MINIREVIEW

De Novo Initiation of Viral RNA-Dependent RNA Synthesis

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RNA viruses use several initiation strategies to ensure that their RNAs are synthesized in appropriate amounts, have correct termini, and can be translated efficiently. Many viruses with genomes of single-stranded positive-, negative-, and double-stranded RNA initiate RNA synthesis by a de novo (primer-independent) mechanism. This review summarizes biochemical features and variations of de novo initiation in viral RNA replication. © 2001 Academic Press

Initiation of viral RNA synthesis is an important regulatory step in the transcription and replication of RNA viruses. The step is also of interest mechanistically because, unlike transcription from DNA templates, viral RNA initiation needs to take place at or near the 3′ end of the viral genome.

Enzymes and terminology. The recent proliferation of reports on the activities of recombinant viral RNA-dependent RNA polymerases (RdRps) necessitates a distinction from studies on enzyme complexes isolated from virions or virus-infected cells. Therefore, the term “replicase” will be used to denote the enzyme complex that contains the RdRp, other viral proteins (sometimes including an RNA helicase), and possible host factors. “RdRp” will be used to refer to the subunit of the replicase that polymerizes nucleotides from RNA templates. The term “viral transcriptase” can be used if the enzyme complex is specific for the synthesis of viral messenger, but not genomic, RNA. Use of these terms will not only help to differentiate the composition of the various enzymes, but also the functional differences. For example, recombinant RdRps are usually not template-specific, but can synthesize RNA from any template that either has a suitable initiation site or is annealed with a primer (Ferrari et al., 1999; Kao et al., 2000). In contrast, a viral replicase can possess the ability to distinguish features in viral templates (e.g., Blumenthal and Carmichael, 1979; Lemm et al., 1998; Kim et al., 2000; Plante et al., 2000; You and Padmanabhan, 1999). Enzymatically active recombinant RdRps are primarily obtained for members of the Picornaviridae (for a summary, see O’Reilly and Kao, 1998), the Flaviviridae (Behrens et al., 1996), and a few other viruses, such as the double-stranded RNA virus, φ6 (Makeyev and Bramford, 2000). Several replicases have been used to study the mechanism of RNA synthesis, including those from bacteriophage Qβ, picornaviruses, flaviviruses, alphaviruses, double-stranded RNA viruses, single and multipartite negative-strand RNA viruses, and many positive-strand plant viruses (for reviews, see Lai, 1998; Buck, 1996; Blumenthal and Carmichael, 1979).

Several terms are commonly used to describe components of the transcription initiation complex. The initiation nucleotide is often abbreviated NTPI. Within the catalytic pocket of the polymerase, the portion that binds NTPI is often called the “i site,” while the one that binds the nucleotide to be added to the growing chain is often called the “i+1” or the “C” site. The template nucleotide used to initiate RNA synthesis has been variously named the N+1, or the T+1, site. We shall use the term T+1.

Initiation mechanisms. RNA viruses initiate RNA synthesis by either of two major mechanisms: de novo initiation or primer-dependent synthesis. For de novo initiation, a nucleoside triphosphate, sometimes referred to as the one-nucleotide primer, provides the 3′-hydroxyl for the addition of the next nucleotide (Fig. 1A). The triphosphates of the NTPI may subsequently participate in the addition of a nucleotide cap by a 5′→5′ linkage to the NTPI of the viral RNA (Shuman and Schwer, 1995). With the enzymes that use primers, the primer could be (1) an oligonucleotide covalently linked to a protein, as is used by picornaviruses (Fig. 1B, for review, see Salas, 1991); (2) oligonucleotides cleaved from the 5′ end of a capped cellular mRNA in a process called cap-snatch...
ing, as is used by many segmented negative-strand RNA viruses (Fig. 1C, Hagen et al., 1995); (3) a leader RNA that was synthesized by the viral transcriptase; (4) the 3' end of the template RNA looping back on itself to serve as a primer for the polymerase. The latter case is variously called template-primed synthesis, loop-back synthesis, covalent loop-back, copy-back, or turn-around synthesis (Fig. 1D). The term "template-primed synthesis" will be used herein. Frequently observed, template-primed synthesis may sometimes be an artifact of in vitro reactions.

With notable exceptions, such as the picornavirus RdRp that absolutely requires a primer, de novo initiation is a common activity of RNA polymerases. However, even RdRps that initiate de novo can often use a short oligonucleotide (of two to five nucleotides) as a replacement for the NTPi in vitro (Chen and Patton, 2000; Downing et al., 1971; Garcin and Kolakofsky, 1992; Honda et al., 1986; Kao and Sun, 1996; Nagy et al., 1997). These short oligonucleotides may be produced by the polymerase during abortive cycling (i.e., repeated rounds of de novo initiated synthesis and release of oligonucleotide nascent RNAs), before the transcribing complex commits to elongative synthesis (McClure, 1985).
A virus may use one or more initiation mechanisms. During infection, RNA viruses must synthesize at least the complement of the genomic RNA as well as the genomic RNA itself. Some also produce subgenomic-length RNAs to translate internal cistrons (for review, see Miller and Koev, 2000). Many viral species use only one initiation mechanism for the synthesis of all classes of RNA. For example, the replicase of phage QB, which initiates RNA synthesis de novo (Blumenthal, 1980), and the poliovirus replicase use a protein–oligonucleotide primer (Paul et al., 1998). Replicases from other viral species can use more than one initiation mechanism. For example, the enzyme from multipartite negative-strand RNA viruses initiates genomic and the complementary antigenomic RNA replication by a de novo mechanism but uses a cap-snatched primer for mRNA transcription (Hay et al., 1982; Honda et al., 1998; Moss et al., 1978; Young and Content, 1971). The enzymes from Nidoviruses likely initiate genomic RNA synthesis de novo, but use a discontinuous primer-dependent mechanism for subgenomic RNA synthesis. (For reviews of current models, see Lai and Cavanagh, 1997 and Sawicki and Sawicki, 1998. See also van Marle et al., 1999 for subgenomic synthesis in arteriviruses.)

In general, viral RNAs without cap-snatched primers or terminal proteins are initiated by a de novo initiation mechanism. In addition to the viral groups already mentioned in this review, alphaviruses and the α-like plant-infecting positive-strand RNA viruses also tend to use a de novo initiation mechanism (Strauss and Strauss, 1994; Goldbach et al., 1991). Viruses that do not contain capped RNA may also initiate RNA synthesis de novo. Examples include dsRNA viruses such as Φ6 (Makeyev and Bramford, 2000; Butcher et al., 2001) and rotavirus (Chen and Patton, 2000), negative-strand RNA viruses such as vesicular stomatitis virus, and members of the Flaviviridae (Testa and Banerjee, 1979; Oh et al., 2000). In the mononegavirales, the de novo initiation of a messenger RNA can be coupled to the termination of the adjacent upstream RNA, indicating that the transcriptase can scan for a downstream initiation site after termination (Barr et al., 1997; Castaneda and Wong, 1989; Stillman and Whitt, 1997; Vidal and Kolakofsky, 1989). A brief discussion of the assays used to assess whether de novo initiation takes place is in the last section of this minireview.

Interactions required for de novo initiation. The catalytic pocket of the polymerase needs to interact with the NTPi (usually either a GTP or ATP), the second nucleotide (NTP), and the template initiation site, a sequence that includes the first template nucleotide, T+1. T+1 is usually a pyrimidine that base pairs to the NTPi (Fig. 1A, Ball, 1995; Blumenthal, 1980; Miller et al., 1986; Siegel et al., 1997; Yoshinari et al., 2000; Yoshinari and Dreher, 2000). The replicases of brome mosaic virus (Kao and Sun, 1996) and vesicular stomatitis virus (Testa and Banerjee, 1979) and the RdRp of the hepatitis C virus (Luo et al., 2000) have a higher $K_m$ for the NTPi than the $K_m$ for the NTPs used in elongation. Different requirements for the NTPi compared to other NTPs indicate that initiation is subject to additional regulations in comparison to other polymerizations catalyzed by the RdRp. Specific recognition of the NTPi by DNA-dependent RNA polymerases (DdRps) has been characterized (Reddy and Chatterji, 1994 and reference therein) and similar specificity should be expected for viral RdRps.

The interactions at the initiation complex need to be sufficiently stable to allow accurate positioning of the initiation complex, but not so stable as to prevent the polymerase within the transcription elongation complex (TEC, containing the polymerase, NTP, template, and nascent RNA) from leaving the initiation site and progressing to elongative synthesis. (For models of the TEC of DdRps, see Toulme et al., 1999; Kozhevnikov et al., 2000.) Two questions relevant to these interactions are (1) What RNA length is required to stably interact with the RdRp? and (2) How many base pairs of the template-RNA duplex can be accommodated during initiation?

In regard to the first question, monomers of the recombinant RdRps of poliovirus (3Dpol) and hepatitis C virus (HCV) protect eight to ten nucleotides of single-stranded RNA from RNase digestion (Beckman and Kirkegaard, 1998; Kao et al., 2000). Templates longer than five to eight nucleotides are needed to stably bind the HCV RdRp (Sun et al., 2000; Zhong et al., 2000b) and to direct RNA synthesis by the bovine viral diarrhea virus RdRp (Kim et al., 2000). Much less information has been uncovered regarding the length of the nascent RNA in the transcriptional complex during initiation. Molecular modeling studies suggest that the HCV RdRp can accommodate up to ten base pairs (Bressanelli et al., 1999), which is in some agreement with biochemical and structural studies of the transcriptional elongation complex of DdRps (Fu et al., 1999; Nucler et al., 1996, 1997). Circumstantial evidence for the length of the nascent RNA comes from studies of the transition from initiation to elongation by the brome mosaic virus replicase. This transition takes place after the synthesis of eight to ten nucleotides, suggesting that this length of a template-nascent RNA duplex can be accommodated in the initiation ternary complex (Sun et al., 1996; Sun and Kao, 1997a,b). Putatively aborted products of up to eight nucleotides are also produced by the turnip crinkle virus replicase in vitro (Nagy et al., 1997).

It is not currently known what signal(s) induces a viral RdRp to make the transition to elongative synthesis. RNA polymerase II achieves this transition by phosphorylating the heptad amino acid repeats found in the C-terminal domain of the largest PolII subunit (Carlson, 1997), a process that requires ATPs that are not incorporated into the nascent RNA (Dvir et al., 1996). The T7 RNA polymerase transitions to elongation when the nascent RNA
reaches a length sufficient to interact with a site in the T7 RNA polymerase, thus triggering a conformational change that leads to processive RNA synthesis (Sousa et al., 1992; Temiakov et al., 2000). While viral RdRps do not have the heptad repeats characteristic of PolII, there is not yet evidence for the nascent viral RNA playing a role in inducing elongation.

The initiation site. Genomic minus- and plus-strand syntheses usually use a T₁₁ nucleotide that is either the 3'–terminal or the penultimate nucleotide (Chapman et al., 1998; Wu and Kaper, 1994). Nucleotides added to the 3' end of the RNA prior to viral RNA synthesis are not present after viral replication, indicating that initiation can take place internal to a 3' extension (Ball, 1994; Miller et al., 1986; Sun et al., 1996; Guan and Simon, 2000). The presence of T₁₁, as the penultimate nucleotide to the 3'-terminal in some viral RNAs raises the question of how the 3'-terminal nucleotide is added to the viral RNA. One scenario is that the viral replicase adds one or more nontemplated nucleotide(s) to the nascent RNA as the TEC dissociates from the template. Indeed, several replicases have been reported to perform terminal nucleotide addition either to the template or to the nascent RNA (Neufeld et al., 1994; Rao et al., 1989; Weber and Weissman, 1970; Guan and Simon, 2000).

Change of the T₁₁ identity in RNAs usually results in either a severe reduction in RNA synthesis or initiation from a pyrimidine near the original T₁₁ (Chapman and Kao, 1999; Kuo et al., 1997; Stillman and Whitt, 1997; Siegel et al., 1997). Some initiation cytidylate moieties required for initiating RNA synthesis by the bovine viral diarrhea virus RdRp were identified using chemically synthesized RNAs containing nucleotide analogs (Kim et al., 2000). In particular, analogs of the T₁₁ cytidine that affected hydrogen bonding to the initiation GTP severely decreased RNA synthesis, indicating that H-bonding between the T₁₁ base and the NTPᵢ contributes significantly to the specificity of initiation (Kim et al., 2000).

Nucleotides adjacent to T₁₁ may also play a role in the recognition required for de novo initiation. Changes to the nucleotides at the +2 and +3 positions generally have a more detrimental effect than changes further downstream from the initiation site (Adkins et al., 1998; Ball, 1994; Kuo et al., 1996a; Sivakumaran and Kao, 1999; Stillman and Whitt, 1997; Yoshinari et al., 2000). RNAs containing deoxynucleotide substitutions at the +2 and +3 positions were ineffective templates for RNA synthesis by the bovine viral diarrhea virus RdRp, suggesting that riboses in RNAs at these positions are needed for interaction with the polymerase (Kim et al., 2000). Another property that may be common to initiation sequences is their tendency to lie in unbase-paired regions (Dreher, 1999). In rotavirus, even though the 5' and 3' termini of the double-stranded rotavirus RNA form a structure required for RNA synthesis, minus-strand RNA synthesis still requires a minimum of two unbase-paired cytidylates at the 3' end of the viral RNA (Chen and Patton, 1998). Deleting the cytidylates or removing the single-stranded region by annealing it with an oligonucleotide severely reduced rotavirus RNA synthesis in vitro (Chen and Patton, 1998, 2000).

Analysis of the transcripts produced by a model minigenome of the monopartite negative-sense respiratory syncytial virus (RSV) revealed some flexibility in the choice of the T₁₁ nucleotide. Changes of the RSV initiation cytidylate to either a uridylic or an adenylic reduced, but did not abolish, RNA synthesis (Kuo et al., 1997). Transcripts produced from these mutant templates were of two classes. The majority class used the altered T₁₁ to incorporate a Watson–Crick complementary nucleotide. The other group unexpectedly initiated with a 5' GTP despite the lack of Watson–Crick hydrogen bonding (Kuo et al., 1997). Transcripts that possess a 5' guanylic were proposed to be the result of initiation from a cytidylate adjacent to the original +1 site followed by a realignment of the nascent RNA in a process called the "prime and align" mechanism (to be discussed below).

The initiation site has been reported to be the primary determinant of template specificity for the replicases of turnip yellow mosaic virus, turnip crinkle virus, and QB (Deiman et al., 1998, 2000; Singh and Dreher, 1997, 1998; Yoshinari et al., 2000). While these RNA viruses require additional regulatory sequences for RNA replication in vivo, cis-acting elements 5' of the initiation site may form relatively generic RNA structures that are highly tolerant of change (Blumenthal, 1980; Chen et al., 2000; Singh and Dreher, 1998). The lack of specific requirement for cis-acting regulatory elements in vitro may help to explain the observations that the QB replicase can synthesize a number of unrelated RNAs in vitro, and, to a lesser extent, in vivo (Avota et al., 1998 and references within). Specificity for the template in some viruses may be coupled with other mechanisms, such as translation. While specificity in some viral systems may not always depend on replicase–RNA interaction, highly specific cis-acting signals have been observed in several systems (e.g., Adkins et al., 1997; Kim et al., 2000; McKnight and Lemo, 1998; Osman et al., 2000; Siegel et al., 1998; Sit et al., 1998; You and Padmanabhan, 1999).

Plus- and minus-strand initiation. Viruses that have a 3' poly(A) tail usually have genomic RNAs with a 5'-terminal purine nucleotide (indicating that a purine nucleotide is used for initiation). However, if the poly(A) tail can direct initiation, then a pyrimidine triphosphate must logically act as the NTPᵢ. This raises the question of whether RdRps need to use the same NTPᵢ (and the same T₁₁) for the synthesis of different classes of viral RNAs. One way to circumvent the need for one replicase to use two different initiation nucleotides is if minus-strand initiation takes place internally, 5' of the poly(A) sequence, and the poly(A) tail is added by a posttranscriptional mechanism. Some RNA viruses do possess a
polyadenylation-like signal (5′AAUAAA3′) 5′ of the poly(A) tail that could direct poly(A) addition (Jupin et al., 1990). Strategies for poly(A) addition in plus-strand RNA viruses are covered more detail in Chapman et al. (1999).

The use of either purine or pyrimidine triphosphates as the NTPi has previously been documented in DdRps (for example, Schibler and Perry, 1977; Reddy and Chatterji, 1994). Some results also indicate that different T₃,₅ nucleotides (and NTPi) may be used by the same RNA replicase (e.g., Kuo et al., 1997). Further, with the positive-strand Semliki Forest virus, Sawicki and Gomatos (1976) found that the 5′ ends of the replication intermediates, but not the virion RNAs, were pyrimidines. The minus-strand RNA of a coronavirus was also reported to contain a uridylylate as the 5′-most nucleotide (Hofmann and Brian, 1991). In vitro, the HCV RdRp and the turnip crinkle virus replicase can use either a purine or a pyrimidine triphosphate as the initiation nucleotide (Guan and Simon, 2000; Oh et al., 2000; Zhong et al., 2000b). The Q6 and brome mosaic virus replicases specifically require GTP as the NTPi (Blumenthal, 1980; Kao and Sun, 1996). These results indicate that the i site of some, but not all, RdRps might be able to accommodate either a purine or a pyrimidine triphosphate.

Lessons from the Flaviviridae. Studies of the monopartite positive-strand Flaviviridae (Rice, 1996) have yielded different interpretations regarding the initiation mechanism. Dimeric-length products were observed by some researchers in reactions using the recombinant HCV RdRp, leading to claims of template-primed RNA synthesis (Behrens et al., 1996; Lohmann et al., 1999). Also, the Dengue virus replicase was reported to use exogenous templates in vitro by a template-primed mechanism (You and Padmanabhan, 1999). The Dengue virus replicase assay likely has biological relevance since RNA synthesis in this assay depends on sequences known to be required in vivo (Hahn et al., 1987; Wengler and Wengler, 1981).

Other mechanisms may account for the production of dimer-length nascent RNAs. One possibility is that the transcription complex may fail to terminate at the end of the first template and continue RNA synthesis on additional template(s) (Kim and Kao, 2001). Template switching has been observed for a number of RNA polymerases, especially when the template is in excess of the polymerase (Arnold and Cameron, 1999; Kim and Kao, 2001; Nuñez et al., 1996).

De novo initiation, rather than a template-primed synthesis, is likely used by the Flaviviridae for several reasons. (1) Dengue virus RNA is capped at the 5′-terminus, and it encodes a protein containing methyltransferase motifs, suggesting that it uses a de novo mechanism of initiation, at least for plus-strand synthesis (Cleaves and Dublin, 1979; Koonin, 1993; Wengler et al., 1978). (2) Fully denatured single-stranded RNAs of genome length were isolated from infected cells, indicating that replication intermediates were not covalently linked and did not arise from a template-primed mechanism (Chu and Westaway, 1985; Gong et al., 1998a,b). (3) Even in reactions where template-primed dimer-length RNA synthesis was observed, a higher GTP concentration was required (Lohmann et al., 1999), consistent with de novo initiation (Kao et al., 2000; Luo et al., 2000). (4) Several groups have independently observed that recombinant Flaviviridae RdRps prefer to initiate synthesis by a de novo mechanism (Kao et al., 1999, 2000; Luo et al., 2000; Oh et al., 2000; Zhong et al., 2000a).

The high-resolution crystal structure of the HCV RdRp revealed a novel structure that may be relevant to initiation (Lesberg et al., 1999; Hong et al., 2001). Template-dependent polymerases have been likened to a right hand, with finger, palm, and thumb domains (Kohlstaedt et al., 1992). Unlike other polymerases, the HCV RdRp has more extensive interactions between the fingers and thumb, making the catalytic pocket more encircled (Lesberg et al., 1999). A β-loop of 12 amino acids was found within the thumb domain, positioned near the site of phosphoryl transfer (Zhong et al., 2000b). Its location led to the speculation that this β-loop may be present to discriminate against primer-dependent RNA synthesis (Lesberg et al., 1999; Zhong et al., 2000b). Consistent with this hypothesis, a comparable structure is absent in the primer-dependent polymerases of poliovirus, Thermus aquaticus, and HIV (Hansen et al., 1997; Li et al., 1998; Kohlstaedt et al., 1992). A β-loop structure is present in the N-terminal domain of the de novo initiating T7 RNA polymerase and plays a role in maintaining the partially denatured DNA template upstream of the site of polymerization (Cheetham et al., 1999).

Using the highly conserved GDD polymerase motif as a landmark, we attempted to identify the putative β-loop in members of the Flaviviridae (Table 1). For the flav-, hepaco-, and pestiviruses listed, a sequence resembling the HCV β-loop was found at approximately 90, 125, and 138 residues from the GDD motif. Chou–Fasman secondary structure prediction analyses revealed that this region tends to form a β-sheet flanked by α-helices. With the hepaci- and flaviviruses, the positions of aromatic amino acids within the putative β-loop seem to be conserved (Table 1).

The ϕ6 ternary RdRp complex. Butcher et al. (2001) recently determined the structure of the ternary complex of the recombinant RdRp from the multipartite double-stranded RNA phage, ϕ6 (for a summary of the ϕ6 replication proteins, see Makeyev and Bramford, 2000 and references therein). This highly significant breakthrough revealed several features of de novo initiation. Similar to the HCV RdRp, the ϕ6 RdRp has an encircled active site (Butcher et al., 2001). The ϕ6 template RNA enters the polymerase through a channel formed by the interaction between the thumb and the fingers while NTPs gain entry to the catalytic site through an exposed
pair with the GTPi. A second GTP then base pairs with N$_{+1}$. Divalent cations connected to the nearby catalytic aspartates then induce phosphoryl transfer between GTPi and GTP2, forming the first phosphodiester bond and releasing pyrophosphate. The third substrate nucleotide enters the catalytic site as the duplexed template and nascent RNA are ratcheted forward. This elegant initiation model provides a wealth of biochemically testable predictions.

Prime and realign. A variation of the de novo initiation mechanism can take place with some negative-strand RNA viruses (Garcin and Kolakofsky, 1990, 1992). The 5’ ends of the genomic and antigenomic RNAs of segmented negative-strand RNA viruses (such as Bunyaviridae, Arenaviridae, and Nairoviridae) contain what appear to be nontemplated nucleotides (Garcin and Kolakofsky, 1992; Garcin et al., 1995; Jin and Elliot, 1993). Arenavirus RNA initiation is hypothesized to take place from an internal templated cytidylate. After the synthesis of a few nucleotides, the nascent RNA is shifted to 3’ of T$_{+1}$, so that the initiation GTP overlaps the end of the template for genomic and antigenomic RNA synthesis (Fig. 2). It is not presently known whether the short primers are released, and then prime synthesis from another transcription complex, or whether the same complex undergoes a conformational shift 3’ of the template. Nonetheless, the prime and realign mechanism nicely explains the extra guanylate present at the 5’ ends of the genomes and antigenomes. This guanylate is a substrate for capping by the vaccinia virus capping enzyme, consistent with the hypothesis that genome-length RNA synthesis can initiate de novo in negative-strand RNA viruses. The prime and realign mechanism may be selected because it allows de novo initiation to take place from a more protected internal nucleotide without loss of viral genetic information. Furthermore, it suggests a highly dynamic interaction between the polymerase, template, and substrate during the initiation of RNA synthesis.

Future challenges. The current active effort to identify cellular and viral factors required for the initiation of RNA synthesis will continue to advance our knowledge of viral RNA replication. Several less active areas that deserve more effort include (1) characterization of the in vivo initiation of genomic and antigenomic RNA synthesis; (2) kinetic analysis of viral RNA synthesis in vitro; (3) elucidation of the biophysical rules that govern ternary complex formation, including the transient interactions between the template and nascent RNAs and the conformational changes during different steps in RNA synthesis. The approaches used to study transcription from DNA templates should be valuable and adaptable for studies of viral RNA synthesis. Another eagerly anticipated event is the elucidation of the structure of a replicase ternary complex. The Q$_B$ replicase should be a leading candidate for structural determination since it can be purified to homogeneity and in sufficient quanti-

### TABLE 1

<table>
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<th>Virus</th>
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<th>β-loop motif?</th>
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* The original β-loop sequence was from Lesberg et al. (1999). RdRp sequences obtained from PFAM (http://pfam.wustl.edu/) were hand curated and analyzed by the Chou–Fasman secondary structure prediction for a structural counterpart to the hepatitis C β-loop motif. The sequences shown are not intended to denote all of the residues within the β-loop structure. Bold letters highlight the abundance of aromatic amino acids in this motif.
ties for crystallization trials and structural analyses. Additional ternary RdRp complexes that can be compared with the existing one from bacteriophage φ6 will also be very useful. Such investigations should reveal targets for antiviral therapies and should allow us to be better initiated in our understanding of viral RNA synthesis.

Appendix: Gathering evidence for de novo initiation.

For many RNA viruses, the mechanism of initiation has not yet been addressed experimentally. Therefore, it may be helpful to summarize the approaches used to demonstrate de novo initiation. In general, a careful examination of the sequence at the 5′ ends of viral RNAs is the best approach for gathering information on the initiation mechanism(s) used by a virus. For de novo initiation, since the γ- and β-phosphates of the NTPi are retained in the nascent RNA, hence the RNA products can be labeled with a γ-32P-NTPi (e.g., Sun et al., 2000). Because there are a number of enzymes that remove phosphates or transfer the γ-phosphate to other nucleotides, these experiments need to be carefully controlled (Kadare and Haenni, 1997; Shapiro and Krug, 1988). Another caution—labeling with γ-32P-NTPi can be inefficient because the NTPi may be needed at such a high concentration that the use of a radioactive NTPi is inconvenient (Kao and Sun, 1996; Luo et al., 2000). However, this very requirement for higher NTPi concentration can be exploited to examine the initiation process. If the NTPi is present in amounts insufficient for efficient initiation but adequate for elongation, RNA synthesis will be limited. The addition of a nucleotide analog that can substitute for the NTPi will increase RNA synthesis without participating in phosphor transfer (Honda et al., 1986; Kao and Sun, 1996). For example, GDP or GMP can sometimes substitute for GTP in initiation, thus allowing analysis of the initiation step.

Modification of the 3′-terminal hydroxyl of the template RNA can be used to help rule out template-primed RNA synthesis. This approach is especially useful if functional templates of minimal lengths have been characterized (Kao et al., 1999, 2000; Luo et al., 2000; Zhong et al., 2000a). De novo initiated RNA could also be tested for the ability to be capped in vitro and/or precipitated by antibody specific for the cap (Venkatesan et al., 1980; Honda et al., 1998).

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