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Review

Initiation of viral RNA-dependent RNA polymerization

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This review summarizes the combined insights from recent structural and functional studies of viral RNA-dependent RNA polymerases (RdRPs) with the primary focus on the mechanisms of initiation of RNA synthesis. Replication of RNA viruses has traditionally been approached using a combination of biochemical and genetic methods. Recently, high-resolution structures of six viral RdRPs have been determined. For three RdRPs, enzyme complexes with metal ions, single-stranded RNA and/or nucleoside triphosphates have also been solved. These advances have expanded our understanding of the molecular mechanisms of viral RNA synthesis and facilitated further RdRP studies by informed site-directed mutagenesis. What transpires is that the basic polymerase right hand shape provides the correct geometrical arrangement of substrate molecules and metal ions at the active site for the nucleotidyl transfer catalysis, while distinct structural elements have evolved in the different systems to ensure efficient initiation of RNA synthesis. These elements feed the template, NTPs and ions into the catalytic cavity, correctly position the template 3′ terminus, transfer the products out of the catalytic site and orchestrate the transition from initiation to elongation.

INTRODUCTION

RNA-dependent RNA polymerases (RdRPs) are central components in the life cycle of RNA viruses. Correct initiation of RNA synthesis is essential for the integrity of the viral genome. Other virus- and cell-encoded components are also often needed in viral RNA synthesis to ensure RdRP activity through the formation of RNA–protein complexes similar to DNA-dependent RNA transcription complexes (Buck, 1996; Lai, 1998, and references therein; Patton *et al.*, 1997). As RNA viruses demonstrate a variety of mechanisms for the initiation of RNA synthesis (Fields *et al.*, 1996), it is expected that RdRPs from different sources will possess distinct molecular adaptations facilitating precise and efficient initiation.

The structures of the RdRPs solved to date show that they all have a basic right hand-like structure with fingers, palm and thumb subdomains (Fig. 1), similar to the DNA-dependent RNA polymerases (DdRPs), reverse transcriptases (RTs) and DNA-dependent DNA polymerases (DdDPs) (Cheetham & Steitz, 2000; Doublie *et al.*, 1999; Ollis *et al.*, 1985).

Several conserved RdRP motifs have been identified (Koonin, 1991; Poch *et al.*, 1989). Most of these motifs are in the palm subdomain, with the motifs A, B and C being most prominent (Ollis *et al.*, 1985; Poch *et al.*, 1989; Hansen *et al.*, 1997). Based on structural similarities and the presence of conserved motifs, it was proposed that all polymerases utilize a common two-metal mechanism of catalysis (Fig. 2). This involves two conserved aspartic acid residues from the A (Asp_A) and C (Asp_C) motifs, respectively, and two divalent metal ions for the formation of the phosphodiester bonds (Joyce & Steitz, 1995; Steitz, 1998). The carboxylates anchor a pair of divalent metal ions, which play the major role in catalysis; one divalent metal ion (Mg^{2+} ion 1) promotes the deprotonation of the 3′ hydroxyl of the nascent strand, while the other (Mg^{2+} ion 2) facilitates the formation of the pentacovalent transition state at the α-phosphate of the dNTP and the exit of the inorganic pyrophosphate group (PP_i).

Here, we will point out that, in addition to the obvious similarities, viral RdRPs have a number of unique features which are important for efficient and accurate initiation of RNA-dependent RNA synthesis in the different viruses.

Diverse terminology has been used in the literature to describe components of the polymerase initiation complex. In this review, the term 'RdRP' will be used to refer to the catalytic subunit of RNA virus polymerases. The template nucleotides at the 3′ end of the template (which is used to initiate *de novo* RNA synthesis) will be called T1, T2,…, in the 3′→5′ direction. Nucleotides of the daughter strand are denoted D1, D2,…, in the 5′→3′ order, such that D1 base pairs with T1, D2 with T2, and so on. In the literature, D1 is

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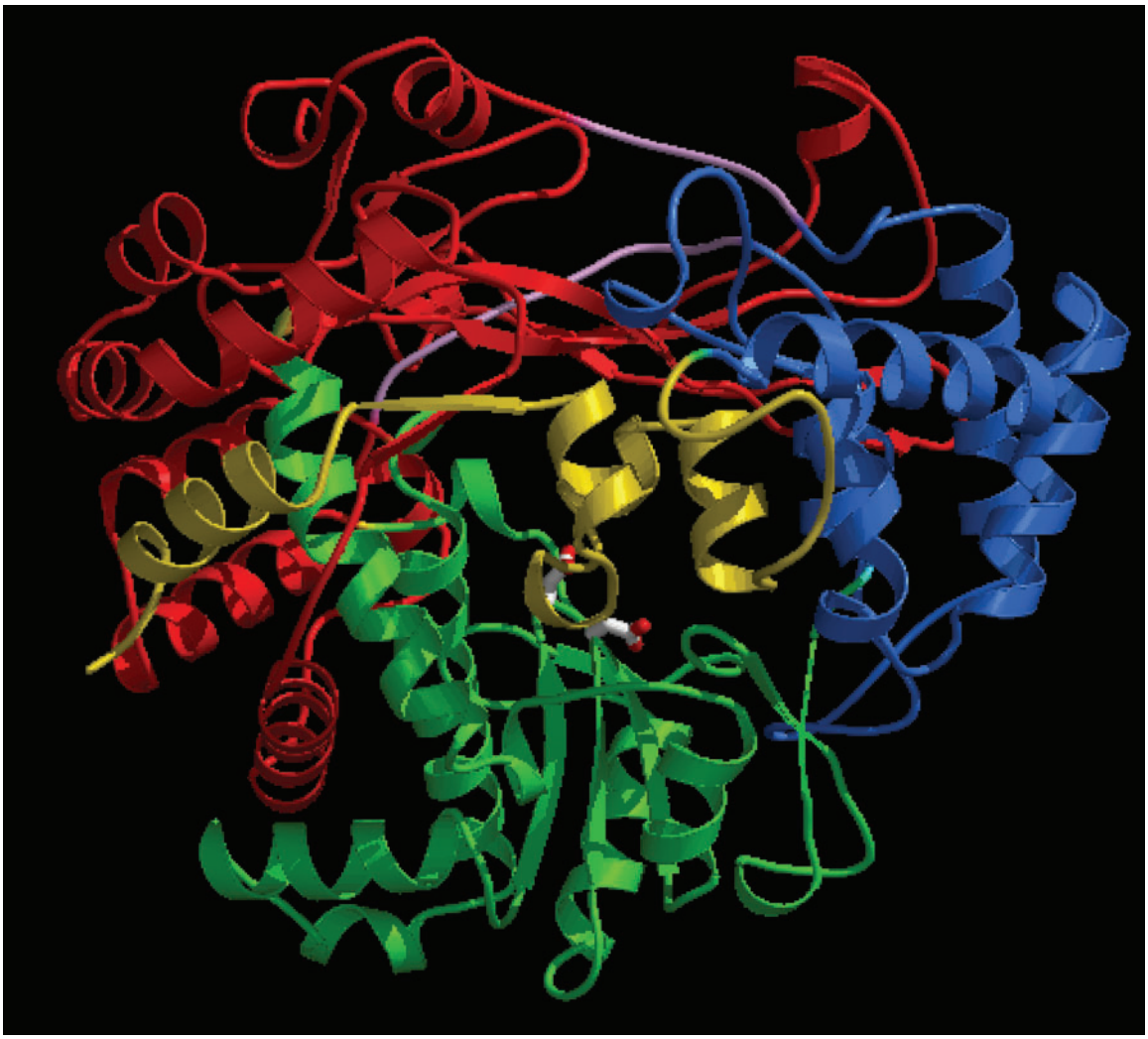

Fig. 1. Basic right hand structure of the bacteriophage ϕ6 polymerase. Red, fingers; green, palm; blue, thumb; and yellow, priming domain. (Courtesy of J. M. Grimes & D. I. Stuart.)

sometimes called the initiation nucleotide (NTPi) and D2 is called NTPi + 1. With regard to the polymerization reaction, we differentiate between replication (synthesis of genomic RNA) and transcription (synthesis of viral mRNAs).

Initiation mechanisms

Although diverse RNA viruses use an amazing variety of replication scenarios, there are only two principally different mechanisms by which RNA synthesis can be initiated (Fig. 3): *de novo* and primer-dependent initiation (reviewed by Kao *et al.*, 2001, and references therein; Ranjith-Kumar *et al.*, 2002b; Paul *et al.*, 1998).

De novo initiation

De novo initiation, also known as primer-independent initiation, requires interactions of at least the following four components: (i) the RdRP; (ii) the RNA template with a virus-specific initiation nucleotide; (iii) the initiation nucleoside triphosphate (D1); and (iv) a second NTP (D2). The first phosphodiester bond is formed between D1 and D2. The initiation nucleotide (essentially a one-nucleotide primer) provides the 3′-hydroxyl for the addition of the next nucleotide. The advantage of *de novo* RNA synthesis for viral RNA replication is that no genetic information is lost during replication and no additional enzymes are needed to generate the primer or to cleave the region between template and newly synthesized RNA. In most cases, the productive *de novo* initiation event is immediately followed by elongation. However, in some instances *de novo* initiation leads to the formation of abortive RNA products (abortive initiation) or gives rise to short RNA oligonucleotides that are subsequently used as primers (prime and realign mechanism).

Abortive initiation. The *de novo* synthesis of short abortive RNAs (two to five nucleotides) has been reported for T7 bacteriophage, *Escherichia coli* and eukaryotic DdRPs

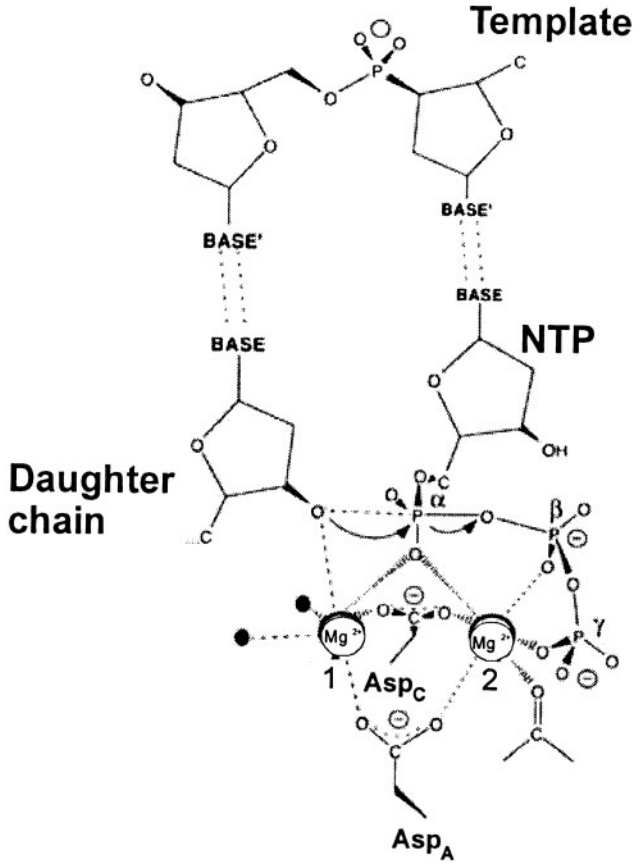

Fig. 2. Catalytic mechanism of nucleotidyl transfer. [Modified from Steitz (1998) with permission.]

(Martin *et al.*, 1988; Carpousis & Gralla, 1980; Ackerman *et al.*, 1983) and viral RdRPs such as those of turnip crinkle carmovirus (Nagy *et al.*, 1997), reovirus (Yamakawa *et al.*, 1981) and rotavirus (Chen & Patton, 2000). Several RdRPs that initiate *de novo* can also use these oligonucleotides to replace D1 *in vitro* (Downing *et al.*, 1971; Garcin & Kolakofsky, 1992; Honda *et al.*, 1986; Kao & Sun, 1996; Nagy *et al.*, 1997).

Prime and realign. With some negative-strand RNA viruses such as *Bunya-*, *Arena-* and *Nairoviruses*, the 5′ ends of the genomic and antigenomic RNAs contain non-templated nucleotides (Garcin & Kolakofsky, 1990, 1992; Garcin *et al.*, 1995; Jin & Elliott, 1993). *Arenavirus* RNA initiation is hypothesized to take place from an internal templated cytidylate. After the synthesis of a few nucleotides, the daughter RNA is shifted to the position 3′ of T1 so that the initiation GTP overhangs the end of the template for genomic and antigenomic RNA synthesis. The prime and realign mechanism explains the extra guanylate present at the 5′ ends of the genomes and antigenomes and may be selected because it allows *de novo* initiation to take place from a protected internal nucleotide without losing genetic information (Kao *et al.*, 2001).

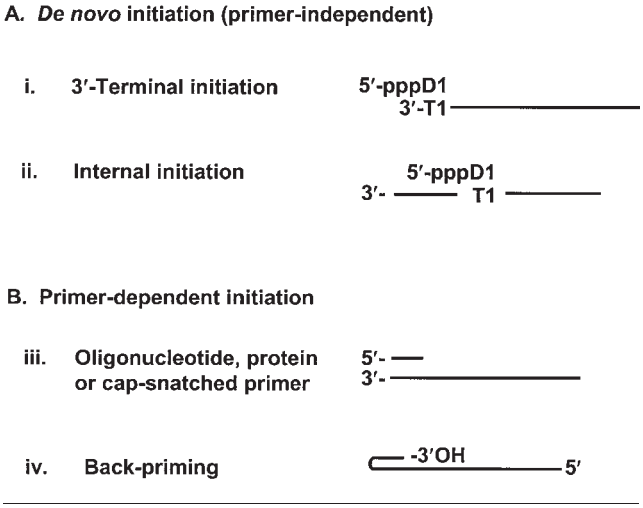

Fig. 3. Schematic diagram of initiation mechanisms of viral RNA-dependent RNA polymerases. (A) *De novo* initiation from the 3′ terminus of the viral genome (i) or internal to the template during subgenomic synthesis (ii). (B) Primer-dependent initiation where the primer can be a protein primer, a snatched cap or an oligonucleotide (iii) or the 3′ terminus of the template that folds back mimicking a template–primer structure (iv).

Primer-dependent initiation

Several viruses initiate RNA synthesis using either an oligonucleotide or protein primer: (i) Oligonucleotides cleaved from the 5′ end of a capped cellular mRNA (cap-snatching) are used by many segmented negative-strand RNA viruses for transcription (Hagen *et al.*, 1995). (ii) A short leader RNA (two to five nucleotides) synthesized by viral RdRPs during abortive cycling (described above) can also serve as primer (McClure, 1985). (iii) With template-primed initiation (also known as loop-back, copy-back, turn-around or back-priming synthesis), the 3′ end of the template RNA loops back on itself to serve as a primer (Behrens *et al.*, 1996; Zhong *et al.*, 1998, 2000b; Luo *et al.*, 2000; Laurila *et al.*, 2002). (iv) With protein-primed initiation, an amino acid provides the hydroxyl group for the formation of a phoshodiester bond with the first nucleotide. This mechanism is used by *Picornaviridae* (Paul *et al.*, 1998) and DNA viruses such as adenoviruses and bacteriophages ϕ29 and PRD1 (Salas, 1991).

RNA viruses can use either one or sometimes both of these mechanisms for initiation of RNA synthesis. *De novo* initiation is used by viruses with positive, negative, double-stranded (dsRNA) and ambisense RNA genomes (Kao *et al.*, 2001). Specific examples include the dsRNA viruses such as the *Cystoviridae* (Makeyev & Bamford, 2000a, b; Yang *et al.*, 2003a) and rotavirus (Chen & Patton, 2000) and negative-strand RNA viruses such as vesicular stomatitis virus (VSV) (Testa & Banerjee, 1979). *De novo* initiation is widely used by positive-strand RNA viruses; examples include plant alphavirus-like viruses (Strauss & Strauss,

1994; Goldbach *et al.*, 1991), members of the family *Flaviviridae*, namely hepatitis C virus (HCV) (Kao *et al.*, 1999, 2001; Luo *et al.*, 2000; Oh *et al.*, 1999; Zhong *et al.*, 2000a), Kunjin (Guyatt *et al.*, 2001) and dengue 2 (Ackermann & Padmanabhan, 2001), as well as bacteriophage Qβ (Blumenthal, 1980). Poliovirus exclusively uses primer-dependent initiation (Paul *et al.*, 1998), while influenza virus employs a combination of the two mechanisms with the choice being determined by the type of RNA to be synthesized (Honda *et al.*, 1986). Back priming, however, appears to be an artefact of *in vitro* reactions. Although RdRPs from HCV and bovine viral diarrhoea virus (BVDV) preferentially utilize back priming *in vitro*, they most likely use *de novo* initiation *in vivo* (Ranjith-Kumar *et al.*, 2002a, and references therein; Kao *et al.*, 2001; Gong *et al.*, 1998a, b).

Most viruses initiate both replication and transcription at the very terminal end of the genome. However, some may employ internal initiation of RNA synthesis for transcription (reviewed by Miller & Koev, 2000, and references therein). Examples include VSV, brome mosaic virus (BMV) and cucumber necrosis virus (CNV). VSV transcription initiates at different positions *in vitro* and *in vivo*. *In vitro*, VSV initiates transcription at the genomic 3′ end (Emerson, 1982), but *in vivo* transcription initiates internally directly at the first gene-start sequence (Whelan & Wertz, 2002). RNA-dependent RNA replicases of the plant viruses CNV and BMV have distinct modes of initiation site recognition for initiation at the 3′ terminus and internal initiation (Panavas *et al.*, 2002; Ranjith-Kumar *et al.*, 2003). Enhancer-like activity of a viral RNA promoter seems to be important in this differentiation.

INSIGHTS FROM THE RdRP STRUCTURES

To date, the structures of six viral RdRPs have been determined (Table 1), namely those of poliovirus (PV) $3D^{pol}$ (Hansen *et al.*, 1997; Hobson *et al.*, 2001), HCV NS5B of the BK strain and HCV NS5B of the J4 strain (Ago *et al.*, 1999; Bressanelli *et al.*, 1999; Lesburg *et al.*, 1999; O'Farrell *et al.*, 2003), bacteriophage ϕ6 protein P2 (Butcher *et al.*, 2001; Salgado *et al.*, 2004), rabbit haemorrhagic disease virus (RHDV) $3D^{pol}$ (Ng *et al.*, 2002) and reovirus protein λ3 (Tao *et al.*, 2002). The structures of initiation complexes have been solved for bacteriophage ϕ6 P2 (Butcher *et al.*, 2001; Salgado *et al.*, 2004), reovirus λ3 (Tao *et al.*, 2002) and HCV HC-J4 NS5B (O'Farrell *et al.*, 2003).

The new structural data confirm the proposal that all nucleic acid polymerases share similarities in structure and the mechanism of catalysis (Joyce & Steitz, 1995). The basic polymerase right hand shape provides the correct geometrical arrangement of substrate molecules and metal ions at the active site for catalysis. However, RdRPs also have specific features that distinguish them from other polymerases. The structural characteristics of viral RdRPs that have been determined closely resemble each other (Hansen *et al.*, 1997; Bressanelli *et al.*, 2002; Butcher *et al.*, 2001; Doublie *et al.*, 1999; Huang *et al.*, 1998; Tao *et al.*, 2002; O'Farrell *et al.*, 2003). One structural attribute that distinguishes most RdRPs from other polymerases is their 'closed hand' conformation, as opposed to the 'open hand' shape of the other known polymerases. The closed conformation is accomplished by interconnecting the fingers and thumb domains with several loops (fingertips) protruding from the fingers. The closed structure creates a well-defined template channel, which might regulate the recognition of the initiation site (Bressanelli *et al.*, 2002; Butcher *et al.*, 2001). The template channel and additional structures near the active site ensure that initiation of minus-strand RNA synthesis takes place at or near the end of the 3′ termini of the HCV and bacteriophage ϕ6 RNAs (Hong *et al.*, 2001; Laurila *et al.*, 2002). The NTP (substrate) tunnel is another common structural RdRP element. The hypothesis is that negatively charged incoming NTPs interact sequentially with positively charged amino acids in the tunnel to reach the active site. DdRPs also have a well-defined channel for nucleotide diffusion (Gnatt *et al.*, 2001; Murakami *et al.*, 2002). Co-crystallization with nucleoside triphosphates and/or with oligonucleotides has mapped substrate-binding sites, while the binding of Mg^{2+} and/or Mn^{2+} has mapped the active site of the enzymes.

The overall structural similarity and the conservation of secondary and tertiary structure elements in the palm and thumb domains of polymerases of the families *Picorna-*, *Flavi-*, *Cysto-* and *Retroviridae* has led to speculation that they may have evolved from a common ancestor (Butcher *et al.*, 2001; O'Farrell *et al.*, 2003). These structural similarities are not predictable by comparative sequence analyses alone (Koonin, 1991; Iyer *et al.*, 2003). The general significance of structural conservation in the context of virus evolution has been discussed elsewhere (Bamford *et al.*, 2002; Bamford, 2003).

Model for the initiation of RNA synthesis of bacteriophage ϕ6

The structures of the initiation complexes of the RdRPs of reovirus (Tao *et al.*, 2002) and bacteriophage ϕ6 (Butcher *et al.*, 2001; Salgado *et al.*, 2004) allow one to elaborate on their initiation mechanisms. Here we will discuss the proposed model for initiation of viral RNA synthesis for bacteriophage ϕ6 RdRP (Butcher *et al.*, 2001). The bacteriophage ϕ6 RdRP is a compact, spherical molecule (Butcher *et al.*, 2001). Its shape is attributed to two elaborations of the basic right hand architecture, a polypeptide chain that connects the fingertips and thumb similar to the chain identified for the HCV RdRP and a C-terminal elaboration, known as the initiation or priming platform, resembling the C-terminal β-hairpin of the HCV RdRP (Ago *et al.*, 1999; Bressanelli *et al.*, 1999; Lesburg *et al.*, 1999).

The following structures have been solved for the ϕ6 RdRP: the RdRP complexed with a DNA (Butcher *et al.*,

Table 1. Information on known structures of viral RNA-dependent RNA polymerases

Polymerase source, virus family, name, PDB identification number	Resolution	RdRP structure	Reference
Poliovirus (*Picornaviridae*) $3D^{pol}$ PDB: 1RDR	2.4 Å	Complete protein crystallized – 461 residues Residues not modelled are 1–12, 38–66, 98–181, 270–290	Hansen *et al.* (1997)
Hepatitis C virus (BK strain) (*Flaviviridae*) NS5B PDB: 1CSJ	2.8 Å	Protein lacks the 55 C-terminal amino acids 536 residues crystallized 531 residues modelled The 5 C-terminal disordered residues (532–536) were not modelled	Bressanelli *et al.* (1999)
Hepatitis C virus (BK strain) (*Flaviviridae*) NS5B PDB: 1GX6 (complex with UTP and Mn^{2+})	1.85 Å	Protein lacks the 55 C-terminal amino acids 531 residues crystallized 531 residues modelled	Bressanelli *et al.* (2002)
Hepatitis C virus (BK strain) (*Flaviviridae*) NS5B PDB: 1QUV	2.5 Å	Protein lacks 21 C-terminal amino acids 578 residues crystallized 553 residues modelled Residues not modelled are 544–547; 557–578 (residues 571–578 are a His tag)	Ago *et al.* (1999)
Hepatitis C virus (BK strain) (*Flaviviridae*) NS5B PDB: 1C2P	1.9 Å	Protein lacks 21 C-terminal residues 576 residues crystallized 566 residues modelled Residues not modelled are 149–153; 567–570	Lesburg *et al.* (1999)
Hepatitis C virus (J4 strain) (*Flaviviridae*) NS5B PDB: 1NB4 (apo-enzyme) PDB: 1NB6 (complex with UTP) PDB: 1NB7 (complex with RNA)	2.0 Å 2.6 Å 2.9 Å	Protein lacks 21 C-terminal amino acids; has a C-terminal His tag 570 residues crystallized Residues not well ordered: residues in a finger-tip surface loop (149–151) and those near the C terminus (567–570) and the His tag	O'Farrell *et al.* (2003)
Bacteriophage ϕ6 (*Cystoviridae*) P2 PDB: 1HI8 (apo-enzyme) PDB1HI0 (complex with DNA)	2.5 Å 3.0 Å	Complete 664 residue protein crystallized All residues modelled	Butcher *et al.* (2001)
Bacteriophage ϕ6 (*Cystoviridae*) P2 PDB: Salgado *et al.* (2004) (complex with RNA)	2.0 Å	Complete 664 residue protein crystallized	Salgado *et al.* (2004)

Table 1. cont.

Polymerase source, virus family, name, PDB identification number	Resolution	RdRP structure	Reference
Rabbit haemorrhagic disease virus (*Caliciviridae*) $3D^{pol}$ PDB: 1KHV	2.5 Å	Complete 516 residue protein crystallized Residues not modelled: 1–4, 181–184 and 15 C-terminal residues (502–516)	Ng *et al.* (2002)
Reovirus (type 3; strain Dearing) (*Reoviridae*) λ3 PDB: 1N35	2.5 Å	Complete 1267 residue protein crystallized 1256 residues modelled Residues not modelled: the flexible loop between residues 957 and 964, one residue at the N terminus and two residues (1266–1267) at the C terminus	Tao *et al.* (2002)

2001) or RNA oligonucleotide representing the 3′ end of the negative-sense strand of the viral genome (Salgado *et al.*, 2004); the RdRP complex with DNA and two GTPs (Butcher *et al.*, 2001); and that of a dead-end initiation complex (Salgado *et al.*, 2004). The latter structure demonstrates that the polymerase is active in the crystal form. The structural details of these complexes complement and extend the biochemical and genetic information on the initiation mechanisms.

The ϕ6 RdRP has two positively charged tunnels that, respectively, allow the access of the RNA template and NTP substrates to the active site. The template RNA enters the polymerase through the channel formed by the interaction between the thumb and the fingers, while NTPs enter the catalytic site through the opening formed primarily by the fingers and palm domains. The template tunnel is wide enough to accommodate single-stranded RNA (ssRNA) but not dsRNA. The distance from the surface to the active site can be spanned by a 5-mer single-stranded oligonucleotide.

Structural and biochemical studies indicate that the C-terminal domain and initiation platform of the ϕ6 RdRP have three functions (Butcher *et al.*, 2001; Laurila *et al.*, 2002). They (i) stabilize the initiation complex by interacting with the initiation nucleotides; (ii) prevent back priming; and (iii) serve as a physical barrier to block the exit end of the template tunnel during initiation.

From the structure of the bacteriophage ϕ6 RdRP–DNA complex, Butcher *et al.* (2001) proposed a sequence of events that could result in the formation of the initiation complex as follows (Fig. 4). Steps I–IV: the template enters the tunnel and interactions with the specificity pocket, S, lock it in place; NTPs occupy site I, presumably in rapid exchange. Step V: the D1 GTP binds to the initiation platform (P), stabilized by three hydrogen bonds to the cytidine of the template and stacking interactions with Tyr-630 of the initiation platform in the C-terminal domain. Step VI: the template ratchets back, facilitated by electrostatic attraction to Arg-268 and Arg-270, freeing T1 from the specificity pocket. Step VII: a second GTP, D2, enters the P site to lock the initiation complex into its active form; catalysis releases pyrophosphate, freeing the nucleotide from Arg-268 and Arg-270 so that it can ratchet down, displacing the C-terminal domain of the protein (step VIII). This may be facilitated by attraction of the phosphates of the GTP in site P to the Mn^{2+} ion. The next NTP slips into the catalytic site, C, from site I, which sets the ratchet for the chain elongation to start.

When RNA oligonucleotides bind in the RdRP template tunnel, the orientation of the bases varies resulting in different oligonucleotide–RdRP interactions (Salgado *et al.*, 2004). The most important difference is in the specificity pocket, where the strictly conserved T1 cytidine is rotated approximately 180°. The T2 RNA nucleotide is stabilized by base stacking with the T3 uracil, an effect not observed with DNA. Overall, the presence of an extra hydroxyl group in RNA changes the sugar conformation of the nucleotides and slightly rearranges the side-chains of interacting polymerase residues, leading to the formation of extra hydrogen bonds between the protein and oligonucleotide, further stabilizing the RNA complex compared with the DNA complex (Fig. 5). This might be the basis for the preference of the bacteriophage ϕ6 RdRP for RNA templates.

Structural features facilitating initiation of RNA synthesis

Structural elements that ensure *de novo* initiation have been identified. The initiation mechanism for bacteriophage ϕ6, where the two initiation nucleotides stack against each other and against a specialized priming platform followed by repositioning of this element to allow egress of the product, may also apply to the RdRPs of HCV (Ago *et al.*, 1999; Bressanelli *et al.*, 1999; Lesburg *et al.*, 1999) and

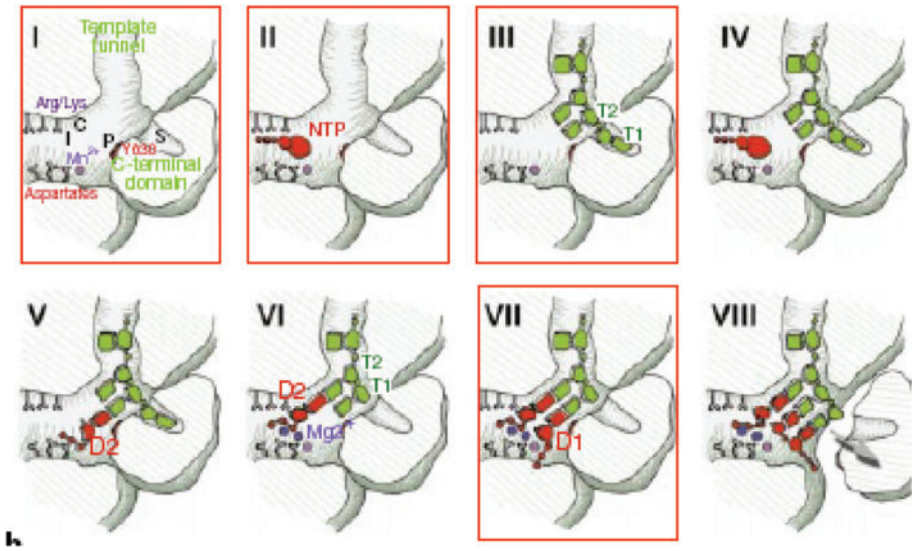

Fig. 4. Model for initiation and chain elongation of the bacteriophage ϕ6 RdRP, illustrating key points in the reaction mechanism. Red boxes highlight experimental results. (I) Apo structure with bound Mn^{2+}. Binding sites are identified in black letters. (II) NTP bound in site I. (III) Template bound. (IV) Template bound and NTP non-productively bound at site I. (V) Initial productive binding at site P. (VI) Template ratchets back. (VII) Second GTP bound at site P. Polymerization can occur. (VIII) Polymerization has occurred, releasing nascent duplex from ordered binding at the active site C. The C-terminal domain moves allowing the duplex to ratchet forward, out of the active site. [From Butcher *et al.* (2001), with permission.]

reovirus (Tao *et al.*, 2002). It may even be used as an initiation paradigm in many RNA polymerases. Structural comparisons of unrelated RNA polymerases have recently revealed general features of initiation (Murakami & Darst, 2003). Similar to the ϕ6 C-terminal domain, the N-terminal domain in the bacteriophage T7 DdRP initiation complex blocks the path of the elongating RNA transcript and also requires conformational changes to facilitate the transition from initiation to elongation. However, the finger domains differ significantly among viral RNA polymerases. This may be a result of specific adaptations to structurally diverse substrates (Hobson *et al.*, 2001). Structural details of individual RdRPs are as follows.

HCV. The fingers domain of the HCV RdRP has a long binding groove, which constitutes a template tunnel (O'Farrell *et al.*, 2003). Other specific structural features of the RdRP include a loop that connects the fingers with the thumb (the Λ or A1-loop) and a β-hairpin protruding from the thumb domain toward the active site at the base of the palm domain (Bressanelli *et al.*, 1999; Lesburg *et al.*, 1999). The A1-loop may be responsible for the closed conformation of the RdRP and the 'clamping' motion of the enzyme (Labonte *et al.*, 2002). Mutation of Leu-30 (which has a strong hydrophobic interaction with the thumb domain) to polar residues was detrimental to RdRP activity, confirming that it is a critical element for enzyme activity (Labonte *et al.*, 2002). The β-hairpin seems to be important in positioning the 3′ terminus of the viral genome for correct initiation of replication. It allows only the single-stranded 3′ terminus of an RNA template to bind productively to the active site and may function as a gate preventing the 3′ terminus of the template RNA from slipping through the active site, thus ensuring terminal initiation of replication (Hong *et al.*, 2001). Recently, a regulatory motif in the C-terminal non-catalytic region of the HCV RdRP has been identified upstream of the membrane anchor domain. It comprises a unique conserved hydrophobic pocket, which protrudes into the RNA-binding cavity. Several functions have been proposed for this motif. One is that, together with the β-hairpin, it forms a rigid bulky loop at the active site that serves as an initiation platform similar to that of bacteriophage ϕ6 (Butcher *et al.*, 2001). Other possible functions are that it could play a role in initiation in ensuring correct RNA replication by preventing primer-dependent and back-primed initiation and recognition of the correct secondary structure at the 3′-terminal end of the HCV genome (Leveque *et al.*, 2003).

Crystal structures of the HCV RdRP (genotype 1b, strain J4) complexed with metal ions and NTPs revealed that the fingers domain forms a positively charged NTP tunnel (O'Farrell *et al.*, 2003). The HCV RdRP–rNTP complexes indicated how incoming NTPs might access the metals by moving along the funnel and how, subsequently, pyrophosphate exits the active site without necessitating large structural changes. The J4 RdRP has a single NTP molecule in the polymerase active site (O'Farrell *et al.*, 2003), while NTP complexes of the RdRP of the HCV BK Δ55 strain accommodated two NTPs (Bressanelli *et al.*, 1999). The difference has been attributed to the deletion of the

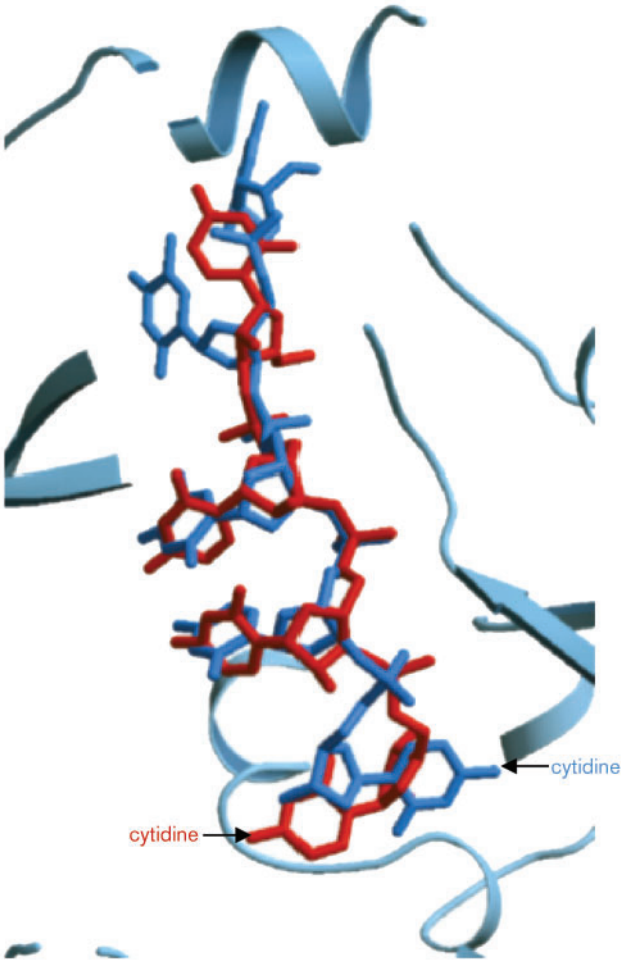

Fig. 5. Superposition of an RNA oligonucleotide and DNA oligonucleotide binding in the template tunnel of bacteriophage ϕ6. In general, RNA (red) and DNA (blue) bind to the template tunnel in a similar way, although they exhibit different base and sugar orientations, particularly in the 3′ and 5′ ends. The presence of the hydroxyl group in the sugar causes rearrangements of the nucleotides and the surrounding residues (not shown). The most important difference observed is in position 1, the conserved cytidine in the specificity pocket (indicated by black arrows). There is a movement of roughly 180° of the base, accompanied by a change in sugar orientation. (Courtesy of P. S. Salgado, J. M. Grimes & D. I. Stuart.)

C terminus in the Δ55 RdRP, which improves solubility (Yamashita *et al.*, 1998; Tomei *et al.*, 2000) but may change its properties. Structurally this deletion causes a shift in the positions of the β-flap backbone atoms, which increases the active site volume and may allow the binding of an extra triphosphate (Bressanelli *et al.*, 1999). Structure-guided site-directed mutagenesis elucidated the role of some basic amino acids in NTP trafficking. The last positively charged amino acid (Arg-222) in close proximity to the active site is critical in delivering NTPs to the active site. Substitution of Lys-151 at the beginning of the NTP tunnel with glutamic acid increased enzyme activity compared with the wild-type due to the formation of more productive pre-initiation complexes (Labonte *et al.*, 2002). Arg-518 in the BVDV RdRP and its HCV RdRP equivalent, Arg-386, were important for *de novo* RNA synthesis (Lai *et al.*, 1999; Hong *et al.*, 2001). HCV Arg-386 proved to be important for dinucleotide-initiated RNA synthesis. These observations support the hypothesis that Arg-386 plays a critical role in stabilizing the interaction between the 3′ terminus of the RNA template and the initiating nucleotide(s) (Hong *et al.*, 2001).

The crystal structure of the HCV RdRP indicates that there is not enough space underneath the β-hairpin to allow the passage of a dsRNA product. Therefore, the β-hairpin should be flexible to give way to the nascent dsRNA (Hong *et al.*, 2001). It was hypothesized that a change in conformation of the thumb domain takes place upon template binding to allow efficient *de novo* initiation of RNA synthesis (Bressanelli *et al.*, 2002).

Reovirus. Structural features of the reovirus RdRP that facilitate initiation of RNA synthesis include a cube-like cage structure, tunnels and a priming loop. The reovirus RdRP polypeptide chain folds into a compact unit with a central cavity. It has three domains: a central polymerase domain, which contains the fingers, palm and thumb subdomains, an N-terminal domain, which bridges the fingers and thumb on one side of the catalytic cleft, and a C-terminal bracelet domain, which covers the catalytic cleft on the other side (Tao *et al.*, 2002). The catalytic site, enclosed in the centre, is accessible to solvent, substrate or template/product through four channels of various sizes at the 'front', 'left', 'rear' and 'bottom'. The front channel is the bracelet opening and is large enough to accommodate dsRNA. The left channel is at the interface of the polymerase and bracelet domains and the rear channel is at the interface of the polymerase and N-terminal domains. All three domains border the bottom channel. Tao *et al.* (2002) suggested that the roles of the channels are as follows. The front channel is for the exit of newly formed dsRNA produced during replication. The left channel is for template entry and the rear channel is the substrate tunnel. The bottom channel in the reovirus RdRP is a structural feature not yet observed in other polymerases. It is most likely the exit route for mRNA produced during transcription.

The reovirus RdRP also has a special priming loop that supports the stacking of the priming NTP, D1. This loop is a unique insertion within a strand present in the palms of all other polymerases. In the fully active polymerase complex, this loop retracts towards the palm with respect to its position in the apo-enzyme and in the initiation complex to fit into the minor groove of the product duplex (Tao *et al.*, 2002).

RHDV and poliovirus. These viruses have a virus-encoded VPg covalently linked to the 5′ terminus of their

genome. *In vitro*, the RHDV RdRP uses back priming from the 3′-hydroxyl end of the genome for minus-strand synthesis (López Vázquez *et al.*, 2001). However, Paul *et al.* (1998) reported that poliovirus VPg facilitates protein-primed RNA synthesis and predicted that this mechanism should function in most plus-strand RNA viruses with protein-linked genomes. The thumb domain of the RHDV RdRP seems to play an important role in RNA synthesis. Comparisons among the structures of the alternative conformational states of RHDV RdRP and RdRPs from HCV and poliovirus suggest novel structure–function relationships (Ng *et al.*, 2002). The RHDV thumb domain can adopt two conformations. Metal ions bind at different positions in the two conformations and suggest how structural changes may be important for enzymic function in RdRPs. The most dramatic difference between the RHDV and poliovirus RdRPs and that of HCV occurs in the loop connecting the third and fourth helices in poliovirus and RHDV RdRPs. In the HCV RdRP, this loop is replaced by a long β-hairpin insert that occludes the active site cleft. The shorter loops seen in the poliovirus and RHDV enzymes are consistent with their ability to utilize dsRNA as templates *in vitro* (Arnold & Cameron, 2000; López Vázquez *et al.*, 2001; Hong *et al.*, 2001) and with the fact that the poliovirus RdRP does not have a structural feature equivalent to the HCV RdRP β-hairpin or the bacteriophage ϕ6 RdRP initiation platform and uses protein-primed initiation for genome replication (Paul *et al.*, 1998).

For bacteriophage ϕ6 and HCV RdRPs, the initiation mechanism of RNA synthesis could be changed from *de novo* to primer-dependent initiation by reducing their initiation platforms. For the ϕ6 RdRP, YKW (aa 630–632) was changed to GSG (Laurila *et al.*, 2002) and the HCV β-hairpin (aa 443–454) from LDCQIYGACYSI to LGGI (Hong *et al.*, 2001).

Specialized features that facilitate dsRNA strand separation

Specialized features have been proposed to facilitate dsRNA strand separation and provide the basis for the mechanism that ensures feeding of the correct strand to the catalytic site for initiation of RNA synthesis. These include the reovirus 5′-cap binding site, which was identified on the surface of the RdRP between the template entrance and exit channels (Tao *et al.*, 2002), and the bacteriophage ϕ6 plough, which is adjacent to a positively charged groove over the polymerase surface (Butcher *et al.*, 2001). Structural comparisons and bioinformatic analysis of the HCV, RHDV and bacteriophage ϕ6 RdRPs also identified the N-terminal region of motif F as possibly being involved in unwinding of dsRNA for transcription (Bruenn, 2003). Whereas replication is a single event for dsRNA viruses, transcription involves multiple rounds of initiation. The mechanism of reinitiation on dsRNA templates is presently unknown. According to some models, it may be facilitated by a helicase suitably positioned in relation to the polymerase to displace the nascent chain from the transcription complex (Butcher *et al.*, 2001).

Active and inactive polymerase conformations

The repeated observations of an open conformation (not to be confused with the general 'open hand' structure of polymerases other than the RdRPs) in three structures of apo forms of RdRPs (RHDV, PV and HCV) suggest that these enzymes may prefer to adopt a catalytically inactive state, which can be transformed to a catalytically competent state by the binding of a primer, template, divalent metal ions and nucleotide triphosphates (Ng *et al.*, 2002).

RHDV. The thumb domain of the RHDV RdRP can adopt either an open or closed conformation (Ng *et al.*, 2002). Under physiological ionic strength conditions, divalent metal ions are bound to the enzyme and the active site cleft is open to the binding of RNA primer–template duplexes. Comparisons between the RHDV RdRP structure and the structures of PV and HCV RdRPs indicate that conformational changes similar to those seen in DNA polymerases may be important to the catalytic mechanism of RdRPs. The closed conformation is probably the active form of the RdRP because the ion coordination closely matches that seen in active enzyme–NTP–primer–template complexes formed by related DdDPs and the human immunodeficiency virus 1 RT (Doublie *et al.*, 1999; Huang *et al.*, 1998).

Reovirus. Crystalline reovirus RdRP is catalytically active. Structures of complexes have been obtained of the enzyme stalled at the initiation complex and of a fully active enzyme performing polymerization (Tao *et al.*, 2002). The structure of the apo form of the RdRP shows that only internal adjustments are required to accommodate substrates. Upon the formation of the first phosphodiester bond, the reovirus priming loop that supports the priming nucleotide D1 through base stacking retracts towards the palm with respect to its position in the apo-enzyme and in the initiation complexes to fit into the minor groove of the product duplex. This allows the newly synthesized RNA to exit the polymerase and facilitates the transition between the initiation and elongation stages of RNA synthesis (Tao *et al.*, 2002).

HCV. The orientation of the nucleotides in the active site of HCV RdRP was identified by superpositioning HCV structures on to the initiation complex of the bacteriophage ϕ6 RdRP (Bressanelli *et al.*, 2002). Density corresponding to the triphosphates of nucleotides bound to the catalytic metals was apparent. A network of triphosphate densities was detected that superimposed on the corresponding nucleotide moieties seen in the ϕ6 RdRP initiation complex, strengthening the proposal that the two enzymes initiate replication *de novo* by similar mechanisms. Three HCV RdRP amino acids that bind the triphosphate moiety of the nucleotide at the priming site,

Arg-158 in the fingers and Ser-367 and Arg-386 in the thumb, are conserved among HCV with different genotypes and in most *Flaviviruses*. A large solvent cavity was noticed in the region where the priming nucleoside moiety should lie, suggesting that a different conformation of the HCV RdRP thumb is needed for efficient initiation, in which the protein, through an element similar to the protein platform provided by residue Tyr-630 of the bacteriophage ϕ6 RdRP, would hold the D1 base in place to make Watson–Crick interactions with the 3′ base (T1) of the template.

Analysis of complexes of the HCV RdRP with NTPs and divalent metal ions revealed a specific GTP-binding site in a shallow pocket at the molecular surface of the enzyme at the interface between fingers and thumb. The position of this site suggested a possible role of GTP either in triggering the conformational change or in stabilizing the active conformation for efficient initiation (Bressanelli *et al.*, 2002). Since no conformational change was observed in the crystal of the enzyme with bound GTP, it was speculated that the presence of both template in the RNA-binding groove and GTP at the surface site might be necessary for such a conformational change. Alternatively, the surface site may provide an oligomerization surface for RdRP, and the oligomeric form could adopt the correct conformation required for efficient initiation. Recently, Qin *et al.* (2002) reported a possible dimerization of the HCV RdRP and identified two surface amino acids as being essential for dimerization and activity.

Template requirements for initiation

Specific template recognition by the replicase is essential for faithful genome replication. In most viral systems, RdRP–RNA interactions account only partially for the overall template specificity. Factors that usually contribute to the template specificity of RNA viruses include: (i) RdRP template preferences determined by the primary sequence and secondary structure at the 3′ end or internal initiation site; and (ii) specific template interactions of other cell- and virus-encoded proteins. For example, the Qβ replicase ensures specificity as a heterotetramer consisting of the viral RdRP and three host proteins (see, for example, Brown & Gold, 1996). In dsRNA viruses such as bacteriophage ϕ6, template specificity is achieved by exclusive packaging of virus-specific RNAs in a polymerase complex comprised of several structural proteins (Mindich, 1999). For some viruses, template specificity may be coupled with other processes, such as translation. Here, we will discuss the significance of the interaction between RdRPs and their cognate templates.

The initiation nucleotide

Initiation efficiency can be affected by the affinity of the polymerase for the initiation nucleotide of the template and the initiation NTPs (D1 and D2). Our structural data show that the specificity pocket of bacteriophage ϕ6 RdRP is designed for 3′-terminal template cytidylates (Butcher *et al.*, 2001; Salgado *et al.*, 2004). Biochemical analysis shows that for the BVDV RdRP the N3- and C4-amino group of the initiation template cytidylate are essential for RNA synthesis (Kao *et al.*, 1999; Kim *et al.*, 2000). Consistently, GTP is the preferred D1 nucleotide for RdRPs of BMV, Qβ, BVDV, HCV, GB virus C (GBV-C) and members of the family *Cystoviridae* (Blumenthal, 1980; Jorgensen *et al.*, 1969; Kao & Sun, 1996; Luo *et al.*, 2000; Sivakumaran & Kao, 1999; Ranjith-Kumar *et al.*, 2002b; Yang *et al.*, 2001, 2003b).

Although they all use *de novo* initiation, the RdRPs of the flaviviruses HCV, BVDV and GBV-C have distinct initiation preferences (Ranjith-Kumar *et al.*, 2002b). The BVDV RdRP prefers to initiate from the 3′-terminal cytidylate, but can also use a penultimate cytidylate. The BMV, turnip yellow mosaic virus and phage Qβ are known to use penultimate cytidylate for initiating RNA synthesis (Deiman *et al.*, 1998; Singh & Dreher, 1998, Sivakumaran & Kao, 1999; Sun *et al.*, 1996; Yoshinari & Dreher, 2000). The ability of RdRP to initiate from penultimate bases may be important for HCV and other RNA viruses whose polymerases can add extra nucleotides to the RNA 3′ end in the process of a terminal transferase reaction (Ranjith-Kumar *et al.*, 2002c).

Although the RdRP seems to contribute to the overall template selection of the replication/transcription machinery by selecting specific T1 or/and D1, it is important to remember that both T1 and D1 requirements may not be very stringent, especially *in vitro*. This may account for some controversy in the RdRP literature. For example, Zhong *et al.* (2000a) observed that BVDV and HCV initiate from a purine nucleotide in the template, while others found that the RdRPs have a specificity for initiation from pyrimidines (Luo *et al.*, 2000; Oh *et al.*, 2000; Sun *et al.*, 2000). In contrast to the observations of Kao and co-workers (2000), Zhong *et al.* (2000a) and Kao *et al.* (1999) demonstrated that short templates containing a 2′,3′-dideoxynucleotide could direct *de novo* initiation by the HCV RdRP and BVDV RdRP, respectively. Two possible explanations have been offered. One is that the two polymerases have different initiation requirements. Another is that the RdRPs are slightly different. Kao *et al.* (2000) used a full-length HCV RdRP, while others used proteins that lacked 20–55 C-terminal residues (Kao *et al.*, 1999; Luo *et al.*, 2000). The C-terminal tail of the HCV RdRP is present in the active site of the crystal structure and has been hypothesized to play a role in template discrimination (Ago *et al.*, 1999).

Some RNA polymerases may prefer pyrimidines in the D1 position (purines in T1) or have a naturally relaxed preference to the initiation nucleotide. The minus-strand RNA of a bovine coronavirus contains a uridylate as the 5′-terminal nucleotide (Hofmann & Brian, 1991). In Semliki Forest virus, the 5′ termini of the replication intermediates, but not the virion RNAs, are pyrimidines (Sawicki & Gomatos, 1976). The use of either purine or

pyrimidine triphosphates as D1 has also been documented in DdRPs (Schibler & Perry, 1977; Reddy & Chatterji, 1994).

De novo initiation of viral RNA synthesis seems to involve a higher K_m for D1 than for other NTPs (Kao & Sun, 1996; Gaal *et al.*, 1997; Joyce, 1997; Kim *et al.*, 2000; Luo *et al.*, 2000; Testa & Banerjee, 1979; Laurila *et al.*, 2002), indicating that initiation is the replication efficiency-limiting step that may be subject to additional regulation. Examples of viruses for which this seems to be the case include bacteriophage ϕ6 (Laurila *et al.*, 2002), Qβ (Blumenthal, 1980), BMV (Kao & Sun, 1996), HCV and BVDV (Kao *et al.*, 1999), and also cellular and viral DdRPs (Losick & Chamberlin, 1976). Increased stability of the ternary complex has been demonstrated in the presence of a high initiation substrate concentration (Gaal *et al.*, 1997). For the BMV replicase, the presence of GTP at the initiation site resulted in the formation of a more stable initiation complex (Sun & Kao, 1997a, b).

Specific template requirements

Beyond the first nucleotide, a stretch of nucleotides proximal to the initiation site may also stimulate initiation by RdRPs in virus systems. The initiation site context serves as a major determinant of template specificity, e.g. in turnip yellow mosaic virus, turnip crinkle virus and Qβ (Deiman *et al.*, 1998, 2000; Singh & Dreher, 1997, 1998; Yoshinari *et al.*, 2000). While these RNA viruses require additional regulatory sequences for RNA replication *in vivo*, *cis*-acting elements adjacent to the initiation site may form generic RNA structures that are highly tolerant of change (Blumenthal, 1980; Chen *et al.*, 2000; Singh & Dreher, 1998). Specific sites within the polypyrimidine tract of the 3′-untranslated region of HCV have recently been identified where RNA synthesis is initiated *de novo* under *in vitro* conditions (Pellerin *et al.*, 2002). Highly specific *cis*-acting signals have been also observed in several other systems (Adkins *et al.*, 1997; Kim *et al.*, 2000; McKnight & Lemon, 1998; Osman *et al.*, 2000; Siegel *et al.*, 1998; Sit *et al.*, 1998; You & Padmanabhan, 1999). An RNA composed of a stem–loop and a single-stranded sequence is a common feature in initiating viral RNA synthesis for replication (Van Belkum *et al.*, 1985; Seal *et al.*, 1994; Netolitzky *et al.*, 2000; Osman *et al.*, 2000; Lahser *et al.*, 1993; Fechter *et al.*, 2001). One function of these 3′-end secondary structures may be to prevent back priming. For transcription, however, dsRNA viruses need to initiate from dsRNA templates. *In vivo*, dsRNA viruses efficiently transcribe dsRNA templates, but *in vitro* initiation from dsRNA templates with RdRPs is much less efficient than with ssRNA templates (Makeyev & Bamford, 2000b; Yang *et al.*, 2001; Laurila *et al.*, 2002). This could be due to a lack of strand-separation ability. When the ϕ6 polymerase complex contains drastically reduced amounts of its normal protein P4 complement, it could perform replication but not initiate transcription (Pirttimaa *et al.*, 2002). However, adding this virus-specific RNA-dependent packaging NTPase (P4) to the bacteriophage ϕ6 RdRP does not stimulate *in vitro* transcription. Therefore, it has been speculated that efficient initiation of transcription requires the structural design of the ϕ6 polymerase complex or that the presence of a complete dsRNA genome in the polymerase complex at completion of replication might induce a conformational change that switches the RdRP from a replicase to a transcriptase (Makeyev & Grimes, 2004). The latter suggestion is supported by biochemical data showing that the large genome segment of bacteriophage ϕ6 regulates the switch between replication and transcription *in vitro* (Frilander *et al.*, 1995; Van Dijk *et al.*, 1995).

HCV. Recombinant HCV RdRP does not exhibit strict template specificity *in vitro*. It catalyses various viral and non-viral RNA templates *in vitro* if the RNA templates have a stable secondary structure and a single-stranded sequence that contains at least one 3′ cytidylate (Behrens *et al.*, 1996; Kao *et al.*, 2000; Luo *et al.*, 2000; Oh *et al.*, 1999, 2000; Zhong *et al.*, 2000b). However, Kim *et al.* (2002) found that native X RNA, which is part of the HCV 3′-untranslated region and plays a major role in the initiation of RNA replication after virus infection, was also an appropriate RdRP substrate, even though it contains a blunt-ended stem at its 3′ terminus. However, *in vivo* the HCV RdRP must discriminate the HCV genomic RNA from other RNAs and catalyse its substrate for viral amplification.

Reovirus. The reovirus polymerase favours a template G at position T2. It provides a carbonyl for a hydrogen bond with Arg-518 and an amino group for a hydrogen bond with the side-chain of Ser-682. U at this position can also interact favourably with Arg-518 but A and C cannot. Given the conserved 3′-terminal sequences of reovirus RNAs (plus, UCAUC-3′; minus, UAGC-3′), the preference for G or U at T2 promotes synthesis of RNAs of either sense (Tao *et al.*, 2002).

Bacteriophage ϕ6 and other *Cystoviridae*. Even though RdRPs of *Cystoviridae* have different template preferences, they all prefer RNAs with one or several 3′-terminal cytosines (Makeyev & Bamford, 2000b; Yang *et al.*, 2001). The replication efficiency seems to be controlled at the initiation step and RdRPs prefer pyrimidine-rich 3′-terminal initiation sites, C-3′ being better than U-3′. The template secondary structure does affect initiation of RNA synthesis. When a stable hairpin–tetraloop structure was added to a template it was replicated one order of magnitude less efficiently than the same template lacking the hairpin (Laurila *et al.*, 2002). For bacteriophage ϕ6 RNAs, the native secondary structure of the 3′ end does not favour the formation of back-primed intermediates. The terminal regions of all three segments have a tRNA-like structure, with the five 3′-proximal nucleotides in a single-stranded form (Mindich *et al.*, 1994). This five-nucleotide terminus can span the template channel of the bacteriophage ϕ6 RdRP (Butcher *et al.*, 2001) but cannot loop back. However, when short 3′-terminal extensions are added to the

RNA, back priming occurs (Laurila *et al.*, 2002). This suggests that the bacteriophage ϕ6-specific 3′-end secondary structure might be a result of evolutionary selection to ensure accurate *de novo* initiation.

It is useful to remember that the template preferences may be obscured when saturating amounts of RdRPs are used *in vitro* and that bacteriophage ϕ6 RdRP and Qβ replicase can synthesize a number of unrelated RNAs *in vitro* – and to some extent *in vivo* (Avota *et al.*, 1998, and references therein; Makeyev & Bamford, 2000a, b, 2001).

ROLE OF DIVALENT CATIONS

Divalent metal ions are essential for the nucleotide polymerization reaction (Fig. 2). In addition, several specific regulatory effects of metal ions on viral RNA synthesis have been described.

Role of Mg^{2+}

The crucial role of Mg^{2+} ions in the catalysis of phosphodiester bond formation has long been known (Steitz & Steitz, 1993; Pelletier *et al.*, 1994; Steitz, 1998). Mg^{2+} has also been implicated in stabilizing the daughter strand in replication complexes of bacteriophage T7 DNA polymerase (Doublie *et al.*, 1998), bacillus DNA polymerase (Kiefer *et al.*, 1998) and ϕ6 RdRP (Salgado *et al.*, 2004). The ϕ6 RdRP Mg^{2+} ions also transiently stabilized the by-product, PP_i, before its release via the substrate pore (Salgado *et al.*, 2004).

Role of Mn^{2+}

Manganese ions are known to stimulate a number of RdRPs including those of Qβ (Blumenthal, 1980; Blumenthal & Carmichael, 1979), HCV (Alaoui-Ismaili *et al.*, 2000; Zhong *et al.*, 2000b), BMV (Sun *et al.*, 1996), poliovirus (Arnold *et al.*, 1999) and members of the family *Cystoviridae* (Makeyev & Bamford, 2000b; Yang *et al.*, 2001, 2003a). Mn^{2+} also stimulates the bacteriophage ϕ6 polymerase complex-based replication and transcription (Emori *et al.*, 1983; Ojala & Bamford, 1995; Van Dijk *et al.*, 1995) and is known to modulate substrate selectivity of DdRPs (Huang *et al.*, 1997; Tabor & Richardson, 1989). The structures of the RdRPs of HCV, bacteriophage ϕ6 and RHDV have revealed that Mn^{2+} can bind in either the catalytic pocket (Bressanelli *et al.*, 2002; Ng *et al.*, 2002) and/or a specific allosteric position (Butcher *et al.*, 2001).

Analysis of the role of metal ions in RNA-dependent RNA synthesis by three flavivirus recombinant RdRPs, GBV-C, BVDV and HCV, showed that only reactions with exogenously provided Mg^{2+} and Mn^{2+} were capable of RNA synthesis. Mg^{2+} and Mn^{2+} affected the mode of RNA synthesis of the three RdRPs. Both metals supported GBV-C RdRP *de novo*-initiated and primer-dependent RNA synthesis. However, Mn^{2+} significantly increased *de novo* initiation by HCV and BVDV RdRPs. In the case of HCV RdRP, Mn^{2+} reduced the K_m for the initiation GTP from 103 to 3 μM. Mn^{2+} increased *de novo* initiation, even at GTP concentrations that are comparable with physiological levels (Ranjith-Kumar *et al.*, 2002a).

However, Mn^{2+} may not play a physiologically relevant role in RNA-dependent RNA synthesis because of its low intracellular concentration (Quamme *et al.*, 1993; Zhang & Ellis, 1989). Furthermore, detecting *de novo* initiation *in vitro* does not by itself prove that this process is biologically relevant. Even poliovirus RdRP, which initiates replication *in vivo* with a protein primer, VPg (Paul *et al.*, 1998), is capable of *de novo* initiation *in vitro* in the presence of Mn^{2+} (Arnold & Cameron, 1999; Arnold *et al.*, 1999). The observation that the HCV RdRP catalytic-site is more occupied when the divalent ion is Mn^{2+} is suggestive of a stabilizing effect of this ion, favouring the binding of both initiating nucleotides in the active site region (Bressanelli *et al.*, 2002). This may lead, for HCV RdRP also, to *de novo* initiation via a non-physiological mechanism in some *in vitro* conditions.

Crotty *et al.* (2003) recently generated the first viruses with a requirement for an alternative polymerase cation. They studied the function of Asn-297 in the poliovirus RdRP, one of the six core amino acid residues that are conserved across all polymerases of positive-strand RNA viruses of eukaryotes. Viable mutants were identified with non-canonical amino acids at this position that exhibited Mn^{2+}-dependent RNA replication and virus growth. This finding is important since it suggests that drugs targeting this region of RdRPs may still be subjected to the problem of drug-resistant escape mutants.

Role of Ca^{2+}

Ca^{2+} inhibits *in vitro* transcription of reovirus (Sargent & Borsa, 1984), RNA polymerase II (Okai, 1982) and bacteriophage ϕ6 (Van Dijk *et al.*, 1995). The crystal structures of ϕ6 RdRP initiation complexes with either Mg^{2+} or Ca^{2+} ions revealed key differences that may explain the inhibitory effect of Ca^{2+} (Butcher *et al.*, 2001; Salgado *et al.*, 2004). In the inhibition complex, the two Mg^{2+} ions that are present in the ϕ6 initiation complex are substituted by Ca^{2+} ions. One of the Ca^{2+} ions (denoted 2 by Salgado *et al.*, 2004) occupies a position equivalent to the corresponding Mg^{2+} in the initiation complex (Butcher *et al.*, 2001) and interacts with D2. The other Ca^{2+} ion (denoted 1) has a different coordination sphere from the equivalent Mg^{2+}: it includes the stabilizing Tyr-630 and the triphosphate and base of D1, instead of the phosphate backbones of the two incoming GTPs. The tyrosine side-chain is rotated, disrupting the stacking between the amino acid and the base of the nucleotide. Furthermore, coordination of the phosphate backbone of D1 displaces it away from D2, preventing the necessary nucleophilic attack. Thus, Ca^{2+} seems to inhibit RNA synthesis by altering the geometry of interactions in the catalytic position.

TEMPERATURE DEPENDENCE

Differentiation between the temperature requirements for initiation and elongation of RNA synthesis has been documented for RdRPs. For the *Cystoviridae*, *de novo* initiation by RdRPs of bacteriophage ϕ6, ϕ12 and a temperature-sensitive mutant of bacteriophage ϕ12 was more sensitive to increased temperatures than elongation (Yang *et al.*, 2003b). In *in vitro* assays, the recombinant dengue virus RdRP can either initiate RNA polymerization *de novo* or extend the template 3′ terminus by back priming. At moderate temperatures, the enzyme predominantly uses *de novo* initiation, whereas back priming dominates at elevated temperatures (Ackermann & Padmanabhan, 2001). Based on these results and the observed structural differences between the poliovirus and HCV RdRP, a steric model was suggested where dengue RdRP can exist in either a closed or an open conformation, with the latter favoured at higher temperatures. The open conformation binds to a fold-back structure at the 3′ terminus of the template with the subsequent elongation producing a dimerized product. Conversely, the closed conformation, favoured at lower temperatures, recognizes the single-stranded 3′ terminus of the template and initiates *de novo* synthesis (Ackermann & Padmanabhan, 2001). The data suggest that *de novo* RNA-dependent RNA synthesis in many virus systems may include a specialized thermolabile state of the RdRP initiation complex.

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