The EMBO Journal Vol.18 No.2 pp.289–296, 1999

EMBO WORKSHOP REPORT

Viruses, microorganisms and scientists meet the nuclear pore

Leysin, VD, Switzerland, February 26–March 1, 1998

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The nuclear pore

The modular architecture of the nuclear pore complex (NPC) consists of a membrane-embedded basic framework (the 'spoke complex') made up of eight multidomain spokes with two rings on each face (Figure 1A). The ring facing the cytoplasm is decorated with eight ~50 nm fibrils extending into the cytosol, and the nuclear ring is capped with a basket-like assembly of eight thin, 50–100 nm filaments joined distally by a 30–40 nm diameter terminal ring. The center of the basic framework harbors the 'gated transport channel', which is involved in signal-mediated bidirectional transport of macromolecules and is plugged to the central 'transporter', whose ultimate structure and functional role in mediated nuclear transport remains to be established. N.Panté and U.Aebi (Basel) reported their approaches to the functional roles of the different NPC components and to dissection of the steps of nuclear import and export at the NPC by direct visualization of gold-labeled proteins and RNAs moving in or out of the cell nucleus through the NPC (Figure 1B).

Since nucleic acids are not transported through the nuclear pore *per se*, the nuclear–cytoplasmic transport of the genomes is mediated by saturable transport receptors shuttling between nucleus and cytoplasm (Görlich, 1997; Mattaj and Englmeier, 1998). These receptors recognize and bind to nuclear localization signals (NLSs) and nuclear export signals (NESs) presented by the transported molecules and mediate their translocation through the NPC. An import receptor, for example, binds to its substrate in the cytoplasm and carries it through the NPC into the nucleus. An exception here may be the plant import receptor (Merkle *et al*., 1996; Smith *et al*., 1997), which is still found connected to isolated nuclei (T.Merkle, Freiburg). On the nuclear side, the imported substrate is released and the receptor returns to the cytoplasm to initiate additional rounds of import (E.Izauralde, Geneva; Figure 2). Conversely, an export receptor binds its export substrate in the nucleus and releases it in the cytoplasm (Fornerod *et al*., 1997; Kutay *et al*., 1997, 1998).

Uptake of viral genomes—trafficking through the cytoplasm, binding to the nuclear pore and nuclear import

Many viruses depend on nuclear host factors for genome replication and, thus, at some stage, the viral genome must enter the nucleus (Whittaker and Helenius, 1998). In dividing cells, the genome may become trapped during reconstitution of the nucleus in late telophase of mitosis (Roe *et al*., 1993). For successful infection of non-dividing cells, viruses must have developed a strategy that mediates active transport of their genome though the nuclear pore (Figure 3). In general, the strategy implies multiple steps after the virus has entered its target cell: the genome-containing subviral structure (i.e. capsids or protein–genome complexes) must (i) move to the nucleus, (ii) bind to the nuclear pore and (iii) be transported through the central channel of the NPC. Due to the size of most viral capsids, which exceed the maximal functional diameter of the nuclear pore, release of the viral genome from the capsid must be assumed prior to nuclear import. The specific mechanisms of different viruses were presented during the workshop. It became evident that all viruses use a variety of host proteins for the delivery of their genomes into the nucleus.

Trafficking through the cytoplasm

Structures >50 nm cannot diffuse efficiently in the cytoplasm (Luby-Phelps, 1994). Consequently, large viral capsids depend upon active transport to reach the nucleus. It became evident during the workshop that the microtubules are a common pathway. Even non-related viruses use this strategy for intracytoplasmic transport, as was shown for non-enveloped polyoma virus VP1 pseudocapsids (N.Krausewitz, London), adenoviruses (U.Greber, Zürich; Dales and Chardonnet, 1973) and herpes simplex virus (HSV) capsids (B.Sodeik, Hannover; Sodeik *et al*., 1997).

Apparently, this strategy encompasses different pathways depending upon the mode of viral uptake. HSV enters the cell by fusion with the plasma membrane. By transiently overexpressing a dynactin subunit, the function of dynein, a minus-end-directed microtubule ATPase, can be disrupted in living cells, and it was thus demonstrated that herpes capsids required functional dynein for their cytosolic transport. In contrast, adenovirus is endocytosed and travels first within the endosomes. After being released, the adenoviral capsid needs microtubules and dynein for final transport to the nucleus, like HSV. By time-lapse fluorescence staining, U.Greber showed that the capsids move with a velocity of 0.4 μm/s and that, surprisingly, transport of the capsids is a rare event (Greber *et al*., 1997).

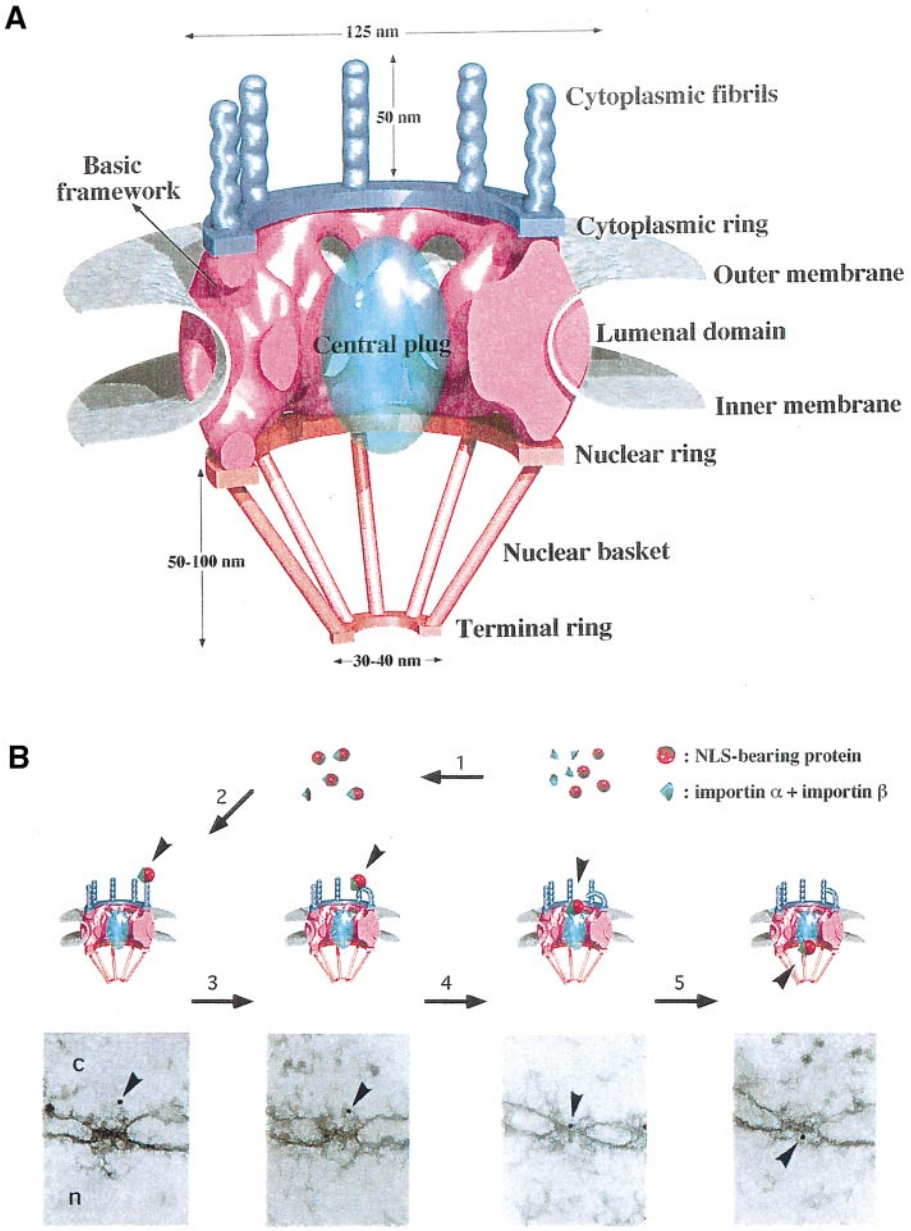

Fig. 1. (**A**) Current consensus model of the membrane-bound NPC. Its major structural components include the basic framework, the central plug or gated channel, the cytoplasmic and nuclear rings, and the cytoplasmic fibrils and nuclear basket. (**B**) Visualization of the different steps of nuclear import of an NLS-bearing protein through the NPC. At the bottom are selected examples of cross-sectioned NPCs with associated nucleoplasmin–gold particles (8 nm diameter), depicted at different stages of transport; at the top is a schematic diagram of the distinct steps visualized by electron microscopy. In the first step of transport, the NLS protein to be imported associates with the NLS receptor (importins α and β) (1). This step takes place in the cytoplasm and does not require physical interaction with any NPC component. In a second step, this 'targeting complex' docks to the distal part of a cytoplasmic filament (2), from where it is delivered to the central gated channel by bending of the cytoplasmic filament (3). Once accumulated at the cytoplasmic entry of the central gated channel (4), the NLS protein, presumably in complex with the NLS receptor, is translocated into the nucleus (5). c, cytoplasm; n, nucleus; bar 100 nm.

With the present state of knowledge, the cytoskeleton appears to be the major transporter within the cytosol. This is not only true of transport to the nucleus but also of viral secretion and intercellular transport. Vaccinia virus induces re-organization of stress fibers into actin tails for leaving the infected cell (Cudmore *et al*., 1995), probably in a regulated manner caused by tyrosine phosphorylation of cortactin, FAK2 and tensin during vaccinia virus infection (M.Way, EMBL, Heidelberg). Intercellular transport of viral components was exemplified by the plant tobacco mosaic virus (TMV) movement protein (MP) which binds to TMV RNA. MP accumulates at the endoplasmatic reticulum (ER), then travels along microtubuli to the plasmodesmata bridging the cell walls to reach the neighboring cell (M.Heinlein, Basel; Heinlein *et al*., 1998).

Binding to the pore and nuclear import

As for the entry of karyophilic proteins, the nuclear import of viral genomes requires docking to the nuclear pore prior to the transport. The different strategies used were discussed during the workshop. However, it is difficult to

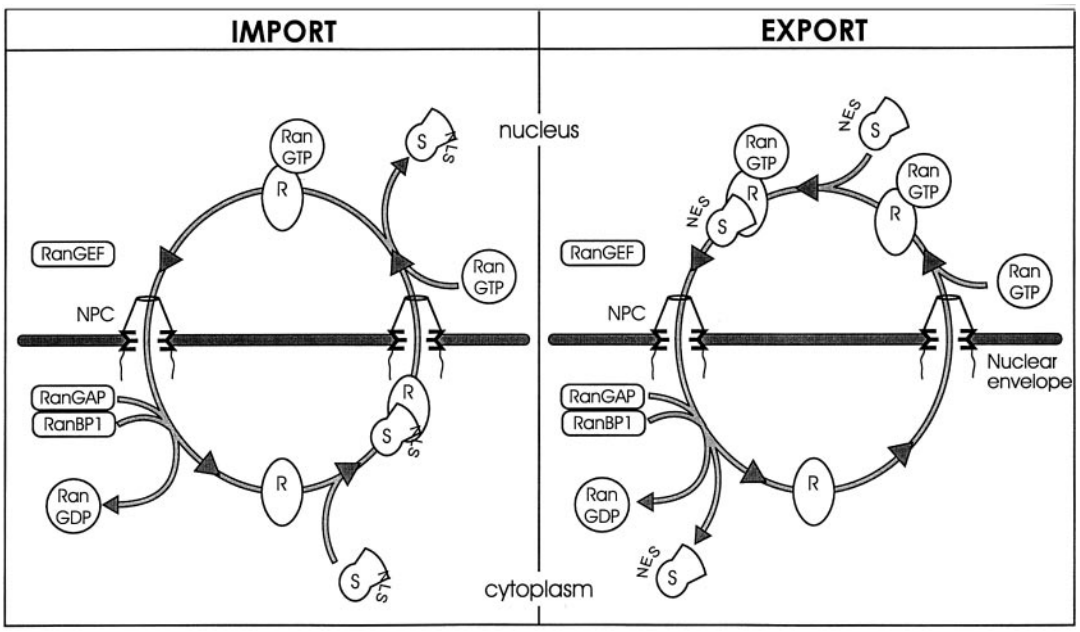

Fig. 2. Nuclear import and export are mediated by shuttling receptors (R) which recognize nuclear localization signals (NLSs) or nuclear export signals (NESs). An import receptor binds its substrate (S) in the cytoplasm, translocates through the nuclear pore complex (NPC) and releases the substrate into the nucleoplasm. The receptor is recycled back to the cytoplasm to initiate another round of import. Conversely, an export receptor binds its cargo in the nucleoplasm and releases it in the cytoplasm. The Ran GTPase plays a central role in nuclear import and export. Together with nucleus- and cytoplasmic-specific proteins which affect its activity, it is a determinant of compartment identity and an effector of substrate binding and release. GAP, GTPase-activating protein; RanBP1, Ran-binding protein 1; GEF, guanine nucleotide exchange factor.

separate docking and transport, and this was only reported for viruses where disassembly of capsids dissects binding of the capsid to the NPC and the nuclear transport of the genome. It became evident that, although different pathways are used, all viruses presented make use of the exposure of some sort of NLS. For hepatitis B virus (HBV; M.Kann, Giessen; Kann *et al*., 1997) and cauliflower mosaic virus (CaMV; D.Leclerc, Basel; Leclerc *et al*., 1999), docking of the capsid via a potential classical NLS of basic amino acids was reported. This implies the use of importin α and β. At least the use of importin β appears to be most probable for the docking of HSV (P.Ojala, Helsinki). However, other types of sequence may be used, as was shown for parvoviruses, where the capsid protein VP2 mediates binding via the amino acid sequence KGKLTMRAKLR; this is not able to mediate nuclear import when fused to a heterologeous protein (J.M. Almedral, Madrid).

Another set of questions concerns the nature of the transported entity: is it DNA alone, a subviral complex or a whole particle? Experiments performed with permeabilized cells and isolated nuclei, as reported at this conference, suggest that the capsids of HBV, CaMV and HSV dock at the nuclear pore but then do not pass the membrane (M.Kann, D.Leclerc and P.Ojala). They may just release DNA through the pore or, as was shown for HBV (Kann *et al*., 1997), genome-bound viral proteins may be involved. However, it may be that the permeabilized cells used in these experiments lacked some factors required to squeeze a capsid through the pore. The *in vitro* observations regarding HSV are in accordance with studies using electron microscopy of HSV-infected cells, where empty capsids at the nuclear pore imply an injection-like transport of the viral DNA into the nucleus without transport of the capsid (B.Sodeik).

For viruses that disassemble prior to the docking of a subviral structure to the nuclear pore, the requirements for the nuclear import of the viral genome have been discussed. All observations of DNA import so far point to proteins as carriers of DNA towards the nucleus, rather than DNA structures themselves. Yet, certain DNA features might increase transport efficiency. In lentiviruses and caulimoviruses, short triple-stranded DNA structures are formed at internal reverse transcription initiation and termination sites. P.Charneau (Paris) pointed out that human immunodeficiency virus type 1 (HIV-1) mutants defective in central initiation and termination, and therefore not able to form the triple-stranded regions, are deficient in DNA translocation through the nuclear pore, regardless of the presence of the karyophilic viral proteins.

However, the necessity for HIV proteins in the cytosolic pre-integration complex has been discussed extensively. The HIV pre-integration complex (Bukrinsky *et al*., 1993) consists of the newly synthesized DNA, nucleocapsid protein, structural protein p6, the accessory protein Vpr, integrase (IN) and several copies of matrix protein (MA). The study of which of these proteins is responsible for DNA transport follows the Hegel principle of 'thesis, antithesis and synthesis', with all opposing views published in leading journals, as is usual in HIV-1 research. Thus, some authors (Gallay *et al*., 1995a) stressed the importance of MA, which has NLS-like sequences, as the key molecule for DNA transport, while others (Freed *et al*., 1997) denied the role of MA and stressed the importance of the IN and Vpr proteins. The compromise presented by D.Trono (Geneva) proposed that all three proteins—MA, Vpr and IN—are able to mediate transport of the genome, thus providing a functional redundancy. However, R.A. Fouchier (Philadelphia, PA) reported evidence that IN is the main transporter acting through its bipartite NLS. Vpr,

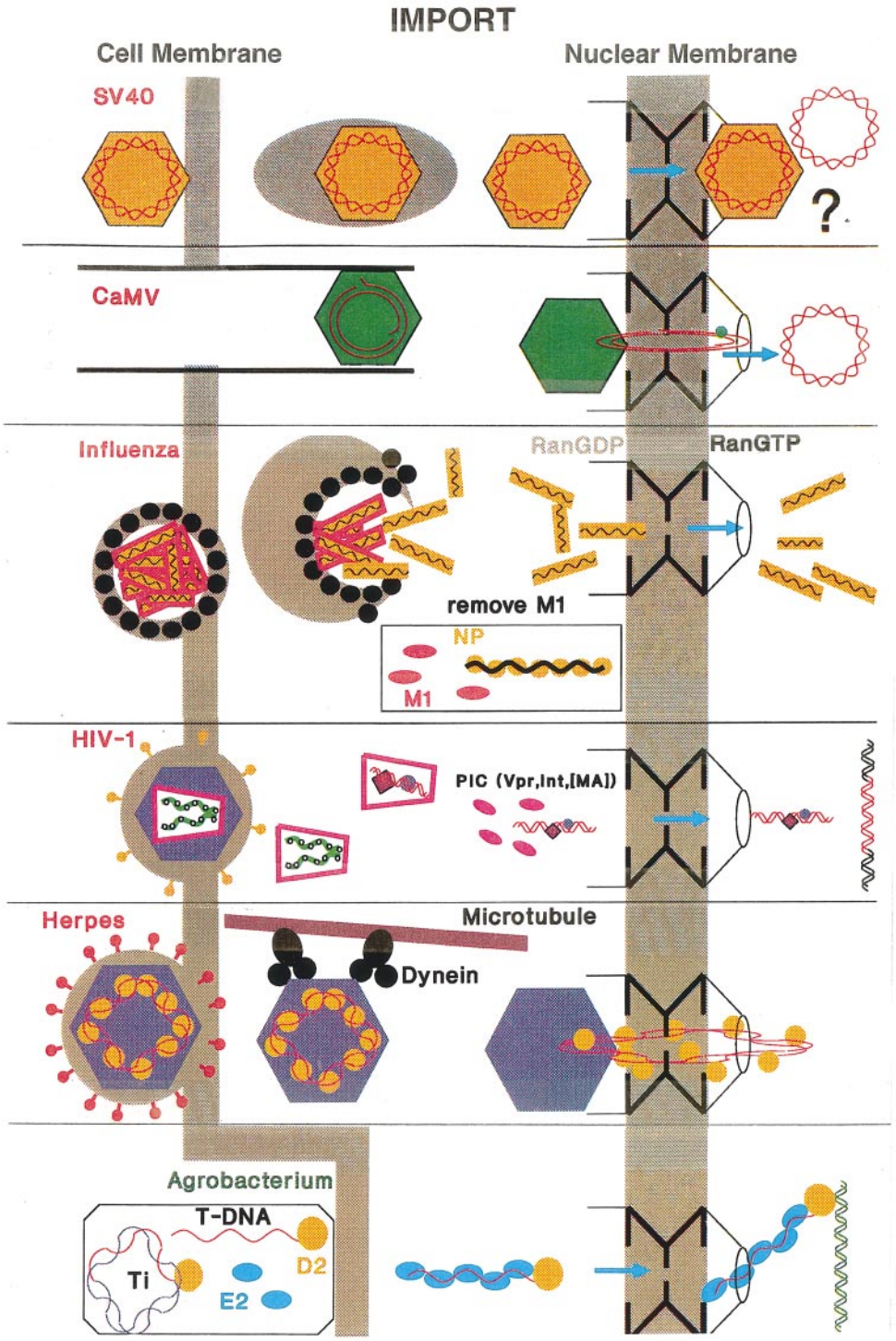

Fig. 3. Nuclear import of viral and bacterial genomes. SV40 virions enter the cell by endocytosis. They are reported to be transported into the nucleus where the nucleic acid is released (Clever *et al*., 1991). Cauliflower mosaic virus enters the cell either from outside by insect transmission (not shown) or from neighboring cells by transport through tubular structures spanning the cell wall. CaMV virions, like hepadnavirus virions (not shown) are thought to dock at the nuclear pore and release their DNA into the nucleus. In both cases, the transferred genome is incomplete, and it becomes 'repaired' in the nucleus to form supercoiled dsDNA and assemble into minichromosomes. Many viruses, such as influenza virus, hepadnaviruses, lentiviruses and herpes virus, enter the cell by endocytosis. In the case of influenza virus, endosomes are formed. The acidic environment of the endosome leads to dissociation of the M1 protein and to fusion of the viral and the endosomal membranes. The set of eight individual ribonucleocapsids are thereby released and transported into the nucleus. Lentiviruses, such as HIV-1, release their nucleocapsids into the cytoplasm, where reverse transcription occurs. The nucleocapsids are dissociated further, yielding pre-integration complexes (PICs) consisting of DNA, Vpr, IN and MA. The proteins cooperate in nuclear targeting and IN integrates the DNA. Herpes virus capsids are transported with the help of dynein along microtubules towards the nucleus, where they release the genome complexed with histone-like virus proteins. Agrobacteria dock at wounds on plant cells. Upon signals derived from the plant, a set of virulence proteins is produced. These are involved in excising single-stranded T-DNA from the Ti plasmid and transporting it into the cell. A complex of T-DNA, VirE2 and covalently bound VirD2 protein is targeted through the nucleus and integrated into the chromosomal DNA.

on the other hand, has no NLS yet it accelerates the transport process (Fouchier *et al*., 1998). It may do this by interacting with phenylalanine–glycine (FG) repeats of the nucleoporins and with importin-α, much like importin-β. In this context, it is interesting that Ty1 also uses IN as a nuclear transporter (Kenna *et al*., 1998; Moore *et al*., 1998).

Caulimo- and hepadnaviruses do not have an integrase and the capsids are fairly stable within the cytoplasm. Therefore, the capsids are the best candidates as mediators of nuclear transport. Their capsid proteins were shown to contain NLSs. These also allow transport of the protein into the nucleus if it is expressed artificially. Why is such a transport of the protein alone rare during a natural infection? Probably because the NLSs of the pararetroviral capsid protein (and the retroviral IN) are regulated. Other pointers to regulation in pararetrovirus infection are the partitioning of genomes in pools transported into the nucleus or into other cells and, in retroviruses, the exclusive use of the nuclear transport mechanism in newly infected cells.

An obvious possibility for regulation of the viral NLS is the original synthesis of polyproteins and preproteins and activation of the NLS upon protein processing. Another possibility would be shielding of the NLS by fast addition of masking proteins or the envelope. Finally, changing the phosphorylation status of the protein could activate an NLS.

In the case of CaMV, the capsid protein is produced as a preprotein containing very acidic termini which are removed upon maturation. D.Leclerc reported that the N-terminal acidic region of the CaMV capsid preprotein in fact masks the NLS located adjacent and downstream of it, and that the NLS becomes activated upon protein maturation by removal of the acidic termini. The N-termini also include substrates for casein kinase II; however, their kinasing status may control protein processing rather than nuclear transport, as was suggested by A.Karsies (Basel).

In the case of hepadnaviruses, the most likely means of control is a change in phosphorylation status. The capsid protein of hepadnaviruses contains several kinasing sites. M.Kann compared the unphosphorylated and phosphorylated capsids of HBV, called ‘cores’ and ‘P-cores’, both expressed in *Escherichia coli*. In digitonin-permeabilized liver cells, only P-cores bind to the nuclear pore complex, mediated by importins α and β. The P-cores are phosphorylated at the fourth serine from the C-terminus and are adjacent to a potential NLS. The use of monoclonal antibodies showed this NLS to be exposed on the surface of only the phosphorylated capsids.

Working with the duck hepatitis B virus (DHBV) in transfected duck hepatocytes, H.Mabit and H.Schaller (Heidelberg) reported on the role of another phosphorylation site. About half of the DHBV capsids are localized in the cytosol, while the other half are associated with the ER or the NPC. Binding to the ER correlates with mature packaged genomes and a low phosphorylation state at the first serine from the C-terminus, while unbound capsids contain immature genomes and are highly phosphorylated at this site. Thus, in both cases, differential phosphorylation of the hepadnavirus capsid protein controls partition of the viral particles and, therefore, ultimately the transport of the DNA into either the producing nucleus or a new one.

Also for HIV-1, control of transport by phosphorylation, in this case the matrix protein, was suggested (Gallay *et al*., 1995b); however, this mechanism remains controversial (Freed *et al*., 1997).

Uptake of bacterial proteins and genomes

Some bacterial pathogens transport part of their components into cells of their eukaryotic hosts. These include pathogens of mammals, e.g. *Yersinia* and *Salmonella*, and of plants, e.g. *Agrobacterium* and *Xanthomonas*. In the plant examples, nuclear transport is also involved. *Agrobacterium* contains a plasmid, the Ti plasmid, bearing the T-DNA sequences. These are transported into the plant cell and then into its nucleus, where they integrate into the chromatin. Virulence (*vir*) genes (Rossi *et al*., 1998) mediate this transport. Some of the *vir* gene products, i.e. VirD2 and VirE2, accompany the transferred DNA. VirD2 is covalently bound to its the T-DNA 5′ terminus and VirE2 coats the entire single-stranded T-DNA. Both of these proteins contain NLSs. Experiments described by B.Hohn and co-workers (Basel) involving *in vitro* nuclear import systems (Merkle *et al*., 1996) and T-DNA complexes assembled *in vitro* showed that both virulence proteins were required for efficient import of T-DNA into plant nuclei. Some plant pathogenic bacteria possess a secretion system encoded by the *hrp* genes. They require corresponding gene products for infectivity in susceptible plants and, as avirulence (*avr*) gene products, to induce programmed cell death (hypersensitive reaction) in resistant plants. G.Van den Ackerveken and U.Bonas reported on an Avr protein (AvrBs3) from *Xanthomonas* which induces the hypersensitive reaction in certain strains of pepper. More than 20 *hrp* genes are required for transfer of this Avr protein. The AvrBs3 protein contains two functional NLSs and an acidic transcription activation domain, both of which are required for induction of the hypersensitive reaction. Yeast two-hybrid experiments revealed that the AvrBs3 protein interacts with importin α. All these findings strongly suggest that the bacterial protein AvrBs3 is in fact transported into the plant nucleus (Bonas and Van den Ackerveken, 1997).

Nuclear export of viral genomes

Viruses can use the host nucleus as an environment for replication to different degrees. In retro- and pararetroviruses, the DNA form of the viral genome is stored and transcribed in the nucleus, while reverse transcription, translation and virus assembly occur in the cytoplasm. A few RNA viruses, such as the influenza virus, replicate their genome in the nucleus and produce there a set of ribonucleoprotein (RNP) particles (nucleocapsids). These are assembled within the cytoplasm. DNA viruses usually replicate their genome in the nucleus; some of them will also assemble in the nucleus and others in the cytoplasm. In the first case, viruses only escape when the host cell dies and/or the nucleus disintegrates. However, herpes viruses leave the nucleus by budding (Baines and Roizman, 1992).

In general, mRNAs are exported in association with several proteins as large RNP complexes (Izaurralde and Adam, 1998; Figure 4). The export is very selective, since

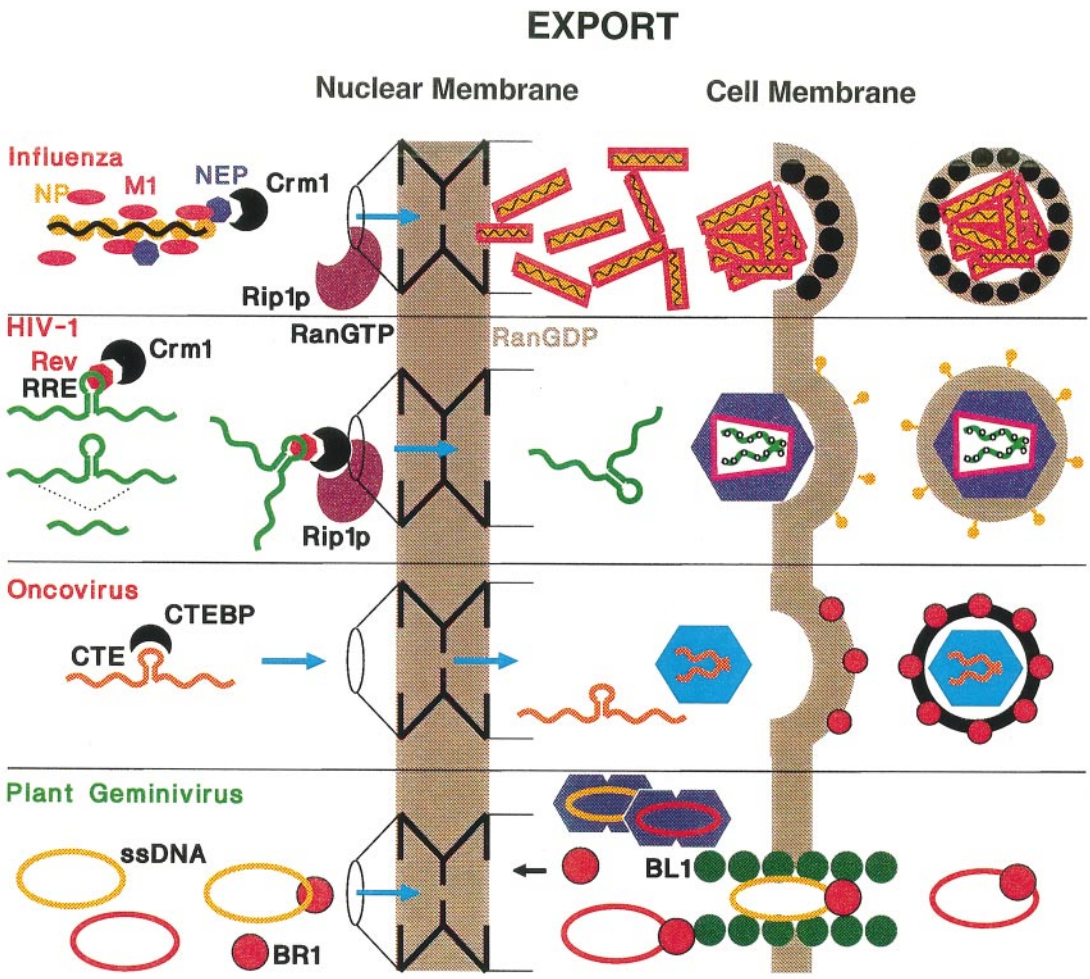

Fig. 4. Nuclear export of virus genomes. Influenza virus nucleocapsids are exported from the nucleus with the help of the virus proteins M1 and NEP. NEP with its export signal presumably interacts with hCrm1 (exportin-1), M1 interacts with NEP and nucleocapsid protein NP interacts with M1. M1 is also the protein that has to be removed from nucleocapsids to allow nuclear import (Figure 3). HIV-1 genomic RNA is exported with the help of the Rev protein, which has a specific binding site for the RRE motif present in unspliced and little-spliced HIV-1 RNA and an NES interacting with hCrm1 (exportin-1). In contrast, many oncoviruses use a constitutive transport element (CTE) on their RNA that interacts with proteins like Crm1. The plant squash leaf curl geminivirus has two viral movement proteins: BR1 is a shuttle protein moving in and out of the nucleus, while BL1, which is associated with tubular structures crossing the cell wall, captures BR1–genome complexes and guides them from cell to cell.

the RNPs are usually retained in the nucleus until they are fully processed. This poses a problem for the export of retro- (and pararetro-) virus genomic RNA, since spliced and unspliced forms co-exist and the requirement for splicing prior to export must be bypassed for a sub-population of molecules. This was clearly illustrated a few years ago by the study of the HIV-1 Rev protein and its ability to export HIV-RNAs. It was found that Rev promotes export of viral RNAs by binding to a specific sequence, the Rev-responsive element (RRE), present on the RNA. Further characterization of this Rev–RRE system led to the identification of the first NES, the Rev-NES, which is also present in several cellular proteins (Fischer *et al*., 1995).

The Rev–RRE system was used to study further interactions involved in RNA export. B.Felber (Frederick, MD) reported that Rev associates in human cells via its NES with protein hCrm1 (exportin-1), and this complex then binds to the nucleoporin Nup98 and to Ran GTPase prior to export. The system seems to be highly preserved, since Rev functions also in yeast, as reported by F.Stutz (Lausanne; Stutz *et al*., 1997). In this host, Rev interacts with the yeast homolog of Crm1 (yCrm1) and this binds to the FG repeat domains of nuclear pore protein Rip1p. Rip1p is a dispensable protein at normal temperatures but becomes essential under heat shock, since it is involved in export of heat shock RNAs (Saavedra *et al*., 1997). Yeast *crm1*$^{-}$ mutants do not transport Rev at either temperature, but providing exogenous Crm1 can compensate this defect.

The export mechanism for influenza virus RNPs seems to be related to the export of HIV-1 RNA. The influenza protein NEP (formerly called NS2) plays a role in export similar to that of HIV-1 Rev (Whittaker *et al*., 1996; O'Neill *et al*., 1998). Both proteins interact with hCrm1 (exportin-1), and NEP can functionally replace the effector domain of HIV-1 Rev in a reporter assay. However, Rev binds directly to RNA, while NEP binds to the viral structural protein 'M1', which itself binds to the viral RNPs (R.O'Neill and P.Palese, NY). M1 seems to be a key regulator in nuclear import/export. After new infections through endocytosis, it is removed from the viral RNPs

in the acidic environment of the endosomes before their release and transport to the nucleus. It is then added in a newly synthesized form to the new RNPs assembled in the nucleus. G.Whittaker (Cornell University, Ithaca, NY) showed, by transient expression employing a recombinant Semliki forest virus vector, that M1 is in fact required for influenza virus RNP export. The inhibition of this export by the protein kinase inhibitor H7 indicates that it is regulated additionally by phosphorylation.

G.N.Pavlakis (National Cancer Institute-FCRDC, Frederick, MD) asked whether only the splicing pathway competes with the Rev–RRE-mediated pathway of HIV-1 RNA export, or whether there is an additional antagonistic principle. He reported the finding of several inhibitory/instability elements (INS) on HIV-1 RNA. One of these is located within the *gag* gene and binds to a poly(A)-binding protein (PAB-1) in a cell-specific manner. He showed by fluorescence labeling that it accumulates in the cytoplasm in cells expressing low amounts of PAB-1, while in those expressing high amounts it accumulates in the nucleus (Afonina *et al*., 1998).

Ordinary retroviruses do not code for a Rev-like protein. Do they still provide for the active transport of unspliced RNA? Simian type D retroviruses have been shown to contain a constitutive transport element (CTE) folding into an extended RNA stem–loop structure. E.Izaurralde (Geneva) reported that this CTE interacts specifically with a protein isolated from HeLa cells and termed it CTE-binding protein (CTEBP). By microinjection into frog oocytes, she showed that CTEBP in fact promotes the export of CTE motif-containing RNAs.

Plant and animal tissues differ. While animal cells can move with respect to each other and only have cell membranes, plant cells are fixed within a solid architecture and also have cell walls. Plant cells are connected by plasma bridges (plasmodesmata), which generally allow passage of small molecules but which can also be manipulated by plant viruses in order to allow passage of their genomes. Furthermore, some viruses initiate the formation of tubular structures crossing the cell walls and use these for virion transport, but plant viruses never move from cell to cell by budding/endocytosis cycles. S.Lazarowitz (University of Illinois, Urbana, IL) reported on the two viral movement proteins of the plant gemini virus, squash leaf curl virus (SqLCV). BR1 is a shuttle protein moving in and out of the nucleus, while BL1, which is associated with tubular structures crossing the cell wall, captures BR1–genome complexes and guides them from cell to cell. BR1 is a multifunctional protein containing two NLSs as well as DNA- and BL1-binding domains. In her contribution, Lazarowitz reported that it also contains a functional NES (Sanderfoot and Lazarowitz, 1996).

The interest in the nuclear pore had brought together a cross-section of virologists, microbiologists, cell biologists, molecular biologists, scientists using plant systems, fungal systems and animal systems, who would normally not meet at conferences. It is therefore only natural that we could all learn a lot from each other. Furthermore, this meeting demonstrated once again how much basic knowledge can be derived from the study of viruses and microorganisms.

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Received July 3, 1998; revised November 13, 1998; accepted November 16, 1998