

# The ins and outs of four-tunneled *Reoviridae* RNA-dependent RNA polymerases

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RNA-dependent RNA polymerases (RdRps) of the segmented double-stranded (ds) RNA viruses of the *Reoviridae* family exhibit distinguishing structural elements, enabling the enzymes to function within the confines of a proteinaceous core particle. These globular, cage-like polymerases are traversed by four well-defined tunnels, which not only allow template RNAs, nucleotides, and divalent cations to access the interior catalytic site, but also provide two distinct exit conduits for RNA templates and products—one leading out of the core and the other back inside the core. Although *Reoviridae* RdRps are intrinsically capable of binding template, their catalytic activities are tightly regulated by interactions with core shell proteins. This intra-particle mechanism of RNA synthesis coordinates genome packaging with replication during the infectious cycle.

## Addresses

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## Introduction

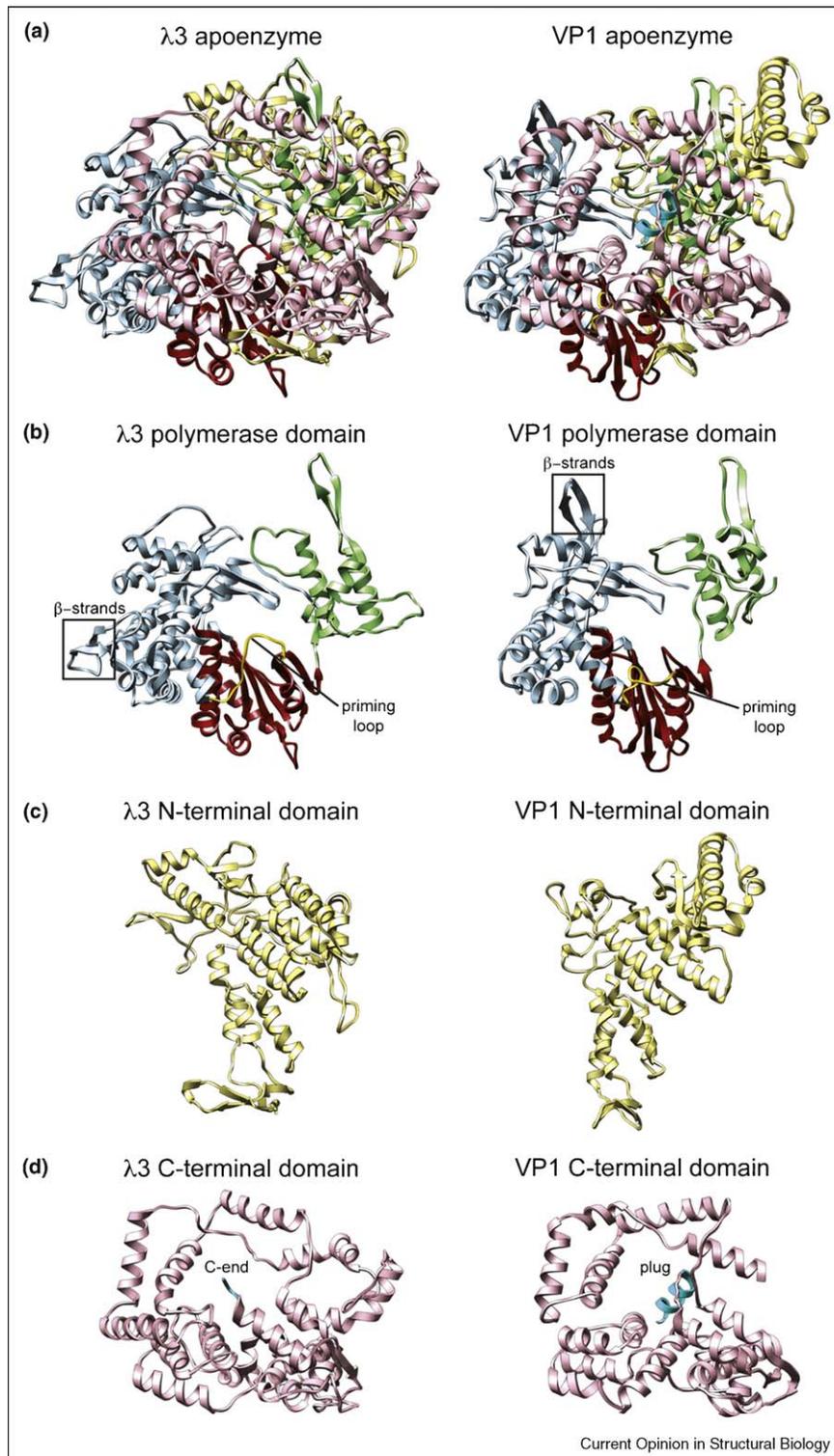
All RNA viruses, with the exception of retroviruses, encode specialized enzymes called RNA-dependent RNA polymerases (RdRps), which catalyze phosphodiester-bond formation between ribonucleotides (NTPs) in an RNA template-dependent manner [1]. These enzymes function either as single polypeptides or in complex with other viral or host components to transcribe and replicate the viral RNA genome [2]. A unique characteristic of segmented, double-stranded (ds) RNA viruses of the *Reoviridae* family is that they retain several copies of their RdRp within a proteinaceous, icosahedral core particle throughout the infectious cycle [3]. Each viral RdRp is anchored inside the core shell, slightly off-center from each fivefold axis and is associated with one of the

10–12 dsRNA genome segments. During viral transcription, *Reoviridae* RdRps use the minus-strand of dsRNA [(-)RNA] as template for the synthesis of multiple copies of plus-strand RNA [(+)RNA]. Following their packaging into an assembling core particle, the RdRps initiate a single round of (-)RNA synthesis on each (+)RNA, creating progeny virions that contain a complete set of dsRNA genome segments. To facilitate the synthesis of RNA within a confined environment, *Reoviridae* RdRps evolved distinctive features not yet seen in other polymerases. This review discusses these features in the context of the high-resolution structures determined for the RdRps of reovirus and rotavirus, two prototypic *Reoviridae* family members [4<sup>\*\*</sup>,5<sup>\*\*</sup>].

## Overall architecture of four-tunneled *Reoviridae* RdRps

Structures of the reovirus and rotavirus RdRp ( $\lambda 3$  and VP1, respectively) have been solved by X-ray crystallography to better than 3 Å resolution, revealing complex multi-domain proteins (Figure 1A) [4<sup>\*\*</sup>,5<sup>\*\*</sup>]. These two enzymes are remarkably alike in their overall architecture, but also exhibit some differences that shed light on how individual family members mediate RNA synthesis. Similar to other polynucleotide polymerases,  $\lambda 3$  and VP1 contain central right-handed polymerase domains ( $\lambda 3$  residues 381–890; VP1 residues 333–778) with fingers, palm, and thumb subdomains (Figure 1B) [1,6]. Distinctive N-terminal and C-terminal domains tightly envelop the polymerase domains creating globular, cage-like enzymes, ~70 Å in diameter, with buried catalytic centers. The N-terminal domains ( $\lambda 3$  residues 1–380; VP1 residues 1–332) help anchor the continuous surface of the fingers and thumb subdomains, effectively closing the enzymes (Figure 1C). The C-terminal domains ( $\lambda 3$  residues 891–1267; VP1 residues 779–1089) are bracelet-like in form, resembling the sliding clamps of DNA polymerases that encircle templates and aid in processivity (Figure 1D) [7]. The surface exposed regions of the N-terminal and C-terminal domains are predicted to form interaction sites with the core shell [8<sup>\*</sup>,9]. For rotavirus, engagement of VP1 by the shell protein VP2 not only anchors the enzyme, but also is essential for its activity as demonstrated by *in vitro* RNA synthesis assays [10<sup>\*</sup>]. Reovirus  $\lambda 3$  has limited *in vitro* poly (C)-dependent poly (G) polymerase activity in the absence of its cognate shell protein  $\lambda 1$ ; yet, this enzyme probably requires  $\lambda 1$  for full activity *in vivo* [4<sup>\*\*</sup>,11]. The RdRp VP1 of blue tongue virus (BTV), another well-studied *Reoviridae* family member, also has limited, non-specific *in vitro* catalytic

Figure 1



Overall architecture of *Reoviridae* RdRps  $\lambda$ 3 and VP1. Ribbon representations of the  $\lambda$ 3 (1MUK) and VP1 (2R7Q): **(a)** apoenzymes; **(b)** right-handed polymerase domains; **(c)** N-terminal domains; and **(d)** C-terminal bracelet domains. In all images, the N-terminal domain is in yellow, the C-terminal domain in pink, and the fingers, palm, and thumb subdomains of the polymerase domain are in pale blue, brick red, and green, respectively. The extreme C-terminus of  $\lambda$ 3 (C-end) and the VP1 C-terminal plug are colored cyan blue. Boxed regions indicate subtle structural differences between the enzymes' polymerase domains, including: a four-stranded  $\beta$ -sheet that protrudes from the surface of the  $\lambda$ 3 fingers and two anti-parallel  $\beta$ -strands near the top of the VP1 fingers. The priming loops (gold) are labeled **(b)**.

activity in the absence of its core shell [12]. While the structure of BTV VP1 remains to be determined, molecular modeling predicts that it has the same three-domain architecture as  $\lambda 3$  and rotavirus VP1 [13<sup>\*</sup>]. By assaying the putative BTV VP1 N-terminal, polymerase, and C-terminal domains, either as individual units or in combination, it was shown that all three domains are required for *in vitro* activity [13<sup>\*</sup>]. This result suggests that intra-molecular interactions between the N-terminal, polymerase, and C-terminal domains of *Reoviridae* RdRps are crucial for the catalytic activity of these enzymes.

The hollow catalytic centers of *Reoviridae* RdRps are connected to the exterior through four well-defined tunnels (Figure 2). Residues of the N-terminal domain and the fingers and thumb subdomains form a template entry tunnel that binds the 3' end of (–)RNA during transcription or (+)RNA during genome replication (Figure 2A and B). Incoming NTPs and divalent cations move into the active site through a narrow tunnel formed by the N-terminal domain and the palm and fingers subdomains; this tunnel is also probably used in release of the pyrophosphate byproducts of catalysis (Figure 2B). At 20 Å in diameter, the largest tunnel in  $\lambda 3$  and VP1 extends through the C-terminal bracelet and serves as a conduit for the exit of both dsRNA replication products and (–)RNA transcription templates (Figure 2B). An interesting feature of rotavirus VP1, not seen in  $\lambda 3$ , is the presence of an  $\alpha$ -helical plug that extends 15 Å into the dsRNA/(–)RNA exit tunnel (Figure 1D) [5<sup>\*\*</sup>]. This plug considerably reduces the tunnel's diameter and would have to be displaced to accommodate dsRNA egress. The significance of the VP1 plug is not known, but mutants containing C-terminal deletions show VP2-dependent *in vitro* activities indistinguishable from wildtype controls, suggesting this structural element is not essential for polymerization [5<sup>\*\*</sup>].

Unlike other known RdRp structures, which only contain template entry, NTP entry, and dsRNA exit tunnels,  $\lambda 3$  and VP1 have a fourth tunnel, created by the bracelet and the fingers and palm subdomains (Figure 2B). Although not experimentally proven, this tunnel probably serves as an exit site for (+)RNA transcripts. By having two product exit tunnels, *Reoviridae* RdRps can ensure that (+)RNAs are effectively shuttled out of the core, while nascent dsRNA gene segments are directed towards the particle interior (Figure 2B). Likewise, the exit of (–)RNA proximal to the template entry tunnel facilitates its re-use during subsequent rounds of (+)RNA synthesis. The observation that the (–)RNA template and (+)RNA product of transcription exit the enzyme via separate tunnels indicates that  $\lambda 3$  and VP1 are capable of internally separating dsRNA duplexes. Because of this, RdRps of *Reoviridae* members are different from three-tunneled RdRps and more similar to the single subunit DNA-dependent RNA polymerases, which synthesize

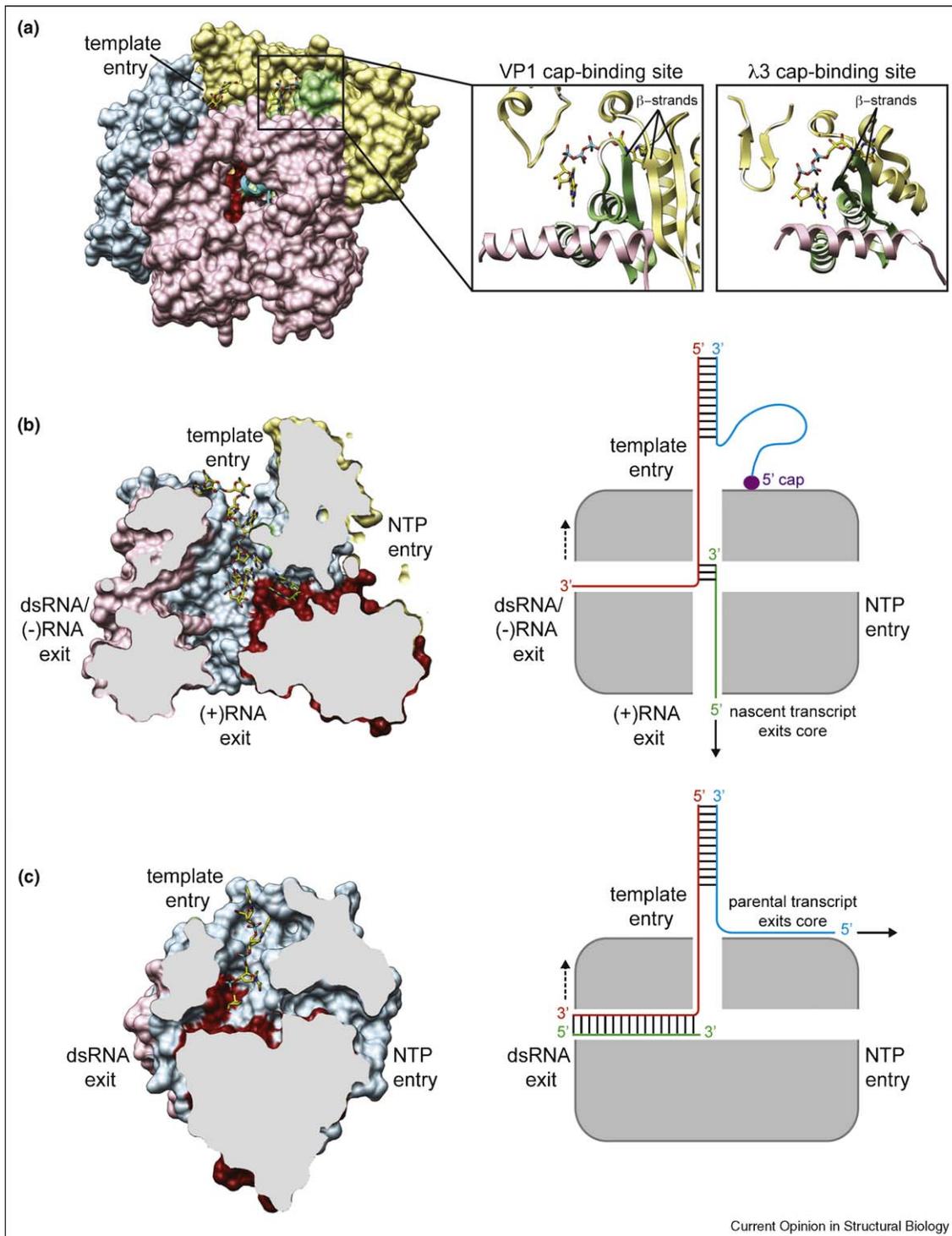
(+)RNA using a melted DNA duplex as template [14]. It is not known how  $\lambda 3$  and VP1 peel apart the nascent RNA duplexes that form within their interiors, but a loop located in the bracelet domain ( $\lambda 3$  residues 1110–1120; VP1 residues 938–943) has been proposed to play a role [4<sup>\*\*</sup>]. In contrast to  $\lambda 3$  and VP1, three-tunneled enzymes of the *Cystoviridae* family of dsRNA viruses (such as  $\phi 6$  P2) only extrude dsRNA products. The (+)RNA transcripts that leave the  $\phi 6$  core are generated by a helix unwinding activity as the (–)RNA of the dsRNA duplex enters the template entry tunnel (Figure 2C) [15<sup>\*</sup>].

A novel feature of *Reoviridae* RdRps is the presence of a N<sup>7</sup>-methyl-GpppG (cap)-binding site near the enzymes' template entry tunnels (Figure 2A and B) [4<sup>\*\*</sup>,5<sup>\*\*</sup>]. A cap analog that was soaked into the  $\lambda 3$  crystals sits in a groove and wraps around two anti-parallel  $\beta$ -strands near the tip of the thumb subdomain (Figure 2A) [4<sup>\*\*</sup>]. The analog is sandwiched in place by a collection of amino acids containing aliphatic side chains and by base-specific hydrogen bonding with each of the purine residues. Similar to  $\lambda 3$ , a binding site for a non-methylated guanosine has also been identified in rotavirus VP1. However, the thumb of VP1 lacks one of the  $\beta$ -strands important for  $\lambda 3$ , but has two additional  $\beta$ -strands formed by a region of the N-terminal domain (Figure 2A) [5<sup>\*\*</sup>]. This three-stranded anti-parallel  $\beta$ -sheet may support the VP1 cap-binding site. Given the 5' to 3' panhandle organization of the capped *Reoviridae* (+)RNAs, the cap-binding site can be presumed to play a role in recruiting the 3' end of (+)RNAs into the template entry tunnel, thereby promoting replication. During transcription, anchoring of the parental (+)RNA strand of a dsRNA gene segment to the cap-binding site may help in recycling the parental (–)RNA template back towards the entry tunnel (Figure 2B).

### Features of the $\lambda 3$ and VP1 polymerase domains

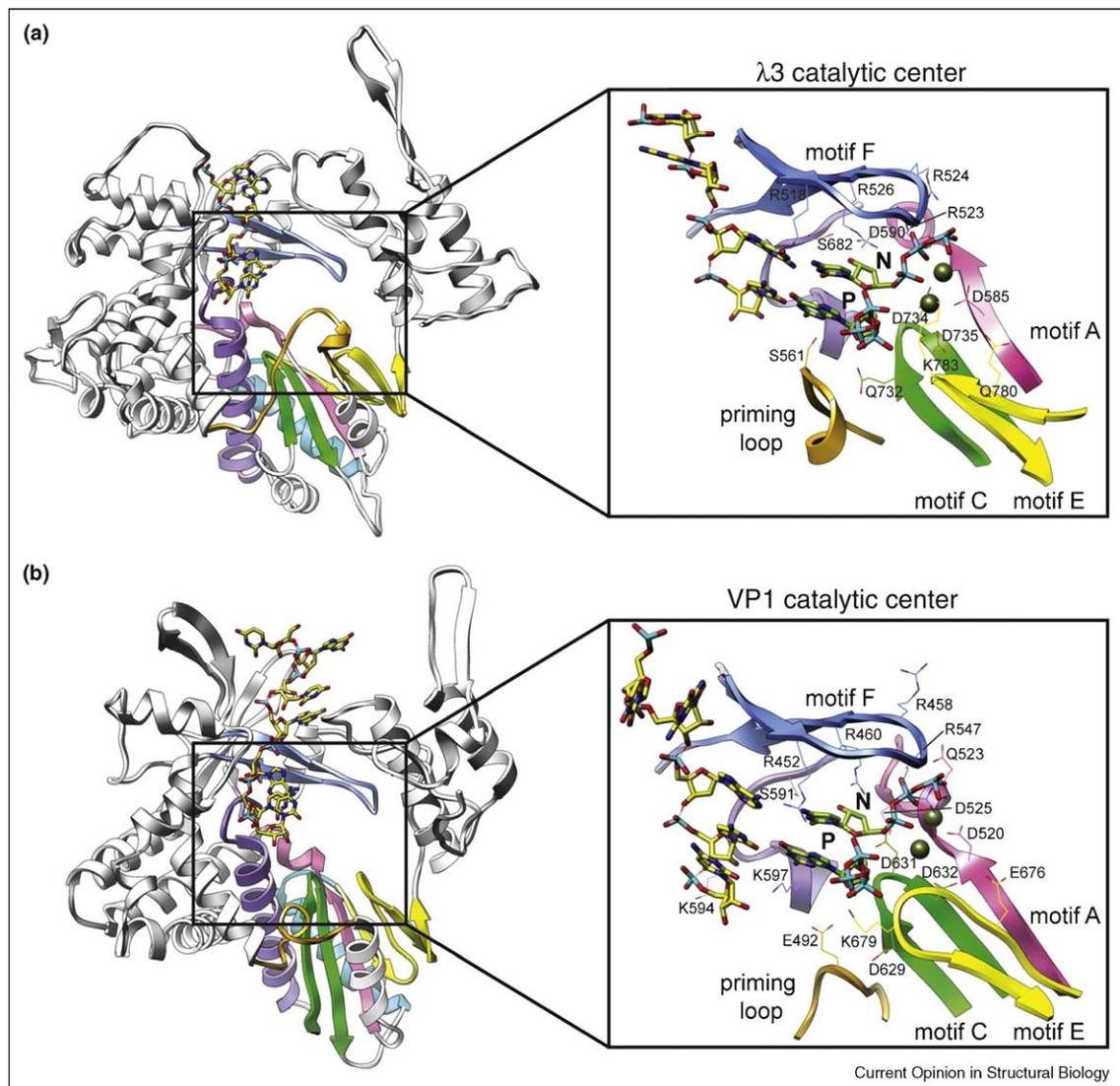
Compared to the architecture of the N-terminal and C-terminal domains, which are unique to *Reoviridae*, the central polymerase domains of  $\lambda 3$  and VP1 are more like those of other RdRps (Figures 1B and 3). The palm subdomains are extremely conserved structurally and include four anti-parallel  $\beta$ -strands flanked by three  $\alpha$ -helices [6]. The fingers subdomains of  $\lambda 3$  and VP1 resemble those of RdRps from *Flaviviridae* and *Cystoviridae* families (HCV NS5B and  $\phi 6$  P2) [15<sup>\*</sup>,16]. The thumb subdomains of both *Reoviridae* RdRps include a  $\beta$ - $\alpha$ - $\alpha$ - $\beta$  arrangement that is similar, but not identical, to the  $\beta$ - $\alpha$ - $\alpha$ - $\alpha$  arrangement of the polymerases of hepatitis C virus, poliovirus, and HIV-1 [16–18]. A distinctive feature of  $\lambda 3$  and VP1 that is absent in other RdRps is an extended loop located between the fingers and palm subdomains (Figures 1B and 3). This so-called 'priming loop' is a flexible element that binds the triphosphates of incoming NTPs during RNA synthesis. In the  $\lambda 3$  initiation complex, the loop is able to serve as a platform

Figure 2



Structure and function of four-tunneled RdRps. All images are colored as in Figure 1. (a) Surface rendering of the VP1 enzyme (2R7W) showing the location of the cap-binding site. An RNA oligo is shown in the template entry tunnel. The boxes on the right show magnified ribbon representations of the VP1 and  $\lambda 3$  cap-binding sites. A cap analog is shown (yellow element wires) on the basis of its location in  $\lambda 3$  (1MWH). The  $\beta$ -strands of VP1 and  $\lambda 3$  important for cap-binding are labeled. (b) Four-tunneled *Reoviridae* RdRps. The left image is a sagittal cutaway of the image in (a), after rotation to the left by  $90^\circ$ . Tunnels are labeled according to their known or putative functions. The right image shows a cartoon of conservative *Reoviridae* transcription. Parental (+)RNA (blue) that is peeled-off the dsRNA segment remains tethered to the cap-binding site of the RdRp. Meanwhile, parental (-)RNA (red) enters the RdRp and is used as template for nascent (+)RNA synthesis (green). The parental (-)RNA (red) and the nascent (+)RNA (green) are separated internally and exit the enzyme via individual tunnels. Nascent (+)RNA transcripts (green) acquire a 5' cap as they exit

Figure 3



$\lambda$ 3 and VP1 polymerase domains and catalytic centers. The images on the left are ribbon representations of the  $\lambda$ 3 (1N1H) and VP1 (2R7R) polymerase domains. Structural and functional motifs are colored as follows: pink (motif A), purple (motif B), green (motif C), cyan (motif D), yellow, (motif E), light blue (motif F) and gold (priming loop). The images on the right are magnifications of the catalytic centers showing residues (colored element wires; labeled) important for initiation of RNA synthesis. Two  $Mg^{2+}$  ions (olive) and free NTPs (green element sticks) are shown in the priming (P) and nucleotide (N) sites on the basis of their location in the reovirus  $\lambda$ 3 (1N1H) initiation complex. Nucleotides (yellow element sticks) of RNA oligos used in soak experiments are shown: (a)  $\lambda$ 3; 5'-UAGC-3' and (b) VP1; 5'-UGUGACC-3'.

for stabilizing a NTP at the priming (P) site (Figure 3A) [4<sup>••</sup>]. In the VP1 crystal structure, the priming loop bends away from the active site by about 90°, leaving it in a retracted state incapable of supporting a NTP and initiating RNA synthesis (Figure 3B) [5<sup>••</sup>]. As a consequence, VP1 crystals do not form a functional initiation complex

upon soaks with template,  $Mg^{2+}$ , and NTPs [5<sup>••</sup>]. Thus, the solved VP1 structure probably represents a pre-initiation complex before activation by the core shell protein, whereas the  $\lambda$ 3 structures capture the subsequent initiation and elongation phases of *Reoviridae* RNA synthesis.

(Figure 2 Legend Continued) the core (solid arrow). The parental (–)RNA (red) is recruited back to the template entry tunnel (dashed arrow) by the tethered (+)RNA (blue). (c) Three-tunneled *Cystoviridae* RdRps. The left image is a sagittal cutaway of  $\phi$ 6 RdRp P2 (1H10). Tunnels are labeled according to their known functions. The right image shows a cartoon of semi-conservative *Cystoviridae* transcription. A dsRNA genome segment is separated into parental (+)RNA (blue) that is shuttled out of the core (solid arrow) and (–)RNA (red) that is used as a template for nascent (+)RNA synthesis (green), made as a dsRNA duplex that is released from P2 via the single RNA exit tunnel. The dsRNA will be directed back towards the template entry tunnel and separated into (+)RNA and (–)RNA upon subsequent rounds of transcription.

The active sites of the enzymes include the highly conserved sequence and structural polymerase motifs (A–F) formed by the palm and fingers subdomains (Figure 3) [8<sup>\*</sup>, 19]. The residues of these motifs and the priming loop coordinate the positioning of two divalent cations and the NTPs in the N and P sites, supporting initiation complex formation. Residues important for anchoring the pair of Mg<sup>2+</sup> ions crucial to phosphodiester-bond formation are present in motif A ( $\lambda$ 3 residue D585; VP1 residues D520 and D525) and motif C ( $\lambda$ 3 residues D734 and D735; VP1 residues D631 and D632). The NTP in the N site of  $\lambda$ 3 is stabilized by several residues, including those of motif A (D590), motif B (S682), and motif F (R518, R523, R524, and R526). Similar residues of VP1 (motif A: Q523; motif B: S591; and motif F: R452, R457, R458, and R460) are predicted to stabilize an NTP in its N site. The NTP in the P site of  $\lambda$ 3 is stabilized by residues of motif C (Q732; D629 of VP1), motif E (K783; K679 of VP1), and the priming loop (S561; E492 of VP1). Appropriate positioning of a NTP in the P site is further supported by its base stacking with the N site NTP. Notably, amino acids of motif B not only interact with NTPs in the catalytic pocket, but also with the terminal residues of the template strand, supporting in-register base pairing. The myriad of interactions between the structural elements and the template RNA and NTPs allow *Reoviridae* RdRps to effectively initiate RNA synthesis in the absence of a primer.

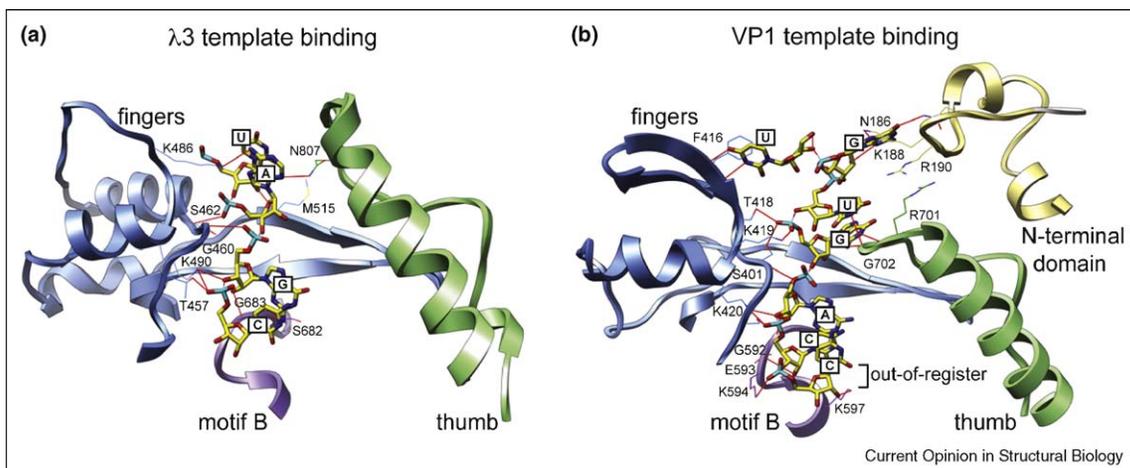
### Template binding by $\lambda$ 3 and VP1

Insight into template binding by *Reoviridae* RdRps was revealed by soaking  $\lambda$ 3 or VP1 crystals with various biologically relevant RNA oligonucleotides (oligos)

(Figure 4) [4<sup>\*\*</sup>, 5<sup>\*\*</sup>]. For both enzymes, the sugar-phosphate backbone of the RNA is largely anchored against the fingers wall through extensive hydrogen bonding, leaving the RNA bases flagging outward. For  $\lambda$ 3, oligos representing the 3' termini of reovirus transcription or replication templates (5'-UAGC-3' or 5'-UCAUC-3', respectively) interact with similar residues of the fingers subdomain: T457, G460, S462, K486, K490, M515, S682, G683, and N807 (Figure 4A). Residues of motif B (S682 and G683) play an important role in stabilizing the 3' end of the template in the catalytic pocket. While no extensive sequence-specific RNA recognition is observed with  $\lambda$ 3, the enzyme interacts preferentially with molecules containing G or U in the penultimate position [4<sup>\*\*</sup>]. Importantly, the interaction of  $\lambda$ 3 with RNA directs the terminal nucleotide of the template to sit in-register with the enzyme's P site, allowing RNA synthesis to initiate at the very end of the molecule.

The non-specific in-register RNA-binding activity of reovirus  $\lambda$ 3 is in contrast to that observed for rotavirus VP1 in complex with an oligo representing the 3' termini of replication templates (5'-UGUGACC-3') (Figure 4B) [5<sup>\*\*</sup>]. Like  $\lambda$ 3, the sugar-phosphate backbone of the RNA is bound non-specifically by residues of the VP1 fingers subdomain (S401, T418, K419, and K420), and the terminal nucleotides of the template are stabilized by motif B (G592, E593, K594, and K597). However, unlike  $\lambda$ 3, residues of the VP1 fingers and thumb subdomains, as well as the N-terminal domain (N186, K188, R190, F416, R701, and G702) directly and specifically recognize of the UGUG bases of the (+)RNA. Unexpectedly, the base-specific recognition of UGUG causes the 3' terminal

Figure 4



Template binding by  $\lambda$ 3 and VP1. Partial ribbon representations of  $\lambda$ 3 (1N1H) and VP1 (2R7R), revealing residues (colored element sticks; labeled) that engage RNA templates. The images include portions of the fingers (pale blue), palm (motif B; purple), and thumb (green) subdomains and, for VP1, the N-terminal domain (yellow). Hydrogen bonds are shown in red. (a)  $\lambda$ 3 residues engage the sugar-phosphate backbone, as well as a few bases in a sequence-independent manner allowing the RNA to sit in-register with the active site. (b) VP1 residues recognize of the UGUG nucleotides and the sugar-phosphate backbone of the (+)RNA, forcing the molecule to bind out-of-register with the active site.

nucleotide of the (+)RNA to overshoot the initiation register (Figure 4B). For proper initiation of RNA synthesis, structural changes must occur in VP1, possibly induced by VP2 binding, to re-position the (+)RNA relative to the active site. Interestingly, specific recognition was not detected when VP1 was soaked with an oligo representing the 3' end of rotavirus transcription templates (5'-AAAAGCC-3'). Because VP1 remains interminably linked to a single dsRNA segment within the confines of a core, the need for extensive sequence-specific recognition of the (–)RNA template during transcription is negated. By contrast, improper recognition of (+)RNA during packaging and genome replication would be catastrophic for the viability of progeny rotavirus particles. Given this notion, why did reovirus  $\lambda 3$  not show VP1-like sequence-specific recognition of its cognate (+)RNA (5'-UCAUC-3') in structural studies? One possibility is that  $\lambda 3$  may recognize bases of the (+)RNA that are upstream of those used in soaking experiments. It is also possible that once the *Reoviridae* RdRps are in an activated form, like what was captured for  $\lambda 3$  crystals, the high-affinity, specific interactions with (+)RNA are lost to allow for promoter clearance. This latter idea is consistent with the hypothesis that the VP1 structure represents a *Reoviridae* RdRp before packaging, while  $\lambda 3$  emulates the activated form of the enzyme following core shell binding.

### RNA synthesis by $\lambda 3$

Crystalline  $\lambda 3$  catalyzes phosphodiester-bond formation when soaked with short template RNAs,  $Mn^{2+}$ , and NTPs [4<sup>••</sup>]. The products of the activity are RNA duplexes, of the type that would be expected to form during genome replication. Indeed, the RNA duplexes are aligned within the polymerase in a manner consistent with their egress through the dsRNA exit tunnel of the C-terminal bracelet domain. Guidance of the dsRNA product towards the exit stems from hydrogen bonds and van der Waals contacts formed between the RdRp and the minor groove of the RNA product.

To accommodate the elongating RNA duplex in the active site, the priming loop is downwardly retracted relative to its extended position in the apoenzyme. The retracted priming loop fits snugly into the minor groove of the elongating RNA duplex, and probably springs back to its extended conformation upon clearance of product RNA from the active site. Failure of the priming loop to shift downward would present a physical barrier to elongation and may lead to abortive initiation. Modeling of longer elongating RNA duplexes within  $\lambda 3$  indicates that unless bent, the product RNA strand would probably collide with a loop extending from the C-terminal bracelet domain [4<sup>••</sup>]. As discussed above, the interaction of the duplex product with this loop may initiate strand separation events during RNA transcription that induce nascent transcripts to move into the (+)RNA exit

tunnel of the RdRp. As measured from the polymerase active site, it takes 8–9 bases for a nascent RNA to reach the possible separator loop of the bracelet and a total of ~13–14 bases before a duplex RNA product begins to extrude beyond the dsRNA product tunnel.

### Conclusions

Combined with previous biochemical studies, the high-resolution  $\lambda 3$  and VP1 structures have allowed for the development of a model for *Reoviridae* RNA synthesis. The RdRps of this virus family function while tethered within an icosahedral particle composed of 60 asymmetric dimers of shell protein [3]. During transcription, *Reoviridae* RdRps are in an activated form owing to their inherent interactions with the core shell. The (–)RNA strand of each dsRNA is non-specifically bound in-register with the enzyme's active site, and the incoming NTPs and  $Mg^{2+}$  ions are positioned appropriately for phosphodiester-bond formation. The (+)RNA products of transcription are separated from nascent dsRNA, exit the enzyme via a unique fourth tunnel, and acquire a 5' cap as they leave the core particle. By contrast, *Reoviridae* genome replication must be coordinated with (+)RNA packaging and is, therefore, a temporally regulated process. For rotavirus, the (+)RNA replication template is specifically engaged by VP1 and positioned out-of-register with the enzyme's active site. In this pre-initiation complex, the priming loop is also retracted, thereby further preventing the start of dsRNA synthesis. Only following packaging of these protein–RNA complexes into an assembling core shell do *Reoviridae* RdRps become activated by structural rearrangements that may include shifting the (+)RNA into register and lifting the priming loop. During elongation, the priming loop must retract again and, for rotavirus VP1, the C-terminal plug must move to allow the nascent dsRNA to exit the enzyme and be deposited in the interior of the particle.

While providing unprecedented insight into the inner workings of *Reoviridae* RdRps, the structures determined for  $\lambda 3$  and VP1 have also raised several important questions: What molecular attributes influence the RdRp to function as a transcriptase versus replicase? How are the activities of individual RdRp units coordinated so they operate simultaneously within a core? What structural changes occur to allow for RdRp activation following core shell binding? Future studies addressing these questions will not only enhance our understanding of *Reoviridae* RNA synthesis, but might also aid in the rational design of therapeutics to inhibit these important pathogens.

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- of special interest
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