Virology 479-480 (2015) 532–544

Contents lists available at ScienceDirect

Virology

journal homepage: www.elsevier.com/locate/yviro

Review

The RNA synthesis machinery of negative-stranded RNA viruses

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ARTICLE INFO

Article history:
Received 19 December 2014
Returned to author for revisions
14 January 2015
Accepted 3 March 2015
Available online 29 March 2015

Keywords:
Negative-stranded RNA virus
Ribonucleoprotein
Nucleocapsid
RNA-dependent RNA polymerase
Transcription
RNA replication

ABSTRACT

The group of Negative-Stranded RNA Viruses (NSVs) includes many human pathogens, like the influenza, measles, mumps, respiratory syncytial or Ebola viruses, which produce frequent epidemics of disease and occasional, high mortality outbreaks by transmission from animal reservoirs. The genome of NSVs consists of one to several single-stranded, negative-polarity RNA molecules that are always assembled into mega Dalton-sized complexes by association to many nucleoprotein monomers. These RNA-protein complexes or ribonucleoproteins function as templates for transcription and replication by action of the viral RNA polymerase and accessory proteins. Here we review our knowledge on these large RNA-synthesis machines, including the structure of their components, the interactions among them and their enzymatic activities, and we discuss models showing how they perform the virus transcription and replication programmes.

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Introduction

The Negative-Stranded RNA viruses (NSV) contain a single-stranded RNA genome that may be non-segmented (nsNSV) or segmented into 2–8 RNA molecules (sNSV). The former constitute

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http://dx.doi.org/10.1016/j.virol.2015.03.018
0042-6822/© 2015 Published by Elsevier Inc.

the order *Mononegavirales* and include the families *Rhabdoviridae*, *Paramyxoviridae*, *Filoviridae, Bornaviridae* and *Nyamiviridae*. The latter contain the families *Arenaviridae*, *Bunyaviridae* and *Orthomyxoviridae*. The most distinctive feature of NSV genomes is their highly structured organisation in the form of ribonucleoprotein complexes or nucleocapsids (RNPs or NCs) in which the genomic RNA is associated to multiple monomers of nucleoprotein (NP/N protein). For the *Mononegavirales*, the viral genes are located along the single RNA genome and expressed sequentially whereas in the sNSVs each genomic RNA segment is contained in a distinct RNP that is functionally independent for transcription and replication.

Many members of the NSV group constitute important human pathogens and produce frequent epidemics of disease, like the influenza, measles, mumps or respiratory syncytial viruses. Other members can produce occasional infections or outbreaks by transmission from various animal reservoirs, like pandemic influenza, rabies, Ebola, Nipah, Machupo, Hanta or Lassa viruses, and are frequently associated to high mortality. All together, the NSV group is responsible for diseases showing high morbidity worldwide with important health and economic implications and, in addition, they constitute a continuous threat for new outbreaks with potentially devastating consequences, as witnessed by frequent WHO reports (http://www.who.int/csr/don/archive/year/2014/en/).

In this review we shall discuss the structure and mechanisms of action of the NSV RNA synthesis machinery. Due to space limitations, many important contributions could not be directly cited. More detailed information is available in recent specific reviews (Albertini et al., 2011; Boivin et al., 2010; Eisfeld et al., 2014; Fodor, 2013; Ivanov et al., 2011; Kranzusch and Whelan, 2012; Martin-Benito and Ortin, 2013; Morin et al., 2013; Reguera et al., 2014; Resa-Infante et al., 2011; Ruigrok et al., 2010; Ruigrok et al., 2011; Zheng and Tao, 2013).

The RNA synthesis molecular machine

The processes of NSV transcription and replication take place in the context of RNPs/NCs complexes containing the template RNA associated to many NP/N monomers, as well as the viral polymerase and other accessory proteins. In this section, the overall architecture of these MDa-sized molecular machines and the structure–function relationships of their components are summarised.

The ribonucleoproteins

The NSV RNPs adopt a general helical conformation, which is generally linear and relatively rigid for the nsNSVs (Schoehn et al., 2004; Tawar et al., 2009) (Fig. 1A and B) while the sNSVs show a more flexible circular conformation (Raymond et al., 2010), as a consequence of non-covalent RNA-RNA interactions between the 5′- and 3′-termini of their RNA segments (Hsu et al., 1987; Raju and Kolakofsky, 1989). In the case of the Orthomyxoviruses this circular conformation is maintained by the concurrence of both, base pairing of the ends and polymerase interaction with the 3′ and 5′ RNA-termini (Klumpp et al., 1997). The structure of influenza virus RNPs is double helical (Fig. 1C) (Compans et al., 1972; Jennings et al., 1983). As discussed below, the NP/N protein is the main determinant of the helical character of NSV RNPs, but the precise location of the viral polymerase and other components

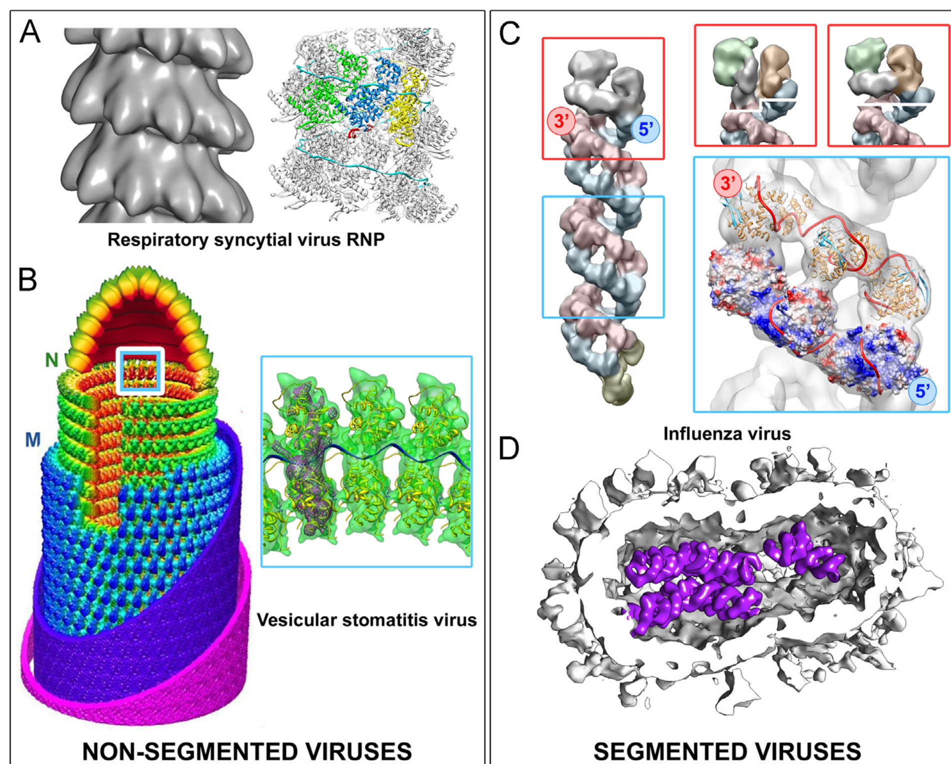

Fig. 1. Structure of NSV ribonucleoproteins. The three-dimensional structures of nsNSVs (A, B) and sNSVs RNPs (C, D) are presented. (A) Cryo-EM reconstruction of RSV RNPs (EMD-1622, shown as left handed structure as corrected in Bakker et al., 2013) and docking of the atomic structure of N protein within the EM structure (Tawar et al., 2009) (pdb 2WJ8). (B) Cryo-electron tomography reconstruction of VSV particle and detail of the RNP structure within the virion (Ge et al., 2010) (EMD-1663) (image courtesy of Dr. Hong Zhou). (C) The structure of influenza virus RNPs was determined by cryo-EM by separate reconstruction of RNP helical regions (blue boxes) (EMD-2205) and RNP termini (polymerase-containing terminus shown in red boxes) (EMD-2206, EMD-2207, EMD-2208) (Arranz et al., 2012). A composite volume is shown on the left; the indicated RNA polarity was deduced from the combination of the data from (Arranz et al., 2012) and (Turrell et al., 2013). Alternative conformations of the polymerase (red boxes) and details on the docking of NP atomic structure (pdb 4BBL) and putative RNA location are presented on the right (blue box). (B) The flexible distribution of individual influenza virus RNPs within virions is shown, as a result of docking RNP structures obtained by cryo-electron tomography (Arranz et al., 2012).

has not been possible except for the influenza viruses. The structure of various influenza functional RNPs have been determined by cryo-electron microscopy (cryo-EM), including circular mini-RNPs containing a short genomic RNA that does not allow a helical conformation (Coloma et al., 2009), recombinant helical RNPs (Moeller et al., 2012) and native full-length RNPs derived from virions (Arranz et al., 2012) (Fig. 1C and D). The structure of virion full-length RNPs was determined by separate reconstruction of the termini (Fig. 1C, red boxes) and the central regions (Fig. 1C, blue boxes) using single particle 3D reconstruction, and by cryo-electron tomography (Arranz et al., 2012) (Fig. 1D). They showed a left-handed, double-helical arrangement of two opposite polarity NP strands (Fig. 1C left; red and blue NP strands) in which a minor groove could be defined by both NP connected strands while a major groove does not show physical contact between strands (Fig. 1C, blue boxes). However, a right-handed helix was reported for the recombinant RNPs (Moeller et al., 2012) and the differences between both reported structures extend to the proposed docking of the NP atomic structures, leaving open questions for further structural analysis at higher resolution.

The viral polymerase

For most of the NSVs the RNA synthesis catalytic activity is included in the very large L protein, a multienzymatic polypeptide responsible for mRNA synthesis and modification, as well as the generation of progeny RNPs (Kranzusch and Whelan, 2012; Morin et al., 2013). As an exception, the Orthomyxoviruses have the required enzymatic activities distributed among three different polypeptides (the PB1, PB2 and PA proteins) that are tightly associated in a heterotrimer (Fodor, 2013; Martin-Benito and Ortin, 2013; Resa-Infante et al., 2011; Ruigrok et al., 2010). Phylogenetic analyses have indicated that NSV RNA polymerases share a common ancestor with RNA polymerases of other origins (Poch et al., 1989) and various L proteins display a series of conserved sequence blocks separated by more variable regions, suggestive of successive functional domains (Poch et al., 1990). Early studies on the architecture of the influenza polymerase heterotrimer indicated a similar organisation, as the subunits are connected in a N-to-C-terminus fashion (Gonzalez et al., 1996; Toyoda et al., 1996) and a single fusion polypeptide containing PA, PB1 and PB2 open-reading frames connected by flexible loops could be efficiently expressed in a soluble form (R. Coloma, Unpublished B.Sc. Thesis, 2001). Consistent with these results, epitope tags or a GFP open reading frame could be inserted into a potential hinge region of the L protein of various nsNSVs, at a position equivalent to the PB1-PB2 connexion in the influenza polymerase complex, with minor alterations in the functionality of the recombinant protein (Duprex et al., 2002; Fix et al., 2011; Ruedas and Perrault, 2009).

The first glimpses on the structure of NSV RNA polymerases were obtained by EM. In the case of the influenza virus polymerase, EM structures are available for the isolated heterotrimer (Moeller et al., 2012; Torreira et al., 2007), for a functional polymerase-template RNA complex devoid of NP (Resa-Infante et al., 2010), for the polymerase complex associated to a mini-RNP (Area et al., 2004), an improved structure of which was latter derived by cryo-EM (Coloma et al., 2009), and for the polymerase present in purified full-length virion-derived (Arranz et al., 2012) or recombinant RNPs (Moeller et al., 2012). So far, the information for the polymerase structure of nsNSVs is less elaborated and derives from 2D analyses of EM images of vesicular stomatitis virus (VSV) and Machupo virus L proteins (Kranzusch et al., 2010; Rahmeh et al., 2010) (Fig. 2A). All together, this low-resolution structural information indicated the presence of a cage structure (Resa-Infante et al., 2010) (P. Resa-Infante, unpublished results), which shows a ring-like appearance in the 2D analyses of nsNSV RNA polymerases (Kranzusch et al., 2010; Rahmeh et al., 2010) (Fig. 2A). This central core might be composed by the PB1 and the large PA domain in the influenza virus polymerase complex (Coloma et al., 2009; Guu et al., 2008) and could be equivalent to the RNA polymerases of double-stranded RNA viruses (Butcher et al., 2001; Tao et al., 2002). From this central structure, flexibly connected domains can be visualised in the projection images of nsNSV RNA polymerases, that have been assigned to the accessory enzymatic activities recognised in the L protein, namely cap-snatching or capping and methylation activities (Fig. 2A) (Kranzusch et al., 2010; Rahmeh et al., 2010)(reviewed in Morin et al., 2013). Although not distinguishable in the EM structures, specific domains of the PA and PB2 subunits of the influenza polymerase heterotrimer could be individually expressed and their structure solved by X-ray diffraction (reviewed in Ruigrok et al., 2010). These results showed that the cap-binding activity reside in the central region of PB2 (Guilligay et al., 2008) whereas the endonuclease activity is located at the N-terminus of PA (Dias et al., 2009; Yuan et al., 2009), in a domain equivalent to the endonuclease domain in the L protein of Arenaviruses or Bunyaviruses (Morin et al., 2010; Reguera et al., 2010).

Very recently, the first detailed structural information of any NSV complex polymerase has been reported. Thus, the atomic structures of the influenza type A and B viruses in complex with the genomic promoter have been solved (Fig. 2B) (Pflug et al., 2014; Reich et al., 2014) and the structure of the type C virus is also available (E. Fodor, personal communication). These active polymerase complexes were expressed as recombinant single polypeptides, as earlier described for the basal Qbeta replicase (Kita et al., 2006), by fusing the three subunits with TEV-cleavable linkers. The polymerase shows a hollow U-shaped structure that could be reasonably fitted into previous low-resolution EM volumes (Coloma et al., 2009; Resa-Infante et al., 2010; Torreira et al., 2007)(unpublished results). The atomic structure reveals a very intricate interaction among subunits (Fig. 2B), not at all limited to the previously described N-to-C-terminus connecting structures (He et al., 2008; Obayashi et al., 2008; Sugiyama et al., 2009). The body of the complex is formed by the tightly associated PB1 and PA subunits, the latter of which is located at the bottom of the structure (Fig. 2B, magenta), while the C-terminal section of PB2 form the left side (Fig. 2B, red) and the C-terminus of PB1 and N-terminus of PB2 border the complex on the right. In addition, two external domains are apparent, which correspond to previously crystallised fragments of the polymerase: the PA endonuclease domain, which is on the top right of the complex (Fig. 2B, endo), tightly bound by interactions with all other subunits, and the PB2 cap-binding domain that is located on the left (Fig. 2B, cap-binding) and is flexibly connected with the rest of the complex (see below). The internal cavity includes the RNA-binding sites for the promoter RNA and the polymerase active site and shows three channels, putative representing the template entrance, nucleoside triphosphate (NTP) entrance and template-product exit sites. The PB1 subunit shows a classical right-handed fold with fingers, palm and thumb subdomains with high structural similarity to hepatitis C virus polymerase (Bressanelli et al., 1999), but includes a number of additional features that will be discussed below. Given the almost identical overall structures of type A and B influenza virus polymerases, it is to be expected that the structure of L proteins could show very similar characteristics. Ongoing work of several groups will soon shed light on this topic.

The nucleoprotein

The NP/N protein is the most abundant element in the NSV RNPs, it provides the basis for their helical structure and is

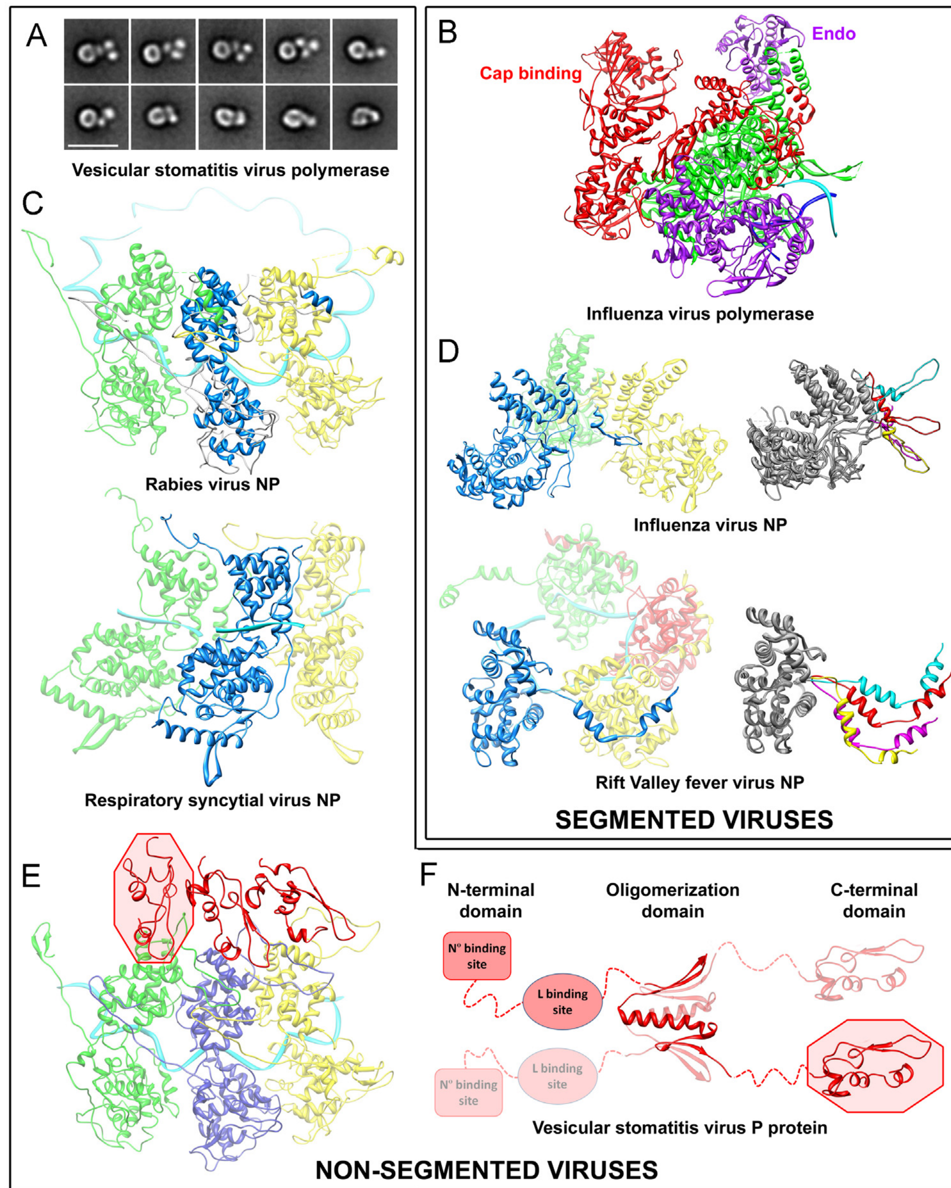

Fig. 2. Structural information on the NSV ribonucleoprotein components. The three-dimensional structures of representative examples of nsNSV and sNSV RNP components are presented. (A) Two-dimensional analysis of purified VSV L protein (Rahmeh et al., 2010) (scale bar represents 20 nm). Although at present no high-resolution information on a L-polymerase has been published, a structure similar to that of the heterotrimeric influenza polymerase could be expected. (B) Atomic structure of the influenza A RNA polymerase complex associated to the vRNA promoter (Pflug et al., 2014)(pdb 4WSB). The PA (magenta), PB1 (green), PB2 (red) subunits and the promoter RNA strands (light and dark blue) are shown. The locations of the cap binding (cap-binding) and endonuclease (endo) domains are indicated. (C) Atomic structures of rabies virus (top, pdb 2GTT) and RSV (bottom, 2WJ8) N proteins associated to RNA. Three N monomers are shown coloured green, blue and yellow, whereas the RNA is depicted in light blue. (D) The atomic structures of influenza virus (top, pdb 2IQH) and RVFV (bottom, pdb 4H5O) NPs are shown, with the connecting structural feature highlighted (blue on yellow). On the right of each structure the superposition of four different structures of influenza (pdb codes: 2Q08 blue, 3TJ0 red, 2IQH yellow and 3TG6 magenta) or RVFV (pdb codes: 4H5P blue, 4H5O red, 4H6F yellow, 3OV9 magenta) NPs are presented to show the flexibility of the connecting features. (E) Atomic structure of the VSV N protein associated to RNA and complexed with the C-terminal domain of P protein (highlighted in a red polygon; pdb 2HHZ). (F) Cartoon showing the structure of dimeric VSV P protein, including the disordered N-terminal region responsible for N° and L binding, the oligomerisation domain (pdb 2FQM) and the C-terminal domain (highlighted in a red polygon; pdb 2HHZ).

essential for the transcription and replication of full-length templates (for reviews see Ivanov et al., 2011; Reguera et al., 2014; Ruigrok et al., 2010; Ruigrok et al., 2011). The NSV NP/N proteins show a general crescent form with two domains. Most of the NSV N proteins contain a N-terminal and a C-terminal domain (Albertini et al., 2006; Ferron et al., 2011; Green et al., 2006; Hastie et al., 2011a; Raymond et al., 2010; Rudolph et al., 2003; Tawar et al., 2009) but each of the domains of the NP from Orthomyxoviruses contain sequences derived from both N-terminal and C-terminal regions of the protein (Ng et al., 2012; Ye et al., 2006; Zheng et al., 2013).

Essential for the function of NP/N proteins is their capacity to oligomerise. In fact, the atomic structures described correspond to various oligomeric forms, except when the oligomerisation domain was mutated (Chenavas et al., 2013). The mechanism for NP/N oligomerisation differs between nsNSVs and sNSVs. In the former, important interactions occur between protein monomers and stabilisation takes place by inter-monomeric contacts involving N-terminal and/or C-terminal protein extensions (Fig. 2C, D) (Albertini et al., 2006; Green et al., 2006; Tawar et al., 2009), leading to viral RNPs with highly regular helical structures (Ge et al., 2010; Schoehn et al., 2004; Tawar et al., 2009) (Fig. 1A, B). In

contrast, oligomerisation of sNSV NPs takes place in a more flexible way and they can form small oligomers, including dimers (Zheng et al., 2013), trimers (Ng et al., 2008; Ye et al., 2006) or tetramers (Ng et al., 2012), in addition to more complex structures and long helices (Ruigrok and Baudin, 1995). This is the consequence of the mechanism of NP–NP association, that in the Orthomyxoviruses relays on the insertion of a protein loop, flexibly connected to the body of NP, into the neighbouring NP monomer (Fig. 2D, top) (Arranz et al., 2012; Chan et al., 2010; Coloma et al., 2009; Ng et al., 2012, 2008; Ye et al., 2006) (reviewed in Martin-Benito and Ortin, 2013). A similar plasticity in the N–N interaction occurs in the Bunyaviruses, in which oligomerisation depends on the insertion of a N-terminal arm of one monomer into a hydrophobic cleft of the neighbouring monomer (Fig. 2D, bottom) (Ferron et al., 2011). This flexibility is reflected in the formation of filamentous RNPs without apparent symmetry (Pettersson and von Bonsdorff, 1975).

The second general property of NP/N proteins of NSVs is their RNA-binding capacity. In many cases we have direct evidence on the RNA binding mechanism, as several NP/Ns have been crystallised as protein-RNA complexes (for instance Albertini et al., 2006; Green et al., 2006; Hastie et al., 2011b; Raymond et al., 2010; Reguera et al., 2013; Tawar et al., 2009) (Fig. 2C). A common feature in these structures is the binding of RNA along a positively charged protein cleft that lies at the interface between the N- and C-terminal domains. Generally, RNA recognition is not sequence-specific, but there are exceptions (Osborne and Elliott, 2000), and most of the protein-RNA contacts occur with the backbone phosphates and/or sugar moieties although some contacts are established with the bases. In most cases the bases are not accessible to solvent and the RNA would need to dissociate from NP/N to act as a template in transcription or replication. Similarly, RNA recognition by influenza virus NP has been proposed to occur via a similar basic protein cleft (Arranz et al., 2012; Chenavas et al., 2013; Ng et al., 2012; Ng et al., 2008; Ye et al., 2006). Although in the case of influenza virus RNA-binding details are not yet clear, the bases are fully exposed to solvent and the RNA is sensitive to RNases (Klumpp et al., 1997), suggesting that minor alterations of the RNP structure would suffice for it to act as a template. Another apparent distinction between Orthomyxoviruses and other NSVs refers to the NP/N-to-RNA stoichiometry. Whereas for the nsNSVs, the Arenaviruses and Bunyaviruses the length of RNA associated per NP/N monomer ranges between 7 and 11 nucleotides, the value for influenza viruses was estimated around 20 by biochemical analysis (Compans et al., 1972), 24 by EM of mini-RNPs (Martín-Benito et al., 2001; Ortega et al., 2000) and these estimations were confirmed by quantitative proteomics of purified virions (Hutchinson et al., 2014). However, biophysical and RNA-binding studies with the ISAV dimeric NP indicated that it contacts around 12 nucleotides (Zheng et al., 2013), suggesting that a fraction of the viral RNA present in an Orthomyxovirus RNP could be not directly associated to NP monomers.

The Phosphoprotein

The Phosphoprotein (P) is a viral factor essential for transcription and replication of nsNSVs that has no direct counterpart in members of the sNSVs. It serves two separate roles, namely (i) recognition of the RNP as a template by the polymerase and (ii) stabilisation of newly synthesised nucleoprotein (N°) as a monomeric protein and blocking its unspecific binding to RNA until it becomes assembled into a nascent RNP.

Despite differences in sequence and structure, all P proteins share some common features that allow them to fulfil these functions: They are elongated proteins that form oligomers and contain a concatenated series of structured domains alternated with intrinsically disordered protein regions (Fig. 2F) (Gerard et al., 2009; Habchi et al., 2010). Three functional regions can be recognised, two of them located in the N-proximal region and involved in the interaction with nascent N° protein and with the polymerase, and one found in the C-terminal section and responsible for interaction with the N-RNA template (Fig. 2F) (reviewed in Ivanov et al., 2011). The oligomerisation domain is central in the protein sequence and is responsible for the formation of dimers in the Rhabdoviruses and tetramers in the Paramyxoviruses (Ding et al., 2006; Ivanov et al., 2010; Tarbouriech et al., 2000). The C-terminal interaction domain is well structured and its mode of interaction with a N-RNA template has been determined. Thus, the P protein C-terminus is inserted between the monomers in the N-RNA template and makes contacts with the C-terminal regions of two successive N proteins, ensuring that this interaction is specific for the N-RNA template and not for monomeric N° (Fig. 2E) (Delmas et al., 2010; Green and Luo, 2009; Mavrakis et al., 2004). In contrast, P binding to N° relays on a N-terminal molecular recognition element (MoRE), which adopts transient structures in solution that become fixed upon recognition of its target (Leyrat et al., 2011), and involve P protein dimerisation (Green et al., 2014; Mavrakis et al., 2003). The VSV P protein element involved in the interaction with the polymerase has been mapped (Emerson and Schubert, 1987). Although it is poorly characterised, its binding has been shown to drive a strong conformational change in the structure of the L polymerase and stimulates its transcriptional activity (Rahmeh et al., 2012). An important consequence of the above mentioned set of interactions is the oligomerisation of the L polymerase, which may be very relevant for its functionality (see below).

As the sNSVs lack an equivalent to the P protein, they have evolved alternative solutions for the regulation of NP/N protein oligomerisation and RNA binding, as well as for the recognition of the template. For the influenza viruses, interaction of viral NP with cellular factor UAP56 has been described (Momose et al., 2001) and this protein has been proposed to act as a chaperone of newly synthesised NP and to avoid its binding to RNA and its multimerisation. In addition, the atomic structure of an oligomerisation mutant of NP revealed that RNA-binding and NP–NP interaction might be co-regulated and phosphorylation of S165 play a role in both regulatory events (Chenavas et al., 2013; Turrell et al., 2014). In the case of the Arenavirus Lassa fever virus, the structure of the N-terminus of NP with and without bound RNA shows a protein conformational change that suggest a regulation of RNA recognition by masking the RNA-binding site until N° is assembled into a nascent RNP (Hastie et al., 2011b). Likewise, two alternative structures have been determined for the N protein of the Phlebovirus Rift valley fever virus (RVFV), a monomeric N (Raymond et al., 2010) and a hexameric N complex (Ferron et al., 2011). The comparison of these structures suggests that the projecting N-terminal arm of the N° protein may either be used for oligomerisation by insertion into the neighbouring N monomer or folded back into its own body to hide the RNA-binding site.

On the other hand, template recognition by sNSV polymerases relays on sequence-specific features (see for instance Tiley et al., 1994) and polymerase oligomerisation appears to occur by direct interactions (Brunotte et al., 2011; Chang et al., 2015; Resa-Infante, 2010).

The Host factors

In addition to the viral proteins involved, a number of host cell factors have been described to play important roles in the generation or functioning of the NSV RNA synthesis machines. Thus, silencing or inhibition of Hsp90 affects the folding and stabilisation of the NSVs RNA polymerases (Chase et al., 2008;

Connor et al., 2007). In the case of influenza viruses, this heat-shock induced chaperone has been shown to bind influenza virus PB1 and PB2 proteins, and its inhibition leads to reduced levels of heterotrimeric polymerase complex (Naito et al., 2007). Likewise, host proteins involved in nucleo-cytoplasmic transport are essential for influenza virus RNA synthesis since, contrary to other NSVs, they transcribe and replicate their genome in the nucleus. Both the parental virus RNPs and the newly synthesised viral polymerase subunits and NP must be transported into the nucleus and they relay on the cellular machinery to overcome this compartmental barrier (Hutchinson and Fodor, 2012), which has been recognised as an important determinant for the adaptation of avian influenza viruses to mammals (Resa-Infante and Gabriel, 2013).

More importantly, a number of host factors have been recognised as direct players in the process of NSV RNA synthesis, although the mechanisms of action for most of them are yet far from clear. For the nsNSV VSV, it has been proposed that the composition of the transcribing and replicating complexes are different: While the replicase complex would only contain the viral proteins L, P and N, the transcriptase would lack N and need the presence of translation elongation factor 1-α and the chaperone Hsp60 (Qanungo et al., 2004). In a similar way, transcription of influenza virus genome requires interaction with the cellular RNA polymerase II, either directly or by means of other transcription regulators, as pTEFb or hCLE (Engelhardt et al., 2005; Huarte et al., 2001; Zhang et al., 2010). In addition, influenza virus mRNA polyadenylation and splicing are specifically regulated by interaction of the polymerase with cellular splicing factors (Fournier et al., 2014; Landeras-Bueno et al., 2011). On the other hand, influenza RNA replication requires the engagement of the polymerase with the minichromosome maintenance complex (MCM) that improves transit from the initiation to the elongation step (Kawaguchi and Nagata, 2007) and the above mentioned UAP56 that may act as a P-like chaperone for NP (Momose et al., 2001).

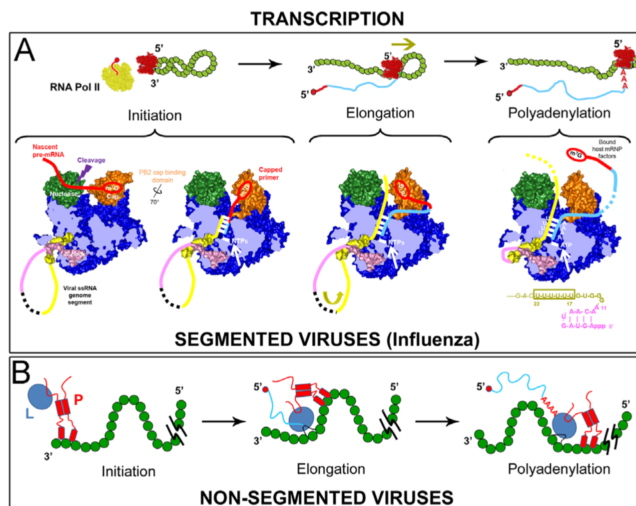

Fig. 3. Models for NSV transcription. (A) Cartoon showing the proposed cis mechanism for influenza virus transcription. The top drawings describe a general outline of the process including the complete RNP, while the detailed mechanism of the polymerase actions is presented at the bottom. Top. The cellular RNA polymerase II (yellow) generates a nascent, capped pre-mRNA (red line), which is snatched by the viral polymerase (red) and used as primer to elongate a viral mRNA (light blue), until it reaches the polyadenylation signal next to the viral 5′-terminus and directs poly A synthesis. Bottom (adapted from Reich et al., 2014). Cross-section of the influenza A RNA polymerase showing the cellular pre-mRNA (red) bound to the cap-binding site (brown). Upon cleavage by the viral endonuclease (green), the cap primer is rotated to the polymerase active site and the 3′-terminus of the template (yellow) is copied in the mRNA (light blue), while the 5′-terminus (pink) remains associated to the polymerase. (B) Cartoon showing the mechanism of transcription of nsNSVs. The L(dark blue)-P(red) complex recognises the 3′-terminus of the N-RNA template (green) and starts to copy at the first gene until it reaches the polyadenylation site. The L-associated capping activities generate the 5′-cap structure (red) co-transcriptionally. For simplicity only the 3′-terminal virus gene of the N-RNA template is presented.

The transcription programme

The polarity of the NSV genomes imposes transcription as the obligatory first step in the virus gene expression programme. Thus, viral mRNAs are first produced from the parental RNPs while RNA replication intermediates are generated at a later stage. These two classes of positive-polarity products are widely different: while viral mRNAs are structurally analogous to their cellular counterparts, i.e. they contain 5′-cap and 3′-poly A modifications (but see below), the antigenome replication intermediates contain unmodified RNAs that are assembled into RNPs much like the genomic RNPs. Hence, the virus transcription and replication programmes are distinct and must be strictly regulated.

As in all transcription processes, transcription of NSVs must follow a series of steps that include template recognition, formation of the initiation complex at the promoter site, initiation (either primer-dependent or de novo), promoter release and elongation, and finally RNA synthesis termination and polymerase release (Fig. 3). In addition, the RNA transcripts must be modified either co- or post-transcriptionaly.

Template recognition

Template RNA recognition takes place by direct polymerase-RNA interaction in the sNSVs. For instance, the influenza viruses bind specifically the promoter structure, constituted by the association of the conserved 5′- and 3′-terminal sequences of each genomic RNA. The main influenza RNA recognition site is found at the 5′-terminus but binding is tighter to the complete 5′-3′ promoter (González and Ortín, 1999a; Tiley et al., 1994). Various structures have been proposed for the influenza promoter, namely the panhandle (Cheong et al., 1999; Hsu et al., 1987), the fork (Fodor et al., 1995) and the corkscrew (Flick and Hobom, 1999; Tomescu et al., 2014) and the recently published structures of the influenza polymerase in the pre-initiation state (Pflug et al., 2014; Reich et al., 2014)(Fig. 2B) indicates that the actual disposition of the promoter within the polymerase represent a hybrid between the corkscrew and fork models. Thus, the predicted 5′-loop is confirmed, albeit slightly different in the fine details, but no 3′-loop is observed. The interaction of both the 5′-terminus and 3′-terminus with the polymerase is very elaborated and involve contacts with all three subunits, in line with the observed stabilisation of the polymerase by promoter binding (Brownlee and Sharps, 2002). Recognition of the 5′-loop is established by PB1 and PA proteins and this interaction probably is important for the folding of the active site, while binding of the 3′-terminus takes place by all three subunits (Pflug et al., 2014; Reich et al., 2014).

In contrast, the nsNSV L protein requires co-operation with the P protein for recognition of the N-RNA template. The L–P binding site lies at the 3′-terminus of the template, within the leader region, overlapping the initiation site (Barr et al., 2002; Cowton et al., 2006; Kranzusch et al., 2010; Morin et al., 2012). Transcription for the sNSVs is independent for each RNP segment (Fig. 3A) while transcription of nsNSVs is sequential and shows a 3′-to-5′ polarity and attenuation (Fig. 3B), as a consequence of a single 3′-terminal entry site (Abraham and Banerjee, 1976; Ball and White, 1976) and rate-limiting steps at the termination and re-initiation sites (Iverson and Rose, 1981). This is best reflected in a general stop-start model whereby the L–P polymerase complex would pause

after termination in each intergenic region and would have an opportunity for release before reinitiating at the downstream initiation site (reviewed in Barr et al., 2002).

Transcription initiation: Capping vs cap-snatching

Initiation of transcription in the nsNSVs takes place de novo, but the precise site of initiation has been a subject of debate over many years, due to conflicting results derived in vitro or in vivo using Rhabdovirus or Paramyxovirus models. In vitro transcription leads to abundant leader RNA and successively less abundant mRNAs of genes downstream, suggesting a single initiation site at the genome 3′-terminus (Emerson, 1982). However, when an engineered short cistron was inserted between the leader and the N gene, transcription in vivo was not sensitive to UV inactivation of the leader region (Whelan and Wertz, 2002). These results, together with the phenotype of a mutant in the N gene that generates an excess of N mRNA over leader RNA (Chuang and Perrault, 1997) suggest that transcription in the nsNSVs initiates internally, at the first gene start site, whereas 3′-terminus initiation would be specific for RNA replication. This model is in agreement with the above mentioned identification of two polymerase complexes from virus-infected cells, namely a transcriptase containing L–P–EF1-α that can initiate at the gene start site and a replicase containing L–P–N and capable of initiating at the 3′-terminus of the genome (Qanungo et al., 2004).

Once the mRNA is initiated it becomes a substrate for the L-encoded RNA modifying activities guanylyltransferase, 2′-O-methyltranferase and guanine-7-methyltransferase. These activities have been associated to specific L sequence signatures (Li et al., 2005; Li et al., 2008) and localised to defined protein domains in the L-protein structure (Rahmeh et al., 2010). The virus-induced capping reaction is mechanistically distinct from the cellular activity (Li et al., 2006; Ogino and Banerjee, 2007) and a conserved His residue in L protein is directly involved in the generation of the L-5′-phosphorylated covalent intermediate (Ogino et al., 2010). In addition, the capping reaction requires elongation of a minimal transcript length to have access to a free 5′-terminus (Tekes et al., 2011) and, conversely, this 5′-modification appears to serve as a quality control to allow further transcript elongation, since its inhibition leads to premature termination (Stillman and Whitt, 1999).

In contrast, transcription initiation in the sNSVs is primer-dependent and mechanistically connected with acquisition of the 5′-cap structure. First described for the influenza viruses (Krug et al., 1979; Plotch et al., 1981) and later extended for Arenaviruses and Bunyaviruses (Jin and Elliott, 1993; Meyer and Southern, 1993; Patterson et al., 1984), the sNSVs polymerases cleave cellular mRNAs to generate cap-containing oligonucleotides to be used as primers for transcription. The influenza viruses, performing transcription in the nucleus, depend on the association of viral RNPs to cellular RNA polymerase II for cap-snatching pre-mRNAs (Engelhardt et al., 2005), whereas Arena- and Bunyaviruses transcribe in the cytoplasm and may utilise preferentially untranslatable mRNAs for cap-snatching (Cheng and Mir, 2012). Therefore, all sNSVs contain a cap recognition activity, that is located within the PB2 subunit of the influenza polymerase (Guilligay et al., 2014; Guilligay et al., 2008) and at the N-terminus of the NP in Arenaviruses (Qi et al., 2010), as well as a cap-dependent endonuclease always found in the RNA polymerase (Dias et al., 2009; Morin et al., 2010; Reguera et al., 2010; Yuan et al., 2009).

In the case of influenza viruses, the availability of the atomic structures for their polymerases (Pflug et al., 2014; Reich et al., 2014) has allowed a better understanding of the cap-snatching mechanism. Thus, the influenza type A cap-binding and endonuclease domains are opposed at a distance of about 50 Å, appropriate for a capped-oligonucleotide of 10–15 nt to be cleaved (Fig. 2B; Fig. 3A, bottom). In the corresponding structure of type B influenza polymerase the cap-binding domain shows a rotation and an extra electron density compatible with the capped primer oriented towards the polymerase active site. These data suggest that, upon endonuclease action, the cap-binding domain rotates to redirect the primer to the active site for elongation to proceed (Fig. 3A, compare structures at initiation step). The capped primer would still be bound to the cap-binding site during the elongation of further 10–15 nt (Braam et al., 1983) and the viral mRNA would emerge from the complex by an exit channel next to the PB2 C-terminal domain, away from the endonuclease site (Fig. 3A, elongation) (Reich et al., 2014).

Transcription termination, polyadenylation and re-initiation

For most of the NSVs, transcription termination is linked to the mRNA polyadenylation process (Hwang et al., 1998). The polyadenylation signal is rich in A/U residues and includes a 5–7 U stretch (Robertson et al., 1981; Schnell et al., 1996) whose composition and length is critical for polyadenylation (Barr et al., 1997; Li and Palese, 1994). Such oligo-U signal is copied multiple times by stuttering of the viral polymerase, as demonstrated by the generation of polyuridylated mRNAs when the signal is mutated to oligo-A in a recombinant virus (Poon et al., 1999). Termination of mRNA synthesis in the Arenaviruses is an exception, as they contain internal intergenic regions (IGR) that function as termination signals and their mRNAs lack poly-A tails and have a 3′-terminal hairpin structure instead (Lopez and Franze-Fernandez, 2007; Meyer and Southern, 1993; Pinschewer et al., 2005).

For the influenza viruses not only the size and composition of the polyadenylation signal are crucial, but also the distance to the 5′-terminus of the template (Li and Palese, 1994). This fact, together with the requirement for binding of the polymerase to the 5′-loop of the promoter (Pritlove et al., 1999), suggest a cis-action of the polymerase bound to the template during polyadenylation (Fig. 3A, polyadenylation) (Jorba et al., 2009; Poon et al., 1998). The situation for Rhabdoviruses is more complex, since transcription of the genome is sequential and the signals for initiation, 5′-capping, polyadenylation and termination are tightly packed and inter-dependent (Fig. 3B). Thus, reinitiation depends on the proper polyadenylation and termination of the upstream gene, capping requires a cis-signal at the gene start and a minimal elongation from the start point and polyadenylation depends on a minimal size of the transcript (reviewed in Barr et al., 2002; Whelan et al., 2004). All together, these data suggest a complex cross talk between the polymerase, the N-RNA template and the newly synthesised transcript to ensure a regulated production of the virus transcriptome.

The amplification of virus ribonucleoprotein templates

The replication of NSV RNA involves not only the synthesis of new RNA molecules of negative polarity but also their assembly into new RNP complexes that can act as templates in new infection events. The amplification of NSV RNPs occurs by two successive steps: (i) the generation of replication intermediates (anti-genomic RNPs or complementary RNPs of positive polarity) and (ii) the production of abundant progeny RNPs using such replication intermediates as templates.

Synthesis of the anti-genome-RNP replication intermediate

The generation of the anti-genome RNP as replication intermediate implies a dramatic change in the activity of the NSV

parental RNPs, early after infection devoted to the transcription programme and later dedicated to RNA replication. Thus, the RNA synthesis initiation, the coupling or not of RNA synthesis with RNP assembly and the RNA termination steps are clearly distinct in the transcription and replication programmes of the same genomic RNP template and therefore essential regulation steps must be in place to allow a normal virus replication cycle.

Transcription vs replication

Models have been proposed for the change from transcription to replication programmes involving either (i) regulation of the initiation step, (ii) alteration of the polymerase complex acting as transcriptase to allow its role as replicase or (iii) combination of the above, including the possible action of specific viral and host protein factors. As there is early evidence that active protein synthesis is required for nsNSVs RNA replication (Davis and Wertz, 1982) and translation inhibition allows continuous influenza transcription but not RNA replication (Mark et al., 1979), it was early proposed that the availability of newly synthesised, monomeric NP/N protein would possibly be a driving force for the switch from transcription to replication (Blumberg et al., 1981; Portela and Digard, 2002). Moreover, the coupled assembly of NP/N protein into nascent antigenomic RNA would avoid polyadenylation and termination events (Beaton and Krug, 1986). Such a basic model could not be supported by more recent data obtained with minimal transcription/replication systems using short negative-strand templates and viral polymerase but devoid of NP/N protein. As these minimal VSV-based systems were capable of correctly initiating (Morin et al., 2012) and the corresponding influenza model systems could normally transcribe and replicate in vitro and in vivo (Resa-Infante et al., 2010; Turrell et al., 2013) (Landeras–Bueno, unpublished results) it might be concluded that NP/N protein is not essential for template activity of short genomic RNAs and that it does not regulate the shift from transcription to replication. However, it must be stressed that NP/N protein can stimulate RNA synthesis activity (Kawaguchi et al., 2011; Newcomb et al., 2009), is present in the VSV replicase complex (Qanungo et al., 2004) and is essential for full processivity in RNA replication (Honda et al., 1988; Morin et al., 2012).

In the case of influenza viruses a new model has been proposed for the transcription vs replication dilemma using an in vivo complementation approach. In these experiments the synthesis of the replication intermediate could be rescued in infected cells under protein synthesis block by pre-expression of NP and a catalytically inactive polymerase (Vreede et al., 2004). These results would be consistent with the viral polymerase synthesised after infection being required for protection of the positive-polarity replication intermediate and not for the proper replication activity, and are compatible with the capacity of virion-derived RNPs to synthesise both mRNA and replication intermediate in vitro (Vreede and Brownlee, 2007). According to these data, the polymerase associated to infecting RNPs would be able to randomly initiate mRNA synthesis by cap-snatching or initiate de novo the synthesis of the replication intermediate, and a newly synthesised polymerase would be in charge of protecting the antigenome RNA and drive the assembly of the replication intermediate RNP. Whether such a mechanism could also operate for the members of the Mononegavirales is still uncertain.

Mechanism of de novo initiation and switch to elongation

The initiation event for RNA replication is independent of a pre-existing primer and requires the correct positioning of the "priming" NTP for incorporation of the incoming NTP. For other RNA replicases of known structure, such as those of dsRNA phage Φ6 and positive-polarity RNA hepatitis C virus, a dedicated structural feature has been described that serves as landing pad by providing an aromatic amino acid on which the "priming" NTP can stack (Bressanelli et al., 1999; Butcher et al., 2001). Such a putative structure has recently been described for the influenza virus polymerases (Pflug et al., 2014; Reich et al., 2014) as a β-hairpin loop in the C-terminus of PB1, containing a conserved His residue at the appropriate position, and future studies will reveal whether similar feature is conserved in L proteins of other NSVs. Surprisingly, the 3′-terminus of the genomic promoter is not properly located at the PB1 active site in the influenza polymerase-promoter atomic structure (Reich et al., 2014) but it rather lays at a specific external site of the polymerase. This implies that a conformational change has to take place to redirect the 3′-terminus of the template into the active site. Alternatively, it is possible that the 3′-terminus is exposed to be more accessible to the active site of a putative trans-acting polymerase (Jorba et al., 2008; Jorba et al., 2009; Resa-Infante et al., 2011)(see below). It will be interesting to determine the location of the 3′-terminus of the replication intermediate promoter within the polymerase, as different initiation sites have been identified for genomic or antigenomic RNAs (Deng et al., 2006).

Once RNA synthesis has been initiated, the polymerase must enter the elongation state, which normally is reflected in a kinetics change (Klumpp et al., 1998), and the accumulation of abortive short RNA products (Kao et al., 2001) that sometimes can be used as primers later by realigning into the initiation site (Garcin and Kolakofsky, 1990). The structure of the influenza virus polymerase in its elongation state has been modelled by docking the poliovirus template-product into the PB1 active site (Reich et al., 2014). Such an exercise led to the recognition of a steric clash for the exit of the template mainly due to blocking by the N-terminal sequences of PB2. These observations suggest that conformational changes must be invoked to permit elongation and are in line with the

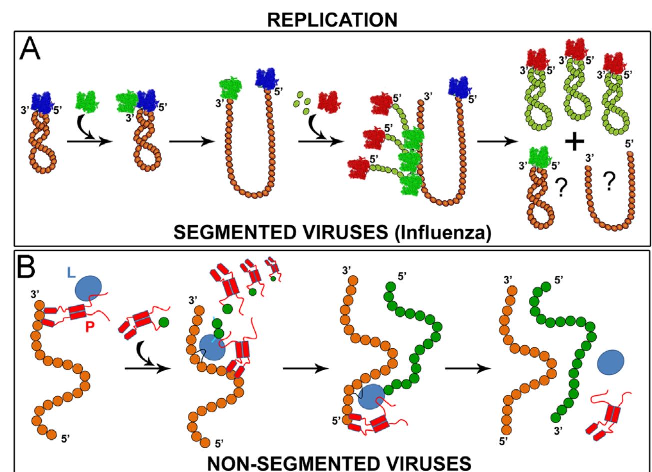

Fig. 4. Models for the amplification of NSV RNPs. (A) Cartoon showing the proposed trans model for the second step in influenza virus RNP amplification. The replicative intermediate (brown) containing a resident polymerase (blue) is activated for replication by interaction with a non-resident polymerase (green), which initiates de novo and produces a progeny RNA. Its 5′-terminal sequence is bound by an additional non-resident polymerase (red), which drives the assembly of NP monomers (green). Hypothetically, multiple initiation events could occur in a single replicative intermediate. Finally, the resident polymerase is displaced allowing the complete copy of the template. (B) Cartoon showing the mechanism of amplification of nsNSV nucleocapsids. The L (blue)-P (red) complex recognises the 3′-terminus of the antigenome N-RNA template (brown) and starts to copy at the 3′-terminus. Concomitant with viral RNA synthesis, monomers of N° protein (green) are assembled by delivery from N°-P complexes. Although protein L dimerisation has been shown biochemically and its relevance for nsNSV amplification has been shown genetically (see text for details), the present evidence does not allow proposing a potential role for L dimerisation in RNP amplification. The colour of the NP/N monomers is only intended to distinguish the polarity of the bound RNA (brown-positive or green-negative).

phenotype of mutants in the PB2 N-terminus that are affected in RNA replication but, intriguingly, not in transcription (Gastaminza et al., 2003; Jorba et al., 2009).

The coupling of initiation, assembly and termination events

During synthesis of the positive-polarity RNA products the initiation, assembly (or not) into RNP and termination steps are coupled in such a way that a mRNA transcript is capped, is not assembled into RNP and (normally) terminates by polyadenylation, whereas a genomic RNA is not modified at its 5′-terminus, becomes assembled into a RNP and is not polyadenlylated.

The transcription programme could be fired in the sNSVs by interaction of the polymerase with the cap-structure of cellular mRNAs, and the presence of this 5′-modification would avoid the recognition of the transcript as a substrate for assembly due to its detection by cellular cap-binding proteins (Bier et al., 2011). Under these circumstances, the termination signals would be recognised and the mRNA would be polyadenylated by the viral polymerase (Fig. 3A). In contrast, de novo initiation by influenza polymerase would first generate the 5′-terminal sequence of the genome, that is specifically recognised by N protein (Osborne and Elliott, 2000) or an additional polymerase complex (Vreede et al., 2004), which would drive the assembly of successive NP monomers onto the replicative intermediate RNA (Turrell et al., 2013) (Fig. 4). Furthermore, RNA product assembly into an RNP structure would help overriding the polyadenylation signal and allow generation of a full copy of the template (Beaton and Krug, 1986). In the case of the nsNSVs, the mechanism responsible for transcription initiation versus synthesis of the antigenome RNP is a matter of debate and several alternatives have been discussed (see for instance Banerjee, 2008; Curran and Kolakofsky, 2008; Whelan, 2008). However, it is worth mentioning that replicative initiation of NSVs requires high concentration of the first two-three nucleotides to be incorporated (Morin et al., 2012; Vreede et al., 2008).

On the other hand, there are accumulating evidences supporting the idea that polymerase complex alterations could account for the variation in the RNA synthesis initiation mechanisms and their coupled RNA modification and/or assembly into RNPs during transcription vs replication. Thus, other viral proteins have been shown to alter the ratio of transcription vs synthesis of the replication intermediate. For example, the NEP protein of influenza virus, known to play a role in the export of progeny RNPs from the nucleus (Boulo et al., 2007; O'Neill et al., 1998), can also stimulate the accumulation of the replication intermediate (Robb et al., 2009) and the VSV P protein promotes the replicative initiation event (Morin et al., 2012), in addition to its role in polymerase–nucleocapsid interaction. On the contrary, respiratory syncytial virus (RSV) M2-1 and Ebola virus VP30 proteins have been shown to stimulate transcription (Fearns and Collins, 1999; Weik et al., 2002). Also cellular factors can participate in the transition between transcription and replication. Thus, in addition to the above mentioned presence of EF1-α in the transcriptase of VSV (Qanungo et al., 2004) it is worth mentioning the association of the MCM complex to the influenza RNA polymerase during elongation of the replication intermediate (Kawaguchi and Nagata, 2007), the interaction of influenza polymerase with RNA polymerase II for virus transcription (Engelhardt et al., 2005) or with the splicing factor SFPQ/PSF for efficient mRNA polyadenylation, but not for RNA replication (Landeras-Bueno et al., 2011). These results are reminiscent of the situation described early on for the RNA replicase of positive-polarity phage Qbeta, which requires the association of the viral subunit of the polymerase with ribosomal proteins S1 and EF Tu-Ts for the synthesis of the replicative intermediate whereas it can perform the synthesis of progeny viral RNA (that acts as a mRNA) with a minimal complex containing only EF Tu-Ts (reviewed in Blumenthal and Carmichael, 1979).

Finally, RNA synthesis by the NSV polymerases is down-regulated by the accumulation of viral matrix proteins, the main drivers of virion egress (influenza M1, Rhabdovirus M, Arenavirus Z, etc), allowing the generation of fully functional virus RNPs for virion encapsidation (reviewed in Kranzusch and Whelan, 2012).

The synthesis of genomic RNPs

The generation of progeny genomic RNPs from the replication intermediates is comparatively simpler than the first step in NSV RNA replication, as there is no alternative transcription event. The promoter activity from the replication intermediates is stronger and consequently there is a bias towards the accumulation of progeny RNPs of negative polarity. In the case of the sNSVs, a distinct initiation mechanism has been described which implies initiation at an internal position in the template and realignment of the primer oligonucleotide to the 3′-terminal position, including or not a non-templated nucleotide addition (Deng et al., 2006; Garcin and Kolakofsky, 1990; Garcin and Kolakofsky, 1992).

One of the commonalities during NSV RNA replication is the capacity of their RNA polymerases to form oligomers. The capacity of the RNA polymerase to self-associate has been shown biochemically (Cevik et al., 2003; Jorba et al., 2008; Sanchez and de la Torre, 2005; Smallwood et al., 2002), and in the case of nsNSVs L protein may be independent of its association to P protein oligomers (Cevik et al., 2004). In addition, it has also been analysed structurally (Chang et al., 2015; Rahmeh et al., 2012; Resa-Infante, 2010) and is functionally relevant for NSV RNA synthesis, as revealed by intragenic complementation (Fig. 4A) (Jorba et al., 2009; Smallwood et al., 2002) and by the dominant-negative properties of specific mutants (Sanchez and de la Torre, 2005). This appears to be a general property of RNA viruses, as it was also described for the positive-stranded poliovirus and hepatitis C viruses (Hobson et al., 2001; Lyle et al., 2002; Wang et al., 2002). Another general property of NSV RNA replication is that not only the NP/N protein but also the polymerase are needed in stoichiometric amounts for RNP amplification. Thus, the polymerase must act enzymatically for RNA replication but must also be incorporated into each virus particle for it to be infectious. For the influenza viruses in particular, each virus RNP contains a copy of the polymerase that has been proposed to act in cis during primary transcription (Jorba et al., 2009; Pritlove et al., 1999) (Fig. 3A) and the synthesis of the replication intermediate (Vreede et al., 2004). However, the question has been raised whether the polymerase associated to the replication intermediate RNP would act in cis for amplification of progeny genomic RNPs or rather would a new, soluble polymerase be in charge of RNA replication in trans (Fig. 4A). Evidence for a trans model of replication came from in vivo complementation experiments in which mutant viral RNPs unable to perform RNA replication could be rescued by expression of genetically marked exogenous polymerase (Jorba et al., 2009). Such a trans model for RNA replication implies that a polymerase distinct from that resident in the replication intermediate RNP performs replicative synthesis and a further non-resident polymerase becomes incorporated into the progeny genomic RNP and drives the assembly of NP monomers (Fig. 4A) (reviewed in Martin-Benito and Ortin, 2013; Resa-Infante et al., 2011). The different modes of replication described for antigenomic and genomic RNPs are in line with the reported differential interaction of the polymerase with positive- and negative-polarity promoters (González and Ortín, 1999b) and the distinct initiation sites identified for either template (Deng et al., 2006). Recent evidence has lent support to the trans model for RNA replication in influenza viruses: (i) Visualisation of recombinant RNPs reconstituted in vivo showed branched structures in which a putative nascent RNP buds from a full-length RNP and a polymerase could

be detected at the branch site (Moeller et al., 2012). (ii) Purification and characterisation of replication intermediate and genomic RNPs by RNA affinity techniques indicated that genomic RNPs could initiate transcription by cap-snatching and initiate replication de novo but the replication intermediate RNPs could only initiate de novo by addition of additional purified polymerase, although intriguingly, a replication mutant polymerase could also trans-activate (York et al., 2013). (iii) The NEP-dependent accumulation of short viral RNAs (svRNAs; 22–27 nt in length corresponding to the 5′-terminus of the genomic RNAs) correlates with an increase in genomic RNA replication (Perez et al., 2010, 2012; Umbach et al., 2010) and these svRNAs might associate with the trans-acting polymerase to direct genomic RNP accumulation.

Concluding remarks

We have recently witnessed a quantum leap in our understanding of the NSV replication machines due to the elucidation of the atomic structures of influenza A and B polymerase complexes in association with their promoter sequences (Pflug et al., 2014; Reich et al., 2014). These data open many new avenues of research, particularly relating to the mechanisms for cap binding, cap snatching and de novo replicative synthesis, and uncover new potential targets for virus inhibition. It is to be expected that this experience will boost similar achievements in the L polymerase proteins from other ns- and sNSVs.

The structural information now available for influenza virus polymerase and that to come for the L proteins will be nuclear to understand how the enzyme is able to access the genomic RNA within the RNP template and how the viral and host factors described to modulate transcription and replication perform their functions. To this aim it will be essential to improve considerably the resolution of the available electron microscopy structures for viral RNPs (Arranz et al., 2012; Coloma et al., 2009; Moeller et al., 2012) and hence unravel the details of the polymerase-NP interaction in a functional machine.

Further to the above developments, we feel that time is ripe to address the mechanics and the kinetics of the polymerase during cap-snatching and RNA synthesis by means of single-molecule techniques, examples of which have been recently reported (Chou et al., 2013; Tomescu et al., 2014).

Acknowledgements

We thank S. Cusack (EMBL, Grenoble) and E. Fodor (University of Oxford) for providing unpublished information and S. Cusack (EMBL, Grenoble) and H. Zhou (UCLA) for permission to use published images. We are indebted to Patricia Resa-Infante for her comments on the manuscript. Work in the author's laboratories was funded by Ministry of Science and Innovation (*Ministerio de Ciencia e Innovación*), grants BFU2010-17540/BMC (JO) and BFU2011-25090/BMC (JM-B), by the EU FLUPHARMproject (FP7-259751) (JO) and Fundación Botín (JO).

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