Structures of the Mononegavirales Polymerases

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ABSTRACT

Mononegavirales, known as nonsegmented negative-sense (NNS) RNA viruses, are a class of pathogenic and sometimes deadly viruses that include rabies virus (RABV), human respiratory syncytial virus (HRSV) and Ebola virus (EBOV). Unfortunately, no effective vaccines and antiviral therapeutics against many Mononegavirales are currently available. Viral polymerases have been attractive and major antiviral therapeutic targets. Therefore, Mononegavirales polymerases have been extensively investigated for their structures and functions. Mononegavirales mimic RNA synthesis of their eukaryotic counterparts by utilizing multifunctional RNA polymerases to replicate entire viral genomes and transcribe viral mRNAs from individual viral genes as well as synthesize 5’ methylated cap and 3’ polyA tail of the transcribed viral mRNAs. The catalytic subunit large protein (L) and cofactor phosphoprotein (P) constitute the Mononegavirales polymerases. In this review, we discuss the shared and unique features of RNA synthesis, the monomeric multifunctional enzyme L, and oligomeric multimodular adapter P of Mononegavirales. We outline the structural analyses of the Mononegavirales polymerases since the first structure of the vesicular stomatitis virus (VSV) L protein determined in 2015 and highlight multiple high-resolution cryo-electron microscopy (cryo-EM) structures of the polymerases of Mononegavirales, namely VSV, RABV, HRSV, human metapneumovirus (HMPV), and human parainfluenza virus (HPIV) that have been reported in recent months (2019-2020). We compare the structures of those polymerases grouped by virus family, illustrate the similarities and differences among those polymerases, and reveal the potential RNA synthesis mechanisms and models of highly conserved Mononegavirales. We conclude by the discussion of remaining questions, evolutionary perspectives, and future directions.
INTRODUCTION

*Mononegavirales*, known as nonsegmented negative-sense (NNS) RNA viruses, are a class of viruses infecting numerous plants, animals, and humans, and many of them cause significant diseases and deaths in humans (1-3). There are currently 11 virus families in the order of *Mononegavirales*: Artoviridae, Bornaviridae, Filoviridae, Lispiviridae, Mymonaviridae, Nyamiviridae, Paramyxoviridae, Pneumoviridae, Rhabdoviridae, Sunviridae, and Xinmoviridae, according to the 2019 taxonomy (4). Recent advances in sequencing technology facilitated the discovery of new families and genera. For example, (a) Pneumoviridae, which used to be a subfamily Pneumovirinae in Paramyxoviridae, became a new virus family (5); (b) A new ebolavirus, three new filovirus genera, and a sixth proposed genus was recently added in Filoviridae (6). Within the order, some *Mononegavirales* circulate within the human population causing (1) respiratory diseases, such as the human respiratory syncytial virus (HRSV) and human metapneumovirus (HMPV) from Pneumoviridae and human parainfluenza virus (HPIV) from Paramyxoviridae (7); (2) common childhood diseases, such as measles virus (MeV) and mumps virus (MuV) from Paramyxoviridae (8-11). Several emerging and reemerging *Mononegavirales* often transmit cross-species and cause severe diseases with high mortality rates, such as (3) NIAID Category A Priority Pathogens, Ebola virus (EBOV) and Marburg virus (MRAV) from Filoviridae, and (4) NIAID Category C Priority Pathogens rabies virus (RABV) from Rhabdoviridae, and Nipah (NiV) and Hendra (HeV) from Paramyxoviridae (1, 12-17). The representative viruses of *Mononegavirales* are listed in Table 1. Currently, no effective vaccine or antiviral therapy is available to prevent or treat many of those NNS RNA viral pathogens (18-29).

*Mononegavirales* are enveloped viruses with various morphology for different families, for example, Rhabdoviridae are bullet-shaped, Paramyxoviridae are pleomorphic or spherical, and Filoviridae are filamentous (30-32). The genome organization and replication of *Mononegavirales* have been extensively studied for decades (1-3). The NNS RNA viral genomes are linear and single-stranded, and the length ranges from 8.9 to 19.0 kilobases (1-3). *Mononegavirales* encode 5 to 10 genes, with four core genes shared by all members: nucleoprotein (N or NP), phosphoprotein (P or VP35), matrix protein (M), and large protein (L). Three out of four shared proteins, N, P, and L, constitute the RNA synthesis machine, suggesting the central role of RNA synthesis in the *Mononegavirales* life cycle (33) (Fig. 1).
Mononegavirales initiate viral infection by delivering into the host cell a virus-specific RNA synthesis machine (33-35). The template for RNA synthesis is not RNA alone, but rather a complex of the viral genomic RNA completely encapsidated by the N or NP, called nucleocapsid (NC) (36). This NC template is copied by the viral RNA-dependent RNA polymerase (RdRP), which comprises L and cofactor P or VP35 (37-43). Additional viral proteins M2-1 in Pneumoviridae, and VP30 and VP24 in Filoviridae, are essential for full processivity (44-48). The L protein has all the enzymatic activities necessary for the transcription of the viral mRNAs, including RNA polymerization, 5’ cap addition, cap methylation, and 3’ polyadenylation, as well as the replication of the viral genome (38, 49-57). Thus, L is the catalytic core of a multi-component and multifunctional RNA synthesis machine.

The RNA polymerase is the sole enzyme of Mononegavirales, and there is a critical need to delineate the molecular and structural basis of the RNA polymerase of Mononegavirales (58). Since the first structure of the L protein alone of vesicular stomatitis virus (VSV) determined in 2015 (59), multiple structures of RNA polymerases of Mononegavirales, including HRSV, HMPV, RABV, HPIV, and VSV, have been reported in recent months, revealing the architectures of L:P complexes and interactions between L and P (60-65). This review illustrates similarities and differences among the polymerases by comparing the structures of those polymerases and reveal the potential RNA synthesis mechanisms of the highly conserved Mononegavirales polymerases.

RNA synthesis of Mononegavirales

Mononegavirales use the negative-sense genomes as the templates for two distinct viral RNA synthesis (1-3): (1) transcription to generate 5 to 10 discrete 5’ capped, methylated and 3’ polyadenylated viral mRNAs, and (2) replication to produce complementary positive-sense antigenomes that act as templates for progeny negative-sense genomes (features highlighted in Fig. 1).

For Mononegavirales transcription: The RdRP initiates de novo RNA synthesis by recognizing a single promoter within the leader (Le) region at the 3’ end of the negative-sense genome and sequentially synthesizes mRNAs of the linear array of genes. The de novo initiation of the RNA synthesis by the RdRP typically involves a priming loop (66, 67). The RdRP first produces a leader (Le) RNA that remains uncapped and non-polyadenylated. After the Le RNA synthesis and before transcription of the first gene, the Le RNA is released by the RdRP. The RdRP then stays on the template, initiates and caps the downstream mRNAs, and terminates and polyadenylates the
upstream mRNAs, in response to the cis-acting gene-start (GS) and gene-end (GE) sequences of viral genes, respectively (33, 68-70). Typically, the RdRP produces a gradient level of viral mRNAs with the attenuation of the downstream mRNAs at each gene junction (71, 72). Recent studies showed the non-gradient and genotype-dependent transcription in HRSV and EBOV, suggesting alternative gene expression strategies (73, 74) (Fig. 1, lower part).

For replication in Mononegavirales, the RdRP initiates at the Le region of the genome and ignores all cis-acting regulatory signals to produce a full-length uncapped RNA antigenome. Consequently, the RdRP initiates at the 3' end of the trailer complementary (TrC) region and replicates the positive-sense antigenome into its negative-sense genome (75). It is known that N protein levels influence the switch from transcription to replication. Unlike transcription, the replication is dependent on a supply of N protein to encapsidate the nascent antigenome and its progeny genome (76, 77) (Fig. 1, upper part).

The multifunctional enzyme - monomeric L

The multifunctional enzyme L protein of Mononegavirales is a single polypeptide of more than 2,000 amino acid residues (except Bornaviridae) in length and larger than 240kDa in size. The sequence of L is conserved among Mononegavirales, and the sequence alignment reveals six conserved regions (CRs), named CR I-VI (78). The CRs are located within three distinct enzymatic domains of Mononegavirales L: CR I-III in the RNA dependent RNA polymerization (RdRp) domain, CR IV-V in the cap addition (Cap) domain, and CR VI in the cap methylation (MT) domain (53, 55, 79) (Fig. 2A).

The Mononegavirales L contains all the catalytic activities necessary for RNA synthesis. The enzymatic activities of L are coordinated in such a way that the nascent mRNA transcript is synthesized and modified during multiple specific stages. A 5'-cap structure is formed after the mRNA transcript reaches a certain length, and failure to make a 5'-cap for mRNA results in the premature termination of RNA synthesis (53, 80, 81). Cap methylation also influences the RdRP activity, and failure to methylate the 5'-cap of mRNA results in hyper polyadenylation of mRNA (55, 79, 82, 83). L also synthesizes a poly-A tail at the 3' end of mRNA by a "stuttering" mechanism using a short U-rich region within the GE sequence of each gene. Thus, the different enzymatic activities of L are linked. However, how the different activities of the L protein coordinate and influence one another remain mostly unclear.
The multimodular adapter - oligomeric P

The multimodular adapter P protein of Mononegavirales is an oligomeric and non-globular molecule in solution (84). Although L contains all catalytic functions, P is the essential cofactor required for L to synthesize RNA effectively (38). P not only is the cofactor of L but also acts as an adapter to coordinate and modulate multiple proteins, including RNA-free N protein, NC complex, and additional regulatory proteins (85, 86). Notably, P forms dimers in Rhabdoviridae (84, 87, 88), trimers or tetramers in Filoviridae (89, 90), and tetramers in Paramyxoviridae and Pneumoviridae (91-95). Each P protomer consists of an intrinsically disordered N-terminal domain (PNTD), an oligomerization domain (POD), and a C-terminal domain (PCTD), connecting with a flexible linker (84). Despite a high diversity in length, sequence, and even in the structural folds of individual domains, this modular architecture is conserved among different Mononegavirales (Fig. 2B). The intrinsically disordered PNTD exhibits a substantial conformational heterogeneity and is essential for its dynamic coordination functions. The key features of P can be revealed as the modular architecture with intrinsically disordered domains and structural domains that interact with different proteins that constitute the RNA synthesis machine (96-100). Interestingly, the length difference seems to correlate with additional functions of the adapter P protein. For example, the linker between POD and PCTD of RABV is longer than that of VSV and contains a dynein light chain 8 (LC8) binding site (101); PCTD of EBOV contains an additional region for RNA binding and innate immune escape (102). Further, P is often phosphorylated by the host kinases, and phosphorylation is essential for its regulation of RNA synthesis (103-108).

Together, P plays several critical roles within the RNA synthesis machine: (1) P is an essential cofactor to regulate the processivity of L. As an adapter, P interacts with NC and bridge in the RNA to thread into the L active sites during transcription and replication (109-114). (2) P acts as a chaperone to maintain a supply of RNA-free N (N0) and delivers to N0 nascent RNA genome or antigenome during replication (99, 100, 115-119). (3) P interacts with other essential cofactors, such as M2-1 in Pneumoviridae, VP30 in Filoviridae, to coordinate the RNA synthesis activities of the RdRP (48, 120-123).

Overview of the structural analyses of the Mononegavirales polymerases

The monomeric L and oligomeric P together constitute the RdRP in Mononegavirales. Due to the large size of L and the oligomeric states of P with intrinsically flexible domains, it is challenging to obtain the crystals of the Mononegavirales RdRPs (124).
microscopy (cryo-EM) offers an alternative way for high-resolution structural characterization of such macromolecular complexes (125).

In 2015, the structure of the VSV L was determined by cryo-EM at 3.8 Å resolution (PDB: 5A22) (59), and that was the first structure of the Mononegavirales polymerases. Although the VSV L was prepared in the complex of the VSV PND, the structure allowed only the de novo model building of the entire L protein, but not the model assignment of PND, despite extra electron density observed (59). Since 2015, there are many attempts for the structural characterizations of the Mononegavirales polymerases. For example, crystal structures of NTD and CTD fragments of L have also been reported (126, 127). In recent months, there were multiple successful cases of the structural characterization of the Rhabdoviridae and Pneumoviridae polymerases by cryo-EM, one for RABV (PDB: 6UEB), one for VSV (PND visible, PDB: 6U1X), two for HRSV (PDBs: 6PZK & 6UEN), one for HMPV (PDB: 6U50), and one for HPIV (PDB: 6V85) (60-64). For consistency, the domain organizations and cartoon representations of the individual structures are colored as follows: RdRp (blue), Cap (green), CD (yellow), MT (pink), and CTD (cyan) for L, and PND (magenta), PDD (red), and CTD (orange) for P, the same as Fig. 2.

**Structures of the Rhabdoviridae polymerases**

A higher 3.0 Å resolution cryo-EM structure of the VSV polymerase (PDB: 6U1X) was reported that enables the visualization of not only the 2,109-residue VSV L but also the bound PND of the 265-residue VSV P (59, 60) (Fig. 3A). The root-mean-square deviation (RMSD) between 3.8 Å and 3.0 Å structures of the VSV L is 1.33 Å (59, 60). All five domains of the VSV L except a few flexible linkers are visible in the structure, including three functional domains, RdRp (35-865), Cap (866-1334), and MT (1598-1892), and two structural domains, the connector domain (CD, 1335-1597) and the C-terminal domain (CTD, 1893-2109) (60) (Fig. 3A). The RdRp domain resembles the classical RNA polymerase fold. The Cap domain folds next to the RdRp domain, and there was no homology for the Cap domain outside of the order of Mononegavirales due to the unique capping mechanism. The CD domain connects the Cap and MT domains, and the CTD domain folds back to be close to the RdRp domain. Three ordered segments, 49-56, 82-89, and 94-105 of PND (1-106), are shown to interact with CTD, RdRp, and CD domains of L, respectively (59, 60) (Fig. 3A).

The 3.3 Å resolution cryo-EM structure of the RABV polymerase closely resembles the VSV polymerase and contains all five domains of the 2,127-residue RABV L and PND of the 297-
residue RABV P (61) (Fig. 3B). Similar to the VSV L, nearly entire RABV L can be modeled in the map, with noticeable flexibility of several interdomain linkers. The RMSD between the RABV and VSV L is 2.10 Å. The domain boundaries are as follows: RdRp, 29-879; Cap, 880-1351; CD, 1352-1627; MT, 1628-1926; and CTD, 1927-2127. Two segments of PNTD (1-91) have been modeled in the structure of RABV polymerase: a short segment (possibly 40-44) interacts with the CTD domain of L, and another long segment (51-87) bridging CTD, RdRp, and CD domains of L (61) (Fig. 3B).

There are 35.05% and 19.22% amino acid identities between VSV and RABV L and P protein, respectively. As expected, the VSV and RABV L share high similarity with nearly complete conservation of secondary structure elements throughout the protein. Despite greater sequence difference, the VSV P and RABV P is also structurally similar to each other. Interestingly, there is a flexible loop (1158-1172 in VSV and 1171-1186 in RABV) in the Cap domain of the Rhabdoviridae L that is against the active site of the RdRp domain. This loop is identified as the priming loop responsible for the de novo initiation of RNA synthesis (59-61). Due to the compact packing of the RdRp and Cap domains, the position of the priming loop appears to block the putative RNA product exit channel. Therefore, it is believed that Rhabdoviridae L adopts an initiation state in the structures, and significant rearrangements of those domains are likely to occur during elongation and other states of RNA synthesis.

**Structures of the Pneumoviridae polymerases**

Multiple cryo-EM structures of the Pneumoviridae polymerases have also been reported in recent months, including a 3.2 Å (PDB: 6PZK) and a 3.67 Å (PDB: 6UEN) resolution structures of the HRSV polymerase and a 3.7 Å resolution structure of the HMPV polymerase (PDB: 6USO) (62-64). Two structures of the HRSV polymerase are nearly identical, with an RMSD 1.48 Å (62, 64) (Fig. 4A). The structures reveal that the RdRp (10-945) and Cap (946-1461) domains of the 2,165-residue L interact with the PO (128-157) and PCTD (158-241) of a tetramer of the 241-residue P. Interestingly, although full-length L and P were used to reconstitute the HRSV polymerases, the EM-densities of MT domain and structural CD and CTD domains of the L and the PNTD are missing in 3D reconstructions (62, 64) (Fig. 4A, missing domains are shown in gray). The integrity of proteins was confirmed by mass spectrometry. The missing EM-densities suggest that the intrinsical flexibility of those domains (62), and PO and PCTD are not sufficient to lock those domains of L into a homogenous conformation. Interestingly, four promoters of the tetrameric HRSV PO and PCTD adopt distinct conformation, and each of the promoters uses
different ranges of residues, 128-182, 128-187, 128-202, and 128-241, to interact with distinct regions of HRSV L (Fig. 4A). Further comparison of structures reveals slightly different intermolecular arrangements among L and tetrameric P, suggesting the plasticity of the L:P interface for structural rearrangements during RNA synthesis (62).

The structure of the HMPV polymerase (PDB: 6U5O) shares a highly similar architecture to that of the HRSV polymerase, which contains the RdRp (8-902) and Cap (903-1380) domains of the 2,005-residue HMPV L and P_{OD} (168-193) and P_{CTD} (194-266) of a tetramer of the 294-residue HMPV P (63). The RMSD between the HRSV and HMPV L is 1.49 Å. The HMPV polymerase also lacks the MT and other structural domains (CD and CTD) of L and P_{NTD} in the 3D reconstructions (63) (Fig. 4B). Similarly, each of the four protomers of the tetrameric HMPV P_{OD} and P_{CTD} adopts distinct conformation, and uses different ranges of residues, 168-219, 168-231, 168-236, and 168-266, to interact with HMPV L (Fig. 4B).

There are high sequence identities between the HRSV and HMPV L and P, 49.12%, and 37.18%, respectively. As expected, HRSV and HMPV polymerases share highly similar architectures between them, including the priming loop. Surprisingly, the priming loop in the Cap domain of the Pneumoviridae L shows a substantial shift and ~37 Å away from the active sites of the RdRp domain, suggesting that L adopts an elongation state in the structures (62-64). Despite the similarities, there are several noticeable differences between the structures of HRSV and HMPV polymerases: (1) HRSV L contains an insertion (134-176) compared to that of HMPV L; (2) HRSV L has a missing connecting helix (660-691), but the equivalent connecting helix of HMPV L can be partially modeled; (3) one protomer of the HRSV P tetramers shows a different arrangement compared to its counterpart protomer of the HMPV P. Those slight differences between two genera, metapneumovirus, and Orthopneumovirus, are likely due to genus-specific features of the RNA synthesis machine, and more detailed comparisons can be found in (128).

**Structures of the Paramyxoviridae polymerase**

Cryo-EM structures of the Paramyxoviridae polymerases have also been reported, including a 4.38 Å (PDB: 6V85) and a 4.63 Å (PDB: 6V86) resolution structures of the HPIV polymerase at two similar stable conformations (65). In the structure, not only all five domains of the 2255-residue HPIV L are visible, including RdRp (1-912), Cap (913-1397), CD (1398-1730), MT (1731-2060), and CTD (2061-2255), but also two domains of a tetramer of the 392-residue HPIV P, P_{OD} (198-271) and P_{CTD} (also called P_{XO}, 346-392), are present to interact with the HPIV L (Fig. 5A).
Interestingly, although all five domains of HPIV L are presented, the CTD adopts a significant domain switch compared to that of the Rhabdoviridae L (Fig. 5B). The two conformations of the HPIV polymerase (L:P) are highly similar, with slightly different orientations of the CD-MT-CTD module with respect to RdRp and Cap (Fig. 5B, right panel). Further, in contrast to Pneumoviridae P, only one protomer of P_{CTD} EM-density is visible in Paramyxoviridae P, suggesting the versatile roles of P in RNA synthesis. It is noticeable that the tetrameric Paramyxoviridae P_{CO} is much longer than that of Rhabdoviridae and Pneumoviridae P_{CO}, highlighting the potential mechanistic differences among those families.

Structural similarities and differences among the Mononegavirales polymerases

The L proteins of Rhabdoviridae, Pneumoviridae, and Paramyxoviridae have similar lengths (2,000-2,300 residues) and share a similar architecture. Indeed, the RdRp domains of Mononegavirales L share a standard right-hand thumb-palm-finger ring-like configuration of RNA and DNA polymerases. Comprehensive comparisons of the RNA/DNA polymerases and viral polymerases have been extensively reviewed elsewhere (129-136). The structural superimpositions of the motifs, namely fingers, palm, thumb, and structural support, of the RdRp domains of the Mononegavirales L, are shown in blue, red, green, and gray, respectively. The active sites (GDN) of the RdRp domains are shown as magenta spheres (Fig. 6A-E). For comparison, we also showed the structural motifs of representative RdRps of reovirus (ReoV) and influenza B (FluB) (Fig. 6F-G), both of which are further discussed in the model section.

The previous studies highlighted the conserved structural motifs A-E of the Cap domain of L (33, 56, 137). Unlike the capping in the host cells, the capping reaction of the Mononegavirales L forms a covalent protein:RNA intermediate linkage between the 5' of the RNA transcript and the active site H residue (motif D), followed by the attack by a guanosine nucleotide. The motifs A-E of the Cap domain of the Mononegavirales L are shown as ribbon diagram in blue, yellow, red, magenta, and green, respectively. Those motifs are centered around the motif D (HR) active site. The proposed priming loops (orange) are next to the motif B (yellow) but exhibit a dramatic conformational rearrangement (Fig. 7).

Despite the high similarities, there are several significant differences between the known structures of Mononegavirales polymerases: (1) all five domains (RdRp, Cap, CD, MT, and CTD) of the Rhabdoviridae and Paramyxoviridae L compared to only two domains (RdRp and Cap) of the Pneumoviridae L are visible in the cryo-EM structures. (2) P forms dimers in Rhabdoviridae

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but tetramers in *Pneumoviridae* and *Paramyxoviridae*. It is thought that P displays distinct structural features due to low sequence identity and different oligomerization states. Interestingly, different domains of P interact with L in the reported structures. In *Rhabdoviridae*, only the P\textsubscript{NTD} interacts with mostly CD and CTD and part of RdRp of L (Fig. 8B). However, in *Pneumoviridae* and *Paramyxoviridae*, the P\textsubscript{OD} and P\textsubscript{CTD} interact with the RdRp domain of L (60-64) (Fig. 8D & 8F). Compared to the oligomeric P shown in *Pneumoviridae* and *Paramyxoviridae*, the lack of the P\textsubscript{OD} in *Rhabdoviridae* resulted in a monomeric P binding to L. (3) The priming loop and the supporting helix of L (Fig. 8, colored in orange) adopt three different conformations: (a) in *Rhabdoviridae* (VSV and RABV), the priming loop together with a supporting helix in the RdRp domain project into the GDN active sites (Fig. 8A) of the RdRp domain and close off a channel towards the Cap domain; (b) in *Pneumoviridae* (HRSV and HMPV), the supporting helix is (partially) disordered, and the priming loop retracts from the RdRp active sites (Fig. 8C) and opens the channel connecting to the Cap domain; and (c) in *Paramyxoviridae* (HPIV), the supporting helix is visible (similar as *Rhabdoviridae*), but the priming loop with a disordered tip is projected away from the RdRp active sites (similar as *Pneumoviridae*) (Fig. 8E).

**Mechanisms and models of Mononegavirales RNA synthesis**

Collectively, the structures of the Mononegavirales polymerases discussed here reveal multiple distinct conformational arrangements of the L and P proteins, as shown in the cartoon diagrams (Fig. 9A). The comparison analyses suggest potential RNA synthesis mechanisms of Mononegavirales, switching of initiation, and elongation associated with priming loop and supporting helix rearrangements (60-64). Based on the structural similarities and differences among the Mononegavirales polymerases, we hypothesize that (1) the polymerases of the *Rhabdoviridae* (VSV and RABV) are likely at the initiation stage of genome replication, and (2) the polymerases of *Pneumoviridae* (HRSV and HMPV) and *Paramyxoviridae* (HPIV) are at different phases, possibly late phase and early phase, of the elongation stages of transcription, respectively.

To better understand the RNA synthesis mechanism by the Mononegavirales polymerases, we superimposed other viral polymerase complexes in the initiation and elongation stages. For the initiation, the superimposition of the reovirus (ReoV) λ3 initiation complex reveals in the presence of the RNA template (yellow), the initiating nucleotide stacks with a Trp (W1167 in VSV L and W1180 in RABV L) residue of the priming loop, which is also similar as the Y630 in hepatitis C virus (NS5B) (60, 61, 138, 139) (Fig. 9B, left panel). The mutation of this Trp residue severely
affects the genome or antigenome end initiation, but not internal initiation or capping (140). For the elongation, the polymerases require the retraction of the priming loop and possibly the support helix to pave the way to accommodate the product. Indeed, the fully retracted priming loops configurations are observed in both Pneumoviridae (HRSV and HMPV) and Paramyxoviridae (HPIV). The superimpositions of the influenza B (FluB) elongation complexes at early and later stages reveal that the RNA transcripts (pink) have sufficient space to extend and pass through a continuous tunnel when the priming loop is entirely retracted (141) (Fig. 9B, middle and right panels). The remaining support helix in Paramyxoviridae (HPIV) results in a partially extruded tunnel, where the missing support helix in Pneumoviridae (HRSV and HMPV) leads to a fully open tunnel, ideal for highly processive transcription.

As highlighted above, the NC is the cognate RNA template for Mononegavirales RNA synthesis. Based on the structures of Mononegavirales RNA polymerases, we propose the models of the initiation, and early and late stages elongation of RNA synthesis, as shown in cartoon diagrams (Fig. 9C). The template RNA (black line) are coated by N at all time except when passing through the active sites of the RdRp domain of L. (1) At the initiation stage, the priming loop of the Cap domain is at the close approximate of the active site of the RdRp domain of L, and a short RNA transcript (blue line) is synthesized (Fig. 9C, left panel). (2) At the early elongation stage, the priming loop is away from, but the support helix stays at the close approximate to the active site of the RdRp domain of L(Fig. 9C, middle panel). (3) At the late elongation stage, the priming loop of the Cap domain of L is away from the active site of the RdRp domain of L, and the CD, MT, and CTD domains of L is flexible when the RNA transcript (blue line) is being extended (Fig. 9C, right panel).

**CONCLUSION**

Many Mononegavirales are significant human pathogens, imposing a tremendous public threat and healthcare burden. However, no effective vaccines and antiviral therapeutics against many Mononegavirales are currently available (18-21, 23, 29, 142-148). Viral polymerases have been attractive and major antiviral therapeutic targets, as seen in multiple drug discovery successes in various viral pathogens, including HIV-1, HCV, and HBV (149-157). Drug design and target search heavily rely on an accurate understanding of the structure and functions of the target molecules. Therefore, various viral polymerases have been extensively investigated for their structures and functions (129, 130). To understand the mechanistic insights of Mononegavirales RNA synthesis, the precise composition and structure of the Mononegavirales polymerases, how
the different activities of the L protein influence one another, and how cofactor regulates RNA synthesis need to be elucidated.

The structures of the Mononegavirales polymerases discussed here, including the L protein in complex with its cofactor P protein of VSV, RABV, HRSV, HMPV, and HPIV reveal three conformations poised for initiation and elongation of RNA synthesis (60-64). The potential channels and the relative locations of multiple catalytic sites of L suggest that L coordinates a distinct capping and methyltransferase reaction with priming for de novo initiation of transcription. The transcription and replication might have different priming configurations and potential different product exit sites. The high similarity between L and P of the Mononegavirales polymerases provides a structural basis for the development of antiviral drugs that inhibit the RNA synthesis in transcription or replication.

This difference might also explain why L shows different architecture in three different families. PNTD is speculated to lock the CD, MT, and CTD domains into a “closed” conformation, which represents L is poised for initiation at the 3’ end of the genome or antigenome, ready for RNA synthesis. The interactions between multiple domains of L and the PNTD reveals how P induces a compact, closed, and initiation compatible state of L and how P positions RNA template and the putative RNA product exit channel.

Several interesting questions arise by comparing and analyzing the known structures of the Mononegavirales polymerases. First, although the mass spectrometry data indicated that the Pneumoviridae L proteins used in structural studies are intact, the mystery of the missing MT domain and structural domains of L remains. Where do the MT and structural domains (CD and CTD) go? How to capture the snapshots of their intermediates? Second, the known structures of the Mononegavirales polymerases are protein only without RNA present in the complex. However, those polymerases are in different initiation and elongation compatible stages. Why the priming-loop and the supporting helix of L adopt different conformations in the protein-only complex? Third, the tetrameric P has a large interaction surface between POD and L in Pneumoviridae and Paramyxoviridae. Given that P is a dimer in Rhabdoviridae, but a tetramer in Pneumoviridae and Paramyxoviridae, is it possible that the dimeric P in Rhabdoviridae may not form a tight complex with L with large interfaces? This may explain that the HRSV, HMPV, and HPIV L need to be co-expressed in the presence of P, but not VSV L, which can be expressed and purified alone.
As an evolutionary perspective, Mononegavirales have evolved to utilize a single multifunctional enzyme to transcribe individual genes (make, cap, and methylate the mRNAs) and replicate the entire genome without capping and methylation. This may be due to reduced evolutionary pressure - typically, this multifaceted process is sensitive to cell state and signaling inputs. These viruses have evolved to drive this process efficiently forward using minimal components. In eukaryotes, RNA transcription (copying the genetic information) is a delicate and complicated process involving many molecular machines, such as DNA dependent RNA polymerases, capping enzymes, and methyltransferases. For example, the eukaryotic counterparts of the RdRp, Cap, and MT domains of the multifunctional enzyme L are 1) RNA polymerase II and polyadenylate polymerase, 2) RNA triphosphatase and guanylyltransferase, and 3) RNA methyltransferase, respectively (158-167). Additionally, Mononegavirales L also mimics the replication of the entire genome by accessing the N protein-coated RNA genome, similar to eukaryotic counterparts of DNA polymerases on the histone assembled DNA genome (168-170).

The structural similarity of the Mononegavirales polymerases agrees with the relatively high sequence conservation. Nonetheless, the structural differences also highlight the virus or genus-specific features. Collectively, the structures of the Mononegavirales polymerases provide significant advance into the molecular architectures, interrelationship, the inhibitors, and the evolutionary implications of the Mononegavirales polymerases. Other polymerases from measles, mumps, Nipah and Hendra in Paramyxoviridae, Ebola, and Marburg in Filoviridae need to be determined for us to fully understand the similarities and differences of the polymerases in Mononegavirales. Further, structures of Mononegavirales polymerases in complex of RNA templates, RNA products, or inhibitors are desired to appreciate the specific protein:RNA interactions and druggable sites.

**FIGURE PREPARATION**

All the figures presenting the structural models were generated using PyMOL (171).

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COMPETING INTERESTS

The author declares no competing interests.
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| Order            | Family            | Genus              | Species                        | Virus (Abbreviation)               |
|------------------|-------------------|--------------------|--------------------------------|------------------------------------|
| Rhabdoviridae    | Vesiculovirus     | Indiana vesiculovirus | vesicular stomatitis virus      | VSV                                |
|                  | Lyssavirus        | Rabies lyssavirus  | rabies virus                    | RABV                              |
| Pneumoviridae    | Orthopneumovirus  | Human              | human respiratory syncytial virus | HRSV                              |
|                  | Metapneumovirus   | Human              | human metapneumovirus           | HMPV                              |
|                  |                   |                    |                                |                                    |
| Mononegavirales  | Henipavirus       | Hendra henipavirus | Hendra virus                    | HeV                                |
|                  | Nipah henipavirus |                    | Nipah virus                     | NIV                                |
|                  | Respirovirus      | Human respirovirus | human parainfluenza virus       | HPIV                              |
|                  | Murine respirovirus |                   | Sendai virus                    | SeV                                |
|                  | Rubulavirus       | Mumps rubulavirus  | mumps virus                     | MuV                                |
|                  | Morbillivirus     | Measles morbillivirus | measles virus                  | MeV                                |
| Filoviridae      | Ebola virus       | Zaire ebolavirus   | Ebola virus                     | EBOV                              |
|                  | Marburgvirus      | Marburg marburgvirus | Marburg virus                  | MARV                              |

Table 1: Taxonomy of the representative Mononegavirales viruses discussed in this review.
FIG 1: The genome organization and RNA synthesis of Mononegavirales. The negative-sense NNS genome is depicted from the 3' end to the 5' end showing the 3' leader (Le, cyan box), genes (gray, blue or green box) flanking with gene-start (GS, white box) and gene-end (GE, black box), and 5' trailer (Tr, yellow box). The essential genes (N, P, L) and necessary cofactors (M2 or p30) for RNA synthesis are colored in blue and green, respectively. The RNA dependent RNA polymerase (RdRP) sequentially produces a gradient level of Le RNA (red line) and viral mRNAs (black, blue, or green line), with the attenuation of the downstream mRNAs at each gene junction. The Le RNA (red lines inside the box) remains uncapped and non-polyadenylated, while the viral mRNAs are 5' capped, methylated, and 3' polyadenylated. The lines under the Le RNA and representative viral mRNAs indicate the abundance and gradient levels of the RNA transcripts. The promoters for transcription and replication are shown with magenta arrows.
FIG 2: The domain organization and architecture of L and P. (A) The domain organization and cartoon representation of the multifunctional enzyme - monomeric L. The conserved regions (CRs) I-VI are labeled in gray boxes. The RNA dependent RNA polymerase domain (RdRp), capping domain (Cap), connector domain (CD), methyltransferase domain (MT), and C-terminal domain (CTD) of L are colored in blue, green, yellow, pink, and cyan, respectively. (B) The domain organization and cartoon representation of the multimodular adapter - oligomeric P. The intrinsically disordered N-terminal domain (PNTD), oligomerization domain (POD), and C-terminal domain (PCRD) are colored in magenta, red and orange, respectively. The interaction regions with other viral proteins, including L, N, RNA-free N (N'), and accessory protein (M2-1), are labeled in gray boxes. The representative P oligomers are shown for representative virus families, Rhabdoviridae, Pneumoviridae, Paramyxoviridae, and Filoviridae.
FIG 3: The cryo-EM structures of the Rhabdoviridae polymerases. (A) Linear domain representation of the L and P proteins of the vesicular stomatitis virus (VSV) polymerase. The cartoon view of 3.8 Å (PDB: 5A22) and 3.0 Å (PDB: 6U1X) cryo-EM structures of the VSV polymerase are shown. (B) Linear domain representation of the L and P proteins of the rabies virus (RABV) polymerase. The cartoon view of 3.3 Å (PDB: 6UEB) cryo-EM structure of the RABV polymerase is shown. The RNA dependent RNA polymerase domain (RdRp), capping domain (Cap), connector domain (CD), methyltransferase domain (MT), C-terminal domain (CTD) of L, and PNTD are colored in blue, green, yellow, pink, cyan, and magenta, respectively. The missing domains are colored in gray. The PNTD is highlighted as spheres, and the terminal residue numbers of the modeled P segments are indicated. The PDB accession codes are underlined.
FIG 4: The cryo-EM structures of the Pneumoviridae polymerases. (A) Linear domain representation of the L and P proteins of the human respiratory syncytial virus (HRSV) polymerase. The cartoon view of 3.67 Å (PDB: 6UEN) and 3.2 Å (PDB: 6PZK) cryo-EM structures of HRSV polymerase complexes. The missing domains compared to the VSV L are shown in the gray meshes. (B) Linear domain representation of the L and P proteins of the human metapneumovirus (HMPV) polymerase. The cartoon view of 3.7 Å (PDB: 6U5O) cryo-EM structure of the HMPV polymerase is shown. The domain colorings are the same as Fig. 2. The terminal residue numbers of the modeled POD and PCTD are indicated. The PDB accession codes are underlined.
FIG 5: The cryo-EM structure of the Paramyxoviridae polymerase. (A) Linear domain representation of the L and P proteins of the human parainfluenza virus (HPIV) polymerase. The side view of the ribbon diagram of the 4.3 Å (PDB: 6V85) cryo-EM structure of the HPIV polymerase complex. (B) The top view of the superimposed VSV L and HPIV L shows the domain switch of the CD-MT-CTD module. The superimposition is based on the RdRp (surface view), and CD, MT, and CTD are shown as the ribbon diagram. The domain colorings are the same as Fig. 2. The VSV L is shown in the left panel (box), and the HPIV L is shown in the right panel. The HPIV L (PDB: 6V85) is colored the same as panel A, and another stable conformation of the HPIV L (PDB: 6V86) is colored in gray. Note the significant location switch of CTD, facing down (VSV) vs. facing up (HPIV L). The PDB accession codes are underlined.
FIG 6: Structural comparison of the RNA dependent RNA polymerase (RdRp) domain. (A-E) The ribbon representations of the RdRp domain of the Rhabdoviridae (VSV, RABV), Pneumoviridae (HRSV, HMPV), and Paramyxoviridae (HPIV) L in conventional orientation. The structural motifs, fingers, palm, thumb, and support region are in blue, red, green, and gray, respectively. The tri-residues (GDN) of the RdRp active sites at a β-hairpin tip of the palm motif are shown in magenta spheres. (F-G) Similarities of the Mononegavirales RdRp domain to other viral polymerases. Structures of the polymerases of reovirus λ3 (ReoV, PDB: 1MUK) and influenza B (FluB, PDB: 4WRT) are shown as the same orientation and coloring scheme as in panel A. The PDB accession codes are underlined.
FIG 7: Structural comparison of the Cap domain. The motifs A-E of the Cap domain of the Rhabdoviridae (VSV, RABV), Pneumoviridae (HRSV, HMPV), and Paramyxoviridae (HPIV) L are shown as ribbon diagram in blue, yellow, red, magenta, and green, respectively. Those motifs are centered around the active site motif D (HR). The proposed priming loop (orange) is next to motif B. The PDB accession codes are underlined.
FIG 8: Structural comparisons of the Mononegavirales RNA polymerases. The active sites of the RdRp and Cap domains of L, GDN, and HR are shown in magenta spheres and sticks, respectively. The priming loops and supporting helix are colored in orange. (A) The structural superimposition of the Rhabdoviridae L. The VSV L is colored the same as Fig. 3A, and the RABV L is colored in gray. (B) The structural superimposition of the Rhabdoviridae P. The VSV P is colored in magenta the same as Fig. 3A, and the RABV P is colored in brown. Only the interacting
domains, RdRp, CD, and CTD of L, are shown as surface. (C) The structural superimposition of the *Pneumoviridae* L. The HRSV L is colored the same as Fig. 4A, and the HMPV L is colored in gray. Note that the supporting helix is missing. (D) The superimposition of the *Pneumoviridae* P. The HRSV P is colored the same as Fig. 4A, and the HMPV P is colored in brown. Only the interacting domain RdRp of L is shown as surface. (E) The structural representation of the *Paramyxoviridae* L. The HPIV L is colored the same as Fig. 5A. (F) The location of the *Paramyxoviridae* P. The HPIV P is colored the same as Fig. 5A. Only the interacting domain RdRp of L is shown as surface. The PDB accession codes are underlined.
FIG 9: Structural models of the Mononegavirales RNA synthesis. (A) The cartoon diagrams of recently reported structures of the Rhabdoviridae (VSV, RABV), Paramyxoviridae (HPIV), and Pneumoviridae (HRSV, HMPV) polymerases. The same color scheme as Fig. 2. (B) The modeled initiation and elongation complexes. The RdRp domain of the L proteins of Rhabdoviridae (VSV), Paramyxoviridae (HPIV), and Pneumoviridae (HRSV) with modeled RNA template from reovirus λ3 polymerase (PDB: 1N1H), FluB polymerase (PDB: 6QCV), and FluB polymerase (PDB: 6QCT), respectively. The same color scheme for the RdRp domain of Mononegavirales L. The priming loop (from the Cap domain) and the support helix (from the RdRp domain) are colored in orange.
The modeled RNA template and RNA transcript are shown in yellow and pink, respectively. (C) 1034

The proposed cartoon models of the initiation and elongation stages on the nucleoprotein (N) encapsidated N:RNA (NC) template. Initiation: the priming loop and support helix are at the close approximate of the GDN active site of the RdRp domain of L. Elongation (Early Stage): the priming loop is away from but the support helix stays at the close approximate to the active site of the RdRp domain of L. Elongation (Late Stage): the priming loop is away from the active site of the RdRp domain of L and the support helix is missing, as well as the CD, MT, and CTD domains of L is disordered and linked by dashed lines. The nucleoprotein (N) protein is shown as the yellow oval. The RNA template, RNA transcript, and the flexible linker are shown in the black, blue, and red lines, respectively. The priming loop and support helix are shown as the thick orange bar and cylinder, respectively. The PDB accession codes are underlined.