Introduction 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 (AspA) and C (AspC) motifs, respectively, and two divalent metal ions for the formation of the phosphodiester bonds (Joyce & Steitz, 1995; Steitz, 1998). The carboxylate anchor a pair of divalent metal ions, which play the major role in catalysis; one divalent metal ion (Mg2+ ion 1) promotes the deprotonation of the 3′ hydroxyl of the nascent strand, while the other (Mg2+ 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 (PPi).

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
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.
(Martin et al., 1988; Carpousis & Gralla, 1980; Ackerman et al., 1983) and viral RdRPs such as those of turnip crinkle carnovirus (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 & Kola, 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).

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, 2003).
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 diarrhea 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 replicates 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) 3Dpol (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) 3Dpol (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. RdRPs 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 Mg2+ and/or Mn2+ has mapped the active site of the enzymes.

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>
<thead>
<tr>
<th>Polymerase source, virus family, name, PDB identification number</th>
<th>Resolution</th>
<th>RdRP structure</th>
<th>Reference</th>
</tr>
</thead>
<tbody>
<tr>
<td>Poliovirus (Picornaviridae) 3D&lt;sub&gt;pol&lt;/sub&gt;</td>
<td>2.4 Å</td>
<td>Complete protein crystallized – 461 residues</td>
<td>Hansen et al. (1997)</td>
</tr>
<tr>
<td>PDB: 1RDR</td>
<td></td>
<td>Residues not modelled are 1–12, 38–66, 98–181, 270–290</td>
<td></td>
</tr>
<tr>
<td>Hepatitis C virus (BK strain) (Flaviviridae)</td>
<td>2.8 Å</td>
<td>Protein lacks the 55 C-terminal amino acids</td>
<td>Bressanelli et al. (1999)</td>
</tr>
<tr>
<td>NS5B PDB: 1CSJ</td>
<td></td>
<td>536 residues crystallized</td>
<td></td>
</tr>
<tr>
<td></td>
<td></td>
<td>531 residues modelled</td>
<td></td>
</tr>
<tr>
<td></td>
<td></td>
<td>The 5 C-terminal disordered residues (532–536) were not modelled</td>
<td></td>
</tr>
<tr>
<td>Hepatitis C virus (BK strain) (Flaviviridae)</td>
<td>1.85 Å</td>
<td>Protein lacks the 55 C-terminal amino acids</td>
<td>Bressanelli et al. (2002)</td>
</tr>
<tr>
<td>NS5B PDB: 1GX6 (complex with UTP and Mn&lt;sup&gt;2+&lt;/sup&gt;)</td>
<td></td>
<td>531 residues crystallized</td>
<td></td>
</tr>
<tr>
<td></td>
<td></td>
<td>531 residues modelled</td>
<td></td>
</tr>
<tr>
<td>Hepatitis C virus (BK strain) (Flaviviridae)</td>
<td>2.5 Å</td>
<td>Protein lacks 21 C-terminal amino acids</td>
<td>Ago et al. (1999)</td>
</tr>
<tr>
<td>NS5B PDB: 1QUV</td>
<td></td>
<td>578 residues crystallized</td>
<td></td>
</tr>
<tr>
<td></td>
<td></td>
<td>553 residues modelled</td>
<td></td>
</tr>
<tr>
<td></td>
<td></td>
<td>Residues not modelled are 544–547; 557–578 (residues 571–578 are a His tag)</td>
<td></td>
</tr>
<tr>
<td>Hepatitis C virus (BK strain) (Flaviviridae)</td>
<td>1.9 Å</td>
<td>Protein lacks 21 C-terminal residues 576 residues crystallized</td>
<td>Lesburg et al. (1999)</td>
</tr>
<tr>
<td>NS5B PDB: 1C2P</td>
<td></td>
<td>566 residues modelled</td>
<td></td>
</tr>
<tr>
<td></td>
<td></td>
<td>Residues not modelled are 149–153; 567–570</td>
<td></td>
</tr>
<tr>
<td>Hepatitis C virus (J4 strain) (Flaviviridae)</td>
<td>2.0 Å</td>
<td>Protein lacks 21 C-terminal amino acids; has a C-terminal His tag</td>
<td>O’Farrell et al. (2003)</td>
</tr>
<tr>
<td>NS5B PDB: 1NB4 (apo-enzyme)</td>
<td></td>
<td>570 residues crystallized</td>
<td></td>
</tr>
<tr>
<td>PDB: 1NB6 (complex with UTP)</td>
<td>2.6 Å</td>
<td>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</td>
<td></td>
</tr>
<tr>
<td>PDB: 1NB7 (complex with RNA)</td>
<td>2.9 Å</td>
<td></td>
<td></td>
</tr>
<tr>
<td>Bacteriophage φ6 (Cystoviridae) P2</td>
<td>2.5 Å</td>
<td>Complete 664 residue protein crystallized</td>
<td>Butcher et al. (2001)</td>
</tr>
<tr>
<td>PDB: 1HI8 (apo-enzyme) PDB1HI10 (complex with DNA)</td>
<td>3.0 Å</td>
<td>All residues modelled</td>
<td></td>
</tr>
<tr>
<td>Bacteriophage φ6 (Cystoviridae) P2</td>
<td>2.0 Å</td>
<td>Complete 664 residue protein crystallized</td>
<td>Salgado et al. (2004)</td>
</tr>
<tr>
<td>PDB: Salgado et al. (2004) (complex with RNA)</td>
<td></td>
<td></td>
<td></td>
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</tbody>
</table>
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 6 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 6 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 6 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 Mn2+ 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 6 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 6, 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

<table>
<thead>
<tr>
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<th>Resolution</th>
<th>RdRP structure</th>
<th>Reference</th>
</tr>
</thead>
<tbody>
<tr>
<td>Rabbit haemorrhagic disease virus (Caliciviridae) 3D&lt;sup&gt;66&lt;/sup&gt; PDB: 1KHV</td>
<td>2.5 Å</td>
<td>Complete 516 residue protein crystallized Residues not modelled: 1–4, 181–184 and 15 C-terminal residues (502–516)</td>
<td>Ng et al. (2002)</td>
</tr>
<tr>
<td>Reovirus (type 3; strain Dearing) (Reoviridae) λ3 PDB: 1N35</td>
<td>2.5 Å</td>
<td>Complete 1267 residue protein crystallized 1256 residues modelled Residues not modelled: the flexible loop between residues 937 and 964, one residue at the N terminus and two residues (1266–1267) at the C terminus</td>
<td>Tao et al. (2002)</td>
</tr>
</tbody>
</table>
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 β6 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 A 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–NTP 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

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**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 Mn²⁺. 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.]
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 LDCQYGACYSI 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 helix that is 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 ion 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 uridylylate as the 5’-terminal nucleotide (Hofmann & Brian, 1991). In Semiliki 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
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 bacteriophages $\phi 6$ (Laurila et al., 2002), Q$\beta$ (Blumenthal, 1980), BMV (Kao & Sun, 1996), HCV and BVDV (Kao et al., 1999), and also cellular and viral DDPRPs (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 DDPRPs 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 Q$\beta$ (Deiman et al., 1998, 2000; Singh & Dreh, 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 & Dreh, 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 observed in several other systems (Adkins et al., 1997; Kim et al., 2000; McKeight & Lemon, 1998; Osman et al., 2000; Siegel et al., 1998; Sitt 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; Fichter 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 DDPRPs 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 $\phi 6$ 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 $\phi 6$ DDPRP does not stimulate in vitro transcription.

Therefore, it has been speculated that efficient initiation of transcription requires the structural design of the $\phi 6$ 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 DDPRP 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 $\phi 6$ regulates the switch between replication and transcription in vitro (Frilander et al., 1995; Van Dijk et al., 1995).

**HCV.** Recombinant HCV DDPRP 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 DDPRP substrate, even though it contains a blunt-ended stem at its 3’ terminus. However, in vivo the HCV DDPRP 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 $\phi 6$ and other Cystoviridae.** Even though DDPRPs 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 DDPRPs 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 $\phi 6$ 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 $\phi 6$ DDPRP (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 Mg²⁺**

The crucial role of Mg²⁺ ions in the catalysis of phosphodiester bond formation has long been known (Steitz & Steitz, 1993; Pelletier et al., 1994; Steitz, 1998). Mg²⁺ 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 Mg²⁺ ions also transiently stabilized the by-product, PP₃, before its release via the substrate pore (Salgado et al., 2004).

**Role of Mn²⁺**

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). Mn²⁺ 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 Mn²⁺ 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 Mg²⁺ and Mn²⁺ were capable of RNA synthesis. Mg²⁺ and Mn²⁺ 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, Mn²⁺ significantly increased de novo initiation by HCV and BVDV RdRPs. In the case of HCV RdRp, Mn²⁺ reduced the Kₘ for the initiation GTP from 103 to 3 μM. Mn²⁺ increased de novo initiation, even at GTP concentrations that are comparable with physiological levels (Ranjith-Kumar et al., 2002a).

However, Mn²⁺ 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 Mn²⁺ (Arnold & Cameron, 1999; Arnold et al., 1999). The observation that the HCV RdRP catalytic-site is more occupied when the divalent ion is Mn²⁺ 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 Mn²⁺-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 Ca²⁺**

Ca²⁺ 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 Mg²⁺ or Ca²⁺ ions revealed key differences that may explain the inhibitory effect of Ca²⁺ (Butcher et al., 2001; Salgado et al., 2004). In the inhibition complex, the two Mg²⁺ ions that are present in the φ6 initiation complex are substituted by Ca²⁺ ions. One of the Ca²⁺ ions (denoted 2 by Salgado et al., 2004) occupies a position equivalent to the corresponding Mg²⁺ in the initiation complex (Butcher et al., 2001) and interacts with D2. The other Ca²⁺ ion (denoted 1) has a different coordination sphere from the equivalent Mg²⁺: 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, Ca²⁺ 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.

**ACKNOWLEDGEMENTS**

We thank Dr Sarah Butcher, Minni Laurila and Paula Salgado for valuable discussions and critical reading of the manuscript. This work was supported by the Academy of Finland (‘Finnish Centre of Excellence Program 2000–2005’, grants 1202855, 1202108 and 172621).

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