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Towards a structural understanding of RNA synthesis by negative strand RNA viral polymerases

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Negative strand RNA viruses (NSVs), which may have segmented (sNSV) or non-segmented genomes (nsNSV) are responsible for numerous serious human infections such as Influenza, Measles, Rabies, Ebola, Crimean Congo Haemorrhagic Fever and Lassa Fever. Their RNA-dependent RNA polymerases transcribe and replicate the nucleoprotein coated viral genome within the context of a ribonucleoprotein particle. We review the first high resolution crystal and cryo-EM structures of representative NSV polymerases. The heterotrimeric Influenza and single-chain La Crosse orthobunyavirus polymerase structures (sNSV) show how specific recognition of both genome ends is achieved and is required for polymerase activation and how the sNSV specific 'cap-snatching' mechanism of transcription priming works. Vesicular Stomatitis Virus (nsNSV) polymerase shows a similar core architecture but has different flexibly linked C-terminal domains which perform mRNA cap synthesis. These structures pave the way for a more complete understanding of these complex, multifunctional machines which are also targets for anti-viral drug design.

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Introduction

Negative stranded RNA viruses such as Influenza, Measles and Respiratory Syncytial Virus (RSV) are responsible for widespread, sometimes severe human diseases that have a large public health and economic impact. Others like Ebola, Rabies, Crimean Congo Haemorrhagic Fever, Hantaan, Lassa Fever and Avian Influenza viruses result in sporadic zoonotic outbreaks with high mortality rates. The RNA-dependent RNA polymerases (RdRp's) of NSVs perform replication and transcription of the single-stranded RNA genome, which may be segmented or not. These large and complex polymerases are multi-functional, not only performing template directed RNA synthesis but also containing customized modules that generate capped and poly-adenylated mRNAs using very different strategies [1]. For these reasons, NSV polymerases are good targets for anti-viral drug development.

Negative strand RNA virus genomes are never free in nature. The functional replication unit is a ribonucleoprotein particle (RNP) in which the genomic RNA is completely coated by viral nucleoproteins and bound to a polymerase [2,3]. The need to maintain such an assembly during all steps of the viral infection presents challenging constraints. First, nucleoproteins bound to the genomic RNA must transiently detach to give the polymerase access to the template. Second, replication has to be coupled to the assembly of a progeny RNP by the incorporation of a new polymerase and nucleoproteins onto the nascent genome copy. Third, *in cis* RNA regulatory sequences, such as the promoter, transcription termination and polyadenylation signals, need to be accessible to modulate specific polymerase functions.

There are two classes of negative strand viruses (NSVs). Non-segmented NSVs (nsNSVs), also known as *Mononegavirales* (e.g. Measles, Rabies, VSV, RSV or Ebola), have a continuous RNA genome, whereas the genome of segmented NSVs (sNSVs) is divided into either two (Family *Arenaviridae*, e.g. Lassa), three (Family *Bunyaviridae*, e.g. Crimean Congo Haemorrhagic Fever, La Crosse, Hanta, Rift Valley) or six to eight fragments (Family *Orthomyxoviridae*, e.g. Influenza, Thogoto, Infectious Salmon Anaemia Virus). In nsNSVs the RNPs form regular helical structures [4••] that in addition incorporate other viral proteins required for efficient RNP transcription and replication, such as the phosphoprotein (P protein). The large (~250 kDa) monomeric polymerase (L protein) carries out genome replication as well as the 5′ cap synthesis and 3′ polyadenylation of mRNA transcripts [5,6]. The genomic segments of sNSV are each packaged into separate, worm or rod-like RNPs which are circularised by the binding of the polymerase to conserved sequences at both ends of the viral RNA [7,8•]. In cytoplasmically replicating *Arenaviridae* and *Bunyaviridae* the polymerase (L protein) is also a single chain, whereas in nuclear replicating *Orthomyxoviridae* the polymerase is heterotrimeric, with subunits PA, PB1 and PB2, but whose total molecular weight is similar to other NSV L

proteins. sNSV polymerases have a radically different way of capping their mRNAs. They employ a unique 'cap-snatching' mechanism for obtaining the cap from cellular mRNA [9–12].

The high divergence among NSV polymerase amino acid sequences and lack of detailed structural information have long hindered understanding of what distinguishes the transcriptase and replicase states of the polymerase and how the different capping mechanisms are coupled to mRNA synthesis. During the last remarkable year, the atomic structures of the sNSV Influenza A, B and C (*Orthomyxoviridae*) [13••,14••,15•], and La Crosse orthobunyavirus (LACV) (*Bunyaviridae*) [16••] polymerases have been determined by crystallography (LACV also by cryo-EM), and that of the nsNSV Vesicular Stomatitis Virus (VSV) (*Rhabdoviridae*) by cryo-EM [17••]. Thus, in one fell swoop, a representative set of structures is now available that relates the common and diverse features of NSV polymerases to their different replication and transcription strategies, as well as revealing their evolutionary relationship to other RNA virus polymerases, such as those of dsRNA (e.g. reoviruses) and positive-strand RNA (e.g. Hepatitis C Virus, HCV). Of particular interest is how these polymerases specifically recognise their genomic RNA, the mechanisms of initiation, elongation, capping and polyadenylation and how polymerase function is regulated by polymerase–vRNA interactions.

Overall structure of NSV polymerases

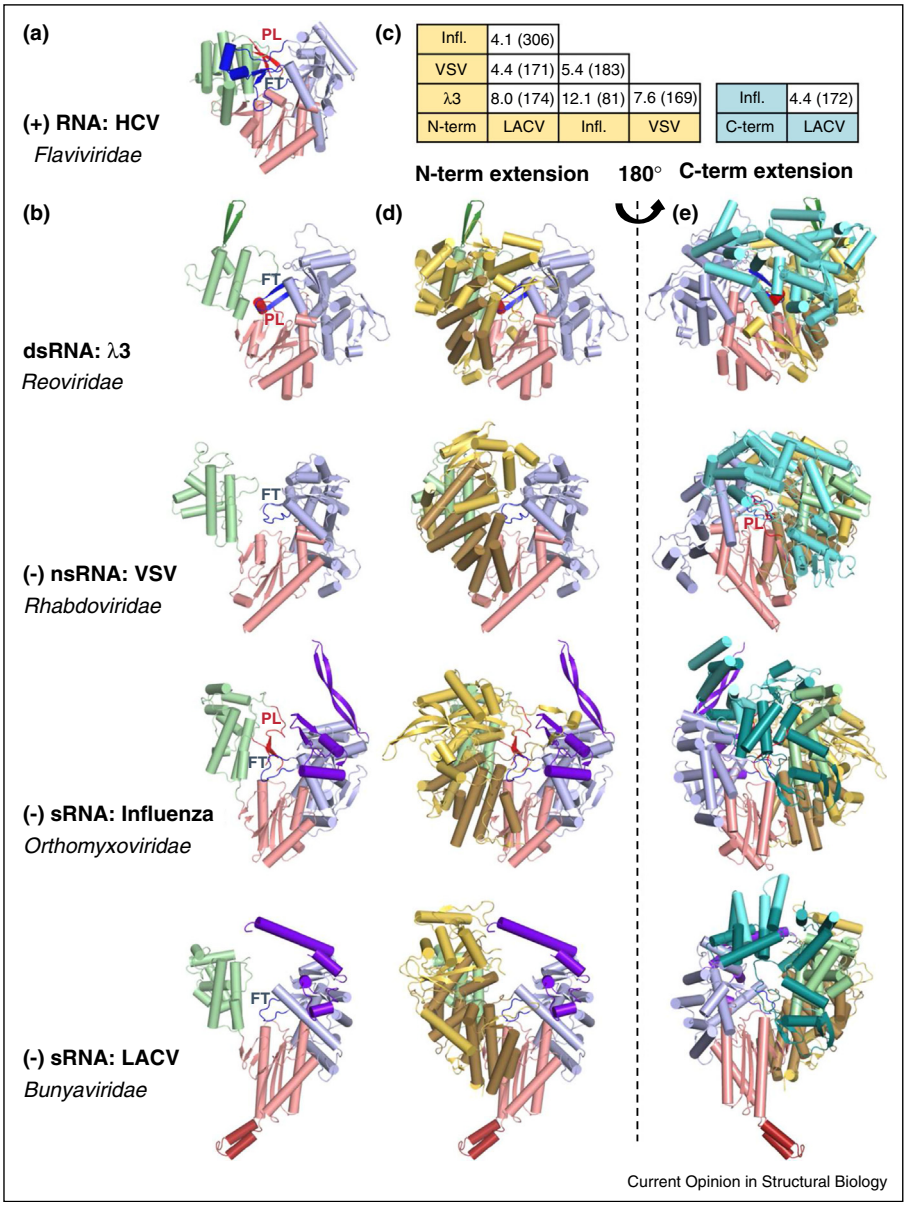
At the core of NSV viruses is the canonical RdRp fold with palm, fingers and thumb domains arranged in a right-handed configuration [18] (Figure 1a,b). The palm is the most conserved domain encompassing the conserved functional polymerase motifs A to E within the β-strands of a central β-sheet, whereas the largely helical fingers and the thumb domains are more variable. The features found in the RdRp core of NSVs are common to many RNA virus polymerases for example that of the positive strand RNA virus, Hepatitis C (HCV) (Figure 1a). These include insertions into the fingers called fingertips (encompassing motif F), which connect across to the thumb forming an enclosed cavity in which RNA synthesis occurs. Another common feature of RNA polymerases which do unprimed ('*de novo*') RNA synthesis is a 'priming' loop that can emerge from different parts of the polymerase (in HCV and Influenza it is from the thumb) and is deployed inside the catalytic chamber to promote formation of the initiation complex (see below).

In NSVs, as in dsRNA viruses (e.g. reovirus), there are substantial N-terminal and C-terminal extensions that buttress the RdRp core. The largely helical N-terminal extensions have sizes between 350 and 500 amino acid residues and form an arc with an extensive interface stretching from the palm domain across one side of the thumb domain to touch the fingers (Figure 1d). In Influenza and LACV, the N extension corresponds to the PA-C (like) domain, which however contains a more elaborate β-sheet region that is involved in 5′-end vRNA binding (see below). Pairwise structural similarities between these regions suggest that nsNSV (VSV) might be the evolutionary intermediate between dsRNA viruses and segmented NSV polymerases (Figure 1c). The C-terminal extension packs on the opposite side of the thumb connecting mainly with fingers. Although this region (PB2-N in Influenza) is clearly structurally similar between Influenza and LACV, this is less obvious with nsNSV (in VSV this corresponds to the capping domain) and dsRNA viruses, but in all cases the extension is in the same spatial location and maintains the similar structural α/β features (Figure 1e). It appears to block exit of the nascent template-product RNA duplex and instead serves to separate the strands into independent template and product exit channels (see below) [14••,16••,19,20].

sNSV polymerases specifically bind both conserved ends of each viral genome segment

A unique feature of sNSVs is that they form pseudo-circular RNPs with both 5′ and 3′ ends (the 'promoter') of the genomic RNA bound to the polymerase. Furthermore, the 5′ and 3′ ends are quasi-complementary such that the replication intermediate cRNA ends can make similar polymerase interactions as the vRNA. The crystal structures of Influenza and LACV polymerases-promoter complexes show that the 5′ and 3′ extremities are bound, not as a panhandle, but as single strands in distinct positively charged binding sites. In neither structure does the 3′ end enter the active site (as might have been expected for the template strand). Instead, they are bound in a sequence specific manner on the protein surface but in quite different ways for LACV and Influenza (Figure 2a,b). In the case of LACV an insertion into the PA-like domain called the 'clamp' (absent in Influenza polymerase) blocks the 3′ end into its binding groove (Figure 2a) [16••]. It is not clear by which mechanism the template is released enabling it to enter the polymerase active site. For both LACV and Influenza, the vRNA 5′ end is bound as a stem–loop structure in a pocket made by insertions into the fingers ('fingernode' or 'PB1 β-turn', respectively) and the N-terminal extension (PA, PA-like) domains ('arch') (Figure 2c,d) [13••,16••]. For LACV it was possible to demonstrate that binding of the 5′ stem-loop led to ordering of the polymerase fingertips, thus explaining how 5′ end binding allosterically activates the polymerase (Figure 2e) [16••]. An additional feature observed in the manner of promoter binding to Influenza is base-pairing between distal parts of the complementary 3′ and 5′ ends, which is known to be required for initiation of replication and transcription of sNSVs [21,22]. The VSV polymerase structure lacks RNA so it remains to be seen how the template is bound. However nsNSVs, but not sNSVs, require a viral phosphoprotein (P) cofactor in

Figure 1



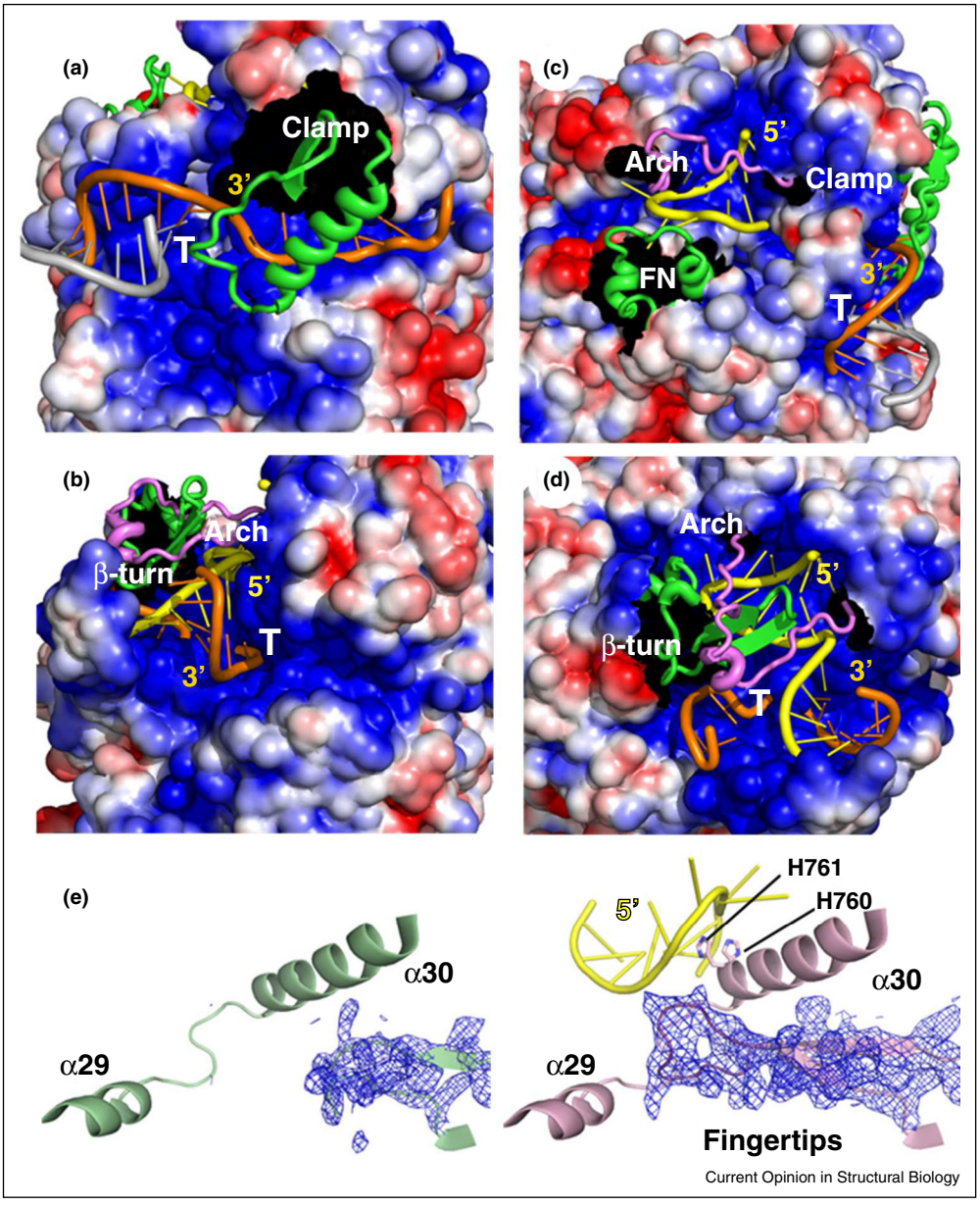
Structures of representative RNA virus polymerases. **(a)** Cartoon model of HCV RdRp showing the right handed arrangement of palm (pink), fingers (violet) and thumb (green) domains. The fingertips (FT) and priming loop (PL) insertions are coloured in dark blue and red respectively. **(b)** Cartoon models of the RdRp core of double-stranded (λ3) and negative-strand (VSV, Influenza and La Crosse bunyavirus) RNA virus polymerases. **(c)** Root mean square deviations (Å) after pairwise structural alignments with DaliLite of the RdRp N-terminal extensions (yellow table) and C-terminal extension (sNSV only, blue table). The number of aligned residues is indicated in parenthesis. **(d)** N-terminal extensions to the RdRp core in dsRNA and NSV polymerases coloured in dark and light yellow respectively for the regions structurally aligning or not with the other polymerases. **(e)** C-terminal extensions to the RdRp core in dark or light cyan for respectively the structurally homologous regions shared by Influenza and LACV and the non-homologous regions.

combination with the nucleoprotein (N) to correctly engage the vRNA with the polymerase to promote transcription and replication [23–25]. Understanding the detailed mechanisms involved requires further structural studies, although the VSV structure does contain a non-resolved fragment of P. More generally, the combination of RNA sequence and secondary structure specific binding of the vRNA to the polymerase is likely to be a general feature for template recognition amongst RNA virus polymerases [26].

NSV transcription and capping mechanisms

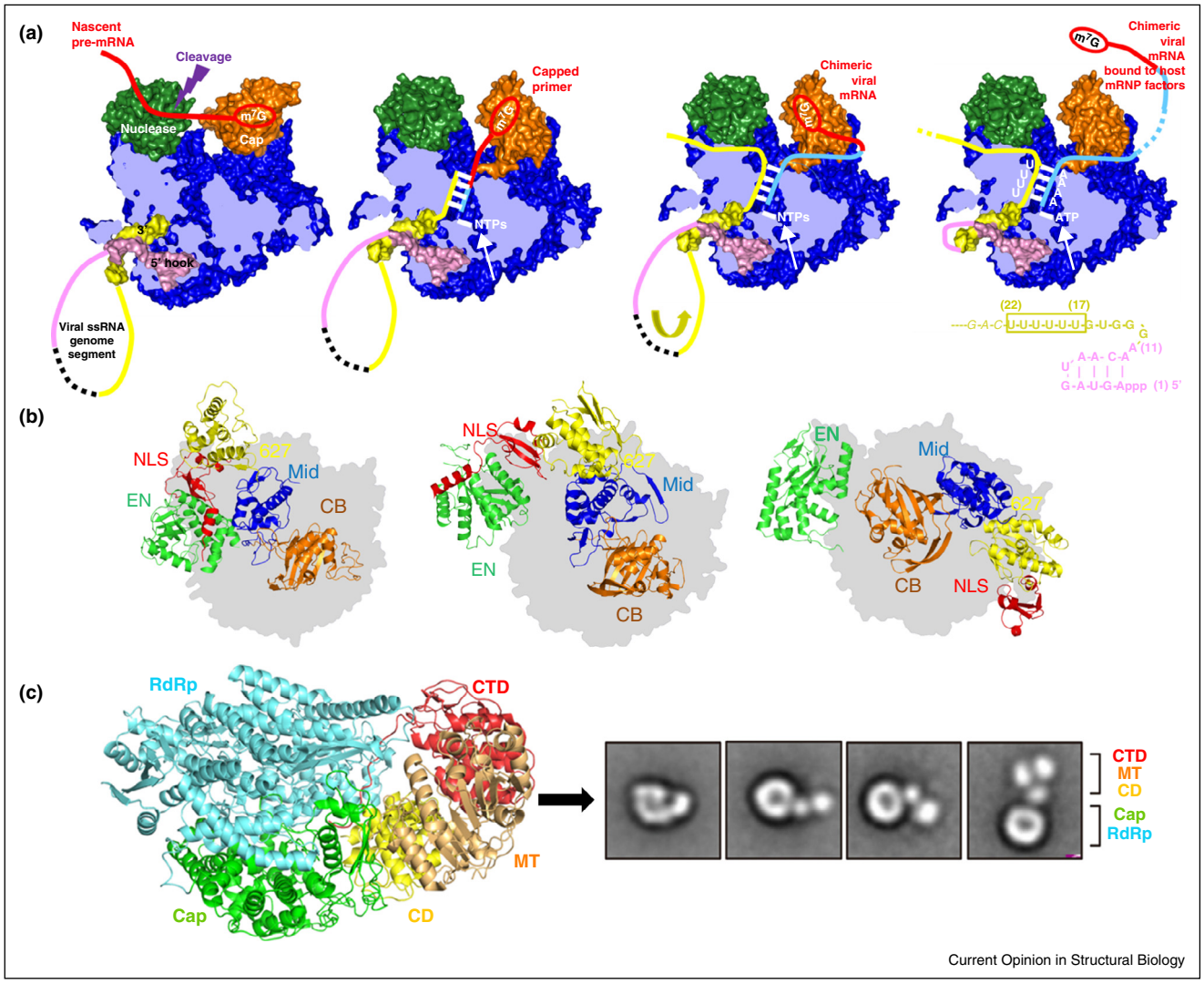
sNSV and nsNSVs have evolved quite different mechanisms for capping of their mRNA transcripts each

Figure 2



sNSV polymerase–vRNA interactions. **(a)** Surface charge representation of the 3′ vRNA binding site of LACV polymerase. The clamp (green) traps the 3′ vRNA end (orange) into a positively charged groove where it binds sequence specifically. For initiation of RNA synthesis the 3′ end must be relocated into the polymerase active site via the template entry tunnel (T). **(b)** Same orientation and colouring as (a) but for Influenza B polymerase structure (PDB: 4WSA) bound to the vRNA 5′and 3′ ends. Differently to LACV, the 3′ RNA is positioned closer to the 5′ binding site allowing base-pairing between the distal parts of the promoter. Influenza has no equivalent to the clamp, however, a positively charged groove at a similar location to the LACV 3′ binding site is maintained. **(c)** Similar to (a) but rotated to show the LACV 5′ vRNA binding site. Insertions denoted the fingernode (FN, green) and the arch (violet) contribute to the binding pocket for the 5′ vRNA end (yellow) which forms a stem-loop or hook structure. **(d)** As (b) but for Influenza polymerase. The 5′ vRNA extremity binds as a hook in an equivalent position to LACV. Whereas the arch is maintained, the LACV fingernode is replaced by a β-turn. **(e)** Allosteric regulation of the LACV polymerase by vRNA 5′ end binding. The structure of the LACV polymerase without (left, green model) and with (right, pink model) nucleotides 1–10 of the 5′ vRNA (yellow) (PDB: 5AMR and 5AMQ respectively). In the absence of the 5′ vRNA there is no electron density (blue mesh, 1.5σ 2FoFc map) for the fingertips (left). Upon 5′ vRNA binding clear density for the loop appears (right). The 5′ vRNA backbone interaction with His760 and His761 raises helix α30 allowing stabilization of an ordered and active configuration of the fingertips loop.

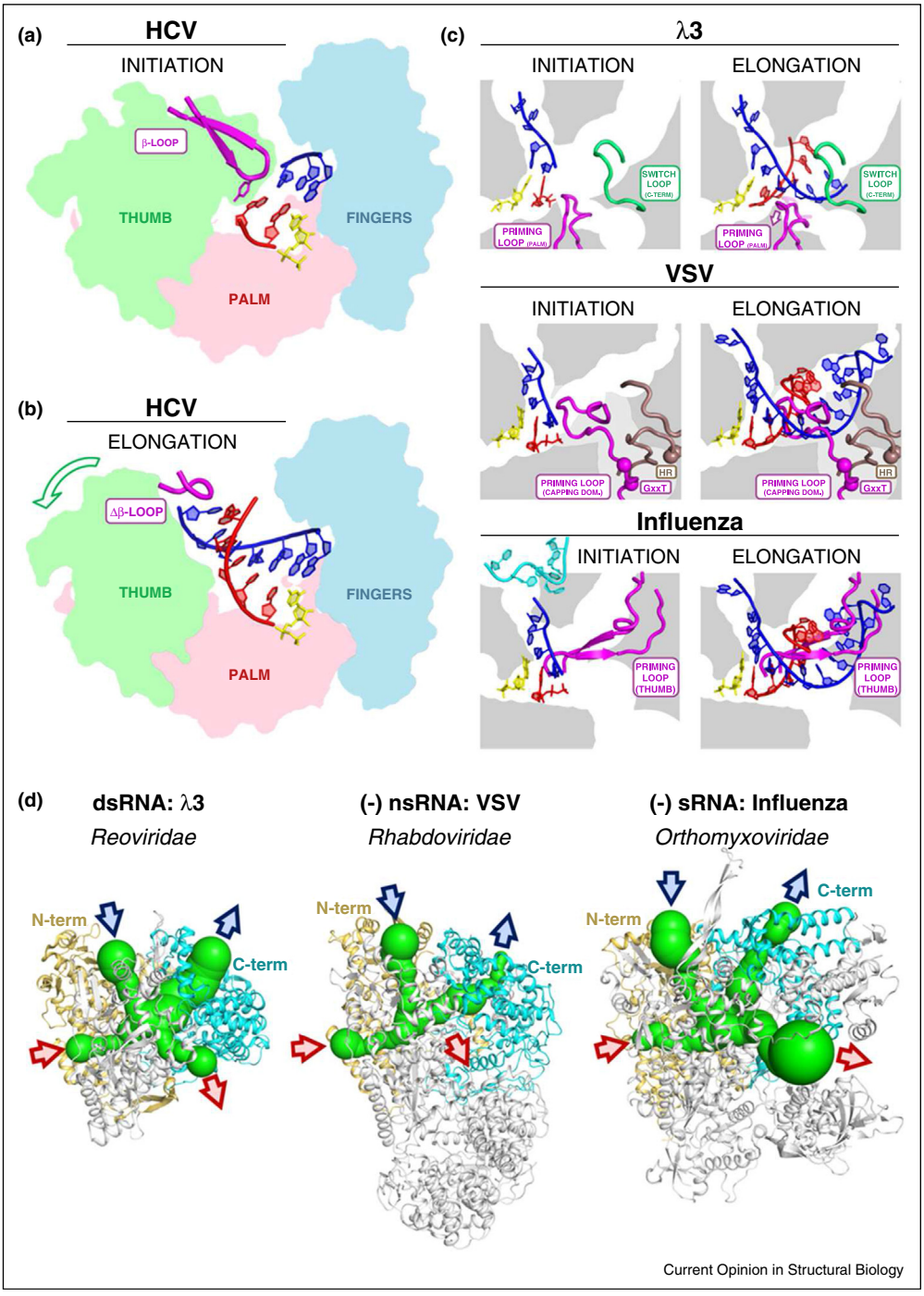
Figure 3



Mobile domains for cap-snatching or capping. **(a)** Influenza polymerase is shown in surface representation with the polymerase core (blue), the endonuclease (green), the cap binding domain (orange) and in yellow and pink the 3′ and 5′ ends of the vRNA promoter. Left: the structure of bat Influenza A (PDB: 4WSB) is consistent with the cleavage of donor pre-mRNA bound to the cap binding domain. Middle left: In the Influenza B structure (PDB: 4WSA) the rotated orientation of the cap binding domain is consistent with the priming step for transcription initiation. Middle right: As elongation proceeds the template and transcript are extruded through different tunnels. Right: After most of the vRNA template has been translocated through the polymerase active site, only a tight turn connects it to the tightly bound 5′-hook. This places the 5′ proximal oligo-U stretch in the active site allowing poly(A) tail synthesis by a stuttering mechanism. The nucleotide sequence of this region is given at the bottom. **(b)** Conformational plasticity exhibited by flexibly linked domains of Influenza polymerase after aligning the invariant polymerase core (grey outline). The cap-snatching endonuclease (EN) at the PA N-term and the mid, cap binding (CB), 627 and NLS domains at the PB2 C-term are dramatically rearranged between the apo-FluC structure (PDB: 5D98, 5D9A) (left), FluB in complex with the 5′ cRNA (PDB: 5EPI) (middle) and the promoter (5′ and 3′ vRNAs) bound FluB/FluA structures (PDB: 4WSA) (right). **(c)** Cryo-EM derived structure of VSV polymerase in cartoon representation (PDB: 5A22) showing the RdRp, capping (CAP), connector (CD), methyltransferase (MT) and C-terminal (CTD) domains. In the right panel, EM class averages show how the flexibly linked CTD, MT and CD can adopt different conformations relative to the RdRp-CAP core. The arrow indicates the class average most similar to the cryo-EM structure.

requiring specialised modules to be added to the core polymerase. sNSVs initiate transcription using a capped primer derived by 'cap snatching' and structures of Influenza polymerase in different states visualise how this works [14••] (Figure 3a). The extreme N-terminal region of PA (orthomyxovirus) or L protein (arena-viruses, bunyaviruses) contains the cap-snatching endonuclease domain [10,27,28], whereas, at least for orthomyxoviruses, the C-terminal two-thirds of PB2, known as PB2-C, includes the flexibly connected cap-binding domain [12]. Nuclear replicating Influenza polymerase is thought to be closely associated with Pol II [29] allowing it to

Figure 4



Initiation and elongation inside the polymerases and RNA trafficking. **(a)** Schematic diagram of HCV polymerase with palm fingers and thumb domains in pink, blue and green respectively. The initiation step (PDB: 4WTL) is stabilized by an apical tyrosine residue from the priming loop (magenta) stacking onto the nascent strand after the first phosphodiester bond formation. **(b)**The structure of the elongation complex (PDB: 4WTA) was only obtained after deletion of the priming loop. The growing duplex is proposed to push away the priming loop inducing a movement of the thumb domain (indicated by the green arrow). **(c)**Models of the initiation and elongation phases of RNA synthesis for λ3 reovirus (top), VSV (middle) and Influenza (bottom). For λ3 both initiation and elongation mode structures contain the RNA (PDBs: 1N1H and 1N35 respectively). For VSV and Influenza the RNA derives from crystal structures of Qβ replicase in initiation and elongation/strand separation modes (PDBs: 3AVT and 3AVY respectively), after superposition with VSV and Influenza A polymerase structures (PDBs: 5A22 and 4WSB respectively). The tunnels are schematically shown white with the polymerase in grey. The putative priming loops, which emerge from diverse polymerase domains (as indicated) are coloured in purple, and in all cases are positioned close to the priming NTP (red sticks) that offers the 3′ OH to the incoming NTP (yellow sticks) for the transfer reaction. The observed position of the template RNA is shown on the initiation panel for Influenza in light blue; during

initiation it has to be relocated into the active site (this is also true for LACV polymerase). During elongation the λ3 priming loop is slightly pushed down by the nascent duplex (arrow). In VSV and Influenza the nascent duplex would clash with the priming loop, implying progressive withdrawal

capture nascent host pre-mRNAs via its PB2 cap-binding domain. The bound pre-mRNA is first directed towards the endonuclease, which cleaves at 10–14 nucleotides from the cap. A subsequent rotation of the cap-binding domain inserts the capped oligomer into the active site for priming of viral mRNA synthesis (Figure 3a). Interestingly, in some orthomyxoviruses (Thogotoviruses) that do not have host sequences at the 5′ ends of their mRNAs, the endonuclease and cap-binding domains are biochemically defunct, suggesting that there is an alternative method of capping in these cases [30]. In the case of *Arenaviridae* and *Bunyaviridae*, which replicate in the cytoplasm and couple translation to transcription [31], the source of capped RNAs and the mechanism of cap-snatching are far less well understood. Structures show a similar endonuclease to Influenza at the N-terminus of the L protein [10,27] but it is not yet known whether a cap-binding domain exits in the C-terminal region, since this part is not present in the truncated LACV polymerase structure determined. In Influenza polymerase, PB2-C is able to adopt, together with the endonuclease, at least two remarkably different domain arrangements, as shown by the recent structure of apo-FluC polymerase [15•] and a new structure of FluB polymerase with only the cRNA 5′ end bound [32•] (Figure 3b). The particular conformation adopted appears to depend on which vRNA ends are bound (and possibly interactions with other viral and cellular factors) and this likely defines whether the polymerase is transcribing, replicating or nucleating progeny RNP assembly. nsNSV such as VSV cap their own mRNAs but use an unconventional, inverse strategy compared to most other eukaryotic and viral systems [33]. For this, extra domains (capping, connector, cap methyl-transferase and C-terminal) are present C-terminally to the polymerase core (Figure 3c). The emerging 5′pppRNA transcript first forms a covalent L-5′pRNA intermediate (with His1227 in VSV), catalysed by a polyribonucleotidyltransferase (PRNTase) in the capping domain. Subsequently the 5′pRNA is transferred onto a GDP generated by a GTPase, whose location is uncertain. H1227 is located in a capping domain loop spatially not far from the GxxT motif that is thought to participate in guanosine nucleotide binding [34]. The methyltransferase domain, structurally similar to those of flaviviruses, is dual functional, methylating first the 2′ OH of the first nucleotide ribose and then the N7 of the cap guanosine, inverting the order of events found in other capping systems [35]. The EM structure of VSV polymerase is thought to correspond to an early initiation state which after synthesis of the first few nucleotides must open up to allow product exit. Since capping only occurs after the synthesis of 31 nucleotides [36] this implies that significant conformational rearrangements of the C-terminal domains are likely to occur to create the active configuration for capping including access to the methyltransferase active site. Consistent with this, EM images of VSV polymerase show that the C-terminal domains are flexibly linked and can adopt alternative configurations (Figure 3c). However understanding the detailed capping mechanism including the requirement for a specific sequence at the 5′ end of the emerging mRNA, clearly requires further structural studies.

Many but not all NSVs poly-adenylate their mRNAs by iterative transcription of poly(U) regions near the template 5′ end before termination (orthobunyavirus mRNAs are not polyadenylated). In Influenza, the structure is fully consistent with the previously proposed mechanism whereby the conserved 5′ end bound tightly as a stem-loop to the polymerase, hinders translocation of the 5′ proximal oligo(U) stretch thus creating the poly(A) tail by stuttering (Figure 3a) [14••,37].

Replication and product and template RNA trafficking

In NSVs replication results in full-length copies of the genome and occurs via a complementary positive strand intermediate. It is initiated '*de novo*' (i.e. without an extrinsic primer) and this is a rate-limiting step in RNA synthesis since two nucleotide triphosphates have to be assembled at the active site together with the template. *De novo* synthesis may occur opposite the first 3′ end nucleotide or, if there are repeated 3′ end triplets by allowing the template to overshoot and then realigning (e.g. hantavirus) [16••,38]. A priming loop may help stabilize the initiation complex, as first described for phage Ø6 [39] and reovirus λ3 [19]. Recently the Hepatitis C Virus (HCV) replication initiation and elongation steps have been structurally characterised (Figure 4a) [40••]. A tyrosine at the tip of the priming loop stabilizes the initiation complex by stacking on the first base-pair. An aromatic residue playing this role is also found at the apex of the priming loop of Ø6 and flavivirus (e.g. Dengue, West-Nile) polymerases [41]. For elongation, the priming loop has to be displaced to make room for the growing template-product duplex to reach the exit tunnel. In HCV, this is coupled to movement of the thumb domain thus allowing a step wise retraction of the priming loop as the initial duplex extends (Figure 4b) [40••].

(Figure 4 Legend Continued) of the priming loop, as observed for HCV, and the separation of the two strands (template in blue and product in red). For VSV, residues essential for capping are shown as spheres in the priming loop and in an additional loop nearby. Since capping occurs after synthesis of 31 nucleotides, and the cavity has only limited capacity, large conformational changes must occur (Figure 3c) to allow product strand exit and to correctly configure the HR and GxxT for capping. **(d)**Representation of the internal tunnels (green) within the RdRps (cartoon) of dsRNA (reovirus), nsNSV (VSV), and sNSV (Influenza), calculated using MOLE 2.0 [48]. The N-terminal and C-terminal extensions to the RdRp core are coloured in yellow and blue respectively. The template RNA entry and exit channels are indicated with blue arrows, and the NTPs entry and RNA product exit with red arrows. In VSV the RNA product exit channel is sealed, consistent with the need for domain movement as shown in Figure 3c.

Although there is not yet a structure of an NSV replication initiation complex, there are reasons to believe that at least for Influenza virus, it might resemble that of HCV since in both cases the priming loop emerges from the thumb domain and is of similar structure and length [14••]. However for other systems the priming loop emerges elsewhere, for example, from the palm domain (dsRNA reovirus) or the capping domain (VSV) (Figure 4c). For VSV, the GxxT motif, important for capping (see above), is at the base of the putative priming loop, suggesting that rearrangement of the priming loop concomitant with emergence of the nascent transcript might induce the enzymatically active configuration for capping (Figure 4c).

Structures of NSV polymerases all show that elements of the C-terminal extension to the core polymerase block partially (lid domain of Influenza and LACV) or totally (VSV capping domain) exit of the product-template duplex. Indeed the likely role of these elements is to force strand separation and to direct the template and product into distinct exit channels. The template turns back to exit close to the entry channel allowing for re-incorporation into the RNP, while the product comes out in the direction of the flexible C-terminal modules (Figure 4c,d). The existence of separate exit tunnels for template and product avoids any interference between processes involving template translocation into and out of the RNA synthesis chamber (coupled to dissociation and re-association of nucleoprotein) and product processing (capping, if an mRNA, incorporation into progeny c/vRNP if a replicate). Thus similar to dsRNA virus polymerases [20,42••] and phage Qβ replicase [43], NSV polymerases have two tunnels in (for template and nucleotides) and two out (for template and product) of the central cavity (Figure 4d). Recent high resolution EM studies of dsRNA reoviruses suggest that the so-called 'switch loop' from the C-terminal extension (positioned similarly to the VSV priming loop), sorts the two strands during transcription or allows double strand exit during replication (Figure 4c) [42••].

Conclusions

After decades of anticipation the first high resolution structures for NSV polymerases are now available revealing a central common structural architecture, similar to the dsRNA virus polymerases. The following picture is emerging, which however needs to be confirmed by further studies. The canonical RdRp core has N-terminal and C-terminal extensions forming an enclosed chamber connected to the exterior by four tunnels. Inside the chamber, the emerging product and template RNA strands are separated at an early stage of RNA synthesis. Template entry and exit tunnels are close to each other facilitating reading the genome with minimal RNP disruption, while the product exit tunnel guides the nascent transcript or replicate towards the C-terminal processing machinery, which is flexibly linked to the core. Specific interactions with the vRNA (e.g. promoter, termination or polyadenylation signals) or product RNA (e.g. 5′ proximal sequences determine capping in VSV) control the functional state of the polymerase. However these new structures are only the starting point for further investigations into how these complex and dynamic machines work. This will involve structural analysis, by crystallography and the new powerful EM technologies, of numerous different functional conformations. Finally, these new structures will be of great use in ongoing efforts to target NSV polymerases for anti-viral drugs, as has been successfully done for HCV [40••]. Examples of recent work in this direction are cap-snatching inhibitors for Influenza [44,45], the broad spectrum RNA virus polymerase inhibitor T705 (favipiravir) in clinical trials for Influenza and Ebola [46] and a new promising inhibitor of RSV polymerase [47].

Conflict of interest statement

Nothing declared.

Acknowledgments

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