The structure of the RNA-dependent RNA polymerase from bovine viral diarrhea virus establishes the role of GTP in de novo initiation

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Contributed by Michael G. Rossmann, January 31, 2004

The bovine viral diarrhea virus (BVDV) RNA-dependent RNA polymerase can initiate RNA replication by a de novo mechanism without a primer. The structure of BVDV polymerase, determined to 2.9-Å resolution, contains a unique N-terminal domain, in addition to the fingers, palm, and thumb domains common to other polymerases. The structure of BVDV polymerase complexed with GTP, which is required for de novo (primer-independent) initiation, shows that GTP binds adjacent to the initiation NTP, suggesting that the GTP mimics a vestigial RNA product. Comparison of five monomers in two different crystal forms showed conformational changes in the fingertip region and in the thumb domain that may help to translocate the RNA template and product strands during elongation. The putative binding sites of previously reported BVDV inhibitors are also discussed.

The function of a polymerase is to synthesize the complementary RNA or DNA molecule from a template strand. Thus, the polymerase requires binding sites for the template, nucleoside triphosphates (NTPs or dNTPs), and the nascent polynucleotide product. Also required are mechanisms to catalyze the addition of NTP or dNTP to the nascent chain, to recognize the next complementary NTP molecule, and to move the template and product by one nucleotide in readiness for the next elongation event. Finally, the polymerase, in conjunction with other viral and cellular enzymes, such as a helicase in a replication complex, requires a mechanism for initiating and terminating synthesis of the new product polynucleotide.

RNA replication in positive-sense single-stranded (ss) RNA viruses is initiated at or near the 3’ end of the template using either primer-dependent or primer-independent (de novo) mechanisms. Poliovirus, for example, utilizes a genome-linked protein as a primer for initiation of RNA synthesis (1). In Flaviviridae, on the other hand, de novo initiation is the likely mechanism used during virus replication in infected cells (2). In de novo initiation, the second NTP is added (with the release of its pyrophosphate moiety) directly to the 3’-OH of the first initiation NTP without the need for a primer. This nucleotidyl transfer reaction is then repeated with subsequent NTPs to generate the complementary RNA product. De novo initiation by Flaviviridae RNA polymerases requires (i) a template RNA with a virus-specific initiation nucleotide at the 3’ end, (ii) a complementary initiation NTP, and (iii) GTP (3–6). Because base pairing between the template RNA and individual NTPs may not be sufficient to allow the formation of a stable initiation complex, it is likely that de novo initiation requires specific molecular interactions among the template RNA, NTPs, the RNA polymerase, and possibly other viral and host proteins. Although not all RNA-dependent RNA polymerases (RdRps) require GTP for initiating RNA synthesis, other ligands or structural components can act similarly as GTP and help to position the 3’-OH group of the priming nucleotide ready for nucleophilic attack.

The Flaviviridae family consists of three genera: the flaviviruses (including dengue, West Nile, and yellow fever viruses), hepaciviruses (hepatitis C virus, HCV), and the pestiviruses (including bovine viral diarrhea virus, BVDV). Pestiviruses are animal pathogens of major importance, particularly to the livestock industry. They show greater similarity in genome structure and translation strategy to hepaciviruses than to flaviviruses (7). Because hepaciviruses, which infect 3% of the world population, are difficult to grow in cell culture, pestiviruses have been used as a model system for investigating hepaciviruses. BVDV, the best-studied pestivirus, has a genome that consists of an ~12.6-kb positive-sense ssRNA (8). The BVDV genome is translated into a single polyprotein represented by NH2-Npro-C-Erns-E1-E2-P7-NS2-NS3-NS4A-NS4B-NS5A-NS5B-COOH, where Npro is the N-terminal autoprotease, C is the capsid protein, Epr is an envelope glycoprotein with RNase activity, E1 and E2 are external envelope glycoproteins, and P7 is a 7-kDa protein of unknown function. The polyprotein is processed into at least four structural (C, Epr, E1, and E2) and six nonstructural (NS2, NS3, NS4A, NS4B, NS5A, and NS5B) proteins required for viral assembly and replication (7, 8). Among the NS proteins, the functions of NS3, NS4A, and NS5B have been most extensively characterized. NS3 contains a viral serine protease domain (sometimes requiring NS4A as a cofactor) and a helicase/NTPase domain. NS5B is an RdRp and is responsible for genome replication as a part of a larger, membrane associated, replicase complex (7).

The crystal structures of RdRps from various families of single- or double-stranded RNA viruses have been determined [HCV (9–13), calicivirus (14), poliovirus (15), d6 (16), and reovirus (17)]. In addition, the structures of several DNA polymerases have been determined, including the DNA-dependent DNA polymerase Klei-now fragment from Escherichia coli (18) and HIV type 1 reverse transcriptase (19–22). Although there is low sequence identity between polymerases (23), they all have roughly the shape of a right hand with fingers, palm, and thumb domains. During elongation, the template and product move along a channel, formed by the thumb and fingers, toward the palm domain, which contains the catalytic residues. The thumb domain might serve to ratchet the template and product over the surface of the hand with its exposed catalytic site (16).

Here, we present the crystal structure of a truncated form of the BVDV polymerase determined to 2.9-Å resolution by x-ray crystallography. The polymerase structure possesses a unique N-terminal domain, in addition to the conserved “core,” common to other polymerases. Like the HCV and d6 polymerases, the BVDV polymerase utilizes a motif in the thumb domain to completely

Abbreviations: BVDV, bovine viral diarrhea virus; HCV, hepatitis C virus; NTP, nucleotide triphosphate; RdRp, RNA-dependent RNA polymerase; SeMet, selenomethionine; ssRNA, single-stranded RNA.

Data deposition: The atomic coordinates have been deposited in the Protein Data Bank, www.pdb.org [PDB ID codes 1S48 (form 1 crystals), 1S4F (form 2 crystals), and 1S49 (form 1 complexed with GTP)].

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encircle the active site cavity. We have now determined the structure of BVDV polymerase complexed with GTP and identified the GTP-specific binding site required for de novo initiation. Comparison of four independent monomers in the crystallographic asymmetric unit of another BVDV RdRp construct shows conformational flexibility that may be important for the translocation of the template and product during RNA elongation.

**Methods**

**Expression and Purification of BVDV Polymerase.** Based on limited proteolysis and multiple sequence alignment, eighty-four truncations of BVDV polymerase were designed representing combinations of variable N and C termini with N- or C-terminal hexahistidine tags. DNAs encoding the variant polymerases were prepared and purified for use in a MAD experiment.

**Selenomethionine Protein Preparation.** Selenomethionine (SeMet) protein was produced and purified for use in a MAD experiment. The Met auxotroph E. coli B834 (DE3) was transformed with a plasmid encoding BVDV polymerase and plasmid RIL encoding rare codon tRNAs. Cells were grown in M9 minimal media (24, 25) (supplemented with 19 amino acids, SeMet, and vitamins) and induced with 1.5 mM isopropyl-β-D-thiogalactoside. Purification of SeMet protein was similar to that of native protein, except that 10 mM DTT was added to all buffers after the Talon column to prevent oxidation of incorporated SeMet. Mass spectrometry of both the native and SeMet proteins verified the presence of 12 SeMet residues, consistent with the 12 Met residues in the protein.

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**Structure Determination.** Crystals (form 1) of the BVDV construct (residues 71–679 with a C-terminal His tag) were transferred to a cryoprotectant solution in a step-wise manner and frozen in liquid nitrogen. A three-wavelength anomalous dispersion data set was collected at the Se fluorescence peak, inflection point, and a remote wavelength. Additionally, a SAD data set was collected at the Se peak. Both data sets were collected at Advanced Photon Source beamline 14ID-B. Data were indexed with DENZO (26) and scaled with SCALEPACK (26). Initial indexing and merging indicated that the crystals belong to the space group P612121, 2 with cell dimensions of a = 99.6 Å, b = 206.1 Å, and c = 102.1 Å.

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**Polymerase Complexes with GTP.** The BVDV polymerase incorporating SeMet (residues 71–679 with a C-terminal hexahistidine tag) was cocrystallized with 10 mM GTP. Data were collected at Cornell High Energy Synchrotron Source beamline FL. The crystals were isomorphous with the form 1 crystals. A difference map contoured

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**Table 1. BVDV data collection and refinement statistics**

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<th>Data processing*</th>
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<td>a = b = 205.7, c = 99.6</td>
<td>a = b = 204.9, c = 96.6</td>
<td>a = b = 412.3, c = 95.6</td>
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MAD, multiple-wavelength anomalous dispersion; SAD, single-wavelength anomalous dispersion.

*The numbers in parentheses refer to the highest-resolution shell.

1 Rmerge = Σhkl(Ihkl − <Ihkl>)/Σhkl(Ihkl).
2 Rmerge = Σhkl(Fhkl − Fo(hkl))/Σhkl(Fo(hkl)).
3 Rmerge = Rrefine, calculated for 10% randomly selected reflections that are not included in the refinement.
4 Friedel opposites are each counted for noncentric reflections.
at 2.5σ showed clear density into which a GTP molecule was easily fitted (Fig. 1).

**Results**

**Crystallization and Structure Determination of BVDV Polymerase.**

BVDV polymerase was expressed in *E. coli* and purified as a soluble protein. A crystallizable protein was obtained by truncation of the first 70 and the last 40 amino acids, which included the highly hydrophobic C-terminal residues where the putative membrane-anchoring region is located. BVDV polymerase crystallized with one monomer in the asymmetric unit forming a dimer across a crystallographic two-fold axis. The final model of BVDV polymerase at 2.9 Å resolution contains 580 of the 601 residues of the truncated molecule. The first 20 N-terminal residues were not observed in the electron density map (Table 1).

**The Fingers, Palm, and Thumb Domains.**

The BVDV polymerase core (residues 139–679) has a roughly spherical shape with dimensions of ~74 × 60 × 58 Å around a central cavity. In addition, there is an N-terminal region (residues 71–138) of which residues 71–91 are disordered (see Fig. 2 for secondary structure nomenclature).

The fingers domain (residues 139–313 and 351–410) consists of 12 α-helices and 11 β-strands. In BVDV RdRp, as in other viral RdRps, the N terminus of the fingers domain, together with a long insert in the fingers domain (residues 260–288), form a fingertip region that associates with the thumb domain. The conformation of the polypeptide chain in the fingertip region differs among RdRps (Fig. 3). For example, whereas BVDV polymerase has a three-
A comparative analysis of RNA polymerase sequences in positive-stranded RNA viruses reveals striking similarities in domain organization. The viral polymerases, which initiate using a template and primer, lack such -hairpin loops (14). These enzymes have a wider channel that can accommodate a template-primer complex. Initial investigations into the polymerase structures have shown that discrimination between primer-dependent and -independent initiation is accomplished by restricting access to the template-binding site.

The ordered part of the N-terminal region (residues 92–138) folds into a separate domain. The N-terminal domain is situated over the thumb domain, interacting with the fingertips region and thumb domain through a -hairpin motif (N83-loop-N84) (Fig. 2A). The -hairpin motif points toward the template channel and partially occludes the channel entrance, possibly explaining why some N-terminal truncated proteins have greater polymerase activity than wild type (33).

The entrance to the template-binding channel created by the -hairpin motif of the N-terminal domain is highly positively charged. An equivalent positively charged surface at the entrance to the channel was found in poliovirus polymerase, where it was proposed to act as a “plough" that unwinds the double-stranded RNA, thus aiding the function of a helicase (16). Similarly, in BVDV polymerase, this positively charged surface may be used to open up RNA secondary structural hairpins before the ssRNA template enters the active center.

Initiation Complex of RNA Polymerase with GTP. A high concentration of GTP (but not any other nucleotide) is required for de novo initiation of RNA synthesis in BVDV (3, 36), as well as in other members of the Flaviviridae family (4–6), regardless of the nucleotide at the 3' end of the RNA template. The structure of BVDV polymerase, complexed with GTP, shows that GTP binds at the catalytic GDD-containing motif, inside the template-binding channel. The GTP molecule interacts extensively with the polymerase through a hydrogen-bonding network, electrostatic interactions, and hydrophobic interactions with residues from all three polymerase domains (Fig. 1A). The orientation of GTP that was fitted into the difference electron density map is supported by the basic environment of the phosphate groups (His-449, Arg-517, Lys-525, Arg-529), by the polar environment of the ribose OH groups (Cys-446, Cys-497, Ser-498), by the hydrophobic environment around the guanine base (Pro-321, Leu-322, Leu-677), and by hydrogen bonds of guanine N1 and N2 with the main-chain carbonyl of Thr-320, as well as O6 with the OH group of Tyr-581. With the exception of the hydrogen bond to N1, these bonds provide specificity for guanine, as opposed to adenine. In contrast, the NTP-binding sites (see below) are created only in the presence of bound template. The GTP-binding site in BVDV polymerase is within ~4 Å of the nearest NTP site, compared to their positions in HCV or -polymerase complex (Fig. 1C). Thus, the polymerase-GTP complex presented here is probably the GTP-polymerase complex required for initiation of RNA synthesis.
Most of the residues involved in the GTP-binding site are in the RdRp motifs III, VII, and VIII. Some of the residues in motifs VII and VIII (including Cys-497, Ser-498, and Arg-517) have been mutated individually to alanine and tested for their ability to direct RNA synthesis via primer-dependent (elongative) and -independent (de novo) mechanisms (33). Primer-dependent RNA elongation was reduced by only 2- to 10-fold, whereas de novo synthesis was almost completely abolished. This discrimination between the two possible modes of initiation was observed only when the three residues mentioned above were mutated, and not when similar mutations were made in other conserved motifs. Similar discrimination has been demonstrated by mutating Arg-386 in HCV polymerase, equivalent to Arg-517 in BVDV polymerase (37), confirming that the GTP-binding site described here is essential for de novo initiation of RNA synthesis in Flaviviridae polymerases.

**Discussion**

**Model for RNA Template Binding.** Of the extensive structural data, the d6 and HCV RdRps proved to be the most useful because of the high degree of structural homology to BVDV RdRp. In the d6 polymerase–template–NTP complex, there are two NTP sites, i [the priming site (16)] and i + 1 (the catalytic site) surrounding the GDD residues essential for RNA synthesis. Both NTPs are base-paired with template bases. The NTP i site, closer to the 3’ end of the template, corresponds to the last base added to the growing strand, paired with template bases. The d6 priming site (16) and

The 3’-OH group of the NTP at the i site is oriented correctly for a nucleophilic in-line attack on the α-phosphate of the incoming NTP at the i + 1 site. Thus, the d6 polymerase structure shows the organization of the template and its base-paired product as expected for elongation.

A model for RNA template binding in BVDV polymerase was based on the d6 polymerase template and polymerase template–NTP complexes (PDB ID codes 1HHT and 1HIO), as well as the HCV polymerase–template complex (ID code 1NB7). Superposition of the conserved palm domains of d6 and HCV polymerase onto the BVDV polymerase palm domain brings the fingers domains of all three polymerases into good agreement. The overall superposition of fingers and palm domains results in rms deviations of 3.2 Å for 234 topologically equivalent Cα atoms of d6 polymerase and 2.5 Å for 247 Cα atoms of HCV polymerase. The position and orientation of the d6 and HCV RNA template strand when placed into the BVDV polymerase structure are similar. In both superpositions, the ssRNA template is easily accommodated by the BVDV polymerase active site with few steric clashes. The entrance to the template channel is restricted by the β-hairpin from the N-terminal domain but is large enough to allow the 3’ end of the template strand to enter the active site. The phosphate backbone of the template interacts with residues mainly from the fingers domain. The RdRp motif II is in contact with the phosphate backbone and bases, indicating the role of this motif in template binding. In addition, motif I is close to the NTP i + 1 binding site, indicating its function for binding the incoming NTP.

When the d6 polymerase–NTP template structure was superimposed onto the BVDV–GTP complex structure, the ribose triphosphate moiety of GTP was found to be positioned as might be expected for an i − 1 site of a synthesized RNA strand (Fig. 1B). The 3’-hydroxyl of the GTP ribose is positioned near the α- and β-phosphates of the NTP at the i site. Thus, the GTP may provide a vestigial synthesized RNA molecule that can initiate elongation of the complementary strand. A similar role for orienting the priming NTP has been proposed for the “priming loop” of reovirus RdRp (17). Various dinucleotides can replace GTP for de novo initiation in pestiviruses and HCV polymerases (36). These dinucleotides have a guanosine at their 3’ end that acts as an initiation nucleotide when eukaryotic host template and a primerase initiation loop. The 5’ end of the dinucleotide to protrude beyond the 3’ end of the template. The ability of these dinucleotides to initiate RNA synthesis could be explained by recognizing that the 3’ end of the dinucleotide would be positioned at the i site and the 5’ end of the dinucleotide would be at the GTP-binding site, the latter thus providing the initiation complex normally associated with the bound GTP.

**Conformational Changes in BVDV Polymerase.** The structure described above (residues 71–679 with a C-terminal hexahistidine tag) has one molecule per asymmetric unit of form 1 crystals. However, a construct consisting of residues 79–678 and an N-terminal hexahistidine tag had four molecules per crystallographic asymmetric unit of form 2 crystals. These four monomers are arranged as two noncrystallographic dimers, AB and CD, each of which is similar to the crystallographic dimer in the smaller (form 1) unit cell. Because each of the four monomers in the asymmetric unit is in a slightly different environment, structural differences between monomers might provide insight into inherent structural flexibility and conformational changes in BVDV polymerase.

Superposition of the structure determined in the smaller (form 1) unit cell onto each of the four monomers in the form 2 crystal gives a rms deviation of <1.0 Å for monomers A, B, and C, but 8 Å for monomer D (calculated over all equivalent Cα atoms). When only the fingers and palm domains were superimposed, monomer D differed from monomers A, B, and C, and the monomer in the form 1 crystal in that the thumb domain was rotated by ~8°, the N-terminal domain was rotated by ~13°, and residues 268–276 in the fingertips region have a changed conformation (Fig. 4). In addition, Ser-532-Glu-537 and Lys-672-Glu-675 in the β-things and residues 277–282 in the finger region, as well as residues 530–532 in the thumb domain of monomer D, are disordered. The finger region contains RdRp motif II, which is involved in NTP binding, and the β-thumb occludes the active site.

Movement of the finger region associated with the binding of each subsequent NTP to be incorporated into the growing RNA product, in conjunction with a rotation of the thumb domain forming part of the template channel, may help translocate the template and product for each elongation step.

**Mutations Conferring Resistance to BVDV Polymerase Inhibitors.** Two inhibitors of BVDV RNA replication that target the polymerase have been reported (38, 39). Baginski et al. (38) describe a small 329-Da noncompetitive inhibitor {3-[(2-dipropylamino)ethyl]-thio]–5H-1,2,4-triazino[5,6-b]indol}, VP32947, which blocks viral RNA synthesis in vitro and in vivo. The potency of the compound against BVDV replication in cell culture (EC50 = ~20 nM) was significantly greater than its activity against purified polymerase in a biochemical assay (IC50 = ~700 nM) (38). Several drug-resistant
variants of BVDV were selected and shown to have Ser substituted for Phe at position 224 in the viral polymerase. When this single amino acid change was introduced into an infectious clone of wild-type virus, replication of the resulting variant virus was resistant to VP32947. Phe-224 is located in a fingers domain surface loop that points toward the fingertip region. The location of Phe-224 is consistent with the noncompetitive nature of the inhibitor, suggesting that the drug-binding site does not overlap with the substrate-binding site. Phe-224, near the putative binding site of VP32947, makes hydrophobic interactions with Ile-390, Ala-392, and Leu-225. Thus, this compound could inhibit the normal flexibility of the fingers (40), which may be required for translocation of the template and product, as previously observed. 

Phe-224 is located in the dimer interface created by a crystallographic two-fold axis, which produces contacts between the N-terminal domain of one monomer and the fingers domain of another in the form 1 crystals. The same interface occurs in the AB and CD noncrystallographic dimers in crystal form 2. Thus, the binding site for VP32947 may be in the dimer interface of the crystalline polymerase, making it difficult for the compound to bind to either of the two crystal forms described here. This is consistent with the absence of bound inhibitor in crystals into which 1 mM VP32947 was diffused for 2 h (Table 1). Dynamic light-scattering and gel filtration experiments showed that BVDV polymerase is a monomer in solution (data not shown). Although poliovirus RdRp forms a dimer (15), the interface is different from that observed for BVDV polymerase; although these results indicate that the crystalllographic dimer is not biologically relevant, the buried surface area created by dimerization is 2,815 Å², suggesting a moderately strong interaction. Thus, it is possible that the dimer might play a significant role when it is a part of the replicase complex.

A second BVDV inhibitor, compound 1453, inhibits replication in the membrane-bound replicase complex but does not inhibit polymerase activity in vitro (39). However, a drug-resistant variant has a Gly substituted for the Glu at position 291, suggesting that Glu-291 may be involved in protein–protein interactions in the replicase complex. Glu-291, which is 7 Å away from Phe-224, is located in a short loop in the fingers domain, close to the fingertip region. The observation that both Phe-224 and Glu-291 are located on the same surface of the fingers domain, coupled with the higher potency of the compounds in replicase complex assays compared to purified polymerase assays, suggests that the top of the fingers domain may be a protein-binding site important for interaction with other proteins, such as the NS3 helicase, in the replicase complex (24, 41, 42). Hence, the antiviral compounds might interfere with the assembly of this complex.

The BVDV Polymerase Mechanism. RNA synthesis by RdRp involves template binding, initiation complex formation, and transition from initiation to elongation. The selectivity of RdRps for template binding and initiating RNA synthesis de novo or using a primer may be controlled by the thumb domain. BVDV, HCV, and other poly-merases, which all use ssRNA templates and initiate de novo, have an elaborate thumb domain architecture, with loops protruding into the active site. In contrast, poliovirus and calicivirus polymerases, which use a protein-linked primer and template, have a smaller thumb domain and, thus, a larger template-binding channel.

Upon binding of the template and GTP, a de novo initiation complex is formed. NTPs complementary to the 3’ end of the template base pair Watson–Crick base pairs. The triphosphate of the incoming NTP is held in place by two metal ions, which are also coordinated by the aspartic residue in the conserved GDD motif (RdRp motif VI). The NTP at the i (priming) site is selected by base pairing with the 3’ end terminal base of the template. A special GTP, required for initiation, mimics a nascent strand at the i-1 site, thereby positioning the 3’-OH of its sugar moiety close to the ribose of the NTP at the i site. This NTP will become the first nucleotide of the new complementary RNA strand. The correctly oriented 3’-OH group of the i site NTP then attacks the α-phosphate of the incoming NTP at the i + 1 (catalytic) site to form a new phosphodiester bond.

The translocation of the template RNA and product RNA may be facilitated by the flexible fingertip region, which contributes to the binding of an NTP at the i + 1 site, in combination with the C-terminal loop of the thumb domain. Subsequently, NTPs are added to the newly synthesized product RNA strand as the nucleotidel chain transfer process is repeated until the template RNA is completely copied.

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