [1,5]. Upon reaching the cellular compartment where viral replication occurs, the viral genome is released from the capsid or nucleoprotein complex, often concurrently with capsid disassembly. After using the cellular machinery for genome synthesis and production of new viral proteins, progeny virions are assembled, and then released from the cell. Release is usually through budding at the plasma membrane or into the endoplasmic reticulum (ER) followed by exocytosis for enveloped viruses; for non-enveloped viruses, it is generally thought that virions are released during cell lysis, although some viruses may also be released by exocytosis [2].

Many viruses, including most DNA viruses and some RNA viruses, depend on nuclear proteins for replication; therefore, their viral genome must enter the nucleus of the host cell (reviewed in [6–9]). Although there are numerous benefits, entry into the nucleus also poses a serious challenge for these viruses, since the nuclear envelope (NE) acts as a barrier between the cytoplasm and the nucleus, and transport of

Abbreviations: AAV2, adeno-associated virus 2; AcMNPV, *Autographa californica* multiple-capsid nucleopolyhedrovirus; cNLS, classical nuclear localization sequence; cPPT, central polypurine tract; CPV, canine parvovirus; ER, endoplasmic reticulum; EM, electron microscopy; GDP, guanosine diphosphate; GTP, guanosine triphosphate; GV, Granulovirus; HBV, hepatitis B virus; HIV-1, human immunodeficiency virus 1; HSV-1, herpes simplex virus 1; INM, inner nuclear membrane; MLV, murine leukemia virus; MVM, minute virus of mice; NE, nuclear envelope; NLS, nuclear localization sequence; NP, nucleoprotein; NPC, nuclear pore complex; NPV, Nucleopolyhedrovirus; Nup, Nucleoporin or NPC protein; ONM, outer nuclear membrane; PIC, pre-integration complex; RNAi, RNA interference; SV40, simian virus 40; vRNP, viral ribonucleoprotein complex

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* Corresponding author. Tel.: +1 604 822 3369 (office), +1 604 822 0664 (lab); fax: +1 604 822 2416.
E-mail address: pante@zoology.ubc.ca (N. Panté).

[1] These authors contributed equally.

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molecules into and out of the nucleus is tightly regulated. Because many viruses make use of the host nuclear transport machinery during infection, we briefly summarize the principles of cellular nuclear transport. We then describe the diverse strategies used by viruses to deliver their genomes into the host nucleus, with emphasis on emerging themes in this field.

2. Nuclear import

The NE consists of an inner nuclear membrane (INM) and an outer nuclear membrane (ONM) separated by the perinuclear space, a regular gap of about 30–50 nm. Embedded in these membranes are the nuclear pore complexes (NPCs) — large protein complexes that act as passageways for the transport of molecules into and out of the nucleus. The mammalian NPC is composed of multiple copies of 30 different proteins, called nucleoporins (Nups), arranged in an octagonal structure that is 120 nm in diameter and has a molecular mass of 125 MDa (reviewed in [10,11]). In addition to the NPCs, a major feature of the NE is the nuclear lamina, a thin (20–30 nm) protein layer that is closely associated with both the INM and the underlying chromatin. The nuclear lamina is composed primarily of A- and B-type lamins, members of the intermediate filament protein family. During cell division of higher eukaryotes, the nuclear lamina and NE are temporarily disassembled to allow the partitioning of chromosomes between daughter cells.

Two general mechanisms have been described for nuclear import: passive diffusion and facilitated translocation. Passive diffusion is for ions and molecules smaller than 9 nm in diameter or proteins smaller than 40 kDa, whereas facilitated nuclear import can accommodate the transport of molecules with diameters of up to 39 nm [12]. The facilitated nuclear import mechanism requires a signal residing on the imported molecule (or cargo), and cytoplasmic receptors (called nuclear import receptors, importins, or karyopherins) that recognize the signal and mediate the translocation of the cargo through the NPC (reviewed in [13–15]). Although there are many types of nuclear localization sequences (NLSs), the first identified and most studied signal consists of one or two short stretches of basic amino acids, called the classical NLS (cNLS). Interestingly, the first identified cNLS was discovered in a viral protein, the large T antigen of simian virus 40 (SV40) [16]. The nuclear import of cNLS-bearing proteins is mediated by a heterodimer import receptor consisting of importin α and importin β. The driving force behind nuclear import is a gradient of the GTPase Ran across the NE. RanGTP abounds inside the nucleus, while RanGDP predominates within the cytoplasm [17]. In the cytoplasm the binding of importins to cargo is favoured, while nuclear RanGTP interacts with importins leading to the dissociation and subsequent release of the cargo from the importins into the nucleus [18,19].

An emerging picture is that different transport routes or pathways exist. In other words, different classes of molecules have different types of NLSs, which are recognized by different importins (with at least 28 different importins in humans). Despite significant progress in identifying NLSs and their receptors (many of which have been crystallized and their structure solved, reviewed in [13]) and in characterizing the molecular basis of the recognition of these molecules, the precise molecular mechanism used by molecules to cross the NPC remains unknown. Several models have been proposed in recent years speculating on the mechanism for the facilitated movement of transport receptors and their cargo through the NPC. These include the affinity gradient, the virtual gating, the selective phase partition, the diffuse permeability and the oily spaghetti models (reviewed in [20,21]). As their names suggest, these models explain some biophysical aspects of the movement of molecules through the central channel of the NPC. They take into account either interactions with or partitioning of transport receptors with Nups containing phenylalanine–glycine repeats (FG Nups) which occupy the NPC central channel (reviewed in [22]). Because viral capsids are among the largest cargos that translocate through the NPC, studies on nuclear import of viruses might provide important information that can be used to test the several proposed models for NPC translocation.

3. Nuclear entry of viruses

The general current understanding is that viruses deliver their genome into the nucleus of their host cells by using the machinery that evolved for the nuclear import of cellular proteins (i.e., NPCs, NLSs, importins, GTP, and Ran). Some of the nuclear transport machinery implicated in the nuclear entry of the viruses discussed in this review is summarized in Table 2. Because the size and structure of viruses vary enormously (for example, herpesviruses are 180–225 nm in diameter [23], but parvoviruses are 18–26 nm in diameter

Table 1
Summary of the major characteristics of the viruses discussed in this review.

Virus family	Virus size (nm)	Structure interacting with NPC and its size (nm)	Genome architecture and size (kb)	References
Enveloped				
Baculovirus -*Autographa californica* multiple-capsid nucleopolyhedrovirus (AcMNPV) -*Trichoplusia ni granulosis* virus	60×300	Rod-shaped capsid (30–60×250–300)	DNA, double-stranded circular (90–180)	[123]
Herpesvirus -herpes simplex virus 1 (HSV-1)	180–225	Icosahedral capsid (120)	DNA, double-stranded linear (152)	[23]
Orthomyxovirus -influenza A	80–120	Helical vRNP (15×50–100)	RNA, negative sense (13.6)	[63,70]
Retrovirus -human immunodeficiency virus 1 (HIV-1) -murine leukemia virus (MLV)	80–130	PIC (size unclear)	RNA, positive sense (7–13)	[159]
Hepadnavirus -hepatitis B virus (HBV)	42–47	Icosahedral capsid (32 or 36)	DNA, double-stranded circular (3.2)	[113,114]
Non-enveloped				
Adenovirus -adenovirus 2	105 (with fibers)	Icosahedral capsid (90)	DNA, double-stranded linear (36)	[96,160]
Polyomavirus -simian virus 40 (SV40)	40–45	Icosahedral capsid or subviral particle (40–45)	DNA, double-stranded circular (5)	[161]
Parvovirus -adeno-associated virus 2 (AAV2) -minute virus of mice (MVM) -canine parvovirus (CPV)	18–26	Icosahedral capsid (18–26) (may not interact with NPC)	DNA, single-stranded linear (5)	[24]

Table 2
Nuclear transport machinery exploited by the viruses discussed in this review.

Protein	Alternative Name(s)	Virus	References
Importin α	Karyopherin α	HIV-1	[43,51,52]
		Influenza A Virus	[72,81,82]
		Adenovirus	[109]
		HBV	[118]
Importin β	Karyopherin β1 p97	HIV-1	[41]
		HSV-1	[87]
		Adenovirus	[109]
		HBV	[118]
		Influenza A Virus	[162]
Importin 7	IPO7 Ran binding protein 7 (RanBP7)	HIV-1	[53–55]
		Adenovirus	[109]
Transportin 1	TNPO1 Importin β2 Karyopherin β2	Adenovirus	[109]
Transportin 3	TNPO3 Importin 12	HIV-1	[56,57]
		Influenza A Virus	[83]
Nup62	p62	HIV-1	[61]
Nup98	Nup98–Nup96	HIV-1	[61]
Nup153		HIV-1	[45,56,59]
		HBV	[122]
Nup155		HIV-1	[60]
Nup214	CAN	HSV-1	[92]
		Adenovirus	[106]
Nup358	Ran binding protein 2 (RanBP2)	HIV-1	[56,59]
		HSV-1	[89]

[24]) and because there are several nuclear import pathways, each virus has evolved a unique strategy to deliver its genome into the nucleus. As indicated in Fig. 1, five general strategies have been identified, which we have ordered according to where in the cell uncoating of the viral genome occurs:

1) Some viruses, such as the retrovirus murine leukemia virus (MLV), gain access to the nucleus during mitosis, when the NE is temporarily disassembled.
2) Some viruses, such as human immunodeficiency virus 1 (HIV-1) and influenza A virus, undergo extensive disassembly in the cytoplasm. The cytoplasmic released components contain NLSs and are thereby able to cross the NPC using the host transport machinery.
3) Some viral capsids use importins or viral proteins to attach to the cytoplasmic side of the NPC. Interaction with the NPC is then used as a cue for disassembly, and the viral genome crosses the NPC and is released into the nucleus, often as a complex with viral proteins. Viruses that use this strategy include herpesviruses (which bind to the NPC via importins) and adenoviruses (which bind directly to the NPC).
4) Some viral capsids, such as those of hepatitis B virus (HBV) and some baculoviruses, are small enough to cross the NPC intact. Genome release then occurs at the nuclear side of the NPC or inside the nucleus.
5) Some viruses, such as parvoviruses, do not use the NPC to deliver their genome into the nucleus; rather, they transiently disrupt the NE and nuclear lamina, and enter the nucleus through the resulting gaps.

Although much progress has been made in characterizing the general nuclear entry strategies of different viruses, many of the molecular details remain obscure. The study of viral nuclear entry is

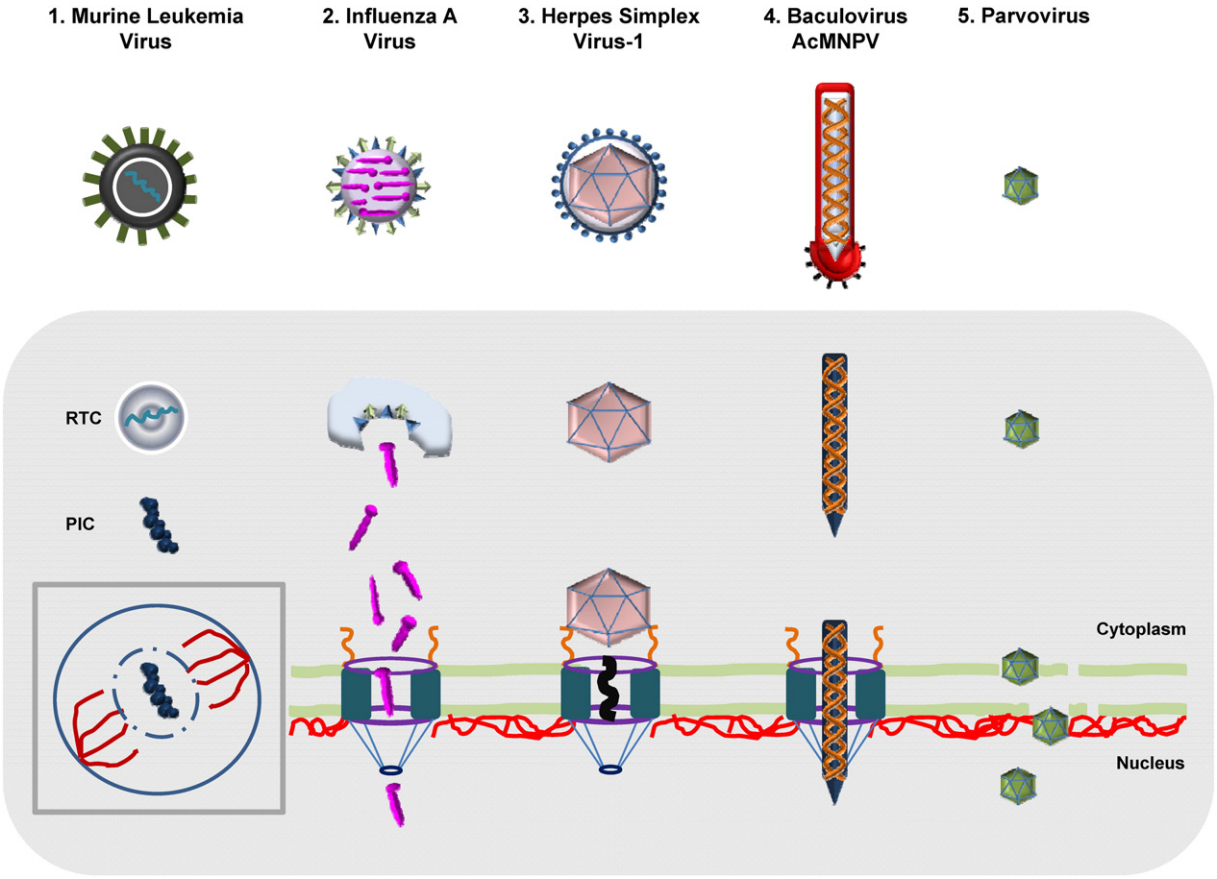

Fig. 1. Schematic representation of different strategies for nuclear entry of viral genomes. (1) The MLV PIC gains access to the nucleus during mitosis, when the NE is temporarily disassembled. (2) Influenza A virus undergoes extensive disassembly in the cytoplasm. The cytoplasmic released vRNPs contain NLSs and are thereby able to cross the NPC using the host transport machinery. (3) HSV-1 capsids use importins to attach to the cytoplasmic side of the NPC. Interaction with the NPC then triggers the release of the viral genome, which then enters the nucleus through the NPC. (4) Capsids of the baculovirus AcMNPV cross the NPC intact. Genome release presumably occurs inside the nucleus. (5) Parvoviruses transiently disrupt the NE and nuclear lamina, and enter the nucleus through the resulting gaps.

complicated by the fact that viral proteins may enter the nucleus multiple times during the virus life-cycle: both as part of an incoming capsid or nucleoprotein, and perhaps also as a newly synthesized protein if assembly of progeny virions occurs in the nucleus. Thus, identification of NLSs and host factors involved in a particular viral nuclear import step can be challenging. Post-translational modifications such as phosphorylation of viral proteins can also play an important role in the exposure of NLSs (reviewed in [25]). This has been studied for HBV (see Section 3.4), but is probably true for other viruses as well. Viral entry into the nucleus and genome release are part of an intricate dance between the virus and host cell, many details of which remain to be elucidated. While some viruses modify or disrupt the cellular nuclear transport machinery during their replication (reviewed in [26]), in the following sections, we discuss the five general strategies of nuclear entry of viral genomes with particular emphasis on the best-studied viruses.

3.1. Nuclear entry during mitosis

Some viruses, such as the retrovirus MLV, can only access the nucleus of a host cell during mitosis, when the NE is temporarily disassembled (Fig. 1; reviewed in [27,28]). While it was initially thought that all non-lentiviral retroviruses required mitosis for nuclear entry of the viral genome, this is now known not to be the case (reviewed in [28]). In addition, there is recent evidence that human papillomaviruses require cell cycle progression in order to establish infection [29]; whether nuclear entry is the barrier in non-dividing cells remains to be determined.

Retroviruses are RNA viruses which reverse transcribe their RNA genomes into DNA; the DNA is then integrated into the host genome, where it serves as a template for the synthesis of new RNA genomes. Retroviruses may enter the cell either by direct fusion of the viral envelope at the cell surface, or by fusion after internalization using an endocytic route [27]. Fusion results in the release of the viral nucleoprotein core particle into the cytoplasm. This is followed by a poorly understood uncoating step and the formation of the reverse transcription complex, which for MLV includes the viral RNA genome, reverse transcriptase, integrase and the capsid protein [30]. Reverse transcription of RNA to DNA produces the pre-integration complex (PIC), which enters the nucleus to integrate into the host genome.

The PIC of MLV is too large to enter the nucleus through the NPC by passive diffusion. Several lines of evidence indicate that MLV waits for NE disassembly in order for the PIC to enter the nucleus. Most retroviruses can only infect actively dividing cells. For MLV, it is thought that the barrier to infection of non-dividing cells is the inability of the PIC to access the nucleus. When the cell cycle is arrested at G_1/S, MLV PICs are present in the cytoplasm, but DNA integration is blocked; if the cell cycle is then allowed to progress to metaphase, the PICs enter the nucleus and integration occurs [31]. In additional support of this idea, random insertion of an NLS into the MLV genome followed by high throughput screening led to the identification of a novel variant that could successfully transduce growth-arrested cells [32], indicating that nuclear entry is in fact the normal barrier. Mutations in the MLV p12 (an MLV-specific cleavage product of the Gag polyprotein) and capsid protein have been shown to specifically block the appearance of nuclear forms of the viral DNA [33–35], indicating that these proteins are likely involved in the inclusion of the PIC within the reforming nucleus after mitosis.

3.2. Genome release in the cytoplasm, followed by entry through the NPC

While similar in many ways to MLV, lentiviruses such as HIV-1 are able to infect terminally differentiated cells in the absence of cell division. HIV-1 entry into cells is similar to the process described above for MLV, although the composition of the resulting PIC is somewhat different. While the MLV PIC includes reverse transcriptase, integrase and the capsid protein, the HIV-1 PIC is composed of reverse transcriptase, integrase, matrix protein, and the accessory protein Vpr, with the capsid protein largely dissociating prior to nuclear entry (reviewed in [28]). It is generally agreed that the HIV-1 PIC enters the nucleus by active transport through the NPC, but the molecular mechanism remains poorly understood.

Every component of the HIV-1 PIC has been suggested to participate in mediating its nuclear entry (reviewed in [28]). Matrix contains NLS-like sequences which target fusion proteins to the nucleus [36–38]. Vpr contains an atypical NLS [39,40], and also interacts directly with Nups [39,41]. In addition, Vpr has been suggested to mediate nuclear entry of the PIC via disruption of the host NE (see Section 3.5). Integrase contains several putative NLSs [42–44], and interacts directly with Nup153 [45]. In addition, a 99-bp triple-strand DNA structure in the centre of the viral DNA called the central polypurine tract (cPPT) or central DNA flap has also been suggested to participate in nuclear entry of the PIC [46,47]. The cPPT has also been proposed to play a role in uncoating of the viral genome, since DNA flap-defective virions failed to disassemble both in infected cells and in an *in vitro* uncoating assay [48].

Interestingly, none of these viral components seems to be absolutely necessary or sufficient for nuclear entry of the PIC. In one study a chimera was created in which the HIV-1 integrase was replaced with the MLV integrase, and all the other described NLSs in matrix and Vpr, as well as the entire cPPT, were eliminated; this chimera was still able to replicate in non-dividing cells [49]. Similarly, an HIV-1-derived virus in which the two NLSs of matrix were mutated and Vpr, the cPPT and a large portion integrase were all removed was still able to enter the nucleus in G_1/S-arrested HeLa cells [50]. However, this mutant virus was partially impaired for nuclear import in non-dividing primary cells, and individual analysis of the mutated components indicated that the cPPT played the most important role. These results suggest that many highly redundant viral components are involved in nuclear transport of the HIV-1 PIC.

Which host factors are involved in nuclear entry of the HIV-1 PIC is also not clear. Members of the importin α family [43,51,52], importin β [41], importin 7 [53–55], and transportin 3 [56,57] have all been shown to be involved in the nuclear import of either individual viral proteins or of the PIC. Depletion of the Nup98 resulted in reduced accumulation of HIV-1 cDNA in the nucleus, suggesting a role for Nup98 in nuclear entry of the PIC [58]. In addition, Nup358 and Nup153 were both identified in two genome-wide RNA interference (RNAi) screens for host factors involved in HIV-1 infection [56,59]. A recent study showed that a single point mutation in the HIV-1 capsid protein could change the nuclear transport requirements of the virus [60]. Wild-type HIV-1 was sensitive to Nup153 depletion, whereas HIV-1 with an N74D mutation in the capsid protein was more sensitive to Nup155 depletion, indicating that HIV-1 may be flexible in its use of host nuclear transport pathways [60]. Thus, the flexibility of HIV-1 in its use of viral and host proteins has made the nuclear entry mechanism of this virus extremely challenging to unravel. In addition, one must use caution when interpreting experiments where the readout is expression of viral proteins. It has recently been shown that the abundance and localization of Nup62 are altered during the late stages of HIV-1 replication [61], indicating that Nups may play important roles during HIV-1 infection at steps other than nuclear entry of the PIC.

Of the viruses that release their genomes in the cytoplasm prior to nuclear entry, the nuclear import of influenza A virus is probably the best studied (Fig. 1). The influenza A virus is an enveloped virus, containing a segmented genome consisting of eight single-stranded negative-sense RNAs. While most RNA viruses replicate in the cytoplasm, influenza replication takes place in the nucleus, likely due to the requirement for cellular splicing machinery present there (reviewed in [62]). Each of the eight RNA segments is separately packed with several copies of the structural nucleoprotein (NP) and a single copy of a trimeric viral RNA polymerase into a viral

ribonucleoprotein complex (vRNP) [63]. In this complex, NP forms a core around which the RNA is helically wrapped [64]. The influenza A virus is internalized into cells via the endocytic pathway using either clathrin- or caveolae-dependent mechanisms [65–67]. The acidic environment of the endosome then triggers the viral fusion machinery, resulting in fusion of the viral and endosomal membranes [68] and disassociation of the viral matrix protein M1 from the vRNPs [69]. This allows the vRNPs to be released into the cytoplasm. Each vRNP has a diameter of about 15 nm and a length between 50 and 100 nm [70]. Thus, vRNPs are too large to enter the nucleus by passive diffusion, and must rather use facilitated translocation. When purified vRNPs are labeled with colloidal gold and microinjected into cells, the vRNPs can be seen threading through the NPCs individually, suggesting that they are transported into the nucleus separately (C. Rollenhagen and N. Panté, unpublished results; Fig. 2).

All four proteins (NP and the three RNA polymerases) of the vRNPs contain NLSs [63]. As newly synthesized NP and viral polymerases undergo transport into the nucleus, where they assemble into new vRNPs, it is not clear whether the NLSs of NP and the viral polymerases are involved in the nuclear import of the newly synthesized proteins or the incoming vRNPs. Nevertheless, NP is thought to mediate nuclear import of the vRNPs. NP contains at least two NLSs: NLS1, also termed the nonclassical or unconventional NLS, spanning residues 1–13 at the N terminus [71,72], and NLS2, also termed the classical bipartite NLS, spanning residues 198–216 [73]. While both NLS1 and NLS2 can contribute to nuclear import of the vRNPs, various types of studies have indicated that NLS1 is a more potent mediator of nuclear import than NLS2 [74–76]. This difference might be due to the location of NLS2 in the intact vRNP. The recent crystal structure of oligomeric NP [77] has indicated that NLS2 might be positioned in an RNA-binding groove, which will make NLS2 less accessible than NLS1. Consistent with this, high-resolution immuno-gold localization of the two NLSs on purified vRNPs revealed that NLS1 is both more highly exposed on the vRNPs, and exposed on a greater number of vRNPs than NLS2 [78]. Although NLS1 and NLS2 are the most studied, experiments with recombinant NP proteins with deletions of both NLS1 and NLS2 have indicated the presence of additional NLSs in NP [71,79]. More recently, a bioinformatics analysis revealed a third NLS in NP, which is located between NLS1 and NLS2 and is present in nine out of 500 NP sequences screened [80]. When fused to GFP, this third NLS was able to import the chimeric protein into the nucleus [80]. The role of this third NLS in the nuclear import of incoming vRNPs remains to be demonstrated.

Consistent with its role in nuclear import, NP binds to a number of human importins α, both *in vitro* and *in vivo* [72,81,82]. Thus, it is thought that vRNPs are transported into the nucleus using the classical importin α/importin β pathway. However, a recent genome-wide RNAi screen identified transportin 3 as a host factor required for influenza virus replication [83], indicating that other nuclear import pathways may play a role as well.

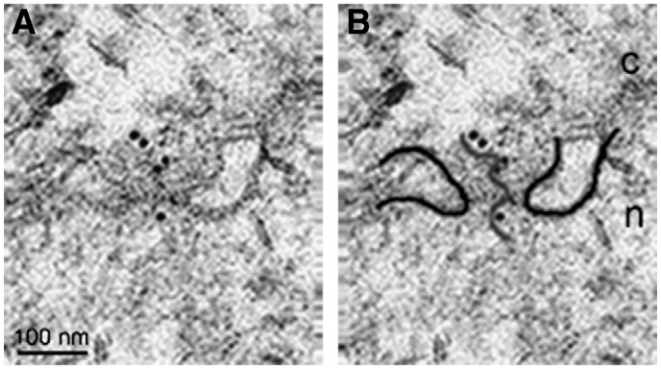

Fig. 2. Import of influenza A vRNPs through the NPC. Electron micrograph of a NPC cross-section from a *Xenopus* oocyte that has been microinjected with influenza A vRNPs conjugated to colloidal gold (9 nm in diameter), and prepared for EM one hour post injection. The vRNPs were conjugated to colloidal gold as described in [157], and microinjection was performed as described in [157,158]. A single vRNP is seen traversing the NPC. (B) Same micrograph as in A, but with the nuclear membrane boundaries and the vRNP outlined. The cytoplasm is indicated by c, nucleus by n.

3.3. Genome release at the cytoplasmic side of the NPC

Herpesviruses and adenoviruses are among the largest and most complex of the viruses that replicate in the nucleus. While each virus has its unique cell entry and disassembly strategy, their capsids – which are released in the cytoplasm during cell entry – attach to the cytoplasmic side of the NPC. However, with diameters of 120 nm (for herpesvirus) and 90 nm (for adenovirus), these capsids are too large to cross the NPC intact; thereby each virus has developed a unique strategy to deliver its genome into the nucleus (Fig. 3).

Herpesviruses are enveloped viruses with an icosahedral capsid containing the viral double-stranded DNA, and a proteinaceous layer (called the tegument) between the capsid and the envelope [23]. The family of herpesviruses is very large; the best characterized member in terms of nuclear import is the human herpes simplex virus 1 (HSV-1). The virion contains more than 30 proteins and its genome is 152 kbp with over 75 open reading frames [23]. HSV-1 enters host cells by fusing its envelope with cellular membranes; either with the plasma membrane (which is thought to be the primary entry pathway) or endosomal membranes after internalization by endocytosis [84]. The capsid with its surrounding tegument is then released into the peripheral cytoplasm. Some of the tegument proteins immediately dissociate from the capsid, others are more tightly attached and remain bound to the capsid. The tegument–capsid structure is then transported by dynein along microtubules to the NPC [85,86]. Electron microscopy (EM) studies using tissue-culture cells infected with HSV-1 [86], as well as *in vitro* binding studies of HSV-1 capsids with isolated nuclei from tissue-culture cells [87] or *Xenopus* oocytes [88], demonstrated that the HSV-1 capsid binds to the cytoplasmic side of the NPC (Fig. 4A). Binding occurs with a distinct orientation: one of the vertices (pentons) of the capsid faces the NPC at a distance of ~50 nm away from the NPC [86,87]. Thus, the capsids are speculated to bind to the filaments that emanate from the cytoplasmic side of the NPC, called NPC cytoplasmic filaments, which are mainly composed of Nup358. A recent study addressing the role of this Nup in HSV-1 nuclear entry showed that capsid binding to the NPC was reduced in cells injected with an anti-Nup358 antibody and in cells depleted of Nup358 by RNAi [89]. NPC-binding of the HSV-1 capsid is importin β-dependent and requires the small GTPase Ran [87]. The viral proteins that mediate the association to the NPC via binding to importin β, however, have not been identified. Tegument proteins have been implicated because removal of these yielded capsids that do not bind to the NPC of isolated nuclei [87]. More recently, it was shown that microinjection of antibodies against the tegument protein VP1/2 inhibited the binding of the HSV-1 capsid to the NPC [89]. It remains to be determined whether VP1/2 binds to importin β.

After binding to the NPC, the HSV-1 capsid releases its DNA into the cell nucleus through the NPC (Figs. 1 and 3). This process leaves intact capsids devoid of the DNA (empty capsids) associated with the NPC [86,87] (Fig. 4B). Very little is known about the mechanisms of DNA release from the HSV capsid, and its transport through the NPC. *In vitro* binding studies of HSV-1 capsids with isolated nuclei demonstrated that DNA release requires the presence of cytosol and energy [87], suggesting that additional cellular factors (beyond those that support binding to the NPC) may be needed. In addition, the tegument protein VP1/2 has long been implicated in DNA release because a HSV-1 temperature-sensitive mutant of this protein (*ts*B7) binds to the NPC, but does not release its DNA at the nonpermissive temperature [90]. More recently, it has been shown that proteolytic cleavage of VP1/2 is required for the release of the viral DNA into the nucleus [91]. At the level of the NPC, Nup214 has

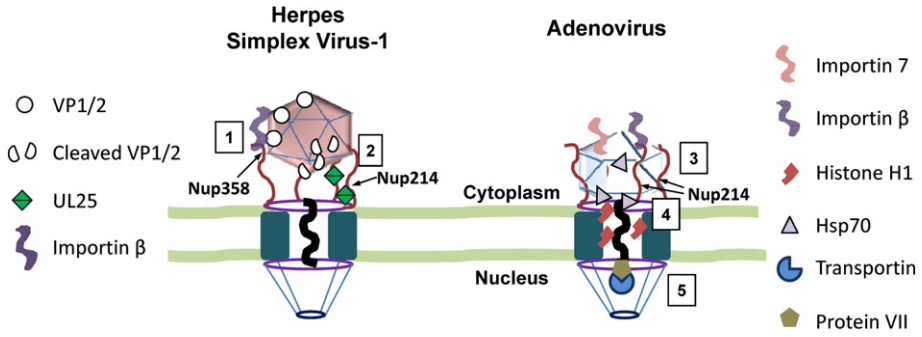

Fig. 3. Schematic diagrams illustrating the protein–protein interactions involved in nuclear entry of the adenovirus and HSV-1 genomes. The HSV-1 capsid binds to the NPC via an importin β-dependent interaction with Nup358; the tegument protein VP1/2 may mediate this interaction (1). Proteolytic cleavage of VP1/2 and interaction of UL25 with Nup214 then trigger DNA release from the intact capsid (2). In contrast, adenovirus capsids bind the NPC via Nup214 in an importin-independent manner (3). The adenovirus capsid then recruits histone H1, importin β, importin 7 and hsp70 (4), which triggers the disassembly/conformational changes required for transportin to bind to protein VII and import the viral DNA into the nucleus through the NPC (5).

been suggested as the cue to trigger DNA release: Nup214 binds to the minor capsid protein UL25, and its depletion by RNAi results in a delay of viral genome delivery into the nucleus [92]. DNA release from the capsid is presumed to occur through the capsid portal, a ring structure formed by 12 copies of the tegument protein UL6 located at a unique capsid vertex [93–95].

The adenovirus capsid has also evolved a nuclear import mechanism in which its genome is released at the cytoplasmic side of the NPC. In contrast to HSV-1, however, the adenovirus capsid completely disassembles at the cytoplasmic side of the NPC (Fig. 3). Adenoviruses are non-enveloped viruses composed of an icosahedral capsid surrounding an inner nucleoprotein core formed by the

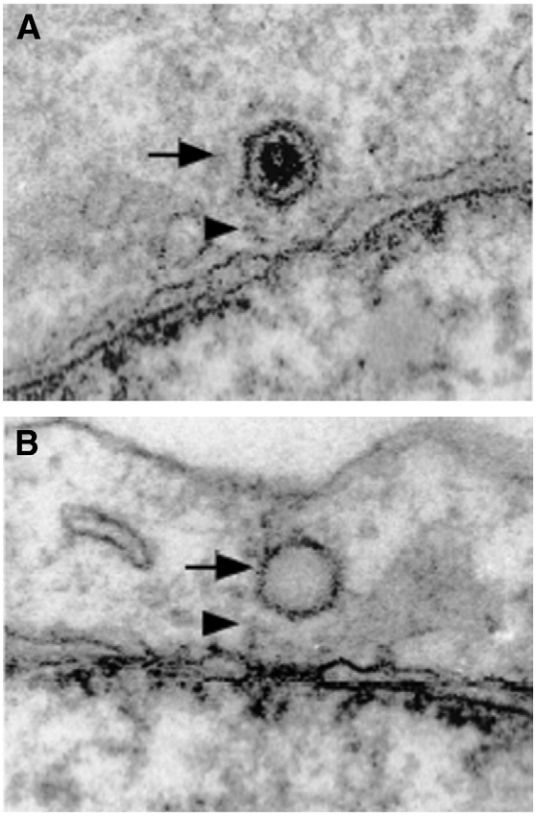

Fig. 4. Docking of the HSV-1 capsid at the cytoplasmic side of the NPC. Electron micrographs of NE cross-sections from Vero cells infected with HSV-1. At later times of infection, both DNA-containing, filled capsids (black arrow in panel A) and uncoated, empty capsids (black arrow in panel B) are located in close proximity to the NPC. Occasionally, capsids can be seen associated with cytoplasmic filaments of the NPC (arrowheads in panels A and B). Reprinted with permission from Ojala et al. [87].

double-stranded DNA genome and several copies of four viral core proteins [96]. A distinct structural feature of adenoviruses is the fibers projecting from the vertices of the capsid. The adenovirus genome comprises about 36 kbp and encodes 45 proteins, of which only 12 are found in the virion [96]. Adenoviruses enter their host cells by receptor-mediated endocytosis and escape the endosome using a capsid component with membrane-lytic activity [97,98]. Both the cellular internalization and the acid environment of the endosome trigger virion disassembly [99,100], which continues in the cytosol. By the time it is delivered to the NPC via microtubule- and dynein mediated motility [101,102], the virion has shed its fibers and several capsid-stabilizing proteins, and some of the remaining viral proteins have been proteolytically processed [100,103]. Upon binding to the cytoplasmic side of the NPC [104,105], adenovirus capsids undergo complete disassembly resulting in the subsequent nuclear import of the viral genome and capsid proteins through the NPC [106].

Experiments using several anti-Nups antibodies demonstrated that the binding of the adenovirus capsid to the NPC is through Nup214 [106], which is located at the base of the NPC cytoplasmic filaments. Thereby, adenovirus is able to dock closer to the centre of the NPC than HSV-1. Strikingly, neither cytosol nor importins α or β are required for binding of the adenovirus capsid to isolated NE [106]. Capsid disassembly is also blocked by antibodies against Nup214 [106], implying that NPC binding is a cue for final capsid disassembly. Fluorescence resonance energy transfer experiments following capsid disassembly in infected cells indicated that the half-time for capsid disassembly is 60 min [107], which coincides with the accumulation of the capsids at the NPC of infected cells [99,105]. Capsid disassembly and nuclear import of the viral genome requires cellular factors, including nuclear import receptors, hsp70 and histone H1 [106,108].

Because nucleic acids are transported through the NPC as nucleoproteins, and because the adenoviral DNA is condensed with viral core proteins, these proteins have been implicated in viral DNA nuclear import [109]. Consistent with this idea, protein VII, the most abundant core protein and the most tightly associated with the viral DNA, has been shown to contain NLSs [109,110] and to bind *in vitro* to several nuclear import receptors including importin α, importin β, importin 7 and transportin [109]. The latter is the nuclear import receptor for several RNA binding proteins (e.g. hnRNP A1), and has recently been shown to mediate the nuclear import of exogenous DNA [111]. The role of transportin in the nuclear import of both protein VII and the viral DNA was recently demonstrated using a permeabilized cell nuclear import assay and recombinant protein VII or purified viral capsids [112]. Taken together these data have led to the current model for nuclear import of the adenoviral genome, which states that after docking to the NPC through Nup214, the adenovirus capsid recruits cellular factors (including histone H1, importin β, importin 7 and hsp70), which triggers the disassembly/conformational changes

required for transportin to bind to protein VII and import the viral DNA into the nucleus through the NPC [112] (Fig. 3).

3.4. Nuclear entry of intact capsids through the NPC, followed by genome release

HBV and baculovirus are among the viruses with capsids small enough to cross the NPC intact. HBV is an enveloped virus with a diameter of 42 to 47 nm, containing a capsid with a single copy of the partially double-stranded DNA genome (3.2 kb) [113]. The capsid is composed of 240 copies of a single type of protein (the core protein, 21 kDa), which is arranged into an icosahedral capsid of 36 nm in diameter [114]. A minor population of capsids with a diameter of 32 nm and composed of 180 copies of core protein also exist. The biological significance of the two different classes of capsids is not clear. Due to the lack of tissue-culture cell lines that can be infected with HBV, the mechanism of HBV cell entry remains uncertain. Analogy with duck HBV suggests that the capsid is released in the cytoplasm after fusion of the viral envelope with a cellular membrane [115] and is transported along microtubules towards the nucleus [116,117]. Studies with recombinant capsids (obtained by expressing the core protein in *E. coli*), and semipermeabilized cells first demonstrated that the HBV capsid binds to the NPC in a phosphorylation- and importin α and β-dependent manner [118]. Phosphorylation of the C-terminus of the core protein is important to expose two cNLSs [118–120]. Following the nuclear import of phosphorylated recombinant capsids after their injection into *Xenopus* oocytes by EM it was demonstrated that the capsid not only binds to the NPC, but is able to cross the NPC without disassembly [12] (Fig. 5). Capsids are, however, not released into the nucleus but get arrested within the NPC nuclear basket – a filamentous structure that extends from the NPC into the nucleus – suggesting that uncoating of the viral genome occurs at the nuclear side of the NPC [12]. This idea was verified by *in-situ* hybridization experiments in semipermeabilized cells [121]. More recently, HBV capsids were shown to bind to Nup153 [122], which resides in the nuclear basket.

HBV uses a unique replication strategy that involves reverse transcription of the initially packaged pregenomic RNA into a partially double-stranded DNA [113]. This process is confined to the interior of intact capsids. Thus, during infection, different types of HBV capsids exist, which have their genome at different stages of maturation. Experiments with capsids containing nucleic acid at different stages of maturation demonstrated that capsids containing the mature genome disassemble at the nuclear basket, releasing their DNA into the nucleus, while those with an immature genome remain bound to Nup153 [121,122]. How Nup153 triggers capsid disassembly and more specifically how it induces disassembly of capsids with mature and not with immature genomes still remains to be established.

In comparison to HBV, the baculovirus capsid is not arrested at the nuclear basket (Fig. 1). Baculoviruses are rod-shaped (30–60×250–300 nm), enveloped viruses with circular double-stranded DNA genomes ranging in size from 90 to 180 kbp with approximately 154 open reading frames [123]. Baculoviruses are unique compared to other viruses because they have two infectious forms: budded virions comprising a single virion enveloped by a plasma-derived membrane, which is involved in cell to cell transmission, and occlusion-derived virions comprising enveloped virions embedded within a crystalline matrix of protein, which are involved in initial host infection when released into the environment upon the death of the host [123]. Although both the occlusion-derived virion and budded virion forms contain rod-shaped capsids enclosed within envelopes of different origins, it is the capsid which eventually gets released into the cytoplasm, is propelled through the cytoplasm by virus-induced actin-polymerization [124–126] and delivers the genome into the nucleus by a mechanism that is largely unknown.

EM studies of baculovirus infected cells have suggested nuclear entry mechanisms that might depend on the type of virus. Baculoviruses are divided into two genera: Granulovirus (GV) and Nucleopolyhedrovirus (NPV). The first study reported capsids from the GV *Trichoplusia ni granulosis* virus docking at the cytoplasmic side of the NPC of cells from infected larvae [127]. Some of the NPC-associated capsids appear partially empty, suggesting a mechanism of DNA nuclear import similar to that of HSV-1, in which the viral genome is released through the NPC, leaving behind empty capsids. Others have detected intact capsids of several types of NPVs at the cytoplasmic side of the NPC and inside the nucleus of cells from larvae inoculated with NPV [128–130]. However, the capsids seen in the nucleus might be newly assembled capsids produced during infection. To clarify this issue a study followed the infection pathway of the NPV *Autographa californica* multiple-capsid nucleopolyhedrovirus (AcMNPV) in tissue-culture cells arrested at G_1/S, and detected AcMNPV capsids at the cytoplasmic side of the NPCs and inside the nucleus [131]. Injection of purified AcMNPV capsids into *Xenopus* oocytes, a cell system in which the virus does not replicate, also reveals capsids docking at the NPCs and inside the nucleus (S. Au and N. Panté, unpublished results; Fig. 6), suggesting that the intact AcMNPV capsid enters the nucleus through the NPC. Thus, the baculovirus genome might enter the nucleus by two separate mechanisms depending on its genus. In any case, the viral and cellular proteins or receptors that are involved in the initial binding step of the capsid to the NPC are largely unknown. Similarly, the viral and cellular components triggering genome release and capsid disassembly remain to be determined.

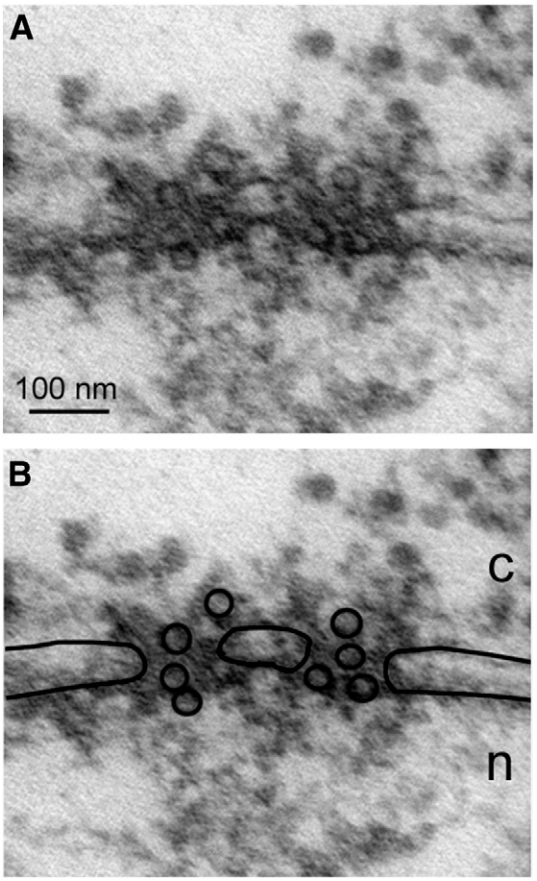

Fig. 5. Nuclear import of intact HBV capsids through the NPC. Electron micrographs of a NE cross-section from a *Xenopus* oocyte microinjected with HBV capsids and prepared for EM one hour post injection, as described in Fig. 2. The HBV capsids can be seen crossing the NPC and lined up along the central channel of the NPC. (B) Same micrograph as in A, but with the nuclear membrane boundaries and the HBV capsids outlined. The cytoplasm is indicated by c, nucleus by n.

Another virus which likely enters the nucleus largely intact is the non-enveloped DNA polyomavirus SV40. SV40 enters the cell by an unusual mechanism: the virus is taken up by caveolar endocytosis, and then traffics to the ER [132]. In the ER, the host chaperone protein ERp29 triggers a conformational change in the capsid [133]. This results in the

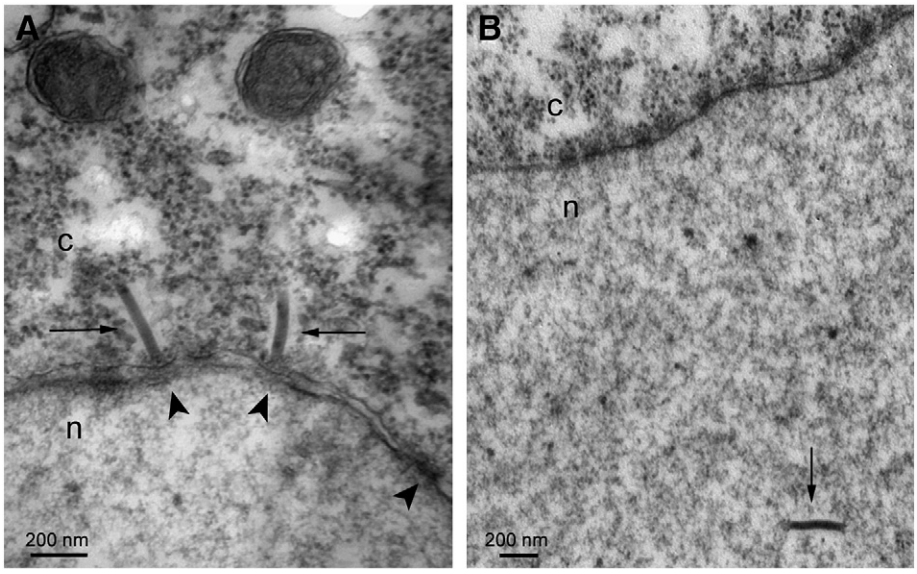

Fig. 6. Nuclear import of baculovirus AcMNPV capsids. Electron micrographs of NE cross-sections from *Xenopus* oocytes microinjected with baculovirus AcMNPV capsids as described in Fig. 2. Oocytes were prepared for EM at four hours post injection. Intact capsids (arrows) can be seen docking at the cytoplasmic side of the NPCs (arrowheads) (A) and inside the nucleus (B). The cytoplasm is indicated by c, nucleus by n.

exposure of the capsid protein VP2, which then integrates into and perforates the ER membrane, releasing the capsid or subviral particle [134]. It is unclear whether the virus escapes from the ER to the cytoplasm and then enters the nucleus through the NPC, or whether it enters the nucleus directly from the ER by penetrating the INM. The current evidence favors the former possibility. When SV40 is microinjected into cells, capsids can be seen traversing the NPCs [135]. However, because microinjection bypasses the normal entry route it is unclear whether this actually occurs during infection. In addition, microinjection of antibodies against SV40 into the cytoplasm inhibits productive infection [136], suggesting that some virions must indeed pass through the cytoplasm. But it is possible that rather than passing through the cytoplasm *en route* to the nucleus, some virions enter the cytoplasm to perform other functions necessary for infection. It is also possible that SV40 can enter the nucleus via multiple routes. Thus, the nuclear entry pathway of SV40 remains elusive.

3.5. Nuclear entry via disruption of the NE

While most viruses that replicate in the nucleus have been shown to make use of the host nuclear transport machinery, including the NPCs, another route is also possible: directly through the nuclear membranes. The small, non-enveloped, single-stranded DNA parvoviruses may use such a mechanism (Fig. 1). Parvoviruses enter the cell via receptor-mediated endocytosis, escape from endosomes with the contribution of a viral phospholipase, and make their way towards the nucleus, possibly by microtubule-mediated transport (reviewed in [137]). Several lines of evidence indicate that the parvoviral genome enters the nucleus in association with an intact capsid after escape from endosomes: microinjection of antibodies against the capsid of the human parvovirus adeno-associated virus 2 (AAV2) into the nucleus inhibits productive infection of tissue-culture cells [138]. In addition, immuno-gold EM has revealed apparently intact capsids of canine parvovirus (CPV) in the nucleus of infected cells in the presence of cyclohexamide, which prevents the synthesis of new capsid proteins [139]. At only approximately 26 nm in diameter [24], parvovirus capsids are small enough to enter the nucleus intact through the NPC, and it has been assumed that this is how the parvovirus genome accesses the nucleus. However, when the parvovirus minute virus of mice (MVM) was microinjected into *Xenopus* oocytes and visualized by EM, unlike influenza vRNPs (Fig. 2) or HBV capsids (Fig. 5), the capsids were not observed in transit through NPCs; instead it was found that MVM induces disruption of the NE [140] (Fig. 7). MVM has also been shown to induce disruption of both the NE and nuclear lamina in infected mouse fibroblast cells [141]. MVM-induced NE and nuclear lamina disruption requires the apoptotic protease caspase-3, and when caspase-3 is inhibited viral capsids accumulate at the NE, suggesting that nuclear entry is via disruption of the nuclear membranes (S. Cohen, A. Marr, P. Garcin and N. Panté, manuscript in preparation). In addition it has been shown that AAV2 enters purified nuclei independently of the NPC [142], suggesting that this nuclear entry mechanism is a common feature of parvoviruses.

While parvoviral capsid proteins do contain functional NLSs [143–145], it is likely that these signals play a role at a different stage of the virus life cycle. For example, the MVM capsid is made up of the capsid proteins VP1 and VP2, in an approximately 1:5 ratio [146]. These proteins are synthesized in the cytoplasm, where they form trimers made up of either VP2 alone, or of two copies of VP2 and one copy of VP1; these trimers are then transported into the nucleus, where capsid assembly occurs [147]. Nuclear import of the trimers is mediated by either an unconventional three-dimensional nuclear localization motif, present in both VP1 and VP2, or by two N-terminal NLSs that are unique to VP1 [147]. However, the nuclear localization motif is buried within the capsid of assembled virions [143,148], and thus cannot be involved in the nuclear entry of incoming capsids. The NLSs in VP1 are also buried within assembled nascent virions; however, there is evidence that the VP1 N-terminus becomes externalized when virions are exposed to acidification in endosomes during the onset of infection [149–151]. Whether the VP1 NLSs are ever sufficiently exposed to interact with importins is unclear. If the VP1 NLSs do become sufficiently exposed to interact with importins, then it is possible that these NLSs play a role in targeting of the parvovirus capsid to the NPC prior to NE disruption and nuclear entry.

In addition to parvoviruses, there are other viruses that may also use a similar strategy. As mentioned above, SV40 may be able to enter the nucleus from the ER by penetrating the INM. This presumably would require disruption of the nuclear lamina. Recent findings show that caspase-6 is activated early on during infection of cells with SV40 [152]. Together with capsase-3, caspase-6 is one of the proteases involved in apoptotic cleavage of nuclear lamins [153]. Thus, caspase-6 activation may be induced by the virus in order to cause lamina disruption, which could then facilitate nuclear entry of the virus via the INM. Lastly, it has been shown that overexpression of the HIV-1

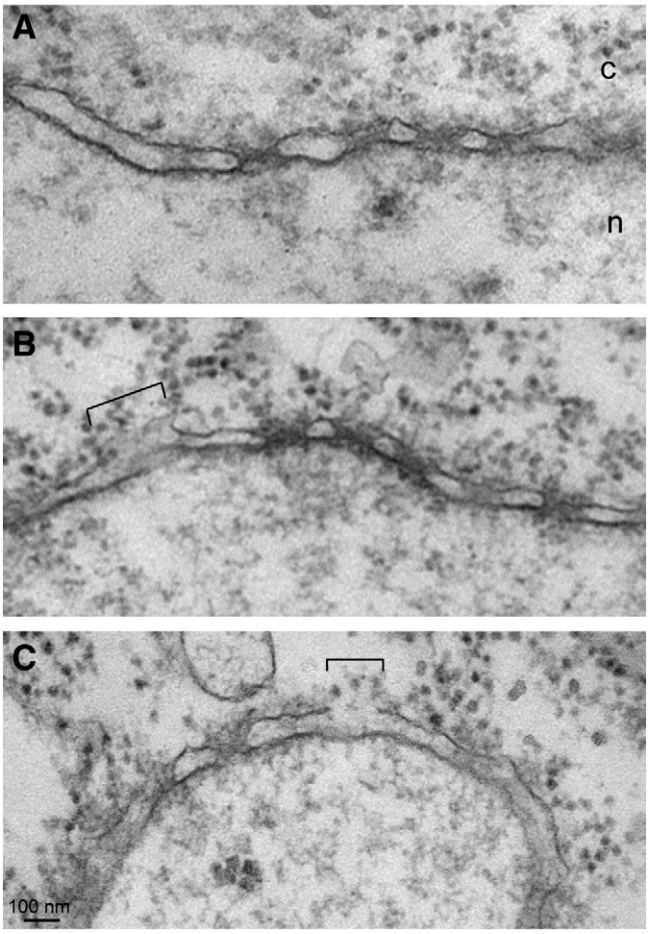

Fig. 7. Parvoviruses induce disruption of the NE. Electron micrographs of NE cross-sections from *Xenopus* oocytes that have been (A) mock-injected, (B) injected with the parvovirus MVM, or (C) injected with CPV, and prepared for EM one hour post injection. Microinjection performed as described in Fig. 2. Disruptions of the NE are indicated by brackets. The cytoplasm is indicated by c, nucleus by n.

protein Vpr induces ruptures of the NE [154]. It has been suggested that these ruptures may mediate entry of the PIC into the nucleus [155]. However, it is unclear whether Vpr-induced NE rupture actually occurs during infection of cells with HIV-1.

4. Concluding remarks

Evidently, viruses have evolved a wide variety of strategies to invade the host cell nucleus. This allows the virus to make use of the cell's machinery for DNA replication and transcription. Virus trafficking and nuclear entry is also intimately linked with virion disassembly. In addition to using the cell's DNA replication machinery, viruses take advantage of compartmentalized cellular cues to ensure that genome release occurs at the correct time. Thus, in addition to cues such as acidification of endosomes, viruses also use binding to NPC proteins, importins or nuclear proteins to trigger genome release.

The different nuclear entry strategies used by viruses depend largely on the size and structure of the virus, and have advantages and disadvantages. The strategy used by MLV – entry during mitosis when the barrier of the NE is absent – has the disadvantage of restricting the virus to infection of dividing cells. Meanwhile, HIV-1 and influenza A undergo extensive disassembly in the cytoplasm. This is likely because the structure of the influenza virion is such that the component released upon viral envelope fusion with the endosomal membrane is a compact and stable vRNP; similarly, it seems that the retroviral PIC must form in the cytoplasm. The consequence is that for both these viruses, the resulting nucleoprotein complex is small enough to traverse the NPC using the host transport machinery. In contrast, for herpesviruses and adenoviruses, the viral component released to the cytoplasm is a large, relatively stable icosahedral capsid. Since the capsid is too large to traverse the NPC, docking occurs at the NPC cytoplasmic side. In both cases, interaction with the NPC is used as a cue to trigger genome release. However, in the case of HSV-1 this involves ejection of the genome from an intact capsid, while the adenovirus capsid disassembles completely. Viruses such as HBV have capsids small enough to traverse the NPC intact. This strategy has the advantage that viral genomes are protected from detection and degradation in the cytoplasm. However, having entered the nucleus intact, disassembly must occur in the nucleus. For HBV, binding at the nuclear side of the NPC serves as a cue for genome release. Lastly, parvoviruses seem to enter the nucleus by inducing disruption of the NE. It is currently puzzling what the advantages might be for a virus to use a nuclear entry strategy that involves disruption of the NE. It is possible that NE disruption results in localized changes in the compartmentalization of cellular proteins in a way that is beneficial for the virus, e.g. cytoplasmic proteins used by the virus for a replication or assembly step are able to leak into the nucleus. It is also possible that disruption of the ONM, which is continuous with the ER, results in release of calcium and that subsequent signaling plays a role in infection.

While significant progress has been made in understanding the general nuclear entry mechanisms used by viruses, much remains to be done. It has become evident that different viruses use different host nuclear import pathways, and viral genomes gain access to the nucleus of their host cells, not only by using the cellular nuclear import machinery, but also components of other cellular pathways, such as proteins of the apoptotic machinery. It is also evident that even viruses using the classical nuclear import receptors have evolved mechanisms to adjust the cell machinery for their needs. Although we now know more about how viruses access the nucleus, many molecular details, such as which viral NLSs are exposed at different times during infection, which viral protein interacts with cellular components, and which host transport factors are involved in each step, remain to be elucidated. For many viruses – non-enveloped viruses in particular – nuclear entry mechanisms have not been studied at all. However, one must use caution when designing experiments to address nuclear entry of viral genomes. Any study on nuclear targeting of viral components must ensure that observed defects are at the level of nuclear entry and not at earlier steps such as transport along microtubules by molecular motors, which can also have an impact on nuclear delivery [156]. In addition, one must design experiments to distinguish between viral NLSs and cellular proteins that play a role in the delivery of viral genomes to the nucleus prior to replication, and those that are important for nuclear import of newly synthesized viral proteins when capsid assembly occurs in the nucleus.

The nuclear delivery of viral genomes is an important field in virology. Discoveries in this field may lead to the development of novel classes of antiviral drugs. We imagine that in the future both viral NLSs and host transport factors could be successfully targeted to limit viral infection and reduce human disease.

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