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The polymerase of negative-stranded RNA viruses

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Abstract

Negative-sense (NS) RNA viruses deliver into cells a mega-dalton RNA-protein complex competent for transcription. Within this complex, the RNA is protected in a nucleocapsid protein (NP) sheath which the viral polymerase negotiates during RNA synthesis. The NP-RNA templates come as nonsegmented (NNS) or segmented (SNS), necessitating distinct strategies for transcription by their polymerases. Atomic-level understanding of the NP-RNA of both NNS and SNS RNA viruses show that the RNA must be transiently dissociated from NP during RNA synthesis. Here we summarize and compare the polymerases of NNS and SNS RNA viruses, and the current structural data on the polymerases. Those comparisons inform us on the evolution of related RNA synthesis machines which use two distinct mechanisms for mRNA cap formation.

Negative-strand (NS) RNA viruses encompass some of the most significant human and agricultural pathogens extant [1,2]. The viruses can be divided into two main groups based on their genomic RNA: non-segmented NS (NNS) RNA viruses and segmented NS (SNS) RNA viruses. The NNS RNA viruses comprise four families, the *Rhabdoviridae* (vesicular stomatitis virus (VSV) and rabies virus), *Paramyxoviridae* (measles and respiratory syncytial viruses (RSV)), *Filoviridae* (Ebola and Marburg viruses) and the *Bornaviridae* (Borna disease virus). The SNS RNA viruses comprise three families, the *Arenaviridae* (lymphocytic choriomeningitis virus and Machupo virus (MACV)), *Bunyaviridae* (Rift Valley fever virus) and *Orthomyxoviridae* (influenza A virus).

The NS RNA viruses share a common replicative machinery comprising a protein–RNA complex in which the viral genomic RNA is found within a capsid protein sheath to form the nucleocapsid (NP) protein–RNA complex. Those NP-RNA templates are copied by the virally encoded RNA dependent RNA polymerase (RdRP) in two synthetic modes: mRNA transcription and genome replication. For the NNS RNA viruses that single genome contains a tandem array of 5–10 genes that are sequentially copied by the viral polymerase, whereas the SNS RNA virus polymerases copy each template into a single mRNA (for review see: [3]). The enzymatic activities necessary for copying of the NP-RNA templates include an RdRP, as well as the enzymes required for mRNA cap formation that are only utilized during mRNA transcription. All of the necessary enzymatic activities reside within a 250 kDa large (L) polymerase protein, except for orthomyxoviruses where 3 separate proteins assemble into a complex of similar size (for review see: [4,5]).

The mechanism of mRNA cap formation, and consequently the enzymatic activities involved differ between the NNS and SNS RNA viruses. The NNS RNA viruses synthesize their mRNA cap-structures. They employ an L encoded polyribonucleotidyltransferase (PRNTase) to transfer nascent RNA onto a GDP acceptor to form a GpppN cap structure, through a covalent L–pRNA intermediate [6,7]. The cap structure is then subsequently modified by an unusual dual specificity methyltransferase that adds both 2'-O and guanine-N-7 modifications to form the 7mGpppNpmNp cap structure [7–9]. By contrast, SNS RNA viruses cannibalize host cell mRNA cap structures to serve as primers of transcription, and employ a cap-dependent endonuclease activity to do so [10–14]. The unique mechanisms of the “cap-snatching” reaction employed by SNS RNA viruses, and the PRNTase employed by NNS RNA viruses hold promise as potential targets for development of antiviral drugs.

The various enzymatic activities required for RNA synthesis have been mapped within the corresponding polymerases. The smaller polymerase fragments of influenza virus facilitated a greater biochemical and structural understanding of this tripartite complex than for NS RNA virus L proteins [15–17]. This in part reflects the large size (~250–450 kDa) of L, and the presence of flexible domains or connecting “hinge” regions that likely separate independent enzymatic activities [18]. Despite those challenges, a combination of sequence analysis, expression and purification of polymerases, *in vitro* biochemistry and low and high-resolution structural studies have provided a map of the different enzymatic activities on the polymerases (Figure 1) [19–23].

The overall architecture of the NNS and SNS polymerases were investigated by single particle electron microscopy. In each case, the start point for these studies was functional enzyme that retains RNA synthesis activity. The first such structures came from studies of the heterotrimeric influenza A virus complex [19,22,23], comprising PA, PB1 and PB2 which assemble into a complex that shares features that were subsequently observed in analyses of the L proteins of VSV, a cap synthesizing NNS RNA virus, and MACV a cap snatching SNS RNA virus [20,21]. The structures of VSV and MACV L reveal a shared ring-like domain that contains the RdRP and this domain is decorated with structurally distinct appendages to which the capping machinery was mapped (Figure 1). For VSV, the PRNTase activity maps to the globular domain adjacent to the ring-like domain, and the very C-terminus of the protein provides the two remaining globules to which the MTase activity maps [21]. Rather than a set of globules appended to the ring-like domain, MACV contains an “arm-like” appendage that likely contains the cap-dependent endonuclease [20]. This EM characterization also demonstrates that the capping activities adopt different orientations in relation to the ring-like domain between different classes of images. Such an observation is consistent with the presence of the flexible linkers or hinge domains present within the L proteins.

The ring-like domain is also reminiscent of the RdRPs of the double-stranded RNA viruses reovirus and rotavirus. The dsRNA virus polymerases are similar in size to the portion of L required to assemble the ring (approximately 130 kDa). The atomic structures of the reovirus and rotavirus polymerases reveal that the RdRP domain is bracketed between an N-terminal bridging domain and a C-terminal bracelet domain that serve to fully enclose the active site [24,25]. This cage like structure provides a secondary RNA exit tunnel that

facilitates the release of the mRNA. Like the L proteins, the RdRPs of the dsRNA viruses engage in two tasks: mRNA synthesis and genome replication. Similar four-tunnel architecture may also be required by NS RNA viruses to facilitate the separation of the template and replication RNAs and permit the simultaneous re-encapsidation of the template strand, and encapsidation of the nascent strand.

The influenza A virus polymerase structures are at considerably higher resolution and permit the docking of the atomic structures of the individual fragments of the influenza proteins (Figure 1) [19,26,27]. The overall dimensions and the presence of a central “ring-like” structure are strikingly similar to those of the VSV and MACV L proteins. The large flexible appendages observed for VSV and MACV L are absent in the influenza structure, although the polymerase adopts a more open conformation in the absence of the NP-RNA template. Intriguingly, those structural rearrangements appear to involve the amino-terminus of PB2 underscoring the flexibility of capping domains in relation to the RdRP. Such flexibility may be essential for discriminating mRNA synthesis from genome replication in which the capping activities are not required and indeed may interfere with encapsidation of the nascent RNA.

X-ray crystal structures of recombinant NP and model NP-RNA complexes reveal the structural basis of RNA coordination (Figure 2) [26–36]. 3D EM reconstructions have allowed the construction of pseudoatomic models of encapsidated NS RNA genomes [19,29,35]. NP from NNS RNA viruses adopts a conserved bi-lobed architecture that forms a positive groove between amino- and carboxyl-terminal globules that coordinates and shields the genomic RNA. Crystallographic structures of NP-RNA complexes from VSV, rabies virus and RSV revealed NP–NP interactions that tightly lock monomers together resulting in the continuous rigid encapsidated structure characteristic of NNS RNA viral NP-RNA templates [28,30,35]. In contrast, NP structures from SNS RNA viruses instead reveal varied structural architecture and less coordinated NP–NP contact consistent with a loosely organized template. The structures also explain why the NP-RNA of the NNS RNA viruses is more resistant to RNase digestion than those of the SNS RNA viruses (for review see: [37]).

The polymerases of NNS and SNS RNA viruses negotiate their corresponding NP-RNA templates by different mechanisms. SNS RNA virus polymerases directly access NP-RNA and produce a single transcript or replication product from each segment. By contrast L proteins of NNS RNA viruses cannot directly engage the NP-RNA but require an accessory factor the phosphoprotein (P) to bridge interactions between L and the NP-RNA. In addition to the cap-synthesis vs cap-snatching properties of the two classes of polymerase a further fundamental distinction for L proteins of NNS RNA viruses is that they sequentially copy their NP-RNA into a series of 5–10 monocistronic mRNAs through a start-stop mode of transcription. Those distinctions may be linked to a requirement for P to dissociate the NP-RNA complex, and to keep L associated with the template during the termination and re-initiation events that occur at gene junctions.

Recent structural studies on VSV have also revealed other functions for the P protein. VSV P forms non-globular homodimers and consists of three domains separated by two

intrinsically disordered linkers [38–41]. The P N-terminal domain which binds nascent RNA-free N (N₀), the central domain (P_{CD}) that constitutes a dimerization interface, and the C-terminal domain (P_{CTD}) which binds the N-RNA (Figure 3A, B and C). The long disordered linkers confer to P a large flexibility in between the different domains important for the functioning of the replicative machinery. The EM studies reveal that P affects the overall structural architecture of L [21] such that the appendage containing the capping activities appears to be reordered and now folds back on the ring-like domain in a general conformation resembling a “6” (Figure 3D, E). That rearrangement in the domain containing the capping activities of L therefore strengthens an involvement of the overall architecture of L in the coordination between polymerization and capping of the newly synthesized mRNA. The ability of P to induce the conformational alterations in L is fully recapitulated by a region of P that extends from residue 41–106 [42].

The structural insights obtained from NS RNA virus templates indicate that the RNA must be displaced from NP to facilitate its copying by polymerase. Such a displacement must involve relatively few molecules of NP at any given time, because for the NNS RNA viruses the template remains resistant to nuclease digestion even during transcription. Those observations catalyzed the development of an improved system to study polymerase biology *in vitro* in which polymerase alone could use naked RNA as template. In agreement with a model of N dissociation, it has been demonstrated *via* a simple *in vitro* system that VSV L, in the absence of NP and P, initiates RNA synthesis on a naked RNA corresponding to the first 19 nucleotides of the genomic RNA (Figure 4) [43]. Thus, it provides the first evidence that the minimal RNA synthesis components of NNS RNA viruses are L and RNA, supporting the model that the RNA is dissociated from NP during polymerization. Similarly, parallel experiments conducted with MACV L demonstrate that this *in vitro* assay is broadly applicable to the study of NS RNA virus polymerases [44].

This new *in vitro* assay is a powerful system to study RNA synthesis initiation by NS RNA virus L proteins and is revealing fundamental new insights into the biology of the RNA synthesis machinery. For the NNS RNA viruses, work with VSV L demonstrates in addition to the structural roles of P in interacting with N and L [21,38], P is a *bona fide* processivity factor for L and an enhancer of initiation (Figure 4A) [43]. The difficulties of obtaining NP-RNA template completely devoid of P, have interfered with a precise analysis of the influence of P on L activity. Results from this new *in vitro* assay convincingly demonstrate that P, independent of its role in engagement of the template-associated NP protein, has direct functional effects on the enzymatic properties of L. The mechanism by which P affects L catalytic activity remains unclear, however EM analysis shows that L undergoes conformational changes on P binding that may facilitate the increased processivity. While P binding increases L processivity, this L–P complex is not fully processive on a naked RNA template. Full processivity requires the template associated NP (Figure 4B), perhaps reflecting a need to keep the template relatively unstructured to not impede the progress of L [43]. The tripartite influenza virus polymerase is also fully processive only in presence of NP demonstrating that all NS RNA viruses polymerases require the template associated NP for full processivity [45]. This simple *in vitro* assay has consequently uncovered previously unappreciated roles for the replication proteins of NS RNA viruses.

RdRPs can initiate RNA synthesis by two distinct mechanisms: primer-dependent and primer-independent or *de novo* initiation. It is clear that both mechanisms operate for the NS RNA viruses. The polymerases of NNS RNA viruses were widely thought to initiate RNA synthesis by a *de novo* mechanism, and work with naked RNA templates provides the first direct evidence for such a mechanism. *De novo* initiation requires a higher K_m for the first nucleotide (NTPi) incorporated during synthesis which engages in specific interactions within the active site (for a review see: [46,47]). Intriguingly, VSV L require high concentrations of the first two nucleotides incorporated during the synthesis suggesting that both nucleotides might bind specifically the active site to generate a stable initiation complex [43]. Engineered templates show that the first two positions at the 3' end of the template are essential for correct initiation by VSV L, with the third position playing an enhancing role. These results suggest that the major requirement of the initiation complex is a double base pair between the first two nucleotides incorporated during the initiation and the first two nucleotides of the RNA template. Although VSV L initiates RNA synthesis in a template-dependent manner, work with RSV indicates that its L uses a non-template initiation mechanism for incorporation the first two nucleotides of the antigenome [48–50]. This raises the possibility that the initiating nucleotides are differently selected by the two polymerases.

Based on the initiation site on the template two mechanisms of *de novo* initiation are known to be used by RdRPs: a 3' terminal initiation and internal initiation. Although VSV L can initiate RNA synthesis by both mechanisms, internal initiation is dramatically less efficient. This suggests that the active site of the RdRP of VSV L is sufficiently flexible to allow the 3' end of the template to overshoot its position and permit a correct initiation. The adaptation of the naked RNA template assay to the study of RSV uncovers efficient initiation at two positions, +1 and +3, at the 3' end of the genome [50,51], lending further support to a distinction in the mechanism of initiation.

The above summarizes advances in our structural understanding of the RNA synthesis machinery of NS RNA viruses that provide an invaluable new framework for further functional analysis. There remain, however, significant gaps in our understanding of both structure and function of these dynamic machines. There remains no complete atomic level structure of any NS RNA polymerases. Such structures should further our understanding of how NP-RNA complexes are transiently dissociated during RNA synthesis as well as how the capping and polymerization activities are coordinated during transcription. The flexible nature of the positioning of the capping and RdRP activities raise the possibility of a significant structural rearrangement of the machinery during replication. As the resolution of structures improves some of these secrets may be revealed. The continued application of newly described *in vitro* assays to study multiple NS RNA viruses demonstrates the broad utility of such approaches. Those assays are already uncovering the mechanisms by which initiation and elongation proceed as well as informing on the mechanism by which polymerase inhibitors function. As some NNS RNA virus polymerases depend upon cofactors in addition to the P protein, notably the M2 protein of RSV, and the VP30 protein of Ebola virus the application of this *in vitro* approach should permit an unequivocal analysis of the function of such proteins.

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Figure 1. Structural architecture and organization of NS RNA viral polymerases
Conserved architecture and domain organization found in nonsegmented (top, purple) and segmented (bottom, orange) polymerases. The linear amino acid sequence of L and the tripartite influenza virus polymerase contain highly conserved regions dedicated to RNA synthesis (blue boxes). L and the influenza virus polymerase also contain blocks of conservation dedicated to 5' cap formation (maroon boxes), including an endonuclease domain for cap-snatching (domain I or PA, segmented NS RNA viruses) or PRNTase / MTase domains for *de novo* cap synthesis (domains V and VI, nonsegmented NS RNA viruses). The regions containing cap formation enzymatic activities are also required for RNA synthesis, and the exact function of domains II and IV in the L protein of segmented NS RNA viruses remains unknown. EM of purified L from Machupo virus and vesicular stomatitis virus reveals a shared structural architecture conserved within NS RNA viral L proteins. L consists of a central ring-like RNA polymerase domain (blue highlight in cartoon) and a large appendage dedicated to 5' cap formation (maroon highlight in cartoon)

attached with a flexible linkage. As described in the text, comparison with EM analysis and 3D reconstruction of the influenza virus polymerase complex provides further insight into the architecture of L[19,22,27]. (Fig. adapted from ref. 4)

Figure 2. Architecture of the NP-RNA complex

(A) EM images of rhabdovirus (NNS RNA viruses, magenta) and arenavirus (SNS RNA viruses, blue) NP-RNA genomic template isolated from purified virions. (Scale bar: 50 nm) [37,52]. (B) Pseudoatomic model of influenza A virus RNP. The tripartite polymerase complex is bound to a truncated NP-RNA template. Atomic structures of the NP are docked inside the EM density. Adapted from (ref). (C) Crystal structure of VSV (magenta), Lassa virus (blue) and influenza A virus NP. The N-terminal (Nt) and C-terminal (Ct) domains are indicated. VSV NP is shown in complex with a RNA oligomer that binds NP in between the N and C-terminal domain of the protein. The full Lassa NP is represented devoid of RNA (left), while the N-terminal domain is shown in complex with a RNA oligomer (right). The influenza A NP is represented free of RNA, the putative RNA binding site is indicated by a dashed arrow.

Figure 3. Molecular architecture of VSV P and L

(A) Schematic organization of VSV P. P consists of three domains connected by two intrinsically disordered regions (Black line). The N-terminal (Nt) and C-terminal (Ct) are indicated. The P N-terminal domain (P_{NTD} , red) contains the RNA-free N (N0) binding region, the central domain (P_{CD} , orange) consists of an oligomerization region and the C-terminal domain (P_{CTD} , green) is involved in the N-RNA binding. (B) Crystallographic structure of VSV P_{NTD} (top), a dimer of P_{CD} (middle) and P_{CTD} (bottom). (C) Structure of P_{NTD} in complex with N0 (magenta) (top) and P_{CTD} in complex with the C-terminal domain of N (magenta) (bottom). (D) EM image (left) and model (right) of L organization. L is arranged with a core ring structure harboring the RdRP domain and a flexible appendage containing the activities necessary for cap formation (capping + methylation). The arrows depict putative flexible linkers. (E) EM image (right) and model (left) of the L-P complex. Upon binding to P or P41-106, the appendage of L undergoes a structural rearrangement. When L binds full length P, the L-P complex is isolated in two forms a monomer and a dimer in which the L pairs are likely bridged by interaction with an oligomer of P (top). The arrow represents the variable orientation of L proteins in the dimers. When L binds P41-106 the L-P41-106 complex exits only as a monomer (bottom).

Figure 4. VSV L *in vitro* RNA synthesis assay

(A) *De novo* RNA synthesis by VSV L, in the absence and presence of P, on a synthetic template corresponding to the first 19 nt of the leader (Le19). Activity assays were set using 0.2 μ M of Le19, 0.2 μ M of protein and [α^{32} P]-GTP as the radio-labeled nucleotide. Reactions were quenched by the addition EDTA/formamide and analyzed on a 20% polyacrylamide/7 M urea gel. *De novo* initiation product sizes are indicated on the left. (B) RNA synthesis by VSV L on a naked 50 (Le50) nucleotides long RNAs, and the encapsidated RNA (N-RNA) templates. (C) RNA synthesis by MACV L on a naked RNA (left), or a naked mutated RNA (right).