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REVIEW ARTICLE

The influenza virus nucleoprotein: a multifunctional RNA-binding protein pivotal to virus replication

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All viruses with negative-sense RNA genomes encode a single-strand RNA-binding nucleoprotein (NP). The primary function of NP is to encapsidate the virus genome for the purposes of RNA transcription, replication and packaging. The purpose of this review is to illustrate using the influenza virus NP as a well-studied example that the molecule is much more than a structural RNA-binding protein, but also functions as a key adapter molecule between virus and host cell processes. It does so through the ability to interact with a wide variety of viral and cellular macromolecules, including RNA, itself, two subunits of the viral RNA-dependent RNA polymerase and the viral matrix protein. NP also interacts with cellular polypeptides, including actin, components of the nuclear import and export apparatus and a nuclear RNA helicase. The evidence for the existence of each of these activities and their possible roles in transcription, replication and intracellular trafficking of the virus genome is considered.

Introduction

If one considers the genes encoded by viruses with negative-sense RNA genomes, it becomes apparent that there is a core of three polypeptides common to all families: an integral membrane glycoprotein, an RNA-dependent RNA polymerase and a single-strand RNA (ssRNA) binding protein, often referred to as a nucleoprotein (NP) (Tordo *et al*., 1992). In simplistic terms, the glycoprotein gains the virus access to host cells, the RNA polymerase transcribes and replicates the virus genome, while the NP encapsidates the virus genome to form a ribonucleoprotein (RNP) particle for the purposes of transcription and packaging. This does not mean that this is the only function performed by NP. The purpose of this review is to illustrate using influenza virus NP as a well-studied example that the molecule is much more than a structural RNA-binding protein but also functions as a key adaptor molecule between virus and host cell processes.

General properties and replication cycle of influenza virus

There are three types of influenza virus: A, B and C, of which influenza A virus is both the best characterized and the most serious threat to public health. Influenza A viruses have genomes comprising eight segments of RNA encoding 10 identified polypeptides. Nine of these polypeptides are incorporated into virions. Three viral polypeptides are inserted into the lipid envelope: the haemagglutinin (HA) and neuraminidase glycoproteins, involved in cell entry and exit, respectively, and M2, a low abundance ion channel involved in uncoating and HA maturation. Underlying the membrane is the matrix or M1 protein, the major structural component of the virion which is thought to act as an adaptor between the lipid envelope and the internal RNP particles and is probably the driving force behind virus budding (Gómez-Puertas *et al*., 2000). Inside the shell of M1 lie the RNPs: these comprise the genomic RNA segments in association with a trimeric RNA polymerase (PB1, PB2 and PA subunits) and stoichiometric quantities of NP (Fig. 1a). Also found in the virion are small quantities of the NEP/NS2 polypeptide whose function will be discussed below.

Virus replication begins with entry of the virus into the host cell through receptor-mediated endocytosis. After fusion of endosomal and viral membranes, the RNPs are released into the cytoplasm and then transported into the nucleus, as, unusually for a virus with no DNA-coding stage, influenza virus transcription occurs in the nucleus (Herz *et al*., 1981). An initial round of transcription produces 5′-capped and 3′-polyadenylated mRNAs (Hay *et al*., 1977). These mRNAs are synthesized by a mechanism unique to viruses with segmented ssRNA genomes: endonucleolytic cleavage of host cell

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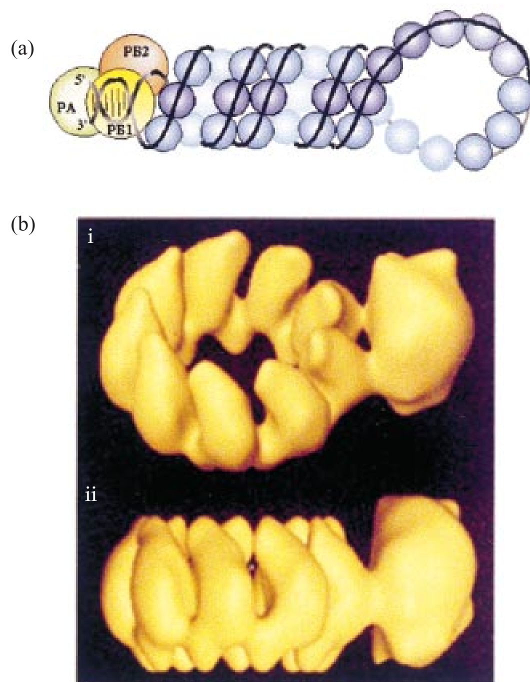

Fig. 1. Structural organization of influenza virus RNPs. (a) Cartoon model of RNP organization. Blue spheres represent NP monomers with associated vRNA molecule (black line). The single-stranded vRNA is coiled into a hairpin structure with a short region of duplex (formed between the 5′ and the 3′ ends) which forms the binding site for the heterotrimeric RNA-dependent RNA polymerase. Adapted from Compans *et al.* (1972). (b) A 27 Å resolution reconstruction of the structure of a recombinant mini-RNP. Oblique (i) and side (ii) views of an RNP consisting of nine NP monomers and one copy of the polymerase. Adapted from Martín-Benito *et al.* (2001) [Three-dimensional reconstruction of a recombinant influenza virus ribonucleoprotein particle (2001). *EMBO Reports*, vol. 2, pp. 313–317] by permission of Oxford University Press.

mRNAs by the viral polymerase results in a short, capped RNA fragment which is then used as a primer. Thus, each viral mRNA contains around 12 bases of heterogeneous host-derived sequence at the 5′ end (Fig. 2a) (Plotch *et al.*, 1981). In the case of influenza viruses, the mRNAs gain a poly(A) tail from repetitive transcription of a short poly(U) stretch near the 5′ end of the viral RNA (vRNA) template (Fig. 2a) (Robertson *et al.*, 1981; Poon *et al.*, 1999). After this primary round of mRNA transcription, the vRNA templates are transcribed into a replicative intermediate RNA (cRNA), which is neither capped nor polyadenylated but, instead, is a perfect copy of the template. These cRNAs then form the template for synthesis of further vRNA segments for amplification of mRNA synthesis and packaging into progeny virions. Both cRNA and vRNA molecules contain a 5′ triphosphate group, leading to the presumption that their synthesis results from unprimed transcription initiation (Young & Content, 1971; Hay *et al.*, 1982). Progeny virions are assembled at the apical surface of the plasma membrane and, therefore, newly synthesized RNPs must be exported from the nucleus and directed to the plasma membrane to allow their incorporation into budding virions. Nuclear export of RNPs has been linked to the M1 and NEP/NS2 polypeptides and the mechanism will be discussed further below.

Structure and activities of influenza virus NP

Influenza A virus RNA segment 5 encodes NP (a polypeptide of 498 amino acids in length), which is rich in arginine, glycine and serine residues and has a net positive charge at neutral pH. However, while the majority of the polypeptide has a preponderance of basic amino acids and an overall predicted pI of 9·3, the C-terminal 30 residues of NP are, with a pI of 3·7, markedly acidic (Fig. 3). In influenza B and C viruses, the length of the homologous NP polypeptide is 560 and 565 residues, respectively (Londo *et al.*, 1983; Nakada *et al.*, 1984). Alignment of the predicted amino acid sequences of the NP genes of the three influenza virus types reveals significant similarity among the three proteins, with the type A and B NPs showing the highest degree of conservation. Phylogenetic analysis of virus strains isolated from different hosts reveals that the NP gene is relatively well conserved, with a maximum amino acid difference of less than 11% (Shu *et al.*, 1993). In addition, the primary sequence of NP is modified by phosphorylation (Privalsky & Penhoet, 1978). All 29 type A strains analysed so far are phosphorylated (Kistner *et al.*, 1989) and, in practically all of these cases, serine is the only phosphorylated residue. The phosphopeptide pattern of NP is strain-specific, dependent on the host cell used for propagating the virus and changes throughout virus replication (Kistner *et al.*, 1985, 1989). In the A/Victoria/3/75 strain, the predominant *in vivo* phosphorylation sites are located near the N terminus at serine 3 and at unidentified serines within the C-terminal 196 residues (Arrese & Portela, 1996). The serine 3 residue is highly conserved in influenza A virus strains and is maintained in all (> 100) but one of the human strains of influenza virus (strain WSN) analysed to date. The role of NP phosphorylation in the virus life cycle remains unclear but its potential role in RNP trafficking is discussed below. Unfortunately, no high resolution structure is available for NP but a 27 Å electron microscopic (EM) model of a recombinant influenza virus RNP generated recently has indicated that the molecule has an elongated, curved, 'banana-like' shape, perhaps comprising two domains (Fig. 1b) (Martín-Benito *et al.*, 2001).

Suggestions that NP contains kinase activity (Skorko *et al.*, 1991; Galarza *et al.*, 1992) have not been confirmed by other laboratories and it is not certain that the protein possesses an enzymatic function. However, NP is able to interact with a variety of other macromolecules, of both viral and cellular origins (summarized in Table 1). As well as binding ssRNA, NP is able to self-associate to form large oligomeric complexes. It also binds the PB1 and PB2 subunits of the polymerase and the matrix protein M1. NP has also been shown to interact with at least four cellular polypeptide families: nuclear import receptors of the importin α class, filamentous (F) actin, the nuclear

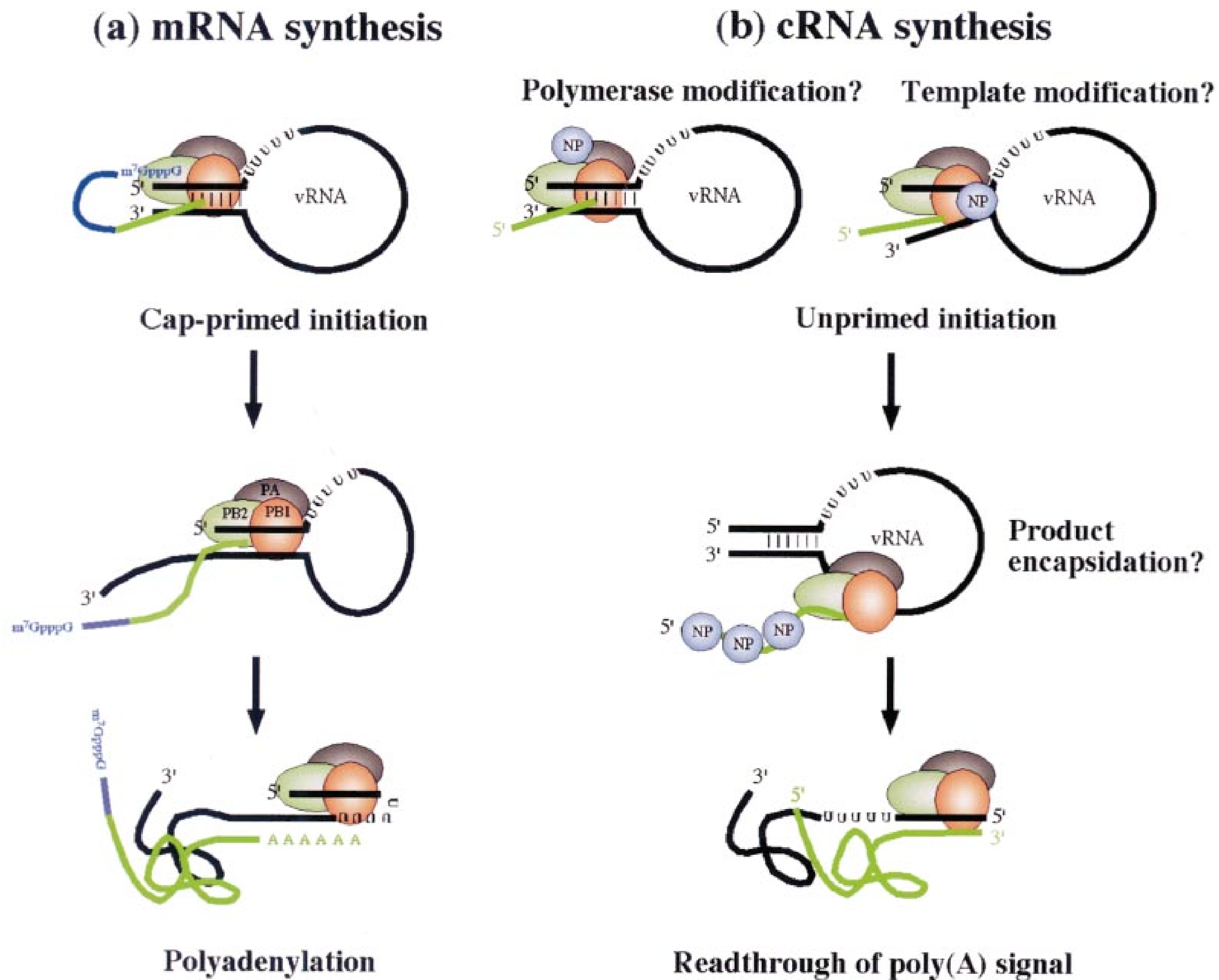

Fig. 2. Models for influenza virus positive-strand RNA synthesis. (a) mRNA synthesis. Binding of the trimeric RNA polymerase to the panhandle structure of vRNA (black line) stimulates its cap-binding and endonuclease activities to generate a short, capped RNA fragment (blue line) from a host cell message which serves as a primer for initiation of virus transcription (green line). The polymerase remains bound to the 5′ end of the vRNA during transcription, leading to an eventual steric block to processive transcription at the polyuridine stretch and consequent synthesis of a poly(A) tail. (b) Possible roles of NP in cRNA synthesis. In the polymerase modification hypothesis, a protein–protein interaction between NP and the polymerase promotes unprimed transcription initiation. In the template modification hypothesis, NP disrupts the panhandle structure and so biases the polymerase towards unprimed transcription initiation. The product encapsidation hypothesis predicts that NP is required to co-transcriptionally coat the nascent cRNA molecule. Synthesis of cRNA terminates after processive readthrough of the polyuridine stretch to produce a full-length copy of the template.

export receptor CRM1 and a DEAD-box helicase BAT1/UAP56. In addition, yeast two-hybrid library screening experiments suggest that this list is not yet complete (Palese *et al.*, 1997). Therefore, it seems reasonable to suppose that the functions of NP in the virus life cycle are mediated through this plethora of binding interactions and the relevant hypotheses will be discussed in the following section. However, the evidence for the existence of each NP activity as well as our current understanding of the regions in the primary structure of NP needed or directly responsible for mediating the interactions will first be reviewed briefly.

RNA-binding activity

Since the earliest recognition that NP binds ssRNA, it has been apparent that it does so with high affinity (K_d of ~ 20 nM) but little or no sequence specificity (Scholtissek & Becht, 1971; Kingsbury *et al.*, 1987; Yamanaka *et al.*, 1990; Baudin *et al.*, 1994; Digard *et al.*, 1999). This is in contrast to the analogous polypeptide from members of the family *Bunyaviridae*, which, in addition to binding ssRNA non-specifically, also display a higher affinity sequence-specific interaction with the 5′-termini of the genome segments (Osborne & Elliott, 2000; Severson *et al.*, 2001). Also unlike the

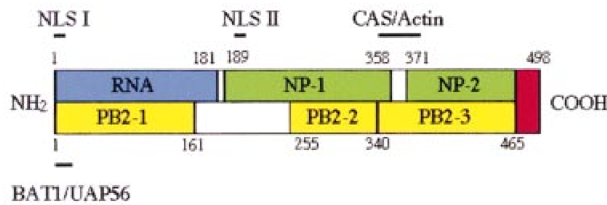

Fig. 3. Functional domains of NP. Sub-fragments of NP identified as capable of binding RNA (blue), NP (green) or PB2 (yellow) are indicated on a linear representation of the NP molecule. Numbers refer to the amino acid co-ordinates. Also indicated is a C-terminal acidic region (red), which acts as a repressor of PB2 and NP binding. Black bars indicate regions shown to be important for binding the cellular polypeptides actin, BAT1/UAP56, importin α (NLS I) and/or function as nuclear localization signals (NLS I and II) or as a cytoplasmic accumulation signal (CAS).

Table 1. Macromolecules bound by influenza virus nucleoprotein

Macromolecule	Function	Reference
Viral		
ssRNA	RNP structure	Pons *et al.* (1969) Baudin *et al.* (1994)
NP	RNP structure	Pons *et al.* (1969) Ruigrok & Baudin (1995) Elton *et al.* (1999a)
PB1	Transcription?	Biswas *et al.* (1998) Medcalf *et al.* (1999)
PB2	Transcription?	Biswas *et al.* (1998) Medcalf *et al.* (1999)
M1	Transcription inhibition	Zvonarjev & Ghendon (1980)
	Nuclear export	Martin & Helenius (1991)
	Assembly?	
Cellular		
Importin α	Nuclear import	O'Neill & Palese (1995) O'Neill *et al.* (1995)
F-actin	Cytoplasmic retention?	Digard *et al.* (1999)
CRM1/exportin-1	Nuclear export?	Elton *et al.* (2001)
BAT1/UAP56	Transcription?	Momose *et al.* (2001)

analogous N proteins from non-segmented negative-sense viruses, NP does not protect RNA from RNase digestion (Duesberg, 1969; Pons *et al.*, 1969; Baudin *et al.*, 1994), although chemical modification experiments suggest that NP binds the phosphate backbone and leaves the Watson–Crick positions of the bases open to the solvent (Baudin *et al.*, 1994). The NP–RNA interaction has been shown to be co-operative (Yamanaka *et al.*, 1990) and the stoichiometry of the interaction estimated at 1 NP per 24 nucleotides of RNA (Compans *et al.*, 1972; Ortega *et al.*, 2000). In addition, comparison of the estimated total length of bound RNA with the dimensions of the RNPs suggests that the RNA is substantially wrapped around each NP monomer (Jennings *et al.*, 1983; Martín-Benito *et al.*, 2001). Analysis of the primary sequence of NP has not identified sequences with similarity to RNA-binding motifs characterized previously. Moreover, despite the basic character of the protein, the basic residues are not clustered in a particular region that could act as an RNA-binding domain. Two independent studies (Albo *et al.*, 1995; Kobayashi *et al.*, 1994) mapped an RNA-binding region to the N-terminal third of the protein by deletion mutagenesis (Fig. 3). This region could be divided further into two smaller regions, which also retained RNA-binding activity (Albo *et al.*, 1995). However, it was noted that, although this N-terminal region functioned in isolation, other sequences were required for the high affinity RNA-binding activity (Albo *et al.*, 1995). More recently, several basic and aromatic amino acids along the length of NP have been shown to be important for RNA-binding by a combination of UV cross-linking, intrinsic fluorescence spectroscopy, chemical modification and mutagenesis experiments (Elton *et al.*, 1999b; Medcalf *et al.*, 1999). Overall, the protein contains an N-terminal region which displays RNA-binding activity but high-affinity binding requires the concerted action of other regions distributed throughout the protein.

Homo-oligomerization

The existence of NP–NP interactions that play a major role in maintaining RNP structure became evident from early work examining the physical characteristics of RNPs after the removal of RNA (Kingsbury & Webster, 1969; Pons *et al.*, 1969). The ability of purified RNA-free NP to form structures resembling authentic RNPs has been demonstrated subsequently by EM analyses (Ruigrok & Baudin, 1995). NP–NP interactions have been estimated to have a K_d of ~ 200 nM in a binding assay, which made use of recombinant immobilized fusion proteins and *in vitro*-translated NP (Elton *et al.*, 1999a). This system was used further to identify NP sequences important for self-association. Deletion mutagenesis identified two independent regions, NP-1 and NP-2 (Fig. 3), capable of forming NP–NP contacts. Interestingly, the C-terminal 23 residues of NP were found to inhibit oligomerization. Point mutations within each of these areas with matching negative and positive effects on oligomerization were also identified (Elton *et al.*, 1999a).

NP–polymerase interactions

In recent years, it has been shown that NP interacts directly with PB1 and PB2 but not with PA, both in virus-infected cells and recombinant systems (Biswas *et al.*, 1998; Medcalf *et al.*, 1999). Consistent with this, a recent EM reconstruction of an RNP clearly shows two regions of contact between the polymerase complex and separate NP monomers (Fig. 1b) (Martín-Benito *et al.*, 2001). In addition, there is genetic evidence for a linkage between NP and PB2. A revertant of a temperature-sensitive (*ts*) virus with a lesion in NP was found to carry an extragenic suppressor mutation, most likely located

in the PB2 gene (Mandler *et al*., 1991). Similarly, an analysis of the compatibility between RNP polypeptides from human and avian strains of influenza virus found a consistent linkage between NP and PB2 (Naffakh *et al*., 2000). Biswas *et al*. (1998) identified three NP fragments (Fig. 3), each of which could interact independently with PB2. They also observed that removal of the last 33 amino acids of NP increased the strength and stability of the NP–PB2 interaction: a finding that is in agreement with the results described for NP–NP oligomerization above.

NP–M1 interactions

Initial studies of disrupted virions indicated an association between M1 and RNPs (Zvonarjev & Ghendon, 1980; Rees & Dimmock, 1981) but, since M1 is an RNA-binding protein (Wakefield & Brownlee, 1989), it was not known if this reflected a direct protein–protein interaction between M1 and NP. However, a recent study that employed mutant M1 molecules unable to bind RNA and RNA-depleted RNPs indicated that M1 does in fact bind to NP directly, although M1–RNA contacts also play a role (Ye *et al*., 1999). However, the M1-binding site on NP has not been identified yet.

NP–importin α interactions

Given our current understanding of the cellular nuclear transport machinery, it now seems self-evident that NP would interact with importin α because of its nuclear localization signal (NLS). However, elucidation of this has not been straightforward. Soon after NP was shown to contain an NLS (Lin & Lai, 1983), amino acids 327–345 (Fig. 3) were proposed to contain a nuclear accumulation signal, based on the behaviour of NP deletion mutants expressed in *Xenopus* oocytes (Davey *et al*., 1985). A decade later, yeast two-hybrid experiments indicated that NP bound members of a family of cellular polypeptides of then uncertain function but which were almost simultaneously identified as components of the nuclear import machinery (O'Neill & Palese, 1995; Gorlich *et al*., 1994; Moroianu *et al*., 1995). However, mutational analysis of NP did not implicate the 'oocyte NLS' as being responsible for this interaction but, instead, identified a short sequence at the N terminus of the protein (Fig. 3, NLS I) which also functioned as a transferable NLS in mammalian cells (Wang *et al*., 1997). Mutation of this NLS in the context of full-length NP did not prevent nuclear import, indicating the presence of other signal(s) in the polypeptide (Wang *et al*., 1997; Neumann *et al*., 1997). Consistent with this, a sequence matching a canonical bipartite cellular NLS has been identified (Fig. 3, NLS II) and shown to be active in the absence of NLS I (Weber *et al*., 1998). However, no evidence has been found to suggest that the oocyte NLS functions as an NLS in mammalian cells (Wang *et al*., 1997; Neumann *et al*., 1997) and, in fact, it has been shown to act in opposition to cause cytoplasmic accumulation of the protein [Fig. 3, cytoplasmic accumulation signal (CAS)] (Weber *et al*., 1998; Digard *et al*., 1999). In addition, systematic deletion analysis of NP suggests the presence of another potential NLS located between amino acids 320 and 400 (Bullido *et al*., 2000).

NP–F-actin interactions

Cell fractionation and co-localization studies suggested that, late in infection, cytoplasmic NP is associated with the cytoskeleton (Avalos *et al*., 1997; Husain & Gupta, 1997). In confirmation of this, purified NP has been shown to bind F-actin *in vitro*, with a K_d of 1 μM and a stoichiometry of 1 NP per actin subunit, and, furthermore, co-localization of NP and *β*-actin has been detected in cells expressing recombinant NP (Digard *et al*., 1999). A discrete sequence in NP capable of binding F-actin has not been identified, but a cluster of point mutations that disrupts the interaction has been characterized (Digard *et al*., 1999). Curiously, these mutations lie within or close to the region identified originally as a nuclear accumulation signal in *Xenopus* oocytes but which has been shown subsequently to promote cytoplasmic accumulation of NP in mammalian cells (Fig. 3, CAS/actin). Accordingly, it has been proposed that the CAS functions as a cytoplasmic retention signal for RNPs by tethering NP to the actin cytoskeleton (Digard *et al*., 1999).

NP–CRM1 interactions

In recent years, it has become evident that, in addition to multiple NLSs, NP also contains signals that work in opposition to cause cytoplasmic accumulation of the polypeptide. Exogenously expressed NP is not static in one cellular compartment but, instead, shuttles between the cytoplasm and the nucleus (Whittaker *et al*., 1996; Neumann *et al*., 1997), suggesting that it contains a nuclear export signal (NES). Even in the absence of other influenza virus proteins, the balance between the import and export signals is not fixed, as, depending on the circumstances, static immunofluorescent 'snapshots' of NP distribution can show the bulk of it in either the nucleus or the cytoplasm, or distributed evenly between the two compartments (Neumann *et al*., 1997; Digard *et al*., 1999). Treatment of NP-expressing cells with the drug leptomycin B (LMB), which specifically inactivates CRM1/exportin-1, a cellular NES receptor (Fornerod *et al*., 1997; Kudo *et al*., 1998), biases NP towards a more nuclear distribution (Elton *et al*., 2001). This suggests that NP contains an NES recognized by CRM1 and, in support of this hypothesis, overexpression of CRM1 biases transfected NP towards cytoplasmic accumulation and the two proteins interact in *in vitro*-binding assays (Elton *et al*., 2001). The identity of the NP NES(s) remains uncertain. Residues 1–38 have been proposed to contain an NES (Neumann *et al*., 1997) but this hypothesis awaits further testing. In addition, the CAS sequence is an alternative or additional candidate for an NES, as the techniques used so far to examine the behaviour of NP molecules bearing lesions within this region do not

formally distinguish between cytoplasmic retention and nuclear export.

NP–BAT1/UAP56 interactions

A cellular splicing factor belonging to the DEAD-box family of RNA-dependent ATPases, BAT1/UAP56, was identified as an NP-interacting polypeptide by a convincing congruence of different techniques in independent laboratories. On one hand, it was identified by fractionation of nuclear extracts from uninfected cells in a search for a stimulatory factor for influenza virus RNA synthesis; on the other hand, by a yeast two-hybrid screen for NP-interacting polypeptides (Momose *et al*., 2001). Deletion analysis of the NP–BAT1 interaction *in vitro* and in yeast led to the identification of a BAT1-binding site in the N-terminal 20 amino acids of NP (Fig. 3) (Momose *et al*., 2001).

Roles of NP during the virus life cycle

Early efforts at defining the roles of individual influenza virus genes relied heavily on a genetic approach of studying conditional-lethal and, in particular, *ts* virus mutants (Simpson & Hirst, 1968). Analysis of viruses containing lesions in segment 5 showed two classes of defects: those where vRNA synthesis failed, indicating an involvement of NP in RNA replication, and those where vRNA and polypeptide synthesis were apparently normal (reviewed by Mahy, 1983). On the basis of negative evidence, the latter group was assumed to be the result of NP defects that interfered with virus maturation (Mahy, 1983). Thus, from the very beginning of the characterization of NP, some evidence existed for its participation at multiple stages of the viral life cycle.

Viral RNA synthesis

NP has long been known to be the major protein component of influenza virus RNPs (Pons *et al*., 1969) and, as these particles were quickly identified as transcriptase complexes (Bishop *et al*., 1971), a role for NP in vRNA synthesis seemed likely. The most obvious role is a structural one of maintaining the RNA template in an ordered conformation suitable for transcription by the polymerase and/or packaging into virions. EM visualization of RNPs reveals rod-like particles, often with loops at one end; these structures have been interpreted as strands of NP–RNA complexes bent into a hairpin and twisted into a helical structure (Fig. 1a) (Jennings *et al*., 1983). This model has been elegantly confirmed by the recent EM reconstruction of a recombinant mini-RNP (Martín-Benito *et al*., 2001) in which the 'barrel' of the RNP is lost because of the artificial shortness of the vRNA molecule, leaving only the terminal loop (Fig. 1b). Formation of these structures depends on a combination of NP–RNA and NP–NP interactions, as discussed in the preceding section. However, in the absence of an identified sequence-specific interaction between NP and the genome segments, it is unclear what directs the specific encapsidation of v- and cRNA but not mRNA (Hay *et al*., 1977). One interesting possibility is that the sequence-specific interaction of the polymerase complex with the 5′ end of vRNAs (Tiley *et al*., 1994) functions as a specific encapsidation signal via ensuing protein–protein interactions between NP and PB1 and/or PB2. Also, host cell proteins may influence RNP formation, as the cellular polypeptide BAT1/UAP56 has been proposed to act as a chaperone for non-RNA-bound NP (Momose *et al*., 2001). NP may also act as a processivity factor for the polymerase, as RNPs which have been stripped of NP by treatment with high concentrations of CsCl retain apparently normal transcription initiation activities but are unable to synthesize long RNA products (Honda *et al*., 1988). This could potentially reflect NP–P protein interactions.

The function of NP during RNA synthesis which has received the most attention is its potential role in the switch from mRNA transcription to genome replication. The form of RNP packaged into virions only synthesizes mRNA *in vitro* (Skorko *et al*., 1991; Seong *et al*., 1992) and, although input vRNA templates are transcribed into cRNA after infection of cells, an initial round of mRNA transcription and subsequent protein expression is essential (Hay *et al*., 1977). While it is possible that host cell polypeptides are necessary, and some evidence has been presented to support this hypothesis (Shimizu *et al*., 1994; Momose *et al*., 1996), multiple lines of genetic and biochemical evidence implicate NP as a major factor. Several NP *ts* mutants have been isolated that are defective for replicative transcription at the non-permissive temperature (Krug *et al*., 1975; Scholtissek, 1978; Mahy *et al*., 1981; Thierry & Danos, 1982; Markushin & Ghendon, 1984). Moreover, infected cell extracts that synthesize cRNA *in vitro* depend on a supply of non-RNP-associated NP for readthrough of the polyadenylation signal to produce a full-length copy of the vRNA template (Beaton & Krug, 1986; Shapiro & Krug, 1988). Similarly, *in vitro* synthesis of full-length vRNA was found to be dependent on a pool of soluble NP (Shapiro & Krug, 1988). Moreover, nuclear extracts from cells infected with the NP mutant A/WSN/33 *ts*56 virus synthesized m-, c- and vRNA *in vitro* at the permissive temperature but only mRNA at the non-permissive temperature (Shapiro & Krug, 1988).

Thus, NP is evidently essential for replicative transcription. However, its mode of action and the precise steps during genome replication which require it remain uncertain. Although the nuclear extract system developed by the Krug laboratory demonstrated the necessity of NP for readthrough of the polyadenylation signal, technical limitations imposed by the nature of the extracts prevented examination of the role of NP in transcription initiation. However, the mode of initiation and termination of positive-sense RNA synthesis is probably coupled *in vivo*, as Hay *et al*. (1982) observed that most full-length transcripts of the viral RNA templates were uncapped, while Shaw & Lamb (1984) found that most polyadenylated vRNAs have host sequences at their 5′ ends. In support of this, transcripts initiated *in vitro* with a capped primer are also

polyadenylated, even in the presence of non-nucleocapsid NP (Beaton & Krug, 1986). Accordingly, several hypotheses have been proposed for the role of NP in the switch between mRNA and cRNA synthesis (Fig. 2b). The encapsidation hypothesis proposes that NP does not have a regulatory function as such but is merely an essential co-factor. In this hypothesis, other factors alter polymerase activity to change the modes of transcription initiation and termination and NP is required to co-transcriptionally coat the nascent cRNA segments (Shapiro & Krug, 1988). This hypothesis perhaps has a precedent in that the analogous process of genome replication in the non-segmented negative-sense RNA viruses is known to depend on co-transcriptional encapsidation of the nascent replicative intermediate by the N protein (Wagner & Rose, 1995). Alternatively, the template modification hypothesis holds that the interaction of soluble (i.e. not already present in the RNP structure) NP with the template RNA alters its structure and, therefore, the modes of transcription initiation and termination (Hsu *et al*., 1987; Fodor *et al*., 1994; Klumpp *et al*., 1997). This is plausible, since the terminal sequences of the vRNA template are partially base-paired to form a panhandle structure (Hsu *et al*., 1987; Baudin *et al*., 1994) and recognition of this structure by the polymerase is intimately connected with the mechanisms of mRNA transcription initiation (Tiley *et al*., 1994; Hagen *et al*., 1994; Cianci *et al*., 1995) and polyadenylation (Pritlove *et al*., 1998; Poon *et al*., 1998). A third more recent hypothesis concerns the ability of NP to bind directly to PB1 and PB2: in this model NP alters the transcriptional function of the polymerase through direct protein–protein contacts (Biswas *et al*., 1998; Mena *et al*., 1999). These hypotheses await definitive testing but circumstantial evidence supports two of the three proposed mechanisms. It has been shown that the *ts* lesions of two NP mutants defective for replicative transcription result in *ts* RNA-binding activity without apparent effects on NP oligomerization or interactions with the P proteins (Medcalf *et al*., 1999). This indicates that an NP–RNA interaction is necessary to support genome replication and is consistent with both the encapsidation and template modification hypotheses.

The regulation of vRNA synthesis may well differ from that of cRNA, as genetic evidence indicates that the roles of NP in c- and vRNA synthesis are mutationally separable (Thierry & Danos, 1982; Markushin & Ghendon, 1984; Mena *et al*., 1999). The role of NP in vRNA synthesis is perhaps simpler, as cRNA templates do not support cap-primed transcription or contain a polyadenylation signal (Cianci *et al*., 1995). Certainly, the premature termination of *in vitro* vRNA transcription in the absence of soluble NP is consistent with the necessity of NP for co-transcriptional encapsidation of the nascent segment (Shapiro & Krug, 1988).

RNP trafficking

Assembled RNPs must be transported in both directions across the nuclear envelope: incoming RNPs from the uncoating virion are imported at the start of the infectious cycle, while later, newly assembled RNPs are exported to be packaged into progeny virions (Fig. 4a). This temporal regulation of RNP trafficking is reflected in the distribution of their major protein component, NP. At early times post-infection, NP is found predominantly in the nucleus of infected cells but, at later times post-infection, substantial amounts accumulate in the cytoplasm (Breitenfeld & Schafer, 1957; Maeno & Kilbourne, 1970). Depending on cell type and, possibly, virus strain, this can take the form of an almost complete reversal of NP distribution, with the nucleus apparently emptying (Fig. 4b). The dramatic change in NP localization must reflect a regulated process and much evidence exists to suggest the involvement of several viral and host polypeptides. Nuclear import of NP and NP–RNA complexes results from the interaction of NP with host cell importin α (O'Neill *et al*., 1995; Wang *et al*., 1997). This trafficking event certainly operates during the early period of the infectious cycle to direct nuclear import of the infecting RNPs (Fig. 4a, i) and of newly synthesized NP (Fig. 4a, ii) to support the process of genome replication. However, later in infection, RNP export becomes dominant, although it is not clear whether nuclear import of NP is turned off or just overridden by the nuclear export process. In support of the former hypothesis, exported RNPs do not apparently shuttle back into the nucleus, perhaps because of interactions with M1 (Martin & Helenius, 1991; Whittaker *et al*., 1996).

The mechanisms responsible for nuclear export of RNPs are less well defined and somewhat more contentious. It is generally agreed that the interaction of M1 with the RNPs is an essential component of the process, as in the context of infected cells, RNP export depends on M1. This has been shown by microinjection of anti-M1 antibodies, the examination of defective viruses and by the use of a drug that blocks late viral gene expression (Martin & Helenius, 1991; Whittaker *et al*., 1995; Bui *et al*., 2000). In addition, the viral NEP/NS2 polypeptide has been implicated in RNP export. Based on the identification of an NES in NS2, the observation that microinjection of infected cells with anti-NS2 serum blocks RNP export and the previous finding that NS2 binds to M1–RNP complexes (Yasuda *et al*., 1993), it was proposed that NS2 is responsible for mediating the interaction of RNPs with the nuclear pore complex and thus directing their nuclear export (O'Neill *et al*., 1998). On these grounds, it was proposed that NS2 be renamed NEP for Nuclear Export Protein (O'Neill *et al*., 1998). This hypothesis has recently received powerful support from the creation of a recombinant virus lacking the NEP/NS2 gene: infectious virus is formed when NEP/NS2 is supplied in *trans* from a complementing cell line but RNP export subsequently fails in normal cells infected with these virions (Neumann *et al*., 2000). However, attractive though the NEP hypothesis is, it does not explain all of the accrued experimental observations, raising the possibility that either the export mechanism is more complex or that more than one

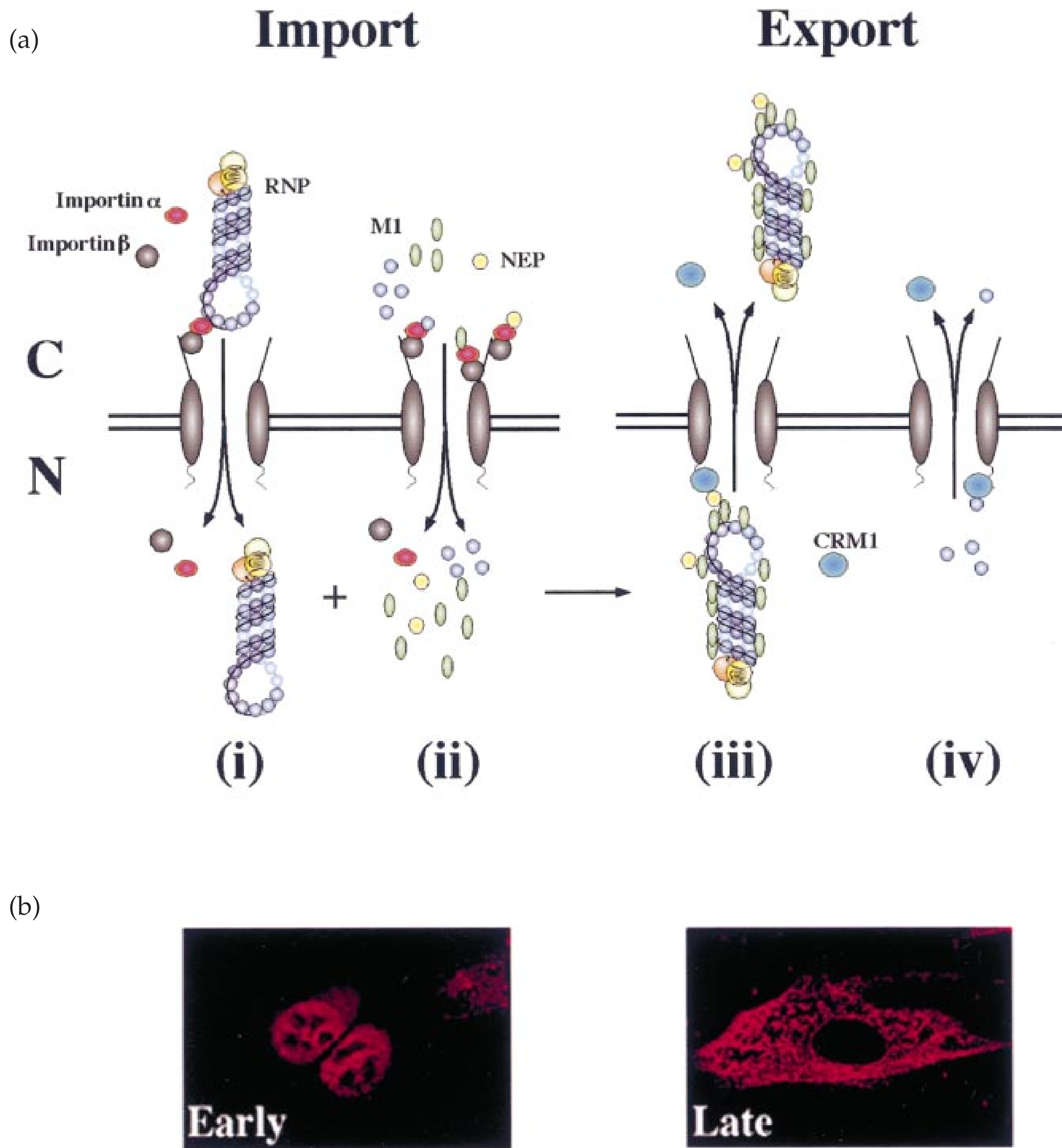

Fig. 4. Influenza virus nucleocytoplasmic trafficking. (a) Cartoon model of import/export mechanisms. (i) RNPs and (ii) monomeric NP, M1 and NEP polypeptides are imported from cytoplasm (C) to the nucleus (N) through NLS-mediated interactions with host-cell importin α followed by importin β docking with the nuclear pore complex (grey ovals). (iii) RNP nuclear export is thought to occur because of the formation of an NEP–M1–RNP complex which interacts with host-cell CRM1 via the NES in NEP. (iv) There is evidence that NP also contains an NES capable of functional interaction with CRM1, but it is not known whether this operates for RNA-bound NP or in infected cells. (b) Immunofluorescent staining patterns of NP early and late in infection. BHK cells infected with influenza virus A/PR/8/34 show predominantly nuclear NP at 4 h post-infection but almost exclusively cytoplasmic staining 5 h later.

pathway exists. Indeed, the original hypothesis of a direct interaction between NEP/NS2 and components of the nuclear pore (O'Neill *et al.*, 1998) has been modified to involve the cellular nuclear export receptor CRM1 (Fig. 4a, iii), as several studies have shown that inactivation of CRM1 with the specific inhibitor LMB leads to nuclear retention of RNPs (Elton *et al.*, 2001; Ma *et al.*, 2001; Watanabe *et al.*, 2001) and that NEP/NS2 does indeed bind CRM1 (Neumann *et al.*, 2000; Paragas *et al.*, 2001). However, to further complicate the issue, as discussed above, NP itself contains an NES, interacts with CRM1 and, in the absence of other influenza virus polypeptides, accumulates in the cytoplasm in response to increased time, expression levels, phosphorylation status of the cell and up-regulation of CRM1 activity (Neumann *et al.*, 1997; Digard *et al.*, 1999; Elton *et al.*, 2001). Furthermore, in the context of virus infection, NEP/NS2 is not required in

stoichiometric quantities, as nuclear export of RNPs occurs in the presence of much reduced or even undetectable levels of NEP/NS2 (Wolstenholme *et al*., 1980; Bui *et al*., 2000; Elton *et al*., 2001). In confirmation that NEP/NS2 might not be obligatory for RNP export under all circumstances, a recent study found that cytoplasmic accumulation of NP depended on the co-expression of M1 and a vRNA segment (Huang *et al*., 2001). This at least raises the possibility that NP or RNP export can occur through a direct interaction between CRM1 and NP (Fig. 4a, iv) (Elton *et al*., 2001). Experimental observations indicate that phosphorylation also plays a role in regulating RNP transport. Treatment of NP-expressing cells with the protein kinase inhibitor H7 biased NP towards nuclear accumulation, while, conversely, the phosphorylation stimulator TPA caused its cytoplasmic accumulation (Neumann *et al*., 1997). Furthermore, treatment of infected cells with H7 also causes nuclear retention of NP and, although this is due in part to a block in viral late gene expression (Kistner *et al*., 1989; Kurokawa *et al*., 1990; Martin & Helenius, 1991), exogenous addition of M1 only restores RNP nuclear export if the inhibitor is also washed out (Bui *et al*., 2000). Although the target(s) of the phosphorylation events remain unclear, even to the point of whether they are viral and/or cellular, there is circumstantial evidence implicating NP as a possibly important target. As already mentioned, a major site of phosphate addition on NP has been mapped to serine 3 located within NLS I (Arrese & Portela, 1996). Mutation of this residue did not drastically affect the transcriptional function of NP (Arrese & Portela, 1996). However, substitution of the serine with acidic residues (thus mimicking the phosphorylated state) biased a GFP fusion protein containing the first 80 amino acids of NP towards cytoplasmic accumulation, suggesting that phosphorylation of serine 3 impairs the functionality of the N-terminal NLS (Bullido *et al*., 2000). However, definitive testing of the role phosphorylation of NLS I plays in the virus life cycle awaits examination of the behaviour of full-length NP, ideally in the context of virus infection.

Future prospects

NP is not just the structural component of the virus transcription machinery but performs multiple essential functions throughout the virus life cycle. So far, most of the research effort has been directed towards characterization of the activities of NP and to the identification of protein domains and specific residues that are required for these functions. Full interpretation of these studies as well as future work would benefit from the determination of an atomic-resolution structure of the protein, a major challenge not yet achieved. In this regard, the recent determination of a 27 Å structure of an *in vivo*-reconstituted mini-RNP is a major development (Martín-Benito *et al*., 2001). In addition, a methodology that allows the rescue of recombinant influenza viruses using entirely cloned cDNA copies of the genome has been described recently (Neumann *et al*., 1999; Fodor *et al*., 1999). This technology has opened the possibility to mutate any residue of the viral genome and examine its effect on virus replication. We expect that the application of this technology to the study of NP function will elucidate further its role in the influenza virus life cycle.

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