

# Control of Template Positioning during *de Novo* Initiation of RNA Synthesis by the Bovine Viral Diarrhea Virus NS5B Polymerase\*

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The RNA-dependent RNA polymerase of the hepatitis C virus and the bovine viral diarrhea virus (BVDV) is able to initiate RNA synthesis *de novo* in the absence of a primer. Previous crystallographic data have pointed to the existence of a GTP-specific binding site (G-site) that is located in the vicinity of the active site of the BVDV enzyme. Here we have studied the functional role of the G-site and present evidence to show that specific GTP binding affects the positioning of the template during *de novo* initiation. Following the formation of the first phosphodiester bond, the polymerase translocates relative to the newly synthesized dinucleotide, which brings the 5'-end of the primer into the G-site, releasing the previously bound GTP. At this stage, the 3'-end of the template can remain opposite to the 5'-end of the primer or be repositioned to its original location before RNA synthesis proceeds. We show that the template can freely move between the two locations, and both complexes can isomerize to equilibrium. These data suggest that the bound GTP can stabilize the interaction between the 3'-end of the template and the priming nucleotide, preventing the template to overshoot and extend beyond the active site during *de novo* initiation. The hepatitis C virus enzyme utilizes a dinucleotide primer exclusively from the blunt end; the existence of a functionally equivalent G-site is therefore uncertain. For the BVDV polymerase we showed that *de novo* initiation is severely compromised by the T320A mutant that likely affects hydrogen bonding between the G-site and the guanine base. Dinucleotide-primed reactions are not influenced by this mutation, which supports the notion that the G-site is located in close proximity but not at the active site of the enzyme.

Despite structural and functional differences among various viral polymerases, previous biochemical and crystallographic

data suggested that several distinct steps define a minimum, general mechanism of DNA or RNA synthesis (1–4). These steps involve binding of the nucleoside triphosphate, a conformational change that traps the nucleotide substrate, formation of the first phosphodiester bond, and the release of pyrophosphate. The addition of the next nucleotide requires that the polymerase translocates a single position further downstream relative to the primer/template. This movement clears the nucleotide-binding site in a processive mode of polymerization before the enzyme dissociates from its nucleic acid substrate. Such a mechanism is likely to be relevant during elongation of a growing DNA or RNA chain; however, the initiation of the reaction is often more complex. Initiation in the absence of a primer, referred to as *de novo* initiation, is of particular interest in this regard.

Viral RNA-dependent RNA polymerases (RdRps)<sup>4</sup> that belong to members of the *Flaviviridae* family, which includes the hepatitis C virus (HCV) and the related bovine viral diarrhea virus (BVDV), were shown to initiate RNA synthesis *de novo* (5–12). Both HCV and BVDV contain a plus-stranded RNA genome, which encodes a single polyprotein that is processed into several structural and non-structural proteins (13). The non-structural protein 5B (NS5B) shows RdRp activity that is required for viral replication (14–18). A minimum mechanism of *de novo* initiation likely involves binding of the initiating nucleotide (NTPi) at a specific site of the enzyme referred to as the priming or initiation site (P-site) (19). For polymerases that recruit a primer, the P-site is occupied by the 3'-terminal nucleotide of the primer. The first NTP substrate (NTPi+1) that is later attached to the primer terminus (primed RNA synthesis) or to the initiating nucleotide (*de novo* initiation, Fig. 1) binds to the nucleotide-binding site (N-site, also referred to as catalytic site or substrate site). Both nucleotides NTPi and NTPi+1 need to be aligned such that the 3'-OH group of the initiating nucleotide in the P-site can attack the  $\alpha$ -phosphate of the nucleotide substrate in the N-site. Following catalysis, the release of pyrophosphate, and translocation, the 3'-end of the newly formed dinucleotide (NTPi+1) occupies the P-site, and

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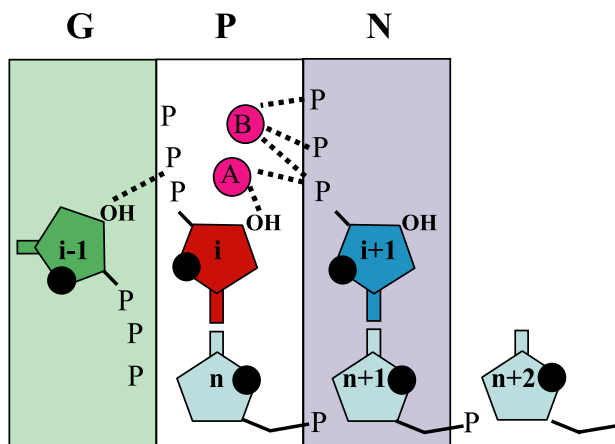
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<sup>4</sup> The abbreviations used are: RdRp, RNA-dependent RNA polymerase; BVDV, bovine viral diarrhea virus; HCV, hepatitis C virus; NS5B, non-structural protein 5B; G-site, GTP-specific binding site; P-site, priming or initiation site; N-site, nucleotide-binding site; NTPi, initiating nucleotide; 3'-dNTPs, 3'-deoxynucleotide triphosphates.

## Template Positioning by BVDV RdRp

### G-site dependent *De Novo* Initiation



**FIGURE 1. Schematic model of G-site-dependent *de novo* initiation of RNA synthesis by the BVDV RdRp.** *De novo* initiation of RNA synthesis involves binding of the initiating nucleotide (GTPi; red) at the priming or initiation site (P-site; white box) and binding of the first NTP substrate (GTPi+1; blue) to the nucleotide-binding site (N-site; purple box). GTP (i-1; green) bound to the GTP-specific binding site (G-site; green box) is positioned in such a way that its 3'-hydroxyl group is located in the vicinity of the  $\alpha$ - and  $\beta$ -phosphates of the initiating nucleotide. Specific binding sites for divalent metal ions (A and B, pink circles) are shown in close proximity to the  $\alpha$ -,  $\beta$ -, and  $\gamma$ -phosphates of the first nucleotide substrate.

the next incoming nucleotide (NTPi+2) gains access to the N-site, which permits another cycle of RNA synthesis (20).

Nucleotide binding to both P- and N-sites is likely to be necessary but not sufficient to efficiently initiate RNA synthesis in the absence of a primer. Crystallographic data suggested that both BVDV NS5B and the HCV enzyme form specific GTP-binding sites, albeit at different locations (21, 22). In the HCV enzyme, a low affinity G-specific binding site is seen 30 Å away from the polymerase active site (21). Possible effects on RNA synthesis, if any, would be mediated indirectly if one considers the long distance. In contrast, the structure of the BVDV polymerase bound to a GTP substrate revealed the existence of a GTP-specific binding site, herein referred to as G-site, that is located  $\approx$ 4 to 6 Å upstream of the P-site (22). This is supported by superimposing the BVDV structural data onto the solved crystal structure of the related  $\phi$ 6 bacteriophage polymerase with bound template and an initiating GTPi (20). The 3'-OH group of the bound GTP is seen in the vicinity of the  $\alpha$ - and  $\beta$ -phosphates of the modeled nucleotide that occupies the P-site. This location suggests a possible role in the process of *de novo* initiation. Based on this model, it has been proposed that the bound GTP helps to position the initiating NTPi in an orientation that facilitates the attack on the first nucleotide substrate NTPi+1 (Fig. 1).

*De novo* initiation requires that the 3'-end of the template interacts specifically with the initiating nucleotide NTPi (23, 24). Here we show that the GTP-specific binding site of BVDV NS5B can control the precise positioning of the template strand. A dinucleotide primer with a GMP at its 5'-end binds preferentially to a 3'-recessed template. Specific binding of the 5'-terminal guanosine to the G-site, and, by extension, specific binding of GTP during *de novo* initiation, appears to prevent overshooting of the template. Based on comparative analyses of *de novo* and dinucleotide-primed reactions, we propose a

model for early steps of G-site-dependent RNA synthesis by BVDV NS5B.

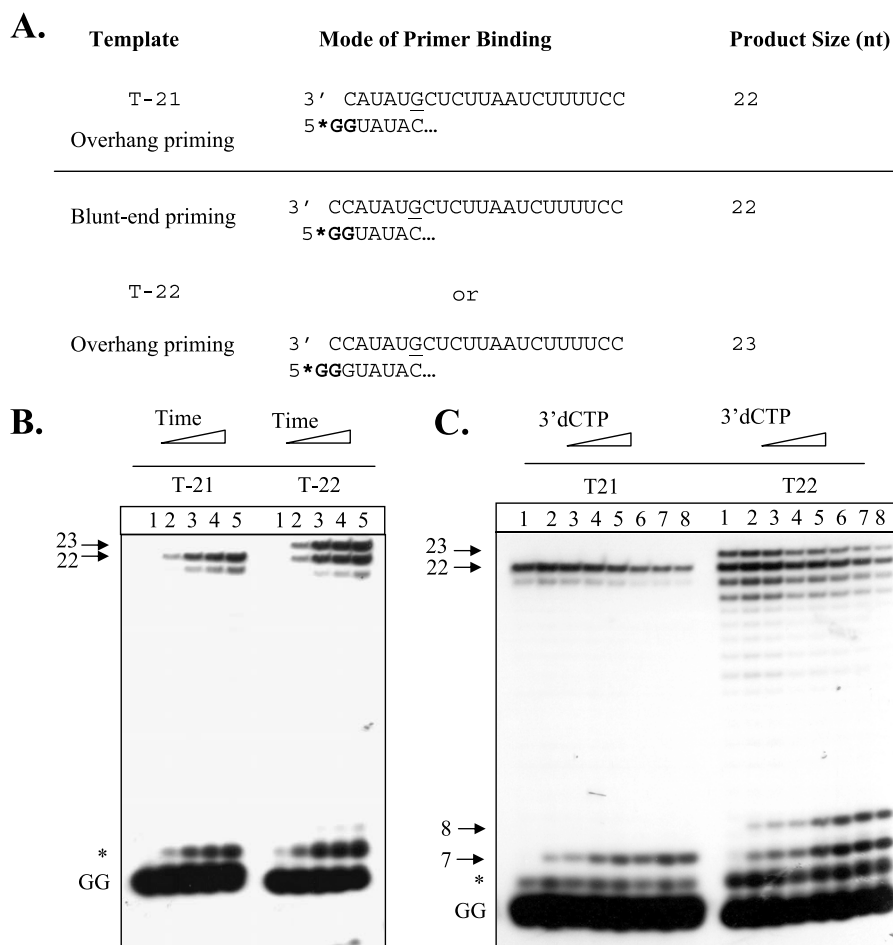
## EXPERIMENTAL PROCEDURES

**Enzymes and Nucleic Acids**—Truncated versions of the BVDV (BVDV $\Delta$ 18) and HCV (HCV $\Delta$ 21) NS5B, which lack the 18 or 21 C-terminal residues, respectively, were generated to facilitate their expression and purification (25–27). The coding sequence of the BVDV enzyme was amplified from an infectious cDNA clone (pACNR/BVDV NADL-XbaI) (28). EcoRI and XhoI sites were engineered into the PCR primers to facilitate cloning into the expression vector pET-21b (Novagen). The BVDV $\Delta$ 18 protein was expressed in *Escherichia coli* and purified as described previously (26). The plasmid encoding the BVDV $\Delta$ 18 was then utilized as the parental clone for the construction of the GTP-binding site mutants. Mutant enzymes T320A and Y581F were generated through site-directed mutagenesis using the Stratagene QuikChange kit according to the manufacturer's protocol. The HCV $\Delta$ 21 NS5B sequence inserted into the expression vector pET-22 (Novagen) was also expressed in *E. coli* and purified utilizing a combination of metal ion affinity and ion exchange chromatography (29). The sequences of all clones were confirmed by sequencing at the McGill University and Genome Quebec Innovation Centre.

The RNA template substrates utilized in this study were as follows: 5'-AACCGUAUCCAAAACAGUCC-3' (T-20 template, HCV), 5'-CCUUUUCUAAUUCUCGUUAUAC-3' (T-21 template, BVDV), and 5'-CCUUUUCUAAUUCUCGUUAUACC-3' (T-22 template, BVDV), while 5'-GG-3' and 5'-GGG-3' served as RNA primers. All RNA oligonucleotides were chemically synthesized. The RNA templates were further purified on 12% polyacrylamide-7 M urea gels containing 50 mM Tris borate, pH 8.0, and 1 mM EDTA, followed by elution from gel slices in a buffer containing 500 mM NH<sub>4</sub>Ac and 0.1% SDS. 5'-End labeling of the RNA primers was conducted with [ $\gamma$ -<sup>32</sup>P]ATP and T4 polynucleotide kinase according to the manufacturer's recommendation (Invitrogen).

**Primer Extension Assay**—Primer extension assay was performed as described previously (30). Briefly, standard reaction mixtures consisted of 0.75  $\mu$ M RNA template (T-21 or T-22), 0.75  $\mu$ M BVDV NS5B, and 0.25  $\mu$ M 5'-end-labeled primer in a buffer containing 20 mM Tris-HCl, pH 7.5, 50 mM NaCl, 1 mM dithiothreitol, and 100  $\mu$ M NTPs. Reactions were initiated by the addition of 0.15 mM MnCl<sub>2</sub> and incubated at room temperature for 30 min, unless otherwise stated. The effect of primer binding on chain termination was monitored in reaction mixtures as described above except that each of the four NTPs was added at a constant concentration of 10  $\mu$ M in the presence of increasing concentrations of 3'-dNTPs (TriLink Biotechnologies). All subsequent chain-termination reactions consisted of 100  $\mu$ M ATP, 100  $\mu$ M GTP, 100  $\mu$ M UTP, and 100  $\mu$ M 3'-dCTP.

The HCV $\Delta$ 21 primer extension assay was conducted with the following modifications: 1  $\mu$ M T-20 template, 1  $\mu$ M HCV enzyme, and 200 nM 5'-end-labeled primer were added to a buffer containing 20 mM Tris-HCl, pH 8.0, 10 mM NaCl, 1 mM dithiothreitol, 0.2 mM MnCl<sub>2</sub>, and 10  $\mu$ M NTPs and incubated at room temperature for 60 min.



**FIGURE 2. Heterogeneous priming of the GG dinucleotide primer during primer-dependent initiation of RNA synthesis.** *A*, schematic representation of the template/primer systems used in this study as well as possible binding positions of the 5'-end-labeled dinucleotide primer (\*GG in *bold*) and expected full-length product sizes. *B*, time course reactions comparing the extension of the 5'-end-labeled GG dinucleotide primer utilizing T-21 (*left panel*) and T-22 (*right panel*) as templates. *Lanes 1–5*, respectively, show reactions after 0, 5, 15, 30, and 60 min. *C*, incorporation of 3'-dCTP at increasing concentrations during heterogeneous priming after a 30-min reaction. *Lanes 1–8*, respectively, show reactions in the presence of 0, 1.5, 3, 12.5, 25, 50, and 100  $\mu\text{M}$  3'-dCTP while maintaining each of the four natural NTPs at 10  $\mu\text{M}$ . *Arrows* denote chain-termination incorporation sites, while the *asterisk* denotes possible unspecific priming events. Note that the numbering refers to the length of the primer.

**De Novo Initiation Assay**—Reaction mixtures for the *de novo* initiation assay are similar to those described for the primer extension assay except that the concentration of NTPs was modified to include 100  $\mu\text{M}$  3'-dCTP, 100  $\mu\text{M}$  GTP, 100  $\mu\text{M}$  UTP, 10  $\mu\text{M}$  ATP, and 1  $\mu\text{Ci}$  of [ $\alpha$ - $^{32}\text{P}$ ]ATP (Amersham Biosciences). Reactions were initiated by the addition of 0.15 mM  $\text{MnCl}_2$  and incubated at room temperature for 30 min, unless otherwise stated. To monitor whether the products obtained during primer-dependent and *de novo* initiation reactions are identical, GMP (Sigma) was added to *de novo* reactions at varied concentrations. For both assays, reaction mixtures were stopped with 100  $\mu\text{l}$  of a solution containing 0.3 M  $\text{NH}_4\text{Ac}$ , 1  $\mu\text{g}$  of bulk tRNA and 90% isopropyl alcohol. Samples were precipitated with ethanol, heat-denatured for 5 min at 95  $^\circ\text{C}$ , and resolved on 12% polyacrylamide-7 M urea gels. Quantification of product bands was performed using a phosphorimager (Bio-Rad Molecular Imager FX).

## RESULTS

**Experimental Design**—Both the BVDV and HCV NS5B are capable of utilizing dinucleotide primers to initiate RNA synthesis (26, 29). Such reactions could mimic early stages of RNA synthesis immediately following the formation of the first phosphodiester bond. Moreover, a complex with a dinucleotide primer has also been compared with an initiation complex that contains GTP and the initiating NTPi in the G-site and P-site, respectively (22, 29). For the BVDV enzyme, the 5'-terminal guanosine of a 5'-GG-3' or 5'-GN-3'-type primer may thus occupy the G-site. Our recent biochemical studies provided support for this notion (30). We have utilized a 5'-end-labeled GG-dinucleotide primer and a short 21-mer RNA template (T-21) as a model system to study the incorporation of chain-terminating 3'-deoxynucleotide triphosphates (3'-dNTPs). The 3'-terminal CA-sequence of the T-21 template provides only partial complementarity with the GG primer; however, the efficiency of RNA synthesis was significantly higher as compared with reactions with the matching GU primer. With the GG primer, we found that chain-terminated elongation products were always a single nucleotide longer than one would expect from the length of the template. These findings show that the 5'-terminal guanosine of the primer can indeed extend beyond the 3'-end of the

template to interact with the G-site, while the 3'-terminal guanosine occupies the P-site (30). At the same time, these data raise the question as to whether G-site binding, and, in turn, “overhang priming” may also occur when the dinucleotide GG primer is perfectly complementary to the 3'-end of the template. To address this issue, we devised a 22-mer as model RNA template (T-22) that contains an additional cytidine at its 3'-terminus and is otherwise identical to T-21 (Fig. 2A). This template is designed to allow both overhang priming, which is indicative of specific interactions between G-site and the 5'-terminal guanosine of the dinucleotide primer, and “blunt-end priming,” which is indicative of perfect Watson and Crick base pairing between the two guanosine residues of the primer and the two 3'-terminal cytidines of the template (Fig. 2A).

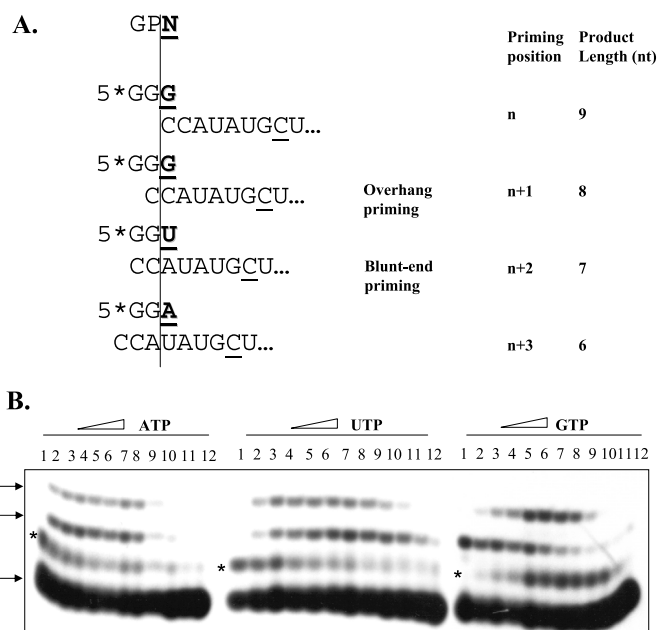
With the T-21 template, the BVDV NS5B enzyme extends the GG primer to generate a major full-length product of 22 nucleotides in length (Fig. 2B, *left panel*). In contrast, we

## Template Positioning by BVDV RdRp

observed a mixture of products containing 22 and 23 nucleotides in length when T-22 was utilized as a template (Fig. 2*B*, right panel). The mixture of products points to differences in priming locations; however, it cannot be excluded that template independent nucleotide additions may have confounded the results (31). Moreover, there are also shorter products that point to truncations at the 5'-end of the RNA template. To control for these problems, we included chain-terminating 3'-dNTPs in the reaction mixture and looked for the appearance of duplet bands that would unambiguously show heterogeneous priming. Increasing concentrations of 3'-dCTP were added to reactions containing either T-21 (Fig. 2*C*, left panel) or T-22 (Fig. 2*C*, right panel). The use of T-21 shows a single band at position +7, while reactions with the T-22 template show two distinct bands at positions +7 and +8, which is indicative of blunt-end and overhang priming, respectively.

**Parameters Affecting the Ratio between Overhang and Blunt-end Priming**—The incorporation of the first nucleotide requires that the NTP substrate binds to the N-site. At the same time, the dinucleotide GG primer occupies the G- and P-site. Both the dinucleotide primer and the nucleotide substrate must bind in this configuration to allow the chemical step. However, the appearance of two distinct reaction products suggests that the template strand is not located at a fixed position. The precise positioning of the template may crucially depend on base complementarity with the incoming nucleotide. To test this hypothesis, we varied systematically the concentration of the incoming nucleotide opposite template positions  $n$ ,  $n+1$ ,  $n+2$ , and  $n+3$  (Fig. 3*A*). The results show that increasing concentrations of GTP (opposite template position  $n$  and  $n+1$ ) provide conditions that favor overhang priming (8-mer product) (Fig. 3*B*, right panel, lanes 5–8). Low concentrations of GTP yield the shorter 7-mer product, which is gradually replaced by the 8-mer product as the concentration of GTP increases (Fig. 3*B*, right panel, lanes 1–5). Longer products are not seen, which indicates that priming cannot take place from template position  $n$ . Two connected template nucleotides are perhaps required to stabilize a fragile alignment between the two reacting residues that are located in the P- and N-sites, respectively.

The effect of the incoming nucleotide on the positioning of the template becomes evident with increasing concentrations of UTP, which is complementary to template position  $n+2$  (Fig. 3*A*). Increasing concentrations of this substrate facilitates RNA synthesis through blunt-end priming (7-mer product) (Fig. 3*B*, middle panel, lanes 5–10). Varying concentrations of ATP, that binds opposite template position  $n+3$ , has no effect on the mode of priming (Fig. 3*B*, left panel). The ratio between overhang and blunt-end priming remains unchanged when increasing the concentration of ATP. This result was expected, since priming from position  $n+3$  would involve a mismatch at the P-site (Fig. 3*A*). Together the data show that the template can freely move between two positions, which allow both overhang priming and blunt-end priming. A third reaction product, that migrates a little faster, is probably the result of an unspecific priming event that could involve G:U misincorporation in the beginning of the reaction (Fig. 3*B*, asterisks). This product is not seen in the absence of GTP, while it becomes dominant when increasing the concentrations of



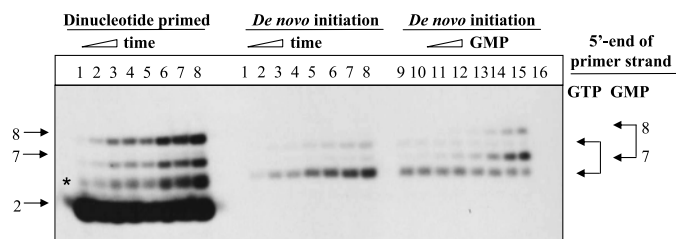
**FIGURE 3. Effect of incoming nucleotide on template positioning.** *A*, schematic description depicting the priming position of the incoming nucleotides tested (**bold**), as well as the expected chain-terminated product lengths. Note that blunt-end priming results in the formation of a 7-mer product, while overhang priming forms an 8-mer product. Site of 3'-dCTP incorporation is underlined. *B*, effect of increasing concentrations of ATP (left panel), UTP (middle panel), and GTP (right panel) on template positioning (lanes 1–12, respectively: 0, 2.5, 5, 10, 19.5, 40, 80, 156, 312.5, and 625  $\mu$ M and 1.25 and 2.5 mM). Reactions were conducted with the T-22 template and incubated for 30 min. Asterisks denote possible unspecific priming events such as G:U misincorporation or internal binding of the GG primer.

GTP (Fig. 3*B*, right panel, lanes 5–10). Alternatively, it is also possible that increasing concentrations of GTP may promote internal binding of the GG dinucleotide.

**De Novo Initiation Versus Dinucleotide-primed Reactions**—A dinucleotide is the first reaction product during *de novo* initiation. After formation of the first phosphodiester bond, the newly synthesized dinucleotide must translocate to clear the N-site for the next incoming nucleotide. The dinucleotide would then occupy the G- and P-site, which raises the question whether *de novo* initiation and dinucleotide-primed RNA synthesis give rise to the same distribution of reaction products. To study which of the two priming modes may adequately mimic events that take place during *de novo* initiation, we looked at product formation in the presence of [ $\alpha$ - $^{32}$ P]ATP (Fig. 4). Time course experiments show a single dominant product and a faint band that migrates a little higher (Fig. 4, middle panel). The spacing between both bands is reminiscent of the spacing seen between the two products generated with the dinucleotide primer (Fig. 4, left panel). However, the corresponding fragments do not co-migrate because the initiating GTP utilized during *de novo* initiation remains attached to the product in its triphosphate form, while the GG-primer contains a (radiolabeled) guanosine monophosphate at its 5' terminus. To confirm whether these products are identical to those obtained during primer-dependent synthesis, we tested the effects of increasing concentrations of GMP during the *de novo* reaction (Fig. 4, lanes 9–16). We found that increasing concentrations of GMP yielded reaction products that co-migrated with the two

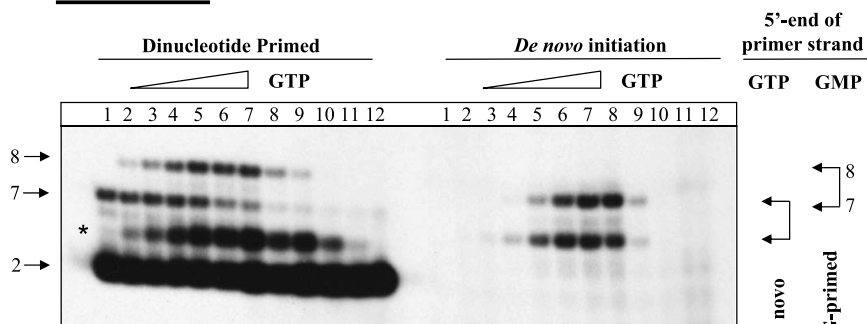
products generated with the dinucleotide primer; however, the ratio of the two reaction products differs significantly. Dinucleotide-primed synthesis favors the 8-mer product, while *de novo* initiation provides conditions that favor the 7-mer product.

**Mutational Analysis of the G-site**—The crystal structure of the BVDV NS5B complex with GTP points to several residues

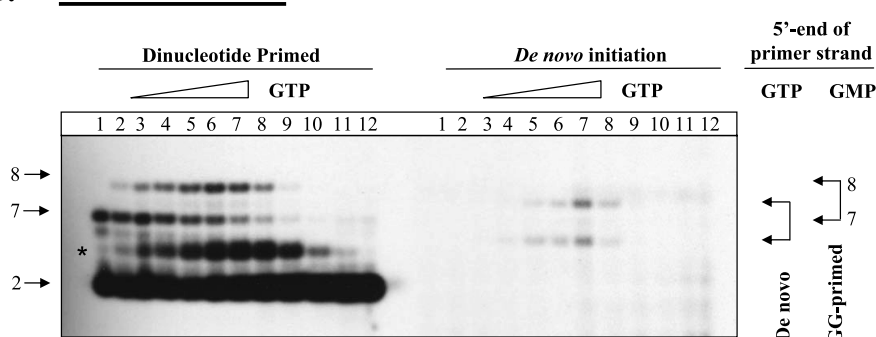


**FIGURE 4. Difference in template positioning during *de novo* and dinucleotide-primed initiation.** Time course experiments comparing the incorporation of 3'-dCTP during dinucleotide-primed (left panel) and *de novo* (middle panel) initiation of RNA synthesis. Reactions were conducted with the T-22 template and were incubated for 0, 5, 10, 15, 30, 45, 60, and 120 min (lanes 1–8, respectively). For *de novo* reactions, a mixture of GTP and UTP was used at 100  $\mu\text{M}$  and ATP at 1  $\mu\text{M}$  to allow for the incorporation of [ $\alpha$ - $^{32}\text{P}$ ]ATP, which is used as a tracer. To confirm whether the products generated between the two priming modes were identical, GMP was added at increasing concentrations during *de novo* initiation for 30 min (right panel). Lanes 9–16, respectively, show reactions in the presence of 0, 19.5, 40, 80, 156, 312.5, and 625  $\mu\text{M}$  and 1.25 mM GMP. Double-arrows indicate the positions of blunt-end (7-mer) and overhang (8-mer) products formed during dinucleotide-primed (GMP) and *de novo* (GTP) reactions. Asterisks denote possible unspecific priming events such as G:U misincorporation or internal binding of the GG primer.

### A. BVDV NS5B



### B. BVDV NS5B T320A



**FIGURE 5. Effect of G-site specific mutation during *de novo* and dinucleotide-primed initiation of RNA synthesis.** Comparison of the effect of a G-site-specific mutation at position Thr<sup>320</sup> with wild-type enzyme (A) and BVDV mutant enzyme T320A (B) on the incorporation of 3'-dCTP during dinucleotide-primed (left panel) and *de novo* (right panel) initiation of RNA synthesis. Lanes 1–12, respectively, show reactions that were allowed to proceed for 30 min in the presence of 0, 2.5, 5, 10, 19.5, 40, 80, 156, 312.5, and 625  $\mu\text{M}$  and 1.25 and 2.5 mM GTP. Double arrows indicate the positions of blunt-end (7-mer) and overhang (8-mer) products formed during dinucleotide-primed (GMP) and *de novo* (GTP) reactions. Asterisks denote possible unspecific priming events such as G:U misincorporation or internal binding of the GG primer.

that lie in close proximity to the guanine base (22). These include His<sup>499</sup>, Arg<sup>517</sup>, Lys<sup>525</sup>, and Arg<sup>529</sup> that are found in close proximity to the phosphate groups, and residues Thr<sup>320</sup>, Pro<sup>321</sup>, Leu<sup>322</sup>, Tyr<sup>581</sup>, and Leu<sup>677</sup> that are found in close proximity to the base moiety. Thr<sup>320</sup> and Tyr<sup>581</sup> appear to interact through hydrogen bonds with positions N-1, N-2, and O-6 of guanine. We generated mutant enzymes to study the effects of structural changes at crucial positions on both *de novo* initiation and dinucleotide primed reactions. The efficiency of RNA synthesis was monitored in the presence of increasing concentrations of GTP (Fig. 5). We found that *de novo* initiation with a T320A change is severely compromised (Fig. 5, right panel). In contrast, the efficiency of dinucleotide primed RNA synthesis remains largely unaffected, which shows that the active site of the enzyme is intact. Thus, this mutation affects selectively the formation of the first phosphodiester bond during *de novo* initiation. We have also tested the Y581F mutant that potentially disrupts hydrogen bonding between guanine O-6 and the hydroxyl group of the side chain. However, this mutant behaves essentially like the wild-type enzyme (data not shown). It appears that the Y581F change alone may not be sufficient to influence GTP binding.

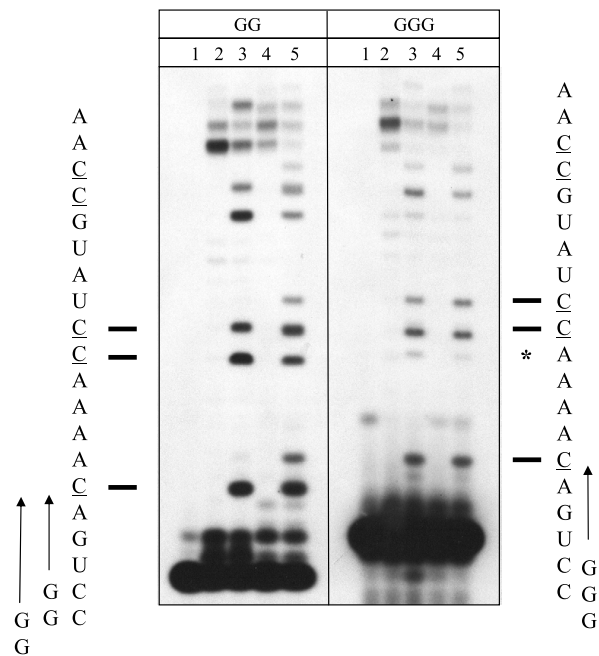
**Template Positioning during Initiation of RNA Synthesis by HCV NS5B**—We next compared early events of RNA synthesis by BVDV and HCV NS5B. We devised another model template, since T-21 and T-22 were poorly accepted by the HCV enzyme.

As for BVDV, we utilized a short synthetic RNA template (T-20), which contains two cytidines at its 3'-end (Fig. 6). We observed the presence of single bands during incorporation of the 3'-dGTP chain terminator, which indicates that the HCV polymerase, unlike the BVDV enzyme, preferentially positions the GG primer to bind complementary to the 3'-end of the template (Fig. 6, left panel, lanes 3 and 5). Overhang priming takes place only when the binding of at least two nucleotides is pre-established, as in the presence of a GGG primer (Fig. 6, right panel, lane 3). Thus, a functionally equivalent G-site, as described for the BVDV enzyme, is not evident in HCV NS5B.

## DISCUSSION

A minimum requirement for *de novo* initiation of RNA synthesis by BVDV and HCV RdRps involves binding of the initiating nucleotide (NTPi) to the P-site and binding of the first NTP substrate (NTPi+1) to the N-site. Crystallographic data have shown that the BVDV polymerase binds an additional GTP to a guanosine-specific binding site, here-

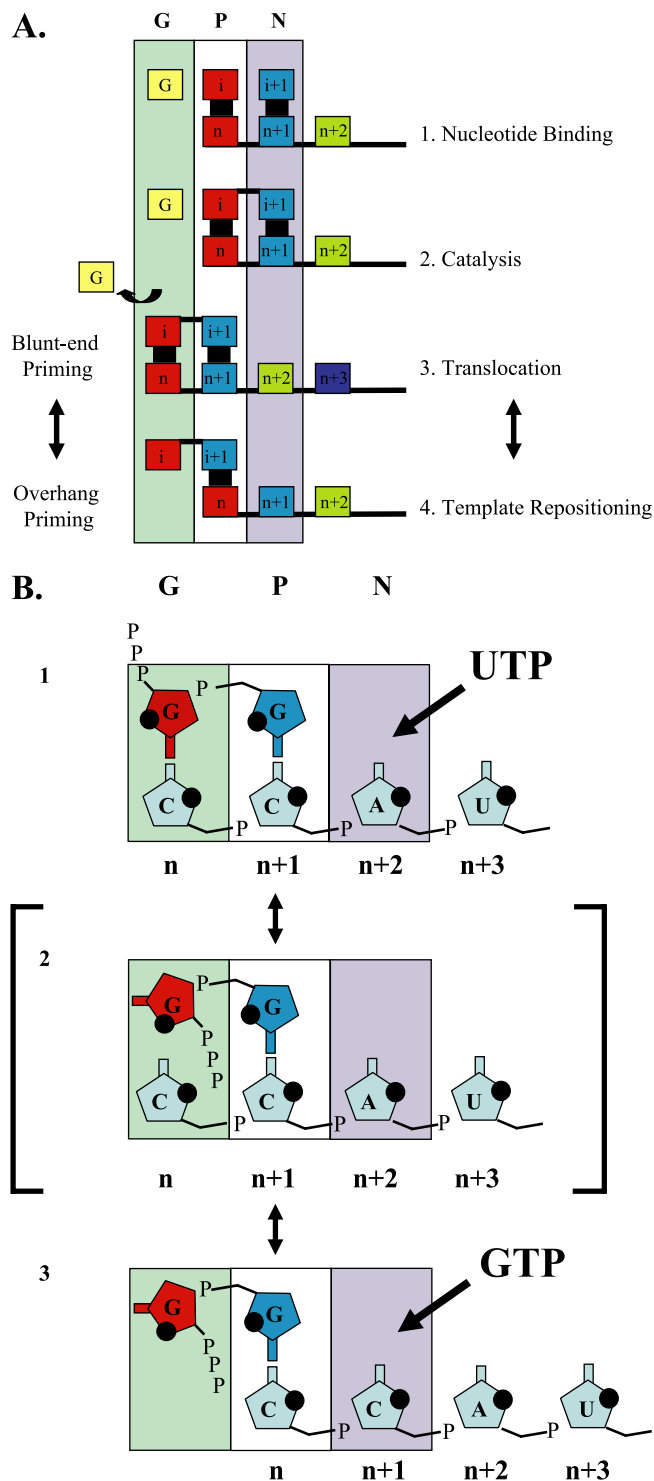
## Template Positioning by BVDV RdRp



**FIGURE 6. Difference in template positioning between BVDV and HCV polymerases.** Each panel shows reactions conducted with the T-20 template and 5'-end labeled dinucleotide (GG, *left panel*) or trinucleotide (GGG, *right panel*) primer during full-length extension (lanes 2 and 4) or the incorporation of 3'-dGTP (lanes 3 and 5) by the HCV (lanes 2 and 3) or BVDV (lanes 4 and 5) RdRp. Lane 1 is a control, which shows unextended primer. Reactions were incubated for 60 or 120 min, depending on whether GG or GGG was used respectively. Template positions for the incorporation of 3'-dGTP are underlined. Possible binding positions of GG and GGG primers are also shown. A minor product, represented by an asterisk, is generated through blunt-end priming of the GGG primer to the 3'-end of the template.

inferred to as G-site, just upstream of the active center (22). However, the functional role of this GTP-binding pocket remains elusive. Based on structural comparisons between the BVDV RdRp-GTP complex, and related polymerases with an RNA template, with and without an incoming nucleotide, it has been suggested that the 3'-OH of the bound GTP may help orient the 3'-OH group of NTPi to attack the first nucleotide substrate (21, 32–36). The biochemical data shown in this study corroborates the existence of a GTP-binding site at this position; however, we invoke an alternative functional role for the bound GTP. We provide strong evidence to show that binding of GTP to the G-site can affect the precise positioning of the template, which leads us to suggest that the bound GTP may facilitate the alignment between the 3' terminus of the template and the priming nucleotide. Both suggestions are not mutually exclusive, and the bound GTP could play a dual role in promoting *de novo* initiation by orienting the priming nucleotide and by controlling the positioning of the template. We developed a model that helps to reconcile the biochemical and crystallographic data. This model covers early stages during *de novo* initiation by BVDV NS5B, including the formation of the first phosphodiester bond, polymerase translocation, and incorporation of the second nucleotide substrate (Fig. 7).

A possible configuration with the bound nucleotides involved in the initiation reaction is schematically shown in Fig. 7A. The G-site, P-site, and N-site accommodate the three nucleotides involved: GTP, NTPi, and NTPi+1, respectively (step 1). The 3'-end of the template is located in close proximity



**FIGURE 7. Model of GTP-facilitated *de novo* initiation of RNA synthesis by the BVDV polymerase.** A, the initial stages of RNA synthesis by BVDV RdRp can be divided into four steps: nucleotide binding (step 1), catalysis (step 2), translocation (step 3), and template repositioning (step 4). In this model, repositioning of the template is triggered by the bound GTP (B). Following enzyme translocation, the 3'-end of the newly synthesized dinucleotide occupies the P-site (white box) and the 5'-end interacts with the complementary 3'-end of the template strand (complex 1). A conformational change at the 5'-terminal guanosine of the primer strand initiates its loss of contact with the templated base and facilitates specific interaction with the G-site (complex 2). The loss of terminal base pairing may trigger repositioning of the template to its original location. RNA synthesis resumes with a 3'-recessed template (complex 3). The isomerization equilibrium is controlled by the concentration of NTPs at template positions  $n+1$  (GTP) and  $n+2$  (UTP).

to the priming position opposite to the initiating NTPi. This model is supported by biochemical data showing that *de novo* RNA synthesis yields products that match the length of the template (blunt-end priming); however, the structural determinants that control the positioning of the template remain to be defined. The study and comparison of dinucleotide-primed reactions and *de novo* initiation of RNA synthesis shed light on this problem.

Following catalysis, the enzyme must translocate relative to the newly synthesized dinucleotide to clear the N-site for the next nucleotide substrate (Fig. 7A, steps 2 and 3). For polymerizing enzymes in the elongation stage, it is assumed that the primer/template substrate forms a stable duplex that moves as a whole relative to the enzyme. During *de novo* initiated reactions, such movement brings the 3'-end of the newly synthesized dinucleotide to the P-site and its 5'-end to the G-site, while the formerly bound GTP is released (Fig. 7A, step 3). The N-site is now accessible for the next nucleotide, and its incorporation yields products that match the size of the template (blunt-end priming). Our data show that blunt-end priming dominates during *de novo* initiation. In contrast, dinucleotide-primed reactions give rise to longer products that originate from overhang priming. To explain these data, we suggest that the template can be repositioned to its initial location following translocation, and both complexes may isomerize to equilibrium (Fig. 7A, step 4). We propose that the existence and occupancy of the G-site promotes template repositioning, which is illustrated in Fig. 7B. Following enzyme translocation, the 5'-terminal G of the newly synthesized dinucleotide is either in contact with the 3'-terminal base of the template (*complex 1*) or with the G-specific binding site (*complex 2*). It is unlikely that the 5'-terminal G can interact simultaneously with the G-site and the complementary 3'-end of the template strand, given that both contacts engage positions O-6, N-1, and N-2 of the guanine base (22). Thus, the repositioning of the template appears to be facilitated by the loss of interactions with the 5'-terminal guanosine of the primer strand (Fig. 7B, complexes 2 and 3). The movement of the template relative to the dinucleotide involves breakage of only a single base pair under these conditions, which is probably compensated by the newly formed interaction between the primer and the G-site. The intermediate complex, shown in Fig. 7B (*complex 2*), may not exist as illustrated. It is conceivable that the conformational change at the 5'-terminal guanosine of the primer might be accompanied by, or even triggers, template repositioning. Regardless of the precise mechanism, the data suggest that the 3'-terminal cytosine of the template is unlikely to be found in the vicinity of the G-site if this pocket is occupied with the 5'-terminal guanosine of the primer. Thus, both the specifically bound GTP during *de novo* initiation, and the 5'-terminal GMP in the context of dinucleotide-primed reaction, could restrict the free movement of the template and lock its 3'-end in close proximity to the P-site.

Our data further support the notion that both complexes can isomerize to reach an equilibrium. We found that the ratio of blunt-end priming and overhang priming depends critically on the concentration of NTPs opposite template positions  $n+1$  and  $n+2$ , which provides strong evidence to show that the tem-

plate can indeed freely move between these locations. The existence of an isomerization equilibrium can also explain why the *de novo* reaction is preferentially blunt-end primed, while dinucleotides are preferentially overhang-primed. We suggest that the incubation of enzyme, dinucleotide primer, and template pre-establishes the equilibrium, and the most stable configuration will predominate. This appears to be the complex with the 5'-end of the primer interacting with the G-site and the 3'-end of the template base-pairing with the 3'-end of the primer (Fig. 7B, *complex 3*). As a result, overhang priming is favored. A dinucleotide product is also generated during *de novo* initiation; however, in this case, blunt-end priming is favored. Thus, binding and incorporation of the next nucleotide is probably faster than the isomerization step and the ensuing repositioning of the template.

Taken together, the results of this study show that the GTP-binding site of the BVDV RdRp can affect the positioning of the template strand, which, in turn, affects the mode of priming. Such effects are not seen with the HCV enzyme (Fig. 6). In this case, *de novo* initiation and dinucleotide-primed reactions give rise to identical products that have been originated from blunt-end priming. Overhang priming is only seen when initiating the reaction with a trinucleotide GGG primer that extends beyond the 3' terminus of the template. It is therefore unlikely that the HCV enzyme contains a functionally equivalent G-specific binding site adjacent to the active site. Here the requirement for high concentrations of GTP may have different reasons, which remains to be addressed (37–41). For the BVDV enzyme, high concentrations of GTP, and, ultimately its binding to the G-site, could help orient the priming nucleotide, and binding of GTP may also help position the 3'-end of the template to facilitate the formation of the first phosphodiester bond. However, the comparison of reactions carried out by BVDV NS5B and the HCV enzyme shows that *de novo* initiation by RNA polymerases is not necessarily based on a common, unifying mechanism.

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**Control of Template Positioning during *de Novo* Initiation of RNA Synthesis by the Bovine Viral Diarrhea Virus NS5B Polymerase**

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