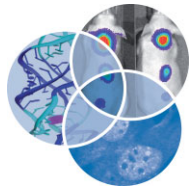
Advanced Review



RNA synthetic mechanisms employed by diverse families of RNA viruses

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RNA viruses are ubiquitous in nature, infecting every known organism on the planet. These viruses can also be notorious human pathogens with significant medical and economic burdens. Central to the lifecycle of an RNA virus is the synthesis of new RNA molecules, a process that is mediated by specialized virally encoded enzymes called RNA-dependent RNA polymerases (RdRps). RdRps directly catalyze phosphodiester bond formation between nucleoside triphosphates in an RNA-templated manner. These enzymes are strikingly conserved in their structural and functional features, even among diverse RNA viruses belonging to different families. During host cell infection, the activities of viral RdRps are often regulated by viral cofactor proteins. Cofactors can modulate the type and timing of RNA synthesis by directly engaging the RdRp and/or by indirectly affecting its capacity to recognize template RNA. High-resolution structures of RdRps as apoenzymes, bound to RNA templates, in the midst of catalysis, and/or interacting with regulatory cofactor proteins, have dramatically increased our understanding of viral RNA synthetic mechanisms. Combined with elegant biochemical studies, such structures are providing a scientific platform for the rational design of antiviral agents aimed at preventing and treating RNA virus-induced diseases.

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INTRODUCTION

Viruses with RNA genomes comprise a vast and assorted group, whose members can differ in their particle architecture, host tropism, and pathological manifestations. RNA viruses infect nearly every type of living organism—from archea to unicellular bacteria and fungi to multicellular plants and animals. Many RNA viruses asymptomatically replicate within their hosts, inducing no obvious disease. Others lead to severe morbidity and/or mortality and therefore have important medical, economic, and social impacts. Specifically, RNA viruses are known to cause devastating diseases in humans, including but not limited to: systemic rashes (measles virus and rubella virus), gastrointestinal diseases (Norwalk virus, rotavirus, and hepatitis A and E viruses), respiratory diseases (SARS-coronavirus, paramyxovirus, rhinovirus, and influenza A virus), chronic hepatitis/liver cancer (hepatitis C virus), hemorrhagic diseases (dengue virus and ebolavirus) and neurological diseases (West Nile virus, poliovirus, bunyaviruses, and rabies virus). In fact, of the 51 distinct families of RNA viruses currently recognized by the International Committee on the Taxonomy of Viruses, approximately 14 of them contain known or suspected human pathogens (http://ictvonline.org).

Investigation of human RNA viruses and laboratory model animal strains from various families has shed light on a fundamental aspect of their

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TABLE 1 | Select RNA Virus Families, Pathogenic Human Virus Members, and RNA Genome Features

		Genome Features		
Virus Family	Pathogenic Human Virus Members	Polarity	Organization	~Size Range (kb)
Caliciviridae	Norwalk virus	(+)RNA	Nonsegmented, VPg-bound	7.5–8.5
Flaviviridae	Hepatitis C virus, dengue virus, West Nile virus	(+)RNA	Nonsegmented	9.6–12.3
Picornaviridae	Poliovirus, rhinovirus, hepatitis A virus	(+)RNA	Nonsegmented, VPg-bound	7.2–9.0
Rhabdoviridae	Rabies virus	(−)RNA	Nonsegmented, RNP	11–15
Orthomyxoviridae	Influenza A virus	(−)RNA	8-segmented, RNP	12–15
Reoviridae	Group A rotavirus	dsRNA	9- to 12-segmented	16–27

lifecycle—how they synthesize new RNA molecules. During infection, an RNA virus must mediate at least two RNA synthetic processes: (1) transcription of message-sense viral RNAs (i.e., mRNAs) that can serve as templates for protein synthesis and (2) replication of viral genomic RNAs for their ultimate encapsidation into nascent virion particles. Both transcription and genome replication are mediated by specialized, virally encoded enzymes called RNA-dependent RNA polymerases (RdRps).[1] RdRps are quite conserved among RNA viral families, and they share distant structural and functional homology to cellular DNA and RNA polymerases. However, these viral enzymes have no counterpart in the host cell, making them ideal targets for antiviral drug development.[2,3] Although RdRps are the sole polypeptides that catalyze phosphodiester bond formation between nucleoside triphosphates (NTPs) in an RNA-templated manner, these enzymes almost never function alone in the context of an infected cell. Instead, they are usually found along with other viral and/or cellular proteins in higher ordered, multisubunit polymerase complexes. Interactions between the RdRp and cofactor proteins in the polymerase complex allow RNA viruses to precisely regulate the type and timing of viral RNA synthesis during infection and coordinate it with other lifecycle events. In this review, we explore the formation and function of polymerase complexes from several diverse RNA viruses. Particular attention will be paid to summarizing the results of high-resolution structural studies of RdRps and regulatory cofactor proteins from six different RNA viral families (Caliciviridae, Flaviviridae, Picornaviridae, Rhabdoviridae, Orthomyxoviridae, and Reoviridae) (Table 1). This review is meant to serve as a general introduction into the complex world of RNA virus transcription and genome replication. Where appropriate, detailed review articles are referenced to provide readers enhanced insight into specific RNA synthetic mechanisms.

TRANSCRIPTION AND GENOME REPLICATION STRATEGIES DICTATED BY RNA GENOME POLARITY

RNA viruses encode within their genomes many of the proteins necessary for their replication, particularly those proteins that are involved in viral RNA synthesis. However, they lack the key machinery to carry out protein synthesis. Thus, viruses are parasites of host cell ribosomes, and they must express their genes as functional mRNA molecules early in infection. The pathways leading from genome to mRNA can vary widely among the different RNA viral families. Nonetheless, the transcription strategy (and thus, the genome replication strategy) of an RNA virus generally depends upon the polarity of its genome (Figure 1).

Positive-Strand RNA Viruses

Viruses with positive-strand RNA [(+)RNA] genomes are abundant in nature, and they are particularly common in plants (http://ictvonline.org). The Caliciviridae, Flaviviridae, and Picornaviridae families are well characterized because they include several notable human (+)RNA viral pathogens (Table 1). A shared feature of (+)RNA viruses is that their genomes are inherently message-sense. In fact, the genomes of these viruses often contain 5′ 7-methylguanosine (m^7G) caps and/or 3′ poly-A tails much like those of cellular mRNAs, allowing them to be efficiently recognized by the host protein synthesis machinery. Members of the Picornaviridae family, such as poliovirus, rhinovirus, hepatitis A virus, and foot-and-mouth disease virus, do not have 5′ capped (+)RNA genomes, but instead they employ RNA stem-loop elements as an internal ribosomal entry sites.[4] Moreover, translation of the Caliciviridae genome is dependent on the presence of a small, virally encoded protein (VPg), which is attached to the 5′ end of the incoming (+)RNA and interacts with components of the host protein

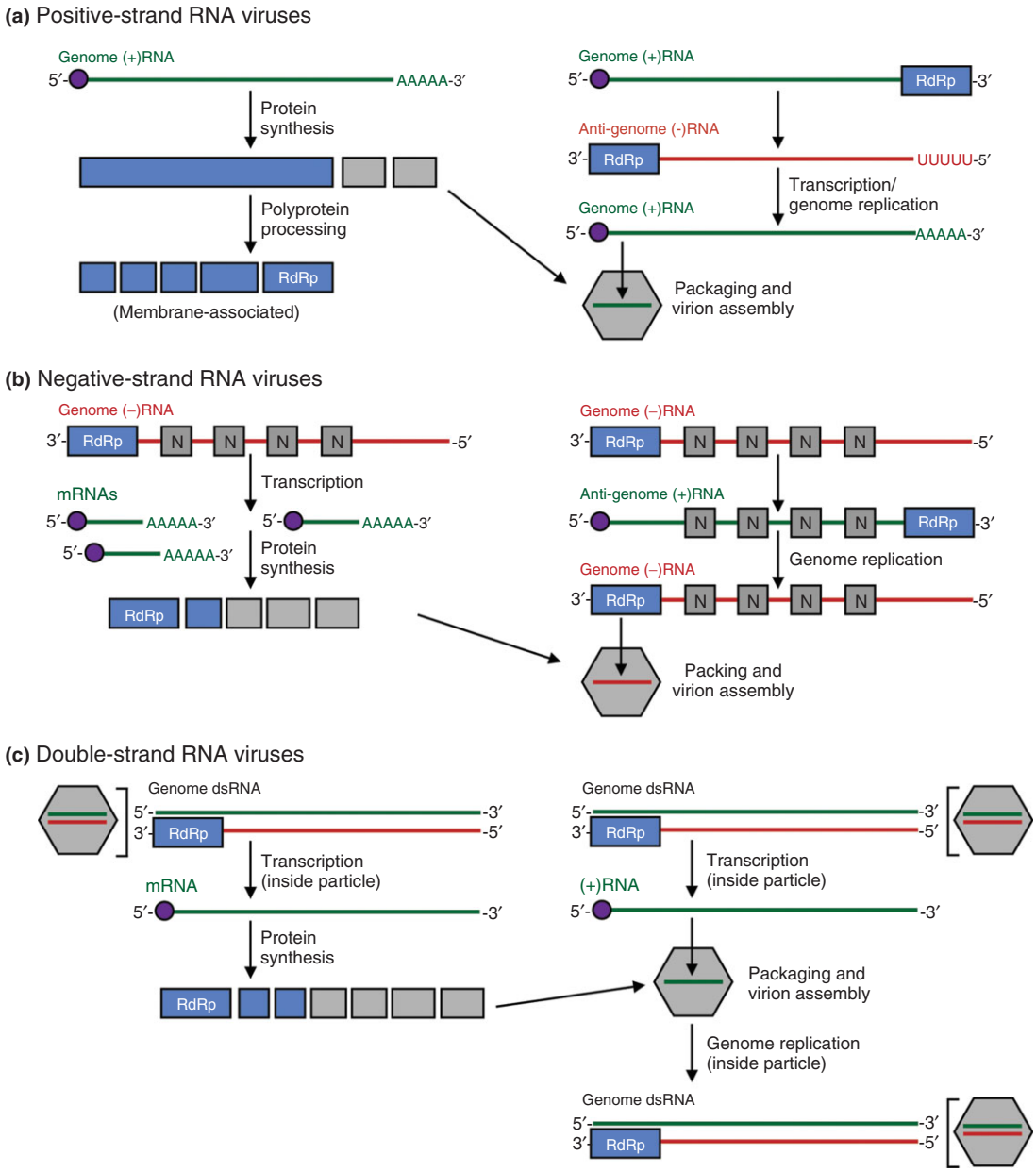

FIGURE 1 | Overview of transcription and replication strategies for various types of RNA viruses. (a) Positive-strand RNA [(+)RNA] viruses. The genomes of (+)RNA viruses are message-sense (green), and they often contain a 5′ m^7G cap (purple circle) and 3′ poly-A tail (AAAAA). Host cell ribosomes translate the genome into one or more polyproteins, which are cotranslationally and posttranslationally processed by virally encoded proteases. Some of the mature polyprotein processing precursors and products include the RNA-dependent RNA polymerase (RdRp; blue rectangle) and cofactors (blue squares) that mediate viral RNA synthesis in association with cellular membranes. Other proteins made by the virus include those that will assemble into viral particles (gray squares). The RdRp mediates the synthesis of negative-strand RNA [(−)RNA] antigenome (red) using the genome as template. The antigenome is then converted into new (+)RNA genome by the RdRp and then packaged into nascent virion particles (gray hexagon). (b) Negative-strand RNA [(−)RNA] viruses. The genomes of (−)RNA viruses are organized as ribonucleoproteins (RNPs) with their RNA molecules (red) bound by the viral polymerase complex (blue rectangle) and nucleocapsid proteins (N; grey square). Transcription is mediated by the RdRp and cofactor proteins, resulting in the synthesis of 5′ capped (purple circle), 3′ polyadenylated (AAAAA) mRNAs (green). The mRNAs are translated by host ribosomes into polymerase complex proteins (blue) and virion structural components (gray). Genome replication occurs when the RdRp produces a full-length, antigenome (+)RNA (green) bound by N. This antigenome is then converted into (−)RNA genome (red) and is packaged as a RNP complex into nascent virion particles (gray hexagon). (c) Double-stranded (ds) RNA viruses. The genomes of dsRNA viruses are maintained inside of particles. The RdRp (blue rectangle) will transcribe (+)RNAs (green) that contain a 5′ cap (purple circle) and serve as protein synthesis templates (mRNAs) or will be packaged into assembling subviral particles (gray hexagon). Once inside the particle, the RdRp will perform (−)RNA synthesis (red) on the (+)RNS template (green) to create the dsRNA genome.

synthesis machinery.[5,6] As described below, VPg also serves an important role in initiation of RNA synthesis for Caliciviridae.

Given the inherent translatability of their genomes, (+)RNA viruses do not need to encapsidate RdRps into their virions. Instead, these viruses utilize host ribosomes to make their RdRps and polymerase complex cofactors once inside the cell (Figure 1(a)). A characteristic trait of (+)RNA viruses is the manner in which their polymerase complexes are formed early in infection. Following entry of a (+)RNA virus into the host cell, the genome is translated into at least one large polyprotein, which is cotranslationally and posttranslationally cleaved by viral proteases into smaller, mature, nonstructural protein products (Figure 1(a)). One of these products is the actual RdRp polypeptide, whereas the other products (and oftentimes precursors) represent regulatory cofactors that play numerous roles during viral RNA synthesis. Most often, the RdRp-containing polyprotein of (+)RNA viruses is intimately associated with membranes of intracellular organelles (e.g., endoplasmic reticulum, mitochondria, endosomes, and lysosomes).[7] In fact, (+)RNA viruses are known to hijack and rearrange cellular membranes in order to fashion scaffolds on which (or within which) the RdRp mediates transcription and genome replication.

Following its synthesis and assembly, the membrane-bound polymerase complexes of (+)RNA viruses will copy genome (+)RNA into full-length, antigenome (−)RNA (Figure 1(a)). Biochemical studies indicate that, for many (+)RNA viruses, the antigenome might remain hybridized to the genome, forming a transient double-stranded RNA (dsRNA) molecule.[8–10] The (−)RNA itself or that of the dsRNA is then copied by the polymerase complex into new, genome (+)RNA that can be packaged into nascent virions (Figure 1(a)). For some (+)RNA viruses, the (−)RNA strand can serve as template for more than one RdRp at a time. As such, numerous (+)RNAs can be simultaneously transcribed from a single (−)RNA.[11–13] Because the (+)RNA genome of these viruses is also a functional mRNA molecule, the processes of transcription and genome replication can be essentially the same (Figure 1(a)). In some cases, however, subgenomic (i.e., smaller than genome) mRNAs are generated, thereby allowing (+)RNA viruses to express multiple genes/proteins from a single polycistronic template.[14]

Negative-Strand RNA Viruses

Viruses with negative-strand RNA [(−)RNA] genomes are found in several different families (http://ictvonline.org). Those commonly known to cause disease in humans are either members of the order Mononegavirales (which includes families Bornaviridae, Filoviridae, Paramyxoviridae, and Rhabdoviridae) or are members of the family Orthomyxoviridae (Table 1). Unlike the genomes of (+)RNA viruses, those of (−)RNA viruses cannot be directly translated into protein. Instead, the genomic (−)RNA must first be copied into mRNA prior to being used as templates by host ribosomes (Figure 1(b)). Thus, all known (−)RNA viruses will package their polymerase complex, including their RdRp polypeptide, inside of the virion particle. Viruses in the Rhabdoviridae and Orthomyxoviridae families are the most well studied in terms of RNA synthetic strategies. These viruses have single-stranded, (−)RNA genomes that are directly bound by a viral polymerase complex. The genomes of Rhabdoviridae and Orthomyxoviridae members are also coated with a nucleocapsid protein, forming a tightly compact ribonucleoprotein (RNP) structure.[15–17] The RdRps of (−)RNA viruses will only catalyze RNA synthesis using RNP templates.[15,18]

Soon after entry of a (−)RNA virus into the host cell, the polymerase complex will commence viral transcription on the bound RNP[18] (Figure 1(b)). The mRNAs generated via this process are not encased by nucleocapsid protein, and they usually contain a 5′ m^7G cap structures and 3′ poly-A tails. As such, the viral mRNAs are translated very efficiently by host ribosomes, generating virion structural proteins and new polymerase complex components. For the Rhabdoviridae member, vesicular stomatitis virus, transcription occurs via a start-and-stop mechanism, whereby the RdRp pauses at regulatory signals in the RNA and then reinitiates at the next gene start.[19] Termination of the RdRp at intergenic regions also includes a slippage on a poly-U tract to generate the poly-A tails of the mRNAs.[19] Genome replication for vesicular stomatitis virus then occurs when the polymerase complex creates a full-length, antigenome (+)RNA by ignoring the transcriptional signals (Figure 1(b)). This antigenome is organized as an RNP, allowing it to be effectively copied into a (−)RNA genome molecule (Figure 1(b)). While influenza A virus and other members of Orthomyxoviridae exhibit a similar RNP-dependent transcription and replication strategy, they do not employ a start-and-stop mechanism. Instead, the influenza A virus polymerase complex creates nearly full-length mRNA copies of each of the eight (−)RNA genome segments.[20,21] Replication of the viral genome is templated by full-length antigenome (+)RNA

intermediates, which are copied into (−)RNA strands and encapsidated into RNPs.[18] Unlike (+)RNA viruses, which perform transcription and genome replication in intimate association with cellular membranes, RNA synthesis for (−)RNA viruses occurs either in cytosolic inclusions (e.g., Rhabdoviridae) or in the host nucleus (e.g., Orthomyxoviridae).

Double-Stranded RNA Viruses

Viruses with genomes comprised of dsRNA are more limited in diversity compared with those having (+)RNA or (−)RNA genomes. Rotaviruses are members of the Reoviridae family and are the most pathogenic dsRNA virus of humans, causing acute gastroenteritis in children[22] (Table 1). Rotaviruses contain 11 dsRNA genome segments and virion-associated polymerase complexes. A unique characteristic of rotaviruses and other Reoviridae members is that they perform all stages of RNA synthesis within a subviral particle.[23] More specifically, upon entry of the dsRNA virus into the host cell cytoplasm, the genome is maintained inside an icosahedral capsid; it is not released into the cytosol like those of (+)RNA and (−)RNA viruses (Figure 1(c)). The RdRps within the capsid transcribe mRNAs using the (−)RNA strands of the dsRNA as templates. A single RdRp is thought to be dedicated to each of the dsRNAs, and it is tethered to the inner surface of the capsid, near the icosahedral vertices.[24] New (+)RNAs acquire a 5′ m^7G cap via viral enzymes as they exit the capsid through aqueous channels. Viral (+)RNAs lack poly-A tails, but nonetheless serve as mRNAs to direct efficient protein synthesis. Two of the rotavirus-encoded proteins induce the formation of inclusions in the host cell cytosol where early particle assembly and genome replication occur.[25] These inclusions are not necessarily membrane-associated, but have been shown to contain markers of autophagosomes and lipid droplets.[26,27]

Within viroplasms, (+)RNAs made by transcriptionally active subviral particles are packaged into assembling viral cores along with several copies of the viral RdRp[28] (Figure 1(c)). Inside a fully or partially assembled core particle, the RdRps perform minus-strand synthesis to convert the packaged (+)RNAs into dsRNA genome segments[29] (Figure 1(c)). A single RdRp is dedicated to each of the packaged (+)RNA segments, but acts in synchrony with the others to simultaneously replicate the dsRNA genome.[30] Thus, at its most minimal, the polymerase complex of a dsRNA virus is comprised of a capsid scaffold upon which the RNA-template-bound RdRps function.[31]

STRUCTURE AND FUNCTION OF THE CATALYTIC RdRp POLYPEPTIDE

Conserved RdRp Structural Features

All RNA viruses, regardless of their genome structure or polarity, encode an RdRp polypeptide that catalyzes phosphodiester bond formation between NTPs in an RNA-template-directed manner.[1] To date, there are more than 60 high-resolution X-ray crystal structures of viral RdRps available in the Protein Data Bank (http://www.rcsb.org/). Although RdRps from different RNA viral families can vary tremendously in their amino acid sequences, they are strikingly similar in their three-dimensional organization. For example, the RdRps of poliovirus, Norwalk virus, hepatitis C virus, and rotavirus (residues 333–778) all exhibit a central cupped, right-handed architecture with fingers, palm, and thumb subdomains[31–35] (Figure 2). This general design is also shared with the distantly related DNA-dependent DNA polymerases, DNA-dependent RNA polymerases, and reverse transcriptases.[1] Unlike these other polymerases, however, which open and close upon templates, RdRps adopt closed structures mainly because of intramolecular interactions between the fingers and thumb subdomains. For (+)RNA viruses in the Caliciviridae, Flaviviridae, and Picornaviridae families, the closed conformation of their RdRps creates three distinct tunnels from the surface of the molecules into the catalytic centers, allowing for (1) the entry of the RNA template, (2) the entry of NTPs and exit of pyrophosphates, and (3) the exit of a transient dsRNA duplex.[36] Indeed, RdRps of (+)RNA viruses have been extensively studied structurally and functionally, followed closely by those of dsRNA viruses. RdRp polypeptides of (−)RNA viruses, such as those in the Rhabdoviridae and Orthomyxoviridae families, have been difficult to express and purify.[17] As such, little high-resolution structural information exists for the RdRps of (−)RNA viruses (see also below).[18,37]

Six highly conserved sequence and structural motifs (A–F) are shared among RdRps and confer specific functions during viral RNA synthesis[38] (Figure 3). Several of these motifs (A–D) are also seen in cellular polymerases and reverse transcriptases, suggesting that they are critical for the nucleotidyltransferase reaction. Motifs A and C are the most well characterized for RdRps, as they contain the aspartic acid residues that coordinate the two metal ions required for phosphodiester bond formation.[39] Specifically, the carboxylate groups of these amino acids anchor a pair of divalent metal ions (usually Mg^{2+}), which play the major role in catalysis. One metal ion promotes the deprotonation of the 3′-OH of the nascent primer,

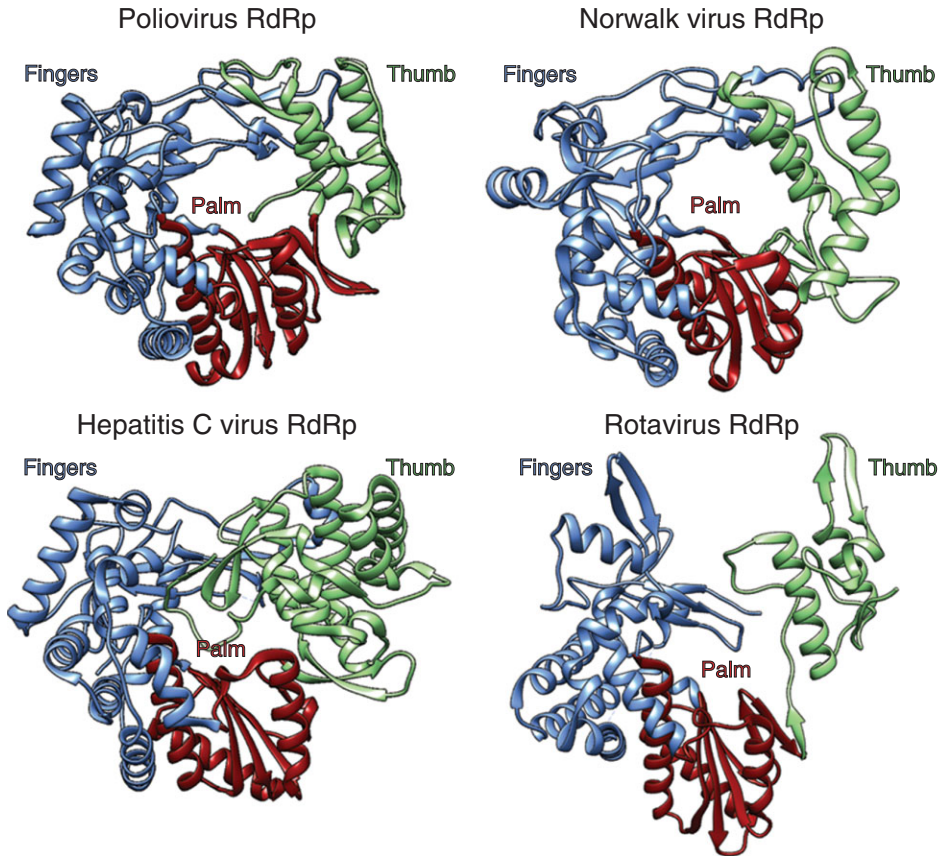

FIGURE 2 | Conserved three-dimensional architecture of RNA-dependent RNA polymerases (RdRps) from disparate RNA viruses. Ribbon representations of the poliovirus RdRp (PDB#1RA6), the Norwalk virus RdRp (PDB#1SH0), the hepatitis C virus RdRp (PDB#1C2P), and the central domain of the rotavirus RdRP (PDB#2R7Q). The fingers, palm, and thumb subdomains are shown in light blue, red, and light green, respectively.

whereas the other ion facilitates the formation of the pentacovalent transition state at the α-phosphate of the NTP and the exit of the inorganic pyrophosphate group.[40] For the poliovirus RdRp, other motifs are known to be involved in nucleotide recognition and binding (A and B), enzyme fidelity (A and D), and initiation of RNA synthesis (E and F)[38,40,41] (Figure 3). While only some of these motifs have been experimentally confirmed for other RdRps, such as those in rotavirus, they are expected to play analogous roles[35,42] (Figure 3). Finally, some argue for the existence of a seventh RdRp motif (i.e., motif G), which is located partially within the fingers subdomain and is implicated in primer and template binding.[43]

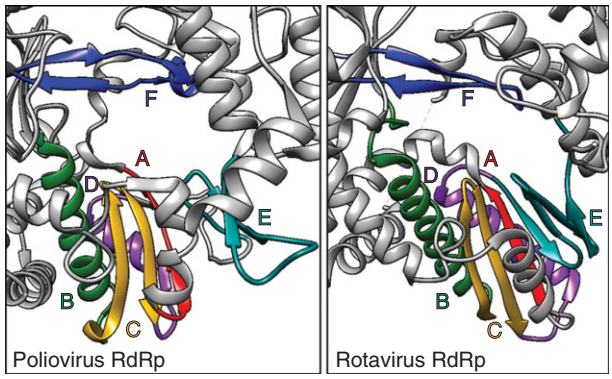

FIGURE 3 | Structural motifs of the RNA-dependent RNA polymerase (RdRp) catalytic center. Ribbon representations of the poliovirus (PDB#1RA6) and rotavirus (PDB#2R7Q) RdRp are shown. Motifs are labeled and colored as follows: motif A (red), motif B (green), motif C (gold), motif D (purple), motif E (cyan), and motif F (blue).

The RdRps of viruses from different families can also show unique structural elements in addition to their shared, canonical right-handed architecture. The RdRps of Reoviridae members, rotavirus and reovirus, are prime examples because they contain a central right-handed polymerase domain as well as two additional domains comprised of the N- and C-termini[35,44] (Figure 4). The globular N-terminal domain of the rotavirus RdRp helps anchor the continuous surface of the fingers and thumb subdomains, effectively closing the enzyme. For the rotavirus RdRp, several residues of the N-terminal domain are involved

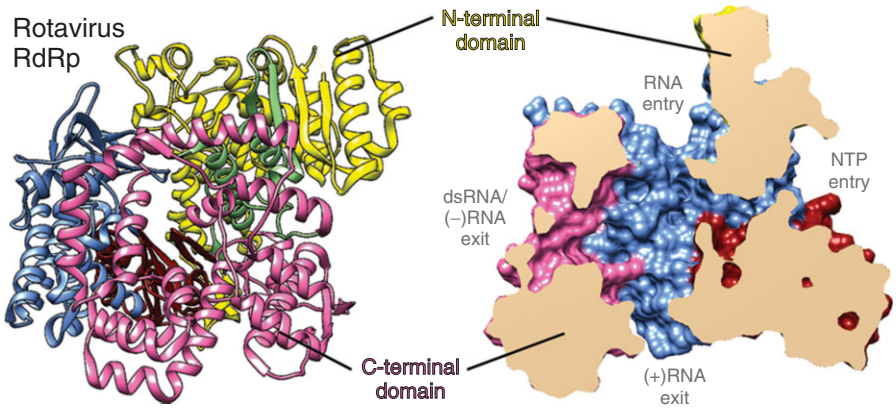

FIGURE 4 | Unique structural features of the Reoviridae RNA-dependent RNA polymerases (RdRps). Left: Ribbon representation of the entire rotavirus RdRp (PDB#2R7Q). The central polymerase domain of the enzyme contains fingers (light blue), palm (red), and thumb (light green) subdomains. This canonical region is surrounded by a C-terminal bracelet-like domain (pink) and an N-terminal domain (yellow). Right: Surface representation of the rotavirus RdRp turned 90° to the left compared to the ribbon representation. This image is also computationally sliced, so as to view the four tunnels involved in RNA template entry, RNA template and product exit, and nucleoside triphosphate (NTP) entry.

in RNA template interactions.[35,45] The C-terminal domain resembles the sliding clamps of cellular RNA polymerases, which encircle DNA templates and aid in processivity.[46] While the precise functions of these additional domains are not understood, they are known to be critical for the catalytic activity of the enzyme. In fact, their presence creates a caged enzyme with a central hollow active site. The C-terminal bracelet helps create a fourth tunnel (along with the fingers and palm subdomains) in the Reoviridae RdRps. Unlike other known RdRps, which only contain template entry, NTP entry/pyrophosphate exit, and dsRNA exit tunnels, those of Reoviridae have a fourth tunnel that likely serves as an exit site for (+)RNA transcripts.[35]

Initiation of RNA Synthesis by Viral RdRps

RNA synthesis by viral RdRps always occurs in a 5′ to 3′ direction, and it can be separated into two distinct steps: initiation and elongation. The primer requirement for the initiation step of RNA synthesis varies among the different viral RdRps. Some viral RdRps can initiate *de novo* (in the absence of primer), whereas others require the presence of a protein or RNA oligonucleotide. During *de novo* initiation, the first phosphodiester bond is formed between the 3′-OH of the initiating NTP and the 5′ triphosphate of the second NTP. In most cases, initiation takes place at the exact terminus of the template RNA, and as such, the initiating nucleotide is dictated by the template. However, for Flaviviridae members, hepatitis C virus and bovine viral diarrheal virus, initiation of RNA synthesis requires a high concentration of GTP, irrespective of the RNA template.[47,48] Thus, GTP does not serve as the initiating NTP, but instead plays a key structural role supporting the initiating NTP.[49,50] The GTP-binding site that provides a *de novo* initiation platform is best described for the bovine viral diarrheal virus RdRp.[51] The GTP is bound inside the template entry tunnel, 6 Å from the catalytic site, and makes contacts with fingers, palm, and thumb subdomains (Figure 5(a)). While there is currently no high-resolution structure of a Flaviviridae RdRp in the midst of initiation (i.e., with divalent ions, GTP, RNA template, and an initiating NTP), it is suggested that the special GTP may position the 3′-OH of the first NTP for nucleophilic attack.[51] The stabilizing GTP is not incorporated into the nascent RNA strand, but rather is thought to be released from the active site during RNA elongation. In addition to this special GTP, a C-terminal loop element is implicated in *de novo* initiation for Flaviviridae RdRps[52] (Figure 5(a)). This loop likely supports the special GTP, but it must move out of the way during elongation so as not to obstruct the egress of the nascent dsRNA.

Reoviridae RdRps also initiate RNA synthesis using a primer-independent mechanism. However, rather than utilizing a GTP molecule and C-terminal loop to support the initiating NTP, these RdRps employ an internal loop, which is located in between the fingers and palm subdomains[35,44] (Figure 5(b)). In fact, both the first and the second NTPs are stacked together by the priming loop from the bottom and by residues of motif F from the top.[35,44] For the reovirus RdRp, the first two NTPs are further supported by base-pair interactions with the RNA template. Following phosphodiester bond formation between these NTPs, a stable dinucleotide is formed, and the

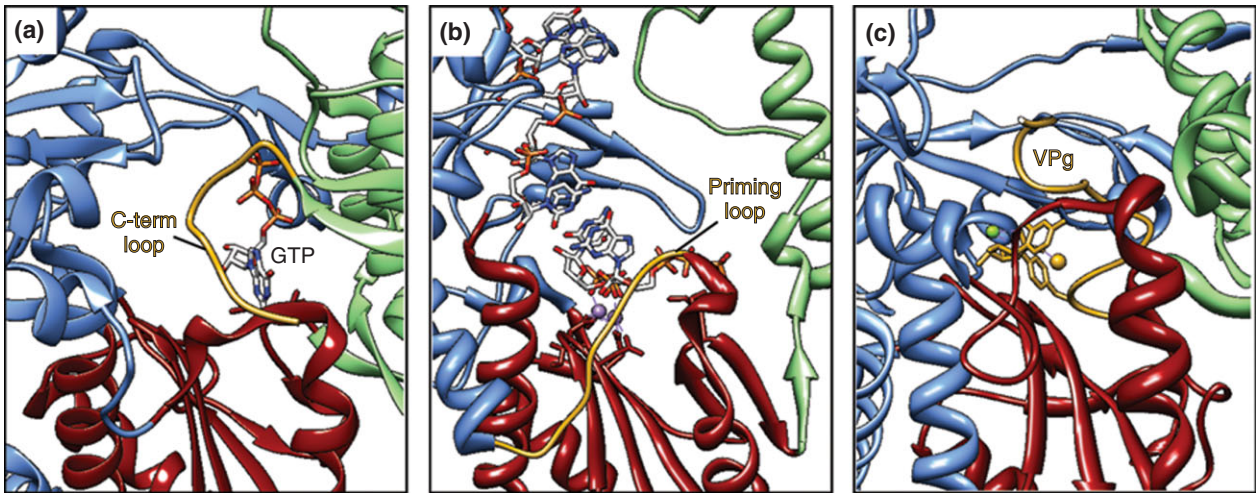

FIGURE 5 | Initiation mechanisms utilized by diverse viral RNA-dependent RNA polymerases (RdRps). Ribbon representations of the catalytic centers of RdRps with different mechanisms of initiation. In all images, the fingers, palm, and thumb subdomains are colored light blue, red, and light green, respectively. (a) *De novo* initiation by the bovine diarrheal virus RdRp (PDB#1S49) as a model for the Flaviviridae family. In this image, the special GTP that stabilizes the initiating nucleotide is shown as element sticks and the C-terminal loop is shown in gold and labeled. (b) *De novo* initiation by the reovirus RdRp (PDB#1N1H) as a model for the Reoviridae family. In this image, the RNA template and nucleoside triphosphates (NTPs) are shown as element sticks and the priming loop is shown in gold. Two divalent cations are shown as lavender spheres. (c) VPg-primed initiation by the foot-and-mouth disease virus RdRp (PDB#2F8E) as a model for Picornaviridae and Caliciviridae. In this image, VPg-UMP is shown in gold and a cation is shown in green.

RdRp transitions to elongation mode.[44] Interestingly, in the rotavirus RdRp apoenzyme structure, this loop is in a retracted state, incapable of NTP binding.[35] It is hypothesized that engagement of the rotavirus RdRp by its cofactor protein induces a structural change in the position of the priming loop, thereby allowing for NTP binding and, thus, the initiation of RNA synthesis (see also below).[35]

In contrast to *de novo* initiating enzymes, RdRps that employ a primer-dependent mechanism of initiation will use either a protein or an oligonucleotide of defined origin. Members of the Caliciviridae and Picornaviridae families, such as Norwalk virus, poliovirus, rhinovirus, hepatitis A virus, and foot-and-mouth disease virus, employ VPg, a small (~20–100 amino acids in length), virally encoded protein, to prime RNA synthesis.[53–55] VPg is covalently bound to the 5′ end of the (+)RNA genome, but it must be modified by nucleotide addition and transferred to the 3′ end of the template prior to its use as a primer. For Picornaviridae members, the VPg protein is uridylated (i.e., several UMP molecules are attached). This uridylation reaction is catalyzed *de novo* by the viral RdRp and is templated by an internal stem-loop element in the genomic (+)RNA. Much of our understanding of VPg-dependent initiation of RNA synthesis is based on structural studies of the Picornaviridae member foot-and-mouth disease virus. In particular, the structures of the foot-and-mouth disease virus RdRp (1) in complex with uridylated VPg and (2) in complex with a template-primer duplex have provided remarkable insight into this initiation mechanism.[56–58] Together, these structures reveal how VPg accesses the RdRp catalytic site from the front part of the molecule and how the first five nucleotides of the oligo (A) template traverse the template entry tunnel (Figure 5(c)). The final base of the RNA template points toward the catalytic site and is in a correct position to be paired with the UMP molecule that is covalently attached to VPg. Essentially, the 3′-OH of the UMP serves as a molecular mimic of a nucleic acid primer. Moreover, it is important to note that, for the foot-and-mouth disease virus RdRp, high-resolution structures of the enzyme in different states of RNA elongation also exist, providing exquisite insight into the molecular basis for the lack of RdRp fidelity.[59]

The influenza A virus RdRp utilizes different initiation mechanisms for transcription versus genome replication.[21] Specifically, transcriptional initiation is primed by small pieces of m^7G-capped oligonucleotides that are stolen from cellular pre-mRNAs.[60] The 10–13 nucleotides of these primers do not base-pair with sequences at the 3′ ends of influenza A virus (−)RNAs. Instead, a GTP is added to the 3′ end of the capped primer (by the RdRp), and this GTP base-pairs with the penultimate C residue at the 3′ end of the viral genome.[61] The viral polymerase complex then

elongates the primer in a template-directed manner until it reaches a poly-U tract, on which it stutters to form the poly-A tail of the nascent mRNA.[21] In contrast to the initiation of mRNA synthesis, initiation of both (+)RNA antigenome and (−)RNA genome synthesis by the influenza A virus RdRp does not require a cellular cap, but instead occurs *de novo*. For (−)RNA synthesis, the *de novo* initiation mode occurs at the terminal template residue, whereas that for (+)RNA antigenome synthesis occurs via an internal residue, requiring template realignment.[62] How the influenza RdRp makes the switch from transcription to genome replication is unclear; however, it has been suggested that the virus creates structurally distinct RdRp complexes (see also below).[63]

COFACTOR PROTEINS THAT REGULATE THE ACTIVITY OF THE VIRAL RdRp

Many viral RdRps are capable of performing transcription and genome replication *in vitro* as single polypeptides. However, in the context of a virus-infected cell, this is rarely the case. More often, viral RdRps are found along with other viral and/or cellular proteins in large, multisubunit complexes. Cofactors within these polymerase complexes modulate the type and timing of RNA synthesis by directly engaging the RdRp or by indirectly affecting its capacity to recognize template RNA. While far from inclusive, the examples below show how the high-resolution structures of RdRps and their cofactor proteins have increased our understanding of RNA virus transcription and genome replication regulation.

Regulation of Poliovirus 3D RdRp Activity by 3CD Proteolysis

As mentioned previously, (+)RNA viruses share the characteristic features of (1) expressing their polymerase complex proteins via a polyprotein cleavage strategy and (2) using cellular membranes as scaffolds for polymerase complex function. One of the simplest human (+)RNA viruses in terms of polymerase complexes is poliovirus, a prototypic Picornaviridae family member. The 7.5-kb message-sense (+)RNA genome molecule encodes a single polyprotein, which is cleaved into three precursors P1, P2, and P3. The majority of products processed from P2 and P3 are polymerase complex proteins, including 2A, 2B, 2C, 2BC, 3AB, 3B, 3C, 3CD, and 3D (Figure 6(a)).

Protein 3AB is believed to be a main anchor for the poliovirus polymerase complex in endoplasmic reticulum-derived, double-membrane vesicles.[64,65]

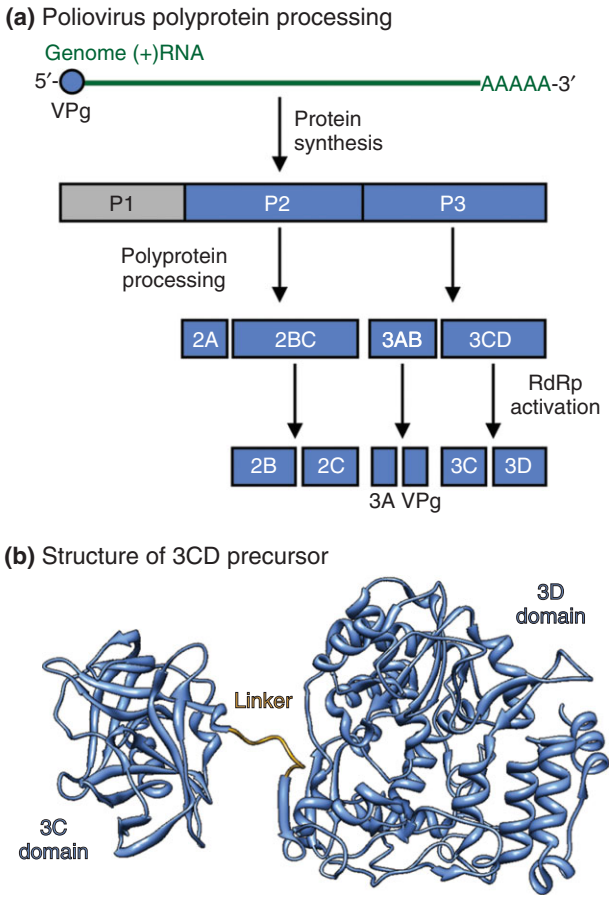

FIGURE 6 | Proteolytic activation of the poliovirus RNA-dependent RNA polymerase (RdRp). (a) The poliovirus genome is message-sense (green), and they often contain a 5′ VPg (blue circle) and 3′ poly-A tail (AAAAA). Host cell ribosomes translate the genome into three polyproteins (P1, P2, and P3), which are cotranslationally and posttranslationally processed by virally encoded proteases (2A, 3C, and 3CD). The polymerase activity of 3D is triggered following cleavage of 3CD. (b) Structure of 3CD. Ribbon representation of poliovirus 3CD (PDB#1VYI) is shown. The flexible linker between the two domains is shown in gold. Upon cleavage, this gold region becomes the N-terminus of 3D and becomes buried inside the RdRp where it helps position the catalytic aspartates.

This protein also has nucleic acid chaperone activity and increases the binding of the viral polymerase to template.[66,67] The 3AB membrane association occurs via a hydrophobic region in the C-terminus of 3A.[64] Additional poliovirus proteins (e.g., 3A, 2B, 2C, and 2BC) are also thought to aid in membrane rearrangements/polymerase complex anchoring in the infected cell.[68–71] The viral protein 3B (alone or possibly in the context of a precursor) represents VPg, the protein primer required for initiation of RNA synthesis (see also above).[55,71,72] Polymerase complex proteins 2C, 3CD, and 3D all interact with viral RNA and with each other in some manner. Moreover,

cellular proteins such as poly-rC- and poly-A-binding proteins (PCBP and PABP, respectively) are also important components of the poliovirus polymerase complex.[73]

Poliovirus 3CD is a multifunctional protein and an essential component of the poliovirus polymerase complex (Figure 6(a) and (b)). The 3CD is an active protease, but it lacks polymerase activity. As such, 3D only becomes an active RdRp following cleavage from 3C. High-resolution X-ray crystal structures of 3D versus 3CD have provided insight into proteolysis-dependent activation of the RdRp.[33,74,75] Initial X-ray crystal structures of 3D contained packing artifacts, because of the inherent (albeit possibly biologically relevant) capacity of the polymerase to oligomerize.[74,75] These 3D–3D interactions obscured the location of the extreme N-terminus of the protein.[74] More accurate structures of 3D were generated using a mutant protein that cannot form oligomers.[76] In that later structure, the N-terminus of 3D is buried in a pocket at the base of the fingers subdomain, where it helps position the catalytic-site aspartate residues to select the incoming 3′-OH group of NTPs. In the structure of the RdRp-inactive 3CD precursor, the 3C and 3D domains do not make any direct contacts with each other and are connected by a seven-residue polypeptide linker[76] (Figure 6(b)). The N-terminal residues of the 3D domain that comprises the linker are relocated in the context of 3CD, thereby preventing catalysis (Figure 6(b)). Thus, the lack of polymerase activity in 3CD is thought to be associated with the preclusion of the N-terminus from the 3D domain. Moreover, in the 3CD structure, the 3D domain makes extensive contacts with the 3C and 3D domains of neighboring molecules.[76] The 3C N-terminus is proximal to the VPg-binding site in 3CD, an arrangement that is consistent with a possible role for these contacts in forming and regulating the poliovirus VPg uridylation complex. Essentially, polyprotein processing allows poliovirus to tightly regulate the stoichiometry of 3CD and 3D in the polymerase complex, and therefore to maximize the functions of these proteins during VPg uridylation, initiation, and elongation.

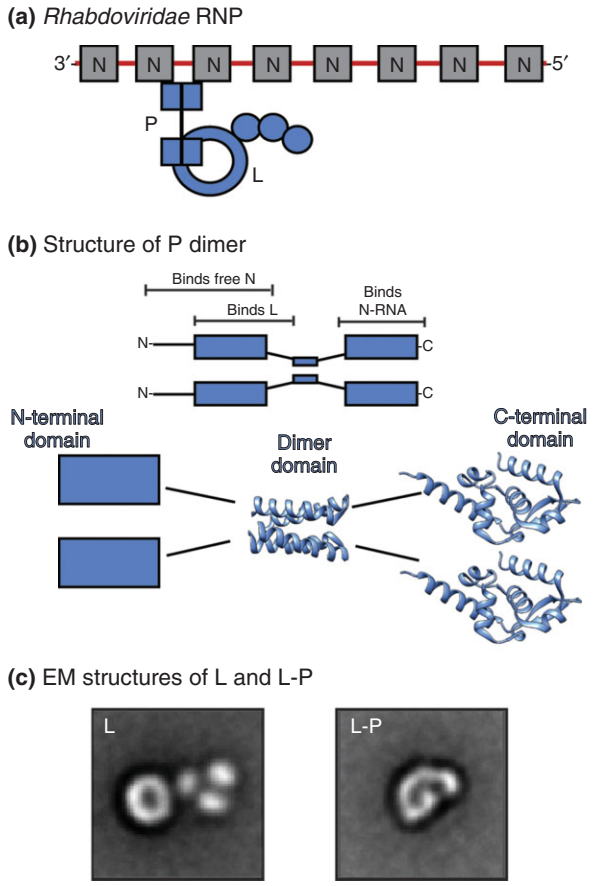

FIGURE 7 | Rhabdoviridae RNA-dependent RNA polymerase (RdRp)–phosphoprotein interactions. (a) The (−)RNA genome of Rhabdoviridae members is bound by nucleocapsid protein (N) and organized as an ribonucleoprotein (RNP). The viral polymerase complex is comprised of a phosphoprotein (P) dimer and the large protein (L). P is able to bind to L and N-RNA simultaneously via distinct N- and C-terminal domains. It is thought that engagement of L by P is required for template recognition by the RdRp. (b) Structure of rabies virus P. The top of this panel shows a cartoon schematic of a P dimer with protein–protein interaction regions labeled. Ribbon representations of the central dimerization domains (PDB# 3L32) and C-terminal domains of a P protein dimer (PDB#1VYI) are shown. The structure of the N-terminal domain of P is not known, nor is the structure of connecting regions between the three ordered domains. (c) Electron micrographs of L and L-P. The images show recombinant L protein of vesicular stomatitis virus in the presence or absence of phosphoprotein. Dramatic rearrangements are seen in L following P binding, particularly in the regions of the protein associated with RNA capping. (Reprinted with permission from Ref 78. Copyright 2012 National Academy of Science, USA)

Rhabdoviridae RdRp Activity Regulated by a Viral Phosphoprotein

The most well-studied members of the Rhabdoviridae family are rabies virus and vesicular stomatitis virus. The 11- to 12-kb (−)RNA genomes of these viruses are encased with nucleocapsid protein (N), forming an RNP structure, and are bound at their 3′ ends with the viral polymerase complex[13] (Figure 7(a)). The polymerase complex is composed of two proteins: a single molecule of the large (L) protein, which has RdRp and 5′ RNA capping activities, and a dimer of the small viral phosphoprotein (P). During both transcription and genome replication, P serves as a noncatalytic cofactor that simultaneously engages L and N (bound to RNA), allowing the RdRp to gain access to the RNA template.[77] While the precise mechanism(s) of

P function are not fully understood, it is thought that P directly binds to L and thereby increases its affinity for RNA template.

P is a modular protein comprised of three distinct ordered domains: (1) an N-terminal domain, (2) a central dimerization domain, and (3) a C-terminal domain[79] (Figure 7(b)). The ordered domains are interspersed by disordered regions, causing the protein to behave as a nonglobular molecule.[80] The P protein forms parallel dimers as a result of interactions between the central domains of individual monomers.[80] The C-terminal domain of P binds to the N protein, only when it is in complex with RNA (i.e., N-RNA).[81,82] In contrast, the N-terminal domain of P binds to free N protein (N^0), possibly preventing it from inappropriately binding to viral or cellular mRNAs.[83] For vesicular stomatitis virus, the N-terminal domain and the central dimerization domain are thought to engage L, the viral RdRp[78] (Figure 7(b)). However, the structure of the N-terminal domain of the P protein has not been determined for any Rhabdoviridae family member. Also, while no X-ray crystal structures of L have been determined to date, recent electron microscopic analyses of the protein show that it contains two domains: (1) a ring-like RdRp domain and (2) a globular appendage domain that harbors the enzymatic activities associated with capping[37] (Figure 7(c)). Interestingly, after complex formation with P, the appendage domain of L rearranges into a more compact structure[78] (Figure 7(c)).

It is not clear exactly how P binding to L and to the N-RNA complex keeps L attached to the template. It is also not understood how the polymerase moves across its RNA template. Studies of closely related Rhabdoviridae members suggest that P might progress along the template in a type of cart-wheeling motion, in which it alternatively associates and disassociates from N.[84] Other studies suggest that P molecules remain bound at regular intervals along the N-RNA and that L jumps between bound P proteins.[85] In this model, the binding of one P dimer to every five N monomers is sufficient to allow one P dimer to 'catch' the viral RdRp and 'transfer' it to the next P dimer. Additional roles of the P protein in regulation of the vesicular stomatitis virus RdRp have been implicated by biochemical studies. Specifically, the same region of P that induces a structural rearrangement (i.e., the N-terminal domain and the central domain) also stimulates the initiation of RNA synthesis and processivity of L *in vitro*.[78] Therefore, (−)RNA viruses of the Rhabdoviridae family employ a multifunctional cofactor to modulate the template binding and enzymatic activities of its RdRp.

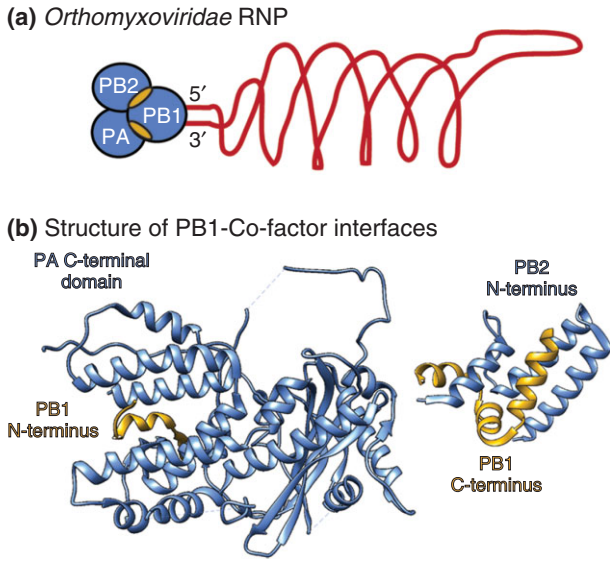

FIGURE 8 | Influenza A virus RNA-dependent RNA polymerase (RdRp)–cofactor interfaces. (a) Each of the (−)RNA genome segments of influenza A virus is bound by nucleocapsid protein (not shown, for simplicity) and organized as a corkscrew-like ribonucleoprotein (RNP). The viral polymerase complex resides at the RNP terminus (made by 5′–3′ base-pair interactions) and is comprised of three proteins, shown as light blue circles: PA (endonuclease), PB1 (RdRp), and PB2 (cap-binding). The interfaces between PA–PB1 and PB1–PB2 for which structure is known are shown in gold. (b) Structure of PB–cofactor interfaces. Left: Ribbon representations of the C-terminal domain of PA (blue) with a small portion of the PB1 N-terminus (gold) (PDB#3CM8). Right: Ribbon representation of very small regions of the PB2 N-terminus (light blue) in complex with the PB1 C-terminus (gold) (PDB#3A1G).

The Heterotrimeric Influenza A Virus Polymerase Complex

Influenza A virus and other Orthomyxoviridae family members maintain their (−)RNA genomes as eight separate segments that are bound by the viral nucleoprotein (NP) and a heterotrimeric polymerase complex (Figure 8(a)). Unlike Rhabdoviridae members, whose RNP is single-stranded with a 3′ end-bound polymerase complex, the 5′ and 3′ ends of Orthomyxoviridae RNPs are base-paired, forming a folded corkscrew-like structure.[16,21] The virus polymerase complex is bound to the double-stranded RNP terminus, and it consists of three proteins: PB1 (RdRp), PB2 (cap-binding protein), and PA (endonuclease), which interact head-to-tail in the order PA-PB1-PB2.[16,20,21] While most RNA viruses transcribe and replicate their RNA in the cytoplasm, the influenza virus RdRp complex only functions within the nucleus of a host cell. The location of RNA synthesis for this virus makes sense in light of its mechanism of transcriptional initiation using 5′ m^7G-capped oligonucleotides stolen

from cellular pre-mRNAs. The PB2 subunit is responsible for binding to the 5′ m^7G cap of a cellular pre-mRNA molecule, while the PA subunit will cleave the structure. Then, PB1 will utilize the stolen capped oligonucleotide as a primer for viral RNA synthesis (see also above).

The influenza A virus polymerase complex has been relatively intractable to high-resolution structural analysis. Of the three subunits, only PA has been expressed as a soluble, recombinant protein. This protein was cleaved into N-terminal and C-terminal domains using trypsin, and the two individual structures were solved by X-ray crystallography. The N-terminal domain of PA has a fold similar to other type II restriction endonucleases.[86,87] In fact, the isolated PA N-terminal domain shows endonuclease activity on single-stranded nucleic acid, suggesting that this region is sufficient for the cap-snatching activities of the influenza A virus polymerase complex.[86] The C-terminal domain of PA has only been crystallized in complex with a short N-terminal peptide of PB1, essentially revealing the interface between these two proteins[88–90] (Figure 8(b)). Biochemical assays have shown that a small peptide corresponding to the N-terminus of PB1 can competitively inhibit polymerase activity, suggesting that the insertion of this region into the PA cavity is necessary for catalysis.[91,92] Aside from interacting with PB1, the function of the C-terminal domain of PA remains to be determined.

Compared to PA, the influenza virus PB2 protein has been even more difficult to express and purify in a manner suitable for structural analysis. However, three small regions of PB2 have been crystallized, revealing the structures of: (1) a nuclear localization region, (2) an independently folded central region containing the 5′ mRNA cap-binding site required for cap snatching, and (3) a putative pre-mRNA-binding region involved in influenza host specificity.[93–96] Moreover, the first 35 N-terminal residues of PB2 have been resolved via a cocrystal with a C-terminal portion of PB1[97] (Figure 8(b)).

To date, no high-resolution structure is available for PB1, the influenza A virus RdRp subunit. Only the subunit interaction interfaces of the PB1 protein are known in atomic detail (i.e., PA–PB1 and PB1–PB2)[89,97] (Figures 6(c) and 8(b)). These subunit interfaces are essential for polymerase function, as mutations in them severely affect transcription and genome replication. Thus, PA and PB2 are essential cofactors that engage PB1 and allow the RdRp to mediate catalysis. This heterotrimeric polymerase complex ensures that PB1 will only elongate viral templates that have been primed with m^7G-capped RNAs via the coordinated activities of PA and PB2.

Core Protein-Dependent Activity of Rotavirus VP1

Like other segmented, dsRNA viruses of the family Reoviridae, rotaviruses perform all stages of viral RNA synthesis within the confines of proteinaceous particles. A single RdRp monomer (VP1) in complex with the RNA capping enzyme is dedicated to transcribing and replicating an associated genome

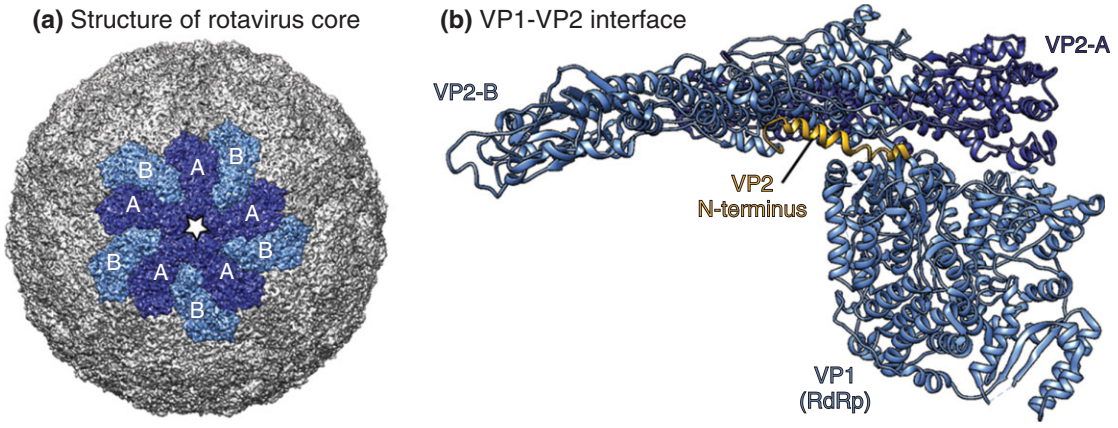

FIGURE 9 | Rotavirus core-shell-RNA-dependent RNA polymerase (RdRp) interactions. (a) The rotavirus VP2 core shell (PDB#3KZ4) is shown alone, with each of the 120 VP2 monomers depicted in surface representation. Five A-VP2 and five B-VP2 monomers of a central decamer are colored dark blue and light blue, respectively. The location of the fivefold axis is depicted with a star. (b) VP1 bound to inner surface of VP2. Two neighboring VP2 monomers (A-VP2 and B-VP2) of a decamer unit (side view; PDB#4F5X) are shown in ribbon representation and colored according to panel a. VP1 (light blue) bound to the inner surface of VP2 is also shown in ribbon representation. The structurally resolved portion of the VP2 N-terminal domain (residues 78–101), which is thought to engage VP1 and aid in enzymatic activation, is shown in gold. No structure yet exists for VP2 N-terminal residues 1–77, but they, as well as regions of the VP2 principal domain, are also important for VP1 activation.

segment, presumably while tethered to the interior of the core shell protein (VP2). However, the role of VP2 extends beyond serving as a scaffold for the viral polymerase. It is also a critical cofactor that causes VP1 to initiate genome replication.[98]

High-resolution structures of the VP2 core shell in the context of a double-layered particle or mature rotavirus virion have been determined by using cryo-electron microscopy or X-ray crystallography.[99–102] In these structures, the VP2 core shell is seen as a smooth icosahedron that is organized into 12 decameric units (Figure 9(a)). Each VP2 decamer is comprised of two chemically identical VP2 conformers (A-VP2 and B-VP2). Five A-VP2 conformers encircle the icosahedral fivefold axes, whereas five B-VP2 confers sit further back from the fivefold axis in between A-VP2 units (Figure 9(a)). A monomer of VP2 is defined into two separate domains: (1) the amino-terminal domain, made up of residues ~1–100 and (2) the principal scaffold domain, made up of residues ~101–880. The VP2 N-terminal domain cannot be fully resolved in the known structures, most likely because it forms a flexible protein region. However, the N-termini of neighboring monomers have been suggested to multimerize beneath the icosahedral fivefold axes.[100]

Recent analysis of the X-ray crystallographic data of rotavirus double-layered particles indicates that VP1 is localized to the inner face of the VP2 principal scaffold domain, just off center from the fivefold axis, with a binding footprint that overlaps the apical subdomains of neighboring A-B VP2 conformers in a decamer[24] (Figure 9(b)). Portions of the VP2 N-terminal domain are also detected near VP1, perhaps forming tethers that support the polymerase. Thus, in the context of a static structure, both the N-terminal domain and the apical subdomains of the VP2 core shell directly bind VP1. These same regions have been shown to be involved in VP1 enzymatic activation *in vitro,* suggesting that they may trigger the capacity of the enzyme to mediate RNA synthesis.[103] It is hypothesized that engagement by VP2 causes structural rearrangements in VP1 that may include lifting the priming loop to allow for NTP binding. The VP2-dependent mechanism of RNA synthesis for rotavirus ensures that dsRNA genome is only made within a particle that is the morphogenic precursor to a mature virion. Interestingly, the RdRps of several Caliciviridae members, Norwalk virus and murine norovirus, also show enhanced enzymatic activity in the presence of their cognate capsid proteins.[104] This observation suggests that coordination of RNA synthesis with virion assembly via polymerase regulation may occur for other families of RNA viruses in addition to Reoviridae.

CONCLUSIONS

This review summarizes the RNA synthetic mechanisms employed by diverse RNA viruses of the families Caliciviridae, Flaviviridae, Picornaviridae, Rhabdoviridae, Orthomyxoviridae, and Reoviridae. While far from inclusive, the examples provided here underscore the various shared and virus-specific features of transcription and genome replication. It is clear from these examples that RNA viruses have evolved distinctive strategies to control the activity of their RdRps and to orchestrate the kinetics of their lifecycle. Indeed, RNA viral RdRps are almost always found along with other viral (and oftentimes cellular) proteins in higher ordered, multisubunit polymerase complexes. In combination with biochemical studies, high-resolution structures are revealing the details of functional protein–protein interactions between the polymerase complex components. Moreover, newer imaging technologies are providing unprecedented views of RNA virus polymerase complexes within or isolated from infected host cells.[105–107] Studies aimed at elucidating the molecular dynamics of these complexes in their most native state during RNA synthesis are expected to provide the 'missing link' between structure and function.[108] The knowledge gained from these studies not only contributes to our general understanding of RNA virus biology but also enhances our ability to develop pharmacological inhibitors of viral RNA synthesis.

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