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Structures and Mechanisms of Nonsegmented, Negative-Strand RNA Virus Polymerases

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Abstract

The nonsegmented, negative-strand RNA viruses (nsNSVs), also known as the order *Mononegavirales*, have a genome consisting of a single strand of negative-sense RNA. Integral to the nsNSV replication cycle is the viral polymerase, which is responsible for transcribing the viral genome, to produce an array of capped and polyadenylated messenger RNAs, and replicating it to produce new genomes. To perform the different steps that are necessary for these processes, the nsNSV polymerases undergo a series of coordinated conformational transitions. While much is still to be learned regarding the intersection of nsNSV polymerase dynamics, structure, and function, recently published polymerase structures, combined with a history of biochemical and molecular biology studies, have provided new insights into how nsNSV polymerases function as dynamic machines. In this review, we consider each of the steps involved in nsNSV transcription and replication and suggest how these relate to solved polymerase structures.

INTRODUCTION

nsNSV: nonsegmented, negative-strand RNA virus

EBOV: Ebola virus

RSV: respiratory syncytial virus

HMPV: human metapneumovirus

sNSV: segmented, negative-strand RNA virus

The order *Mononegavirales*, otherwise known as the nonsegmented, negative-strand RNA viruses (nsNSVs), includes a large collection of human pathogens, as well as viruses that infect animal and plant hosts. The human pathogens range from rare, highly pathogenic viruses, such as Ebola virus (EBOV), Marburg virus, and Nipah virus, to highly prevalent respiratory viruses, such as respiratory syncytial virus (RSV), human metapneumovirus (HMPV), and parainfluenza viruses that exact a steady toll on the young and elderly. A greater understanding of the molecular mechanisms underlying nsNSV replication could aid in the quest to develop countermeasures against these viruses. Central to the nsNSV replication cycle is the viral polymerase, an incredible multifunctional molecular machine, capable of messenger RNA (mRNA) synthesis, capping, cap methylation, and polyadenylation, as well as viral genome replication. Thus, activities that are performed by a multitude of different proteins in other biological systems are encapsulated in a single nsNSV polymerase protein. The last decade has seen significant progress in characterizing nsNSV polymerases, both structurally and functionally. In parallel, there have been remarkable advances in our understanding of the polymerases of a related group of viruses, the segmented, negative-strand RNA viruses (sNSVs), a group of viruses that includes the influenza viruses and bunyaviruses, among others. The capture of snapshots of sNSV polymerases in complex with RNA ligands representing different stages of transcription and genome replication has led to an appreciation of the significant conformational changes that these polymerases undergo to accomplish their different activities (1–4). While most of the nsNSV polymerase structures have been solved in apo form, polymerases from different nsNSVs have been captured in different conformations. By drawing analogies with the sNSV polymerases and integrating nsNSV polymerase functional studies with the available structures, we can suggest how different conformations relate to function. In this review, we provide an overview of nsNSV transcription and replication and then focus on individual steps in these processes, providing models for how these steps relate to polymerase structure.

OVERVIEW OF nsNSV TRANSCRIPTION AND REPLICATION

At the time of writing, the order *Mononegavirales* includes 11 different virus families (5); those that have been researched most extensively are the families *Rhabdoviridae*, *Paramyxoviridae*, *Pneumoviridae*, and *Filoviridae*, and it is the literature on these families that shaped this review. The nsNSV genome is a ribonucleoprotein complex, consisting of RNA encased with multiple copies of nucleoprotein or nucleocapsid protein (referred to here as NP) to form a helical nucleocapsid (6). The nsNSV genes are arranged sequentially on the viral genome and are transcribed in 3′ to 5′ order (reviewed in 7–11). The polymerase transcribes individual, subgenomic mRNAs by responding to gene start and gene end signals that lie at the boundaries of each gene (**Figure 1**). The nsNSV polymerase initiates transcription at a promoter at or near the 3′ of the genome and first synthesizes a short transcript, called le+, that corresponds to the leader promoter region (**Figure 1*a***). The le+ transcript is released from the polymerase with the polymerase remaining template bound. The polymerase can then scan the template to locate the first gene start signal and reinitiate RNA synthesis. Following initiation, the polymerase adds a methylated cap to the pre-messenger RNA (pre-mRNA) and then continues in an elongation mode until it reaches a gene end signal where it polyadenylates and releases the mRNA but again remains attached to the template. The polymerase then scans the genome template to locate the next gene start signal and reinitiates RNA synthesis (**Figure 1*a***). It continues in this manner for the length of the genome, with some attrition at each of the gene junctions. The nsNSV genomes are replicated via a replicative intermediate antigenome RNA, which in turn acts as a template for synthesis of genome sense RNAs (7–11). Replication is also initiated at the 3′ end of the genome, but in this

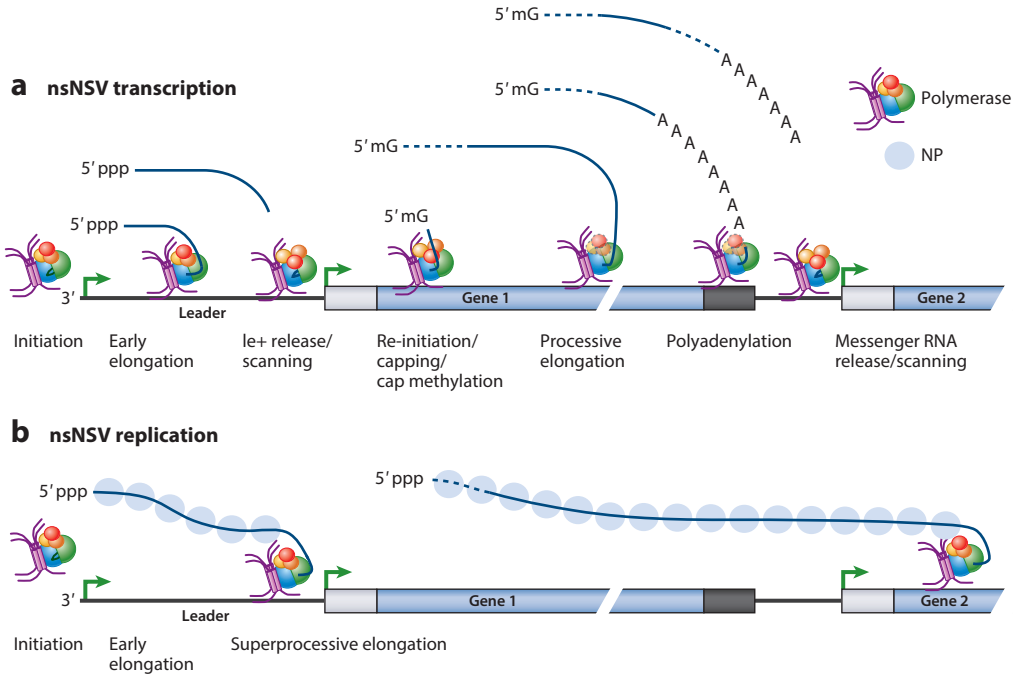

Figure 1

Schematic diagrams illustrating the different steps that occur during nonsegmented, negative-strand RNA virus (nsNSV) transcription (*upper panel*) and replication (*lower panel*). The 3′ portion of an nsNSV genome is shown, with the leader region, *gene 1*, and part of *gene 2* illustrated. Gene start and gene end signals are represented with white and black boxes, respectively. Initiation sites are indicated with green arrows. The RNAs generated contain either a 5′ triphosphate (5′ ppp) or 5′ methylguanosine (5′ mG) cap. For clarity, the nucleocapsid protein (NP) coating the genome template is not shown.

case, the polymerase does not release the le+ transcript and is superprocessive, continuing RNA synthesis to the end of the genome. Productive replication differs from transcription in that it depends on concurrent encapsidation of the replication product with soluble NP, which is delivered to the replicating polymerase as a complex with the viral P protein or VP35 (in the case of the filoviruses), and this might account for polymerase superprocessivity (12) (**Figure 1*b***).

OVERVIEW OF THE nsNSV POLYMERASE STRUCTURE

Cryo–electron microscopy (cryo-EM) structures have been solved for intact or largely intact polymerases of representatives from four families of nsNSVs: the rhabdoviruses vesicular stomatitis virus (VSV) and rabies virus (RABV), the pneumoviruses RSV and HMPV, the paramyxoviruses parainfluenza virus type 5 (PIV-5) and parainfluenza virus type 3 (PIV-3; in preprint form), and a large fragment of the filovirus (EBOV) polymerase (13–21). In addition, a structure is available for the EBOV polymerase fragment in complex with an RNA template-primer duplex (21). The overall structures of these polymerases are very similar (22). The polymerase is a complex of the large polymerase subunit (L) and cofactor phosphoprotein (P) or VP35. The L protein contains five domains: the RNA-dependent RNA polymerase (RdRp), capping, connector, methyltransferase, and C-terminal domains (18, 22) (**Figure 2*a***). The RdRps of the nsNSVs have similarities to other RdRps, including those of the sNSVs, with thumb, palm, and finger subdomains (22, 23). The polymerase has a catalytic cavity where phosphodiester bond formation is catalyzed, and feeding

VSV: vesicular stomatitis virus

RABV: rabies virus

PIV-5: parainfluenza virus type 5

PIV-3: parainfluenza virus type 3

RdRp: RNA-dependent RNA polymerase

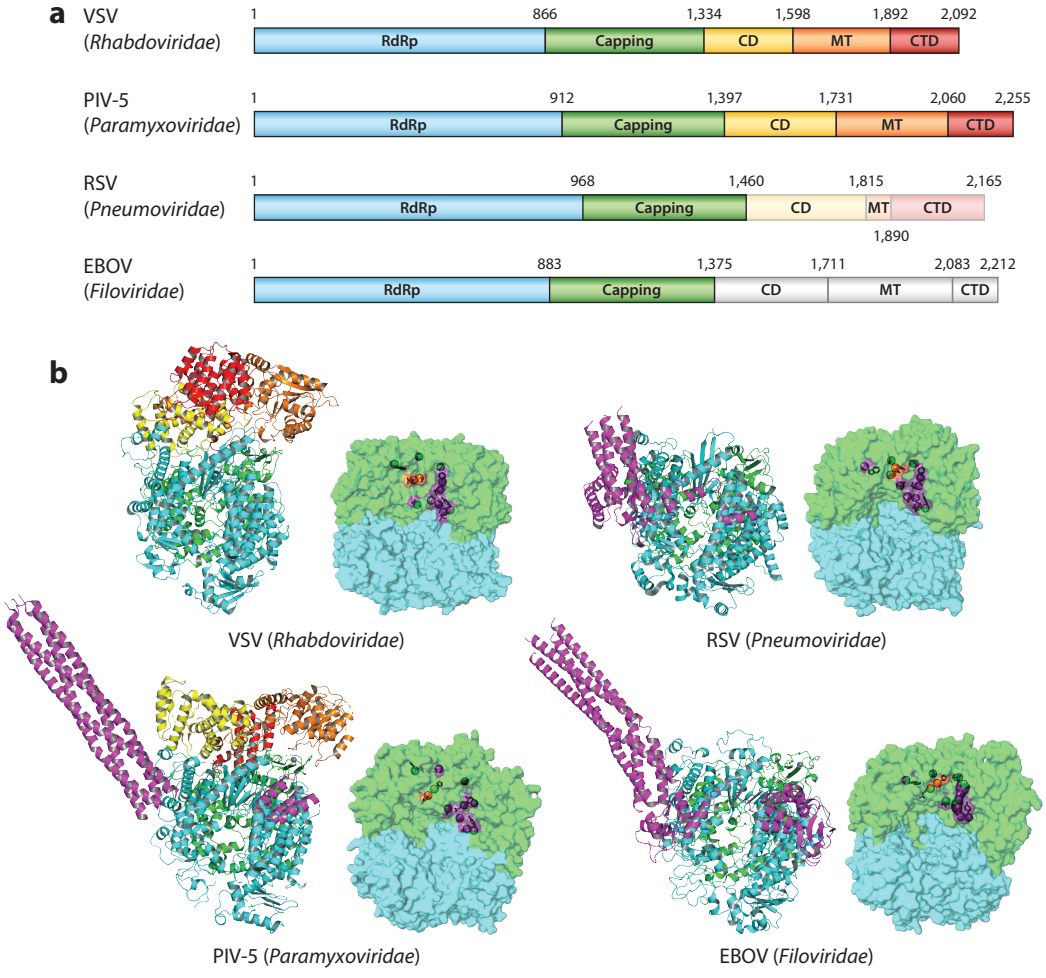

Figure 2

Examples of nonsegmented, negative-strand RNA virus (nsNSV) polymerases. The figure shows representative polymerases of four viral families. (*a*) Linear organization of the large polymerase subunit L proteins showing the RNA-dependent RNA polymerase (RdRp) domain, capping domain, connector domain (CD), methyltransferase domain (MT), and C-terminal domain (CTD). In structural studies of the vesicular stomatitis virus (VSV) and parainfluenza virus type 5 (PIV-5) polymerases, all domains of L were visible. In structural studies of the respiratory syncytial virus (RSV) polymerase, the CD, MT, and CTD were present but not visible due to their flexibility (*lighter color shading*). In the structural study of the Ebola virus (EBOV) polymerase, a fragment of the polymerase was solved. The missing regions are shown in gray. (*b*) Structures of the L proteins, solved by cryo–electron microscopy. The color coding matches panel *a*. The P or VP35 protein is shown in purple. For each polymerase, a ribbon model of the polymerase is shown as a side view alongside a top view of a space-filled model of the RdRp and capping domains. The space-filled model shows the location of residues involved in messenger RNA capping, determined biochemically for VSV polymerase, and correlated by sequence alignment for the other polymerases. Buried residues are shown as circles. The ribbon and space-filled models are not at the same scale. Protein Data Bank identifier numbers used to construct the images are 5A22 (VSV), 6V85 (PIV-5), 6PZK (RSV), and 7YES (EBOV + RNA).

into and out of this central cavity are template entry and exit channels, an NTP entry channel, and a product exit channel, although some channels are occluded in some polymerase structures, as described in more detail in subsequent sections (22, 23). The nsNSV capping domain contributes to the RdRp function by enclosing the catalytic cavity and by creating template and transcript exit channels (16). As described in more detail below, the capping domain also contains catalytic

residues required for cap addition and affects RNA elongation, le+ and mRNA release, and RNA replication. The connector, methyltransferase, and C-terminal domains form appendages that are flexible relative to the RdRp-cap core and each other (16, 24) (**Figure 2*b***).

TEMPLATE AND PROMOTER RECOGNITION

The first step in transcription or replication is polymerase recognition of the promoter within the template RNA. It is thought that the genome and antigenome RNAs are coated along their entire lengths with NP, and in the paramyxoviruses and filoviruses there is evidence that NP can contribute to promoter recognition (25–28). Thus, the RNA must be dissociated from NP for the polymerase to access the 3′ promoter. The P or VP35 component of the polymerase complex is responsible for this activity. These proteins have disordered C-terminal extensions that can elicit conformational changes in the NP chain to release the RNA (29, 30). While the *cis*-acting sequences required for productive RNA synthesis can be extensive, biochemical studies of filoviruses, rhabdoviruses, paramyxoviruses, and pneumoviruses have shown that the core promoter, required for initiation of RNA synthesis, is located within the 3′ terminal 12–20 nucleotides of the genome or antigenome template (31–36). Evidence indicates that the core promoter at the 3′ end of the genome signals initiation of both transcription and genome replication (37, 38) (**Figure 1**).

INITIATION AND EARLY ELONGATION AT THE 3′ END OF THE TEMPLATE

The nsNSVs use a de novo initiation mechanism in which two initiating NTPs enter the catalytic chamber and, following their correct positioning relative to the template, the polymerase catalyzes the formation of a phosphodiester bond between the NTPs (39). This dinucleotide product then acts as a substrate for further nucleotide addition, and the RNA is elongated and extruded (40). An initiation complex at the 3′ end of a template is less stable than an initiation complex formed at an internal site (41). However, 3′ initiation complex stability can be increased by a polymerase feature referred to as a priming loop containing a priming residue (42). A priming loop can stabilize the template 3′ terminus, preventing it from folding into a hairpin structure (43, 44), and the priming residue is typically an aromatic or other ring-based residue that can form base-stacking interactions with the initiating NTPs to help stabilize them in the correct position (42). This mechanism is used by the influenza virus polymerase, which initiates RNA replication from the 3′ end of the genome sense templates by a de novo initiation mechanism that is aided by a proline priming residue at the tip of a flexible priming loop (41).

There is no structure available for an nsNSV polymerase in the process of initiation, but we can infer which features of the polymerase are significant for this process. The nsNSVs have two loops that extend from the capping domain into the RdRp catalytic cavity, referred to as the priming and intrusion loops. In the rhabdovirus polymerase structures, the priming loop is positioned in the cavity, whereas in the paramyxovirus structures, the intrusion loop occupies the space (13, 16–18, 20). Modeling indicates that the two loops could not both extend into the cavity simultaneously (13). It is thought that both loops occupy the cavity at different stages of RNA synthesis, with the priming loop aiding RNA synthesis initiation at the 3′ end of the template (13, 16–18, 20, 22) and the intrusion loop playing a different role. This is because the rhabdovirus polymerase priming loop has an aromatic tryptophan residue near the tip that is oriented toward the active site (16, 17) (**Figure 3**). Biochemical studies showed that this tryptophan is required for initiation at the 3′ terminus of the genome, but not for initiation at an internal site, consistent with it functioning as a priming residue (45).

In the other nsNSV apo polymerase structures in which the priming loop could be visualized, it was folded into the capping domain (13–15, 19, 20). These observations are consistent with the

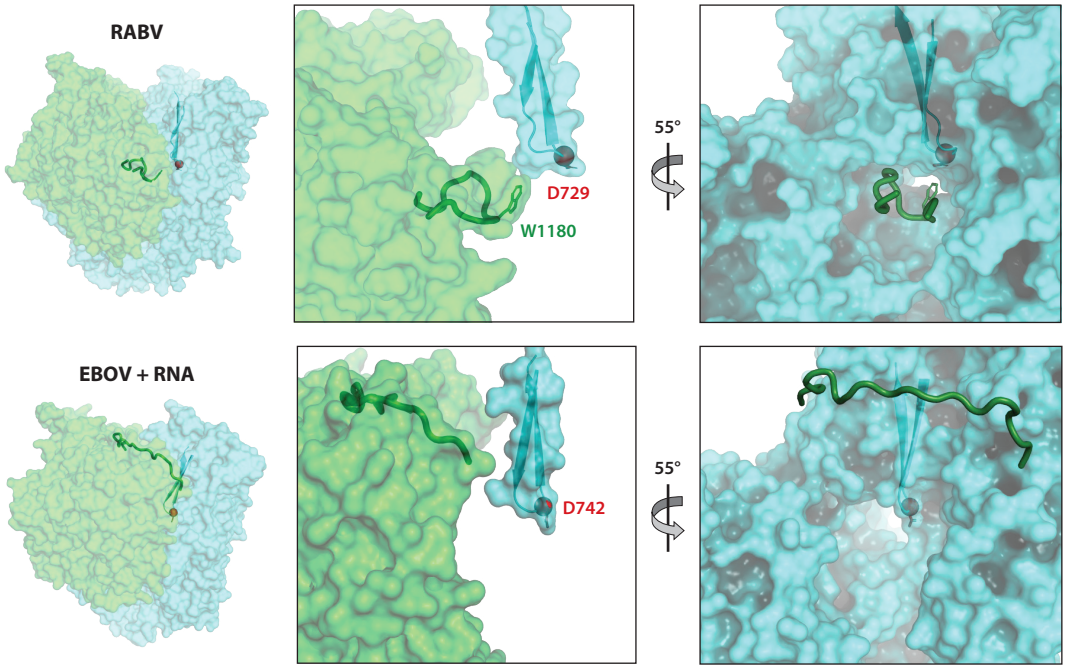

Figure 3

Comparison of the structures of the rabies virus (RABV) polymerase in apo form and the Ebola virus (EBOV) polymerase in complex with a template-primer duplex to illustrate possible structural changes that occur during the transition from initiation to elongation. In each case, the priming loop that extends from the capping domain is highlighted as a green ribbon. In addition, the RABV priming residue Trp1180 is labeled in green. In each case, the beta-hairpin containing the catalytic GDN motif of the RNA-dependent RNA polymerase (RdRp) is highlighted in blue with the catalytic aspartic acid residue labeled in red. The figure shows that the RABV priming loop occludes the transcript exit channel. Protein Data Bank identifier numbers used to construct the images are 6UEB (RABV) and 7YES (EBOV + RNA).

priming loop being a flexible structure, as would be expected. However, much of the priming loop sequence is highly divergent between nsNSV families, and there is no clear conservation of a ring-based residue corresponding to the priming tryptophan that was identified in the rhabdoviruses (46). Importantly, ebolaviruses and pneumoviruses have internal initiation mechanisms that might diminish their dependence on a priming loop and residue, such that the role of this loop could differ between nsNSVs. Studies of the ebolaviruses showed that they initiate RNA synthesis at position 2 of the promoter (47), and while the polymerases of the pneumoviruses initiate RNA replication at position 1, they initiate transcription at position 3, with initiation at position 3 being the dominant initiation site (48). Mutational analysis of the ring-based residues of the RSV putative priming loop identified a proline residue that affected initiation from both positions 1 and 3, with initiation at position 3 being affected to a lesser extent, consistent with this proline playing a role in stabilizing the initiation complex (46). However, this residue is not essential for initiation at either position 1 or 3 and instead appears to play an accessory role (46). An intriguing feature of the RSV polymerase is that it can select the initiating NTPs for position 1 replication initiation independently of the template sequence, indicating that there are specific interactions between the polymerase and these NTPs (49, 50). It is plausible that this reduces the need for a priming residue for initiation from position 1. Therefore, it is possible that in the ebolaviruses and pneumoviruses, the priming loop serves mainly to occlude the transcript exit channel, with a proline residue aiding

initiation complex stability in the case of RSV. This hypothetical model bears some analogy with the bunyaviruses, which initiate RNA replication using a prime-realign initiation mechanism that involves initiation at an internal site, rather than at the 3′ end of the genome (2). In these viruses, there is a poorly conserved prime-realign loop that helps to position the 3′ end of the template but that does not have a priming residue to stabilize the initiation complex (2, 51).

TRANSITION FROM INITIATION TO EARLY ELONGATION

Following initiation of RNA synthesis at the promoter, the polymerase begins to elongate the nascent RNA. Initiation of RNA synthesis typically involves conformational changes in a polymerase, and this is borne out in the structural studies of sNSV polymerases. In influenza virus, the transition from preinitiation to early elongation involves multiple changes in the RdRp domain (52, 53). Most significantly, the priming loop retracts to reveal a template exit channel, and as RNA elongation proceeds, a structure referred to as a lid separates the duplex to enable the template and nascent RNA to exit via separate channels (52, 53). In the case of bunyavirus, the prime-realign loop retracts, providing space for the template-product duplex, and another loop, referred to as a template plug loop, moves out of the template exit channel (51, 54). As noted above, the nsNSV priming loop appears to be flexible and adopts different conformations, consistent with it retracting during early elongation. Consistent with this hypothesis, in the apo structure of the EBOV polymerase, the priming loop is unresolved, whereas in the template-primer complex structure, the priming loop is folded into the capping domain, revealing a transcript exit channel (21) (**Figure 3**). Additional changes occur in the RdRp domain. In the rhabdovirus apo polymerase structure, there is a supporting helix in the RNA synthesis cavity of the RdRp domain that would clash with template-product RNA (22). In the EBOV apo polymerase structure, this helix is partially disordered, whereas in the template-primer complex it is anchored out of the way, creating additional space for the template-product duplex (21). In the RABV polymerase structure, the template exit channel is blocked by several small loops, which could be displaced by the RNA template being extruded from the active site (16).

As noted above, the priming and intrusion loops extend from the capping domain. Consistent with structures that indicate that these loops reorganize during the transition from initiation to elongation, functional studies have shown that the capping domain is involved in this transition. Mutations in the RSV and VSV priming and intrusion loops, and a small molecular inhibitor that binds adjacent to the interface of the priming loop region, the connector domain, and the RdRp domain of RSV polymerase impaired different stages of early elongation (46, 55–58). These changes also depend on the globular domains, as truncated polymerases lacking these domains are unable to elongate beyond ~9 nucleotides (21, 58). Thus, together the structural and biochemical studies indicate that the transition from initiation to early elongation requires a series of conformational rearrangements, including movement of the priming loop, changes in a supporting loop in the RdRp, and (in the case of RABV) opening of the template exit channel, and that at least some of these changes are dependent on the globular domains.

Having initiated RNA synthesis at the 3′ promoter, the polymerase transcribes le+ RNA (**Figure 1*a***). The le+ RNAs are not terminated precisely and are heterogeneous in length for a given virus (48, 59–62). What elicits release of the le+ RNA is not clear, but it appears to be dependent on the length of the transcript, independent of any *cis*-acting release signal (34, 59). One factor that might contribute to transcript release is the fact that the le+ transcript is not capped (48). As described in more detail in the next section, cap addition is required for the polymerase to become fully processive, and failure to add a cap could mean that the polymerase is in a conformation that is highly prone to transcript release. A small molecule inhibitor that elicits resistance in the capping domain of the RSV polymerase inhibits release of the le+ transcript

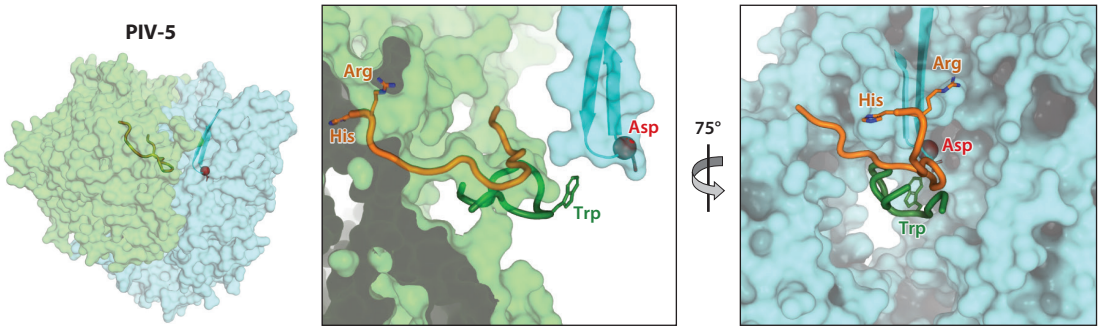

Figure 4

Structure of the parainfluenza virus type 5 (PIV-5) polymerase showing the positioning of the intrusion loop in the transcript exit channel. The catalytic histidine of the polyribonucleotidyltransferase (PRNTase) and the adjacent arginine are shown. The rabies virus (RABV) priming loop, containing the tryptophan priming residue, is superimposed on the PIV-5 polymerase structure to illustrate that the priming and intrusion loops would clash if they were to adopt the same conformations in the two virus polymerases. The catalytic aspartic acid of the RNA-dependent RNA polymerase is also indicated. Protein Data Bank identifier numbers used to construct the images are 6V85 (PIV-5) and 6UEB (RABV).

(55), indicating that the capping domain not only is involved in initiation and early elongation but also is involved in regulating polymerase processivity.

SCANNING AND INITIATION OF PRE-MESSENGER RNA SYNTHESIS AT THE GENE START SIGNAL

Having released the le+ RNA, the polymerase needs to remain attached to the template and locate the first gene start signal to initiate mRNA synthesis (**Figure 1*a***). Although there is no definitive structure of the polymerase in a scanning mode, we can surmise that the template entry and exit channels are occupied during this process with the transcript exit channel being devoid of RNA. Initiation at the gene start signal is unlikely to require a priming loop given that priming residues are not typically required for internal initiation and there is no need to stabilize the 3′ end of the template. Therefore, the priming loop is probably not involved in gene start signal initiation. Interestingly, in the PIV-5 polymerase structure, the priming loop is retracted into the capping domain, the template exit channel is open, the supporting helix of the RdRp partially occludes the active site, and the intrusion loop is protruding toward the active site, blocking the transcript exit channel (**Figure 4**). It is possible that this structure represents a conformation of the polymerase during scanning prior to initiation at the gene start signal. Following initiation of pre-mRNA synthesis at the gene start signal, the intrusion loop could be expelled by the nascent transcript (13). As the intrusion loop contains the catalytic histidine required for a key step in the capping reaction, as described below (**Figure 4**), it is possible that intrusion loop expulsion from the transcript exit channel by the nascent RNA is coincident with formation of the capping active site–pre-mRNA substrate complex.

CAP ADDITION AND CAP METHYLATION

The nsNSV polymerases are capable of capping the 5′ ends of their pre-mRNAs, and this occurs through a mechanism that is biochemically distinct from the typical guanylyltransferase activity and that is specific to the nsNSVs (63). Because this capping activity is distinctive, there is no precedent from which to make inferences as to how the polymerase structure relates to its capping function. However, extensive mutational analysis of nsNSV polymerases has been performed, allowing mapping of key residues (**Figures 2*b*** and **5**). In addition, while there is no parallel for the

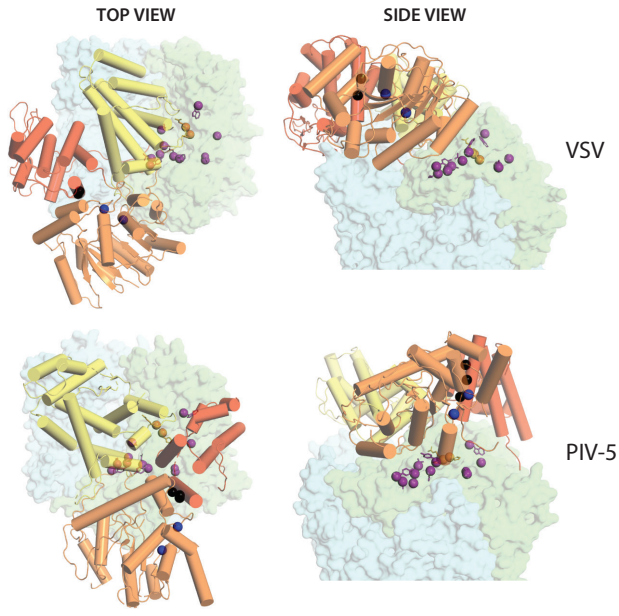

Figure 5

Comparison of the vesicular stomatitis virus (VSV) and parainfluenza virus type 5 (PIV-5) polymerases illustrating the different arrangements of the methyltransferase and C-terminal domains. This figure shows that the residues involved in capping activity are in closer proximity to each other in the PIV-5 polymerase structure than in the VSV polymerase structure. Residues involved in polyribonucleotidyltransferase (PRNTase) and GTPase activity and in the K-K-G motif are in purple, blue, and black, respectively. These residues were identified in mutagenesis studies of the VSV and human metapneumovirus polymerases and extrapolated by sequence alignment to the other polymerases. Protein Data Bank identifier numbers used to construct the images are 5A22 (VSV) and 6V85 (PIV-5).

capping and cap methylation reactions in the sNSVs, studies of sNSV polymerases have revealed that the cap binding and endonuclease appendages that enable the cap-snatching mechanism of these viruses are highly flexible relative to the polymerase core (3); likewise, there is considerable flexibility in the methyltransferase and C-terminal domain appendages of the nsNSVs (**Figures 2*b*** and **5**). As described in more detail below, this flexibility might provide a mechanism for switching cap addition on and off and for enabling capping and cap methylation to be coupled.

Cap addition is a two-step reaction that involves GTPase and GDP polyribonucleotidyltransferase (PRNTase) activities that are both performed by the polymerase (64). Using GTP as a substrate, the GTPase generates GDP, which then acts as a substrate for the PRNTase (64). The PRNTase recognizes the 5′ end of triphosphorylated pre-mRNA and, via linkage to the catalytic histidine residue in the intrusion loop, transfers monophosphorylated RNA to GDP, creating a guanosine cap (GpppRNA) (64, 65). The significance of the capping domain was first identified by the location of a cluster of mutations that provided resistance against an RSV polymerase capping inhibitor (66). Subsequent mutational and biochemical studies of the VSV polymerase capping domain identified key residues in the body of the capping domain, priming and intrusion loops that are conserved across nsNSVs and that are required for PRNTase activity (45, 65, 67, 68) (**Figures 2*b*** and **5**). The location of the GTPase has proven to be more elusive. Studies with a C-terminal fragment of the HMPV L protein, consisting of the methyltransferase and C-terminal domains (but lacking the PRNTase domain), showed that it has GTPase activity (69). However, mutation analysis identified only a few amino acid residues in this C-terminal fragment that

PRNTase: polyribonucleotidyltransferase

affected GTPase activity, and the GTPase enzyme activity of the fragment was slow, suggesting that the remainder of the L protein is required for it to be efficient. Consistent with this, mutations in conserved residues in the priming and intrusion loops of the VSV polymerase inhibited GTPase activity (68), indicating that they are either directly involved in GTPase activity or required to facilitate domain reorganization necessary for the GTPase to be fully active.

In addition to receiving a guanosine cap, the pre-mRNAs of most nsNSVs are methylated by the viral polymerase at the 2′-O and guanine N7 sites (69–73). The methyltransferase and C-terminal domains of the polymerase function together to perform 2′-O and G-N-7 methylation (69, 74, 75). X-ray crystallography analysis of an L fragment containing these domains of the HMPV polymerase showed that the methyltransferase structure resembles that of other 2′-O methyltransferases (69). The methyltransferase domain contains a pocket for the S-adenosyl methionine substrate and a K-D-K-E catalytic motif. It was also shown that a groove between the methyltransferase and C-terminal domains binds the RNA substrate (69, 75). The methyltransferase domain also contains a nucleotide binding pocket (not found in methyltransferases outside the nsNSVs) that might bind to the guanosine cap (69). These features could also be detected in a crystal structure of the Sudan ebolavirus L fragment and in the rhabdovirus and paramyxovirus polymerase structures (13, 16–18, 76).

Cap addition occurs as the pre-mRNA is being synthesized, occurring within 31 nucleotides of elongation in VSV (77). It has been shown that cap addition depends on the sequence at the 5′ end of the pre-mRNA (the complement of the gene start signal) (48, 64, 78, 79), suggesting that the polymerase might adopt the active capping conformation only when in complex with the appropriate RNA ligand. Methyltransferase activity is also dependent on the sequence of the RNA substrate (69, 71–73, 80). It is not known if the same RNA binding pocket is used for both capping and methyltransferase enzymatic reactions, but studies with the paramyxovirus parainfluenza virus type 2 (PIV-2) hint that it is. Specifically, a semiconserved K-K-G motif in the groove between the methyltransferase and C-terminal domains was shown to be required for PIV-2 mRNA synthesis, suggesting an involvement in capping (81). As noted above, the methyltransferase and C-terminal domain appendages differ in their positioning in cryo-EM structures of different viruses (**Figure 1**). In the PIV-5 polymerase structure, these domains are positioned directly adjacent to the capping domain (**Figure 5**). This brings the residues involved in GTPase, PRNTase, and K-K-G motifs in much closer proximity to each other than in the rhabdovirus polymerase structures (13) (**Figure 5**). This arrangement could potentially form a complete capping enzyme, with both GTPase and PRNTase sites in close proximity to each other, and it could allow the 5′ end of the pre-mRNA to transfer readily from the capping to the methyltransferase active sites. Thus, this is consistent with the PIV-5 polymerase being in a conformation prior to gene start initiation, with the mRNA capping and methylation-C-terminal domains poised to accept the RNA product.

TRANSITION TO PROCESSIVE ELONGATION

Addition of a cap serves to regulate a checkpoint, such that if the RNA is not capped, the polymerase aborts RNA synthesis and releases the pre-mRNA transcript (55, 57, 67, 68, 78, 82). These findings suggest that cap addition enables the polymerase to transition from an early elongation to a processive elongation conformation (**Figure 1*a***). It is thought that the structures of the RSV and HMPV pneumovirus polymerases might represent this processive elongation conformation (14, 15, 19). Of the nsNSV polymerase structure, the RdRp active site is the most open in these pneumovirus structures, which could correlate with a high degree of processivity (22). In addition, the connector, methyltransferase, and C-terminal domain appendages were not visible, despite being present, indicating that they were highly flexible (14, 15, 19) (**Figure 2**). This flexibility could have been elicited by the conformational rearrangements of the priming loop displacing the connector

domain (20), and their flexibility might correlate with the fact that these appendage domains are unlikely to be required when the polymerase has completed capping and cap methylation and is in a fully processive mode.

GENE END RECOGNITION, POLYADENYLATION, AND MESSENGER RNA RELEASE

The nsNSV polymerase elongates the pre-mRNA to the gene end signal, where the RNA is polyadenylated and released (**Figure 1*a***). The gene end signals are short, self-contained sequences that include a poly(U) tract (11). Analysis of polycistronic mRNA transcripts (produced when the polymerase fails to release the mRNA at a gene end signal) has identified poly(A) tracts at the gene junction site, indicating that polyadenylation occurs as the mRNA is being synthesized and prior to mRNA release (83, 84). Thus, nsNSV mRNAs are polyadenylated by a stuttering mechanism that is akin to the polyadenylation mechanism of the influenza virus polymerase. A key difference is that in the case of influenza virus, the polymerase is stalled at the polyadenylation signal because another site on the polymerase remains bound to a 5′ hook structure at the 5′ end of the template throughout mRNA synthesis (53). As the influenza polymerase reaches the polyadenylation signal, the connection to the 5′ hook creates tension that, together with the repetitive U sequence, enables the polymerase to stutter and polyadenylate the RNA (53). In the case of the nsNSVs, another factor must cause the polymerase to stall and stutter. VSV studies showed that the polymerase can efficiently respond to a gene end signal only if the gene end signal is more than ~70 nucleotides from the gene start, indicating that the polymerase needs to have adopted a processive elongation conformation to be stalled by the gene end signal (85). Mutations in the RSV capping domain can inhibit gene end recognition (86, 87), again implicating this domain in regulating polymerase processivity. It is not known what governs the release of the mRNA following polyadenylation, but intriguingly the methyltransferase appears to play a role. Experiments in which the by-product of methyltransferase activity, S-adenosylhomocysteine, is added to the reactions or artificially increased in infected cells showed synthesis of hyperpolyadenylated mRNAs, as did experiments with polymerases containing mutations in the methyltransferase domain (88–91). These findings suggest that the structure of the methyltransferase domain is important in facilitating transcript release.

GENOME REPLICATION

For most nsNSVs, replication is initiated in the same way as described for transcription, opposite the 3′ terminal nucleotide of the template (or opposite position 2 in the case of EBOV), with the initiation process being the same for both antigenome and genome RNA synthesis. Therefore, it is likely that the polymerase undergoes the same sequence of events during initiation and early elongation, as described above (**Figure 1**), and because the gene start complement sequence is not present at the 5′ ends of the antigenome and genome (or le+) RNAs, they are not capped (64). However, in the case of productive replication, the polymerase does not release the le+ transcript. It is thought that this is due to concurrent encapsidation of the nascent RNA occurring by association of NP with P or VP35 of the polymerase complex (12) (**Figure 1*b***). In the case of viruses that initiate transcription and replication from the same initiation site, the switch from le+ synthesis and transcription to replication is thought to be due to accumulation of soluble NP (12, 92–94). In the case of the pneumoviruses, which initiate transcription and replication from distinct sites, it is thought that the additional two nucleotides at the 5′ end of the replicative RNA facilitate encapsidation (48, 50). It is also possible that the polymerase adopts a different, more processive conformational state for replication. Precedence for this comes from influenza virus, in which an asymmetrical polymerase dimer is believed to facilitate encapsidation; one polymerase of

the dimer is responsible for RNA synthesis, and the other enables encapsidation (4, 95). There are several lines of evidence indicating that nsNSV polymerases can form dimers. Mutational analysis of paramyxovirus and filovirus polymerases showed evidence for L-L interactions (96–98), and negative-stain EM images of VSV and PIV-3 polymerases and a cryo-EM structure of PIV-3 polymerase have revealed dimeric forms (20, 24). However, further experiments are required to determine if these dimers function similarly to those of influenza virus replicase.

CONCLUDING REMARKS

The precedence set by structure-function studies of sNSV polymerases, coupled with the functional data obtained regarding nsNSV transcription and replication, provides strong support for the notion that the nsNSV polymerases undergo multiple structural transitions to perform their multiple activities. The data that are currently available are consistent with the models described above, elements of which have also been put forward by others (22, 23), in which the different virus polymerase structures capture the polymerase at different functional stages: (*a*) poised for initiation at the 3′ terminus of the template (RABV, VSV), (*b*) in early elongation (EBOV + RNA), (*c*) in a scanning mode poised for initiation at the gene start signal (PIV-5), and (*d*) in a transcription elongation mode (RSV, HMPV). However, it should be cautioned that in the absence of RNA ligands representing different stages of transcription and replication, these are only speculative models. Importantly, the question arises, why would different viral polymerases adopt distinct functional conformations in the absence of RNA or other ligands? While the answer to this is not known, the structures are consistent with polymerase behavior. For example, a paramyxovirus polymerase can tolerate 3′ terminal extensions of more than 200 nucleotides (99), suggesting that it can readily adopt a scanning mode. The RSV and EBOV polymerases are both capable of engaging in back-priming activities in which the 3′ end of the template folds into a hairpin and is extended by a few nucleotides (47, 100), and this might correlate with the more open structures of these polymerases. Finally, ready conversion between different conformational states could explain some apparent anomalies in the literature regarding VSV transcription initiation. Specifically, the VSV transcription process has been shown to begin at the 3′ end of the genome (as posited above) or directly at the first gene start signal, depending on the experimental conditions (101–105). It is possible that the local environment can change the polymerase between the 3′ initiation and scanning conformations and that this affects where the polymerase begins transcription, a model that could also apply to other nsNSVs. The next decade of research on these fascinating proteins promises to yield exciting information as structures of nsNSV polymerases in association with ligands are obtained and functional properties are analyzed with more structure-directed approaches. This will allow them to be more fully characterized as dynamic, multifunctional, molecular machines.

NOTE ADDED IN PROOF

Since this article was submitted for publication, the structure of the paramyxovirus Newcastle disease virus polymerase has been published, providing further evidence for the dynamic nature of the nsNSV polymerases (106).

SUMMARY POINTS

1. Transcription and replication of nonsegmented, negative-strand RNA virus (nsNSV) genomes are carried out by the same polymerase from the same promoter at the 3′ end of the genome.

2. The transcription and genome replication processes of nsNSVs can be subdivided into multiple different steps that likely correspond to different polymerase conformations.
3. Transcription of nsNSV messenger RNAs (mRNAs) involves repeated cycles of template scanning, initiation, early elongation, capping and methylation, processive elongation, polyadenylation, and mRNA release, enabling each gene on the genome to be transcribed into an mRNA.
4. Capping and cap methylation are dependent on *cis*-acting signals at the 5′ end of pre-mRNAs, explaining why mRNAs are capped, whereas genome and antigenome RNAs are not.
5. Large-scale rearrangement of polymerase domains could bring together or disperse the residues that comprise the capping and methyltransferase active sites, turning these enzyme activities on and off.
6. Replication involves a superprocessive form of the polymerase that is able to disregard the gene junction signals, likely due to concurrent encapsidation of the nascent RNA.

DISCLOSURE STATEMENT

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85. Paper presenting evidence that the polymerase must elongate a certain distance from the gene start signal before it can recognize a gene end signal, consistent with it undergoing a conformational change following cap addition.

91. Paper presenting the first evidence that perturbation of the methyltransferase domain can lead to hyperpolyadenylation, suggesting a link between the structure of the methyltransferase domain and mRNA release.