Bacteriophage \( \phi6 \) RNA-dependent RNA Polymerase

MOLECULAR DETAILS OF INITIATING NUCLEIC ACID SYNTHESIS WITHOUT PRIMER

Like most RNA polymerases, the polymerase of double-strand RNA bacteriophage \( \phi6 \) (\( \phi6 \)pol) is capable of primer-independent initiation. Based on the recently solved \( \phi6 \)pol initiation complex structure, a four-amino acid-long loop (amino acids 630–633) has been suggested to stabilize the first two incoming NTPs through stacking interactions with tyrosine, Tyr\(^{630}\). A similar loop is also present in the hepatitis C virus polymerase, another enzyme capable of \textit{de novo} initiation. Here, we use a series of \( \phi6 \)pol mutants to address the role of this element. As predicted, mutants at the Tyr\(^{630}\) position are inefficient in initiation \textit{de novo}. Unexpectedly, when the loop is disordered by changing Tyr\(^{630}\)-Lys\(^{631}\)-Trp\(^{632}\) to GSG, \( \phi6 \)pol becomes a primer-dependent enzyme, either extending complementary oligonucleotide or, when the template 3'-terminus can adopt a hairpin-like conformation, utilizing a “copy-back” initiation mechanism. In contrast to the wild-type \( \phi6 \)pol, the GSG mutant does not require high GTP concentration for its optimal activity. These findings suggest a general model for the initiation of \textit{de novo} RNA synthesis.

Enzymatic synthesis of nucleic acids can be initiated using two distinct mechanisms. All DNA and some RNA polymerases are strictly primer-dependent. These enzymes add nucleotides to the free hydroxyl group of an appropriate polynucleotide or protein primer. In contrast, most RNA polymerases initiate RNA synthesis \textit{de novo}, that is without a primer (1, 2). In this case, the 3’-OH group of the first NTP molecule acts as an acceptor for the second nucleotide. Nucleotidyl transfer is then expected from the amino acid sequence comparison. This suggests an evolutionary link between the polymerases of the dsRNA viruses infecting bacteria and the positive-sense ssRNA viruses of animals (22). In contrast to many polymerases that have the “open right hand” architecture, with fingers, thumb, and palm subdomains (23–26), \( \phi6 \)pol and homologues from the \textit{Cystoviridae} family (3, 4). Another group of enzymes comprises the NS5B polymerase of hepatitis C virus (HCVpol) and homologous proteins from other members of \textit{Flaviviridae} (5–12).

Purified \( \phi6 \), \( \phi8 \), and \( \phi13 \) polymerases have been shown to act as replicases and transcriptases \textit{in vitro} utilizing single-stranded RNA (ssRNA) and dsRNA substrates, respectively (3, 4, 13). As documented for \( \phi6 \)pol, cystoviral polymerases initiate RNA synthesis at the very 3’-end of the template employing a primer-independent initiation mechanism (3). HCVpol as well as the related bovine viral diarrhea virus polymerase are also capable of initiation \textit{de novo} (10, 14–17). However, under \textit{in vitro} conditions, these enzymes preferentially utilize a “back-priming” or “copy-back” initiation mode. In this case, the 3’-end of the template loops back to form a hairpin structure, which is subsequently extended with the polymerase (5, 8, 15, 18). This type of initiation is obviously deleterious for the virus replication \textit{in vivo}, because the newly produced daughter strand remains covalently bound to the template strand (2).

High resolution structures of HCVpol and \( \phi6 \)pol have been recently determined (19–22). The structures of the two enzymes are considerably similar (418 of the Ca atoms of \( \phi6 \)pol (665 aa total) can be superimposed on HCVpol with a root mean squared deviation of 3.5 Å). The structural similarity is not expected from the amino acid sequence comparison. This suggests an evolutionary link between the polymerases of the dsRNA viruses infecting bacteria and the positive-sense ssRNA viruses of animals (22). In contrast to many polymerases that have the “open right hand” architecture, with fingers, thumb, and palm subdomains (23–26), HCVpol and \( \phi6 \)pol appear as a cupped right hand with the fingers and thumb strongly interconnected (21, 22). Overall, both enzymes appear as compact spherical molecules with internally located active sites, and two positively charged tunnels allowing the access of the RNA template and NTP substrates to the polymerase interior (Refs. 19–22 and Fig. 1A).

In addition to the apoenzyme, the \( \phi6 \)pol initiation complex structure has been solved. This provides a detailed view of the enzyme associated with an oligonucleotide template and two NTPs complementary to the template 3’-end (22). This information is not available for the HCVpol. One intriguing feature of the \( \phi6 \)pol initiation complex is a chain of stacking interactions encompassing the bases of the two initiatory NTPs, Tyr\(^{630}\) and perhaps Trp\(^{632}\). Both residues are located in the C-terminal loop 630–633 that has been referred to as the “initiation platform” (Ref. 22 and Fig. 1B). This type of stacking is likely to be preserved in the \( \phi8 \) and \( \phi13 \) polymerases, because both proteins have aromatic residues at the equivalent positions (4, 27, 28). It has been suggested that Tyr\(^{630}\) of \( \phi6 \)pol could stabilize the NTPs in the process of initiation. Following the initia-

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‡ The abbreviations used are: RdRp, RNA-dependent RNA polymerase; \( \phi6 \)pol, RNA-dependent RNA polymerase of bacteriophage \( \phi6 \); aa, amino acid(s); dsRNA, double-stranded RNA; HCVpol, hepatitis C virus polymerase; ssRNA, single-stranded RNA; WT, wild-type.
tion step, the C-terminal domain containing this loop is believed to move, allowing the exit of the newly synthesized ssRNA product. Interestingly, an analogous structural element containing a tyrosine residue is also present in HCVpol (19–21) but not in the RdRp subunit of poliovirus (26). The latter enzyme is known to utilize a protein primer to initiate RNA synthesis (29). Furthermore, it has been observed (2) that the critical tyrosine is conserved at least across flaviviral and pestiviral polymerases. The proposed initiation platform (β-hairpin aa 443–454) of HCVpol has been shortened from LDC-QIYGACYSI to LGGI (30), leading to an increased propensity of the polymerase to initiate from an internally annealed primer, as compared with the wild-type enzyme that could only utilize short primers complementary to the template 3′-end. It was concluded that the β-hairpin acts as a gate preventing the 3′ terminus of the template RNA from slipping through the polymerase active site and ensuring terminal initiation of replication. However, the primer-independent initiation mode, crucial for the in vivo initiation, has not been studied in the HCVpol using mutated polymerases.

Here, we use a series of 6pol mutants to address the role of the 630–633 loop (the tentative initiation platform) in the de novo initiation of RNA replication. Mutations affecting conserved aromatic residues implicated in stacking interactions significantly decrease de novo initiation on 6pol-specific (+) sense ssRNAs. Nevertheless, the mutants replicate rather efficiently some 3′-modified (+) sense RNAs using “back-priming” initiation mechanism, reported earlier for other viral RdRps (5, 8, 11, 15, 18, 31–33). When the initiation platform of 6pol is disordered by the mutation YKW (630–632) to GSG, back-priming becomes the major mode of initiation. Overall, our results extend the HCVpol data and suggest a model where the de novo initiation is assured by a specialized element of the polymerase polypeptide chain and is further controlled by the secondary structure on the 3′-end of viral RNA.

**EXPERIMENTAL PROCEDURES**

**Plasmids**—Plasmid pLM659 (34) was used to produce (+) sense ssRNA copies of the small 6 genomic segment (s+). Plasmid pEM15 containing the 6 s+ segment with an internal deletion (13) was used to prepare sΔ s′ RNA and its 3′-terminally modified variants (sΔ13 and sΔ14p). Plasmid pEM19 was derived from pEM15 by inserting two duplexes of annealed phosphorylated oligonucleotides TL1/TL2 and TL3/TL4 at the XhoI-SacI sites (see Table I for oligonucleotide sequences). The plasmids encoding for the 6pol mutants were derived from the wild-type 6pol expression plasmid pEM2 (3). First, a short fragment of 6pol gene was PCR-amplified using Pfu polymerase (Stratagene) and oligonucleotide seq3_p2 as an upstream primer. The downstream primers p2_Y630F, p2_Y630A, and p2_GSG were designed to introduce corresponding mutations into the initiation platform loop. The PCR products were digested with NruI-NsiI and ligated with the large fragment of the similarly cut pEM2. The resultant plasmids pSJ4 (encoding for the Y630F mutant), pSJ5 (Y630A mutant), and pEM28 (YKW (630–632) to GSG mutant) were partially sequenced to verify the mutations.

**Preparation of ssRNA Substrates**—Synthetic ssRNAs were produced by run-off transcription in vitro with T7 RNA polymerase (3). Templates for the T7 transcription were prepared by either cutting plasmid DNA with restriction endonucleases or by PCR amplification. RNAs sΔ13 and sΔ14p were transcribed from the Smal cut plasmids pEM15 and pEM19, respectively. The sΔ fragment was PCR-amplified from pEM15 using Pfu DNA polymerase and oligonucleotides T7,1 and 3′ end, as upstream and downstream primers, respectively. RNA s− was produced as described by Ref. 3. All ssRNAs were dissolved in sterile water, and the RNA concentration was measured (A260). The quality of each preparation was checked by electrophoresis in 1% agarose gels.

**6pol Polymerase Assay**—Both wild-type and mutated 6pol polymerases were expressed in Escherichia coli BL21(DE3) containing the appropriate expression plasmid at 20 °C for 15 h and purified to homogeneity as described previously (3). The replication activity of wild-type 6pol and 6pol mutants were typically assayed in 10-μl reaction mixtures containing 50 mM HEPES-KOH, pH 7.8, 20 mM ammonium acetate, 6% (w/v) polyethylene glycol 4000, 5 mM MgCl2, 1 mM MnCl2, 0.1 mM EDTA, 0.1% Triton X-100, 1 μM each NTP (Amersham Biosciences, Piscataway, NJ), and 0.8 unit/μl RNase A. The reaction mixture was incubated for 1 h at 30 °C and treated for further analysis as described below.

**Agarose Gel Electrophoresis**—Standard agarose gel electrophoresis was used to achieve separation of the positive-sense ssRNA and the corresponding dsRNA segments (3, 35). It was carried out in 1.2% agarose gel 3% MetaPhor agarose gels containing 0.25 μg/ml ethidium bromide and buffered with 1× TBE (50 mM Tris borate, pH 8.3, 1 mM EDTA, and 0.25 μg/ml ethidium bromide). The 6pol replication reaction was stopped with an equal volume of U2 buffer (10 mM EDTA, 0.2%
SDS, 0.05% bromphenol blue, 0.05% xylene cyanol FF, 6% (v/v) glycerol, and 7–8 μl urea). For strand-separation gels, the samples were boiled for 2 min and then incubated on ice for 3 min before loading into the gel. After RNA separation (5 V/cm), gels were photographed under UV light exposure, dried on Whatman 3 filter paper or Hybond-N+ membrane (Amersham Biosciences, Inc.) followed by autoradiography and/or phosphorimaging (Fuji BAS1500) analysis of the product bands.

**RNAse I Digestion—**6pol replication reactions were assayed as described above but in a 20-μl reaction volume. The reactions were stopped after 1 h at +30 °C by adding EDTA to a final concentration of 10 mM. NH4Ac was added to a final concentration of 0.2 M, and the RNase digestion of the reaction products was initiated by adding 0.2 unit of RNAse I (RNase ONE reaction buffer, Promega). An equivalent volume of 1× reaction buffer (RNase ONE) instead of the RNAse I was added to the reactions without RNase digestion. After incubation of 1 h at 30 °C, the RNAse I reactions were stopped with 0.1% SDS and purified by phenol extraction and gel filtration (Amersham G-50) according to the manufacturer's instructions. The reaction products were processed further as described above for strand separation gel electrophoresis.

**Primer Extension Assay**—The oligonucleotide anti s-117 used in primer extension assays was designed to be complementary (in the 3’–5’ direction) to nt 117–136 of the s* segment (36). Primer extension reactions were done according to a previous study (37) with some modifications: 2 μl of s or s* RNA (1.4 μg) was combined with 2 μl of the hybridization buffer (0.25 mM K-HEPES, pH 7.0, 0.5 mM KCl), 0.1 mM EDTA, and 4 pmol of γ-[32P]ATP, Amersham Biosciences, Inc., 3000 Ci/mmol) in a final volume of 9 μl. Tubes were incubated at 65 °C for 1 min and slowly cooled to 30 °C over a period of 30 min. The primed RNA templates were mixed with the rest of the 6pol polymerase assay components (total volume 20 μl) and incubated at 30 °C for 1 h. For the control reaction 6pol control buffer (50 mM Tris-HCl, pH 8.0, 100 mM NaCl, 0.1 mM EDTA) was added instead of polymerase. The reactions were stopped by adding EDTA to a final concentration of 10 mM, purified with gel filtration (Amersham Biosciences, Inc., G-50), vacuum-dried, and dissolved into the sample buffer (95% formamide, 20 mM EDTA, 0.05% bromphenol blue, and 0.05% xylene cyanol FF). Sequencing lanes (A, C, G, and T) were produced with T7 Sequenase 2.0 (Amersham Biosciences, Inc.) from cloned cDNA of the s* segment using the same primer, anti s-117, as in the primer-extension reactions. The primer extension mixtures were incubated at 100 °C and sequencing reactions at 80 °C for 2 min and analyzed in a 6% polyacrylamide gel containing 7.5% urea. After electrophoresis, the gels were dried and exposed to phosphorimaging and autoradiography analysis. If the primer anti s-117 was not labeled, [α-32P]UTP (Amersham Biosciences, Inc., 3000 Ci/mmole) was added to the reaction in a final concentration of 0.1 mCi/ml. The reactions were stopped by adding EDTA to a final concentration of 10 mM, purified with gel filtration (Amersham Biosciences, Inc., G-50), and U2 buffer was added for analysis in standard or strand separation agarose gel electrophoresis as described above.

### RESULTS

The Initiation Platform Mutants Fail to Replicate Efficiently 6pol (3) Sense ssRNAs—To examine the role of the C-terminal loop 630–633, three 6pol mutants were constructed and purified (Fig. 2A). In two of the mutants, Tyr630 was substituted with either alanine (Y630A) or phenylalanine (Y630F). In the third case, three bulky amino acids YKW (aa 630–632) were changed to considerably smaller residues GSG (6pol(GSG)). All mutants were expressed and purified according to the protocol described for the wild-type 6pol (3). RNA-synthesizing activities of the purified enzymes were initially assayed using the 710 nucleotide (nt)-long ssRNA template...
sΔ−, which is a plus (+) sense copy of the d6 small genomic segment (s−) containing an extensive internal deletion (38). The assays were carried out as specified under “Experimental Procedures” and subsequently analyzed by gel electrophoresis followed by autoradiography. The results revealed significant differences between the wild-type and the mutated d6 polymerases (Fig. 2B). None of the tested mutants could utilize the sΔ− substrate efficiently, whereas the wild-type d6pol control contained a readily detectable amount of the full-length dsRNA product.

The Initiation Platform Mutants Employ a Back-priming Initiation Mechanism—The platform mutants were also assayed using chimeric ssRNAs templates. One of these templates, sΔ−13, was similar to sΔ+ RNA but contained a 13-nt extension...CUAGAGGAUCCCC-3′ originating from the plasmid polylinker. Both the wild-type and mutated polymerases accepted this template producing a full-length dsRNA product (Fig. 2B). Mutants Y630F and Y630A demonstrated relatively low activity, but d6pol(GSG) mutant replicated sΔ−13 more efficiently than the wild-type enzyme. There are two principal explanations for this difference: (i) The mutated polymerases might initiate de novo on the 3′ terminus of sΔ−13 but not sΔ+. Indeed, it has been shown that the addition of one or several cytosines to the template 3′ terminus stimulates initiation by the wild-type d6pol (13). (ii) The d6pol mutants might use an alternative initiation mechanism on sΔ−13, which differs from the de novo mechanism of the wild-type enzyme. The 3′ terminal regions of these two ssRNAs have been suggested to form a tRNA-like secondary structure. In sΔ−, this tRNA like element contains a 5-nt single-stranded 3′ tail, which apparently does not form any stable intramolecular base pairs (39). Conversely, the 13-nt longer tail of sΔ−13 has a potential to form a transient hairpin structure, which might be used by the polymerase mutants to prime RNA synthesis (Fig. 2C).

Because back-primed synthesis should result in the daughter strand covalently attached to the template, we analyzed heat-denatured RNA polymerization products by agarose gel-electrophoresis. In the case of the de novo initiation, the complementary strands of the duplex RNA molecule would migrate as single-stranded after denaturation. However, if RNA synthesis is initiated by the back-priming mechanism, the hairpin-like dsRNA product should re-annex immediately after the denaturation step and appear in the gel at the position of dsRNA (Fig. 3A). As expected, wild-type d6pol produced daughter strands, which could be almost completely separated from the sΔ−13 template upon heat denaturation (Fig. 3B, compare lanes 1 and 9). On the contrary, most replication products of d6pol(GSG) mutant could not be converted to the single-stranded form (Fig. 3B, lane 11). In the case of Y630A and Y630F mutants, approximately half of the RNA products appeared as double-stranded after denaturation (Fig. 3C, lanes 25 and 27). This indicates that all three mutants have a substantially increased propensity to generate back-primed RNA products consisting of covalently linked template and daughter strands.

To confirm that the dimer-sized RNA products observed after the denaturation were indeed hairpin-like species, we introduced an RNase digestion control. RNase I of E. coli readily hydrolyzes single-stranded and partially double-stranded RNA but not perfect RNA duplexes (40). The loop at the 3′-end of the hairpin product should not be base-paired and is, therefore, RNase-sensitive. As expected, RNase digestion had almost no effect on the wild-type d6pol replication products (Fig. 3B, lanes 1, 5, 9, and 13). In the case of mutated polymerases, RNase digestion converted heat-resistant double-stranded...
into RNA chains initiated de novo but not into back-primed products (Fig. 4A). This allowed us to selectively detect the de novo initiated dsRNA products. Accumulation of dsRNA products reaches steady state within ~0.5 min and is linear for at least 5 min for all four polymerases (not shown). We therefore measured the amount of dsRNA products produced within the first 5 min as an approximation for the initial velocity of de novo initiation. The velocities, thus determined, were plotted as a function of GTP concentration for all 6pol mutants (Fig. 4B). The resultant curves are distinctly S-shaped, which indicates a cooperative binding of the initiating GTPs. Consequently, the data were fitted to the Hill equation, \( V_0 = V_{\text{max}} \frac{[S]^{n}K_{50}^{n}}{K_{50}[S]^{n} + [S]^{n}} \), where \( V_0 \) is the initial velocity; \( V_{\text{max}} \), the maximal velocity; \( [S] \), initial substrate concentration; \( n \), Hill cooperativity coefficient; and \( K_{50} \), the half saturation constant, using non-linear regression software EZ-Fit (Perrella Scientific, www.JLC.net/~fperrel). The apparent \( K_{50} \) and relative \( V_{\text{max}} \) values calculated from the regression analysis are presented in Fig. 4C. The Hill coefficient was ~2 for all polymerases. It is obvious that, compared with the wild-type, all three mutants have increased \( K_{50} \) and decreased \( V_{\text{max}} \) values.

The GSG Mutant Polymerase Accepts ssRNA Substrates with a Preformed Hairpin Loop at the 3’ Terminus—Because the 6pol platform mutants were capable of back-priming on the 3’-end of the template, we further tested whether the mutants could initiate on the 3’-end containing a pre-formed hairpin. For this purpose, a very stable hairpin structure . . .

![Image of Fig. 5](image-url)
of the wild-type 6pol-directed RNA synthesis (Fig. 5A, compare lanes 1 and 5). This is not surprising, because GTP is used here as a priming nucleotide for initiation de novo. Phospho-
ing analysis revealed that the GTP concentration sufficient for a half-maximal level of RNA synthesis was ~0.2 mM, as measured with the sΔ−13 template (Fig. 5B). The overall RNA synthesis was detected by the non-denaturing agarose gel analysis. In contrast, the 6pol(GSG) mutant was less dependent on the GTP concentration with either the sΔ−13 or the sΔ−1P ssRNA templates (Fig. 5A, compare lane 3 with 7 and lane 4 with 8). In the case of the sΔ−13 template, the GTP concentration for a half-maximal synthesis by the GSG mutant was only ~0.02 mM (Fig. 5B). The requirements of the two enzymes do not differ in respect to the other NTPs (Table II).

6pol(GSG) Can Utilize Oligonucleotide Primers—The platform mutants were also tested for their ability to extend a complementary oligonucleotide primer. For this purpose, 5′-labeled primer (anti s-117) complementary to nt 117–136 of the 6pol s− segment (2948 nt long) was annealed to the corresponding ssRNA, and this primed template was used in wild-type and mutant 6pol assays. The reaction products were analyzed in a denaturing polyacrylamide gel along with the sequencing lanes produced with the same anti s-117 primer and cDNA of the of s− segment (Fig. 6). The reaction with 6pol(GSG) resulted in ~20-fold more efficient initiation from the primer than the wild-type 6pol reaction (Fig. 6, compare lanes 1 and 2). 6pol mutants Y630A and Y630F were able to initiate from the primer but clearly not as efficiently as the GSG mutant. Mutant Y630A was more prone to primer-dependent initiation than Y630F. In the control reaction no RNA synthesis occurred on the primed template incubated with buffer instead of 6pol (Fig. 6, lane 5). The adjacent sequencing lanes show that the primer extension products were full-length, initiated accurately from the primer (Fig. 6, lanes A, C, G, and T).

The following experiment was carried out to estimate the efficiency of the 6pol(GSG)-directed primer extension reaction compared with the wild-type-directed de novo initiation. Unla-
beled anti s-117 primer was annealed to the sΔ− ssRNA tem-
plate, and this was used in the 6pol-directed RNA synthesis reactions containing wild-type 6pol (lane 1), 6pol(GSG) (lane 2), 6pol(Y630A) (lane 3), 6pol(Y630F) (lane 4), or buffer (lane 5) assayed in the primer extension experiment with a labeled anti s-117 primer, complementary to the s− segment. The dRNA reaction products were analyzed in a denaturing polyacrylamide gel along with the dideoxynucleotide termination sequencing lanes (A, C, G, and T) produced with T7 Sequenase 2.0 (Amersham Biosciences, Inc.) using cloned cDNA of the s− segment (pLM659) and the anti s-117 primer. The 136-nt-long 6pol primer extension products are indicated with the arrow, B, a histogram showing the amount of synthesized dRNA product in primer-extension reactions by wild-type 6pol and 6pol mutants. The numbers are based on phosphorimaging quantification of the gel (Fuji BAS1500).

**Discussion**

This report provides direct experimental insights into the mechanism of de novo initiation of RNA-dependent RNA polymerization. The high resolution structure of the 6pol quaternary complex with a template and two NTPs provides an excellent ground for biochemical studies on the primer-independent initiation mechanism of RNA synthesis. Based on the structural data, the C-terminal platform (aa 630–633) of 6pol forms stacking interactions with the initiator NTPs, thus suggesting that this structural element might be critical for the de novo initiation (Ref. 22 and Fig. 1). This idea is further supported by the fact that a similar polypeptide loop is present in the HCVpol, which is also capable of initiation de novo (19–21). Furthermore, this element is absent from the poliovirus RNA-dependent RNA polymerase of poliovirus that is strictly primer-dependent (26, 29). The recent work by Hong et al. (30) addressed the role of the C-terminal β-hairpin (aa 434–454) of HCVpol in the terminal initiation, but its role in the de novo initiation remains elusive.

Here, the platform was minimized by changing Tyr630-
Lys631-Trp632 to GSG. Alternatively, point mutations were introduced that only affected Tyr630, immediately involved in stacking interaction with the initiator NTPs (mutants Y630A

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**Table II: Primer-independent Initiation of RNA Synthesis**

| NTP  | WT   | GSG |
|------|------|-----|
| GTP  | 175  | 18  |
| ATP  | 6    | 12  |
| CTP  | 7    | 14  |
| UTP  | 6    | 7   |

The final concentration of the other NTPs in the reaction mixture was 1 mM.
and Y630F). As predicted, these platform mutants were found to be inefficient in the initiation on \( \phi6 \)-specific (+) sense ssRNA. The impaired de novo initiation of Y630F is somewhat surprising, because this mutant only lacks the phenolic hydroxyl compared with the wild-type enzyme. We notice, however, that this OH group is located close to the side-chain carboxyl of Asp\(^{624} \), suggesting that this potential hydrogen bonding is somehow important for an adequate stacking interaction with the incoming nucleotide. This conclusion is further supported by the presence of Asp\(^{624} \) in the polymerases of \( \phi6 \)-related viruses \( \phi8 \) and \( \phi13 \).

However, the mutated enzymes were fully functional with some chimeric ssRNAs (Fig. 2B). We noticed that the 3’-end of these ssRNA (s\(^{\Delta-13} \)) can fold back and form a hairpin structure, which may allow the polymerase to utilize the back-priming initiation mode. To address this hypothesis, we heat-denatured the replication reaction products before gel electrophoresis and confirmed the results using RNase digestion. Unlike the dsRNA products of the wild-type \( \phi6 \)pol, which could be converted to the single-stranded form by heat denaturation, most of the RNA species produced by the \( \phi6 \)pol mutants migrated as dsRNA even after extensive boiling. Only after the RNase I pretreatment, the mobility of these species was shifted to that of ssRNA thus confirming the hairpin-like nature of the replication products (Fig. 3, B and C). The \( \phi6 \)pol(GSG) mutant clearly prefers back-priming initiation mode to the de novo initiation whereas Y630A and Y630F mutants can use both initiation mechanisms with almost equal efficiencies. Kinetic analysis of the de novo initiation on the s\(^{\Delta-13} \) ssRNA template corroborates the idea that stacking interaction between the aromatic side chain of Tyr\(^{630} \) and incoming nucleotide (GTP) stabilizes the initiation complex. Interestingly, when compared with the wild-type, mutations in the platform loop both increase \( K_{\text{m}} \) and decrease \( V_{\text{max}} \) for the de novo initiation (Fig. 4). \( K_{\text{m}} \) of the Y630F is closer to the wild-type than Y630A and GSG, consistent with the notion that the phenylalanine side chain can still stack against GTP base. All mutants are characterized by substantially reduced relative \( V_{\text{max}} \) values, GSG having somewhat higher value than the point mutants. Additional studies are clearly needed to rationalize this dramatic decrease in \( V_{\text{max}} \). The calculated Hill coefficient is \(-2\) for all four polymerases, which probably reflects cooperative binding of two GTP molecules to the initiation complex. This observation is supported by our previous structural data (22).

Several laboratories have previously reported that HCVpol can initiate RNA synthesis in vitro using back-priming mechanism, in addition to the de novo initiation mode (5, 15, 18). In fact, studying de novo initiation by HCVpol requires specific measures to be taken to prevent back-primed initiation. Several other viral RdRps that also utilize back-priming initiation in vitro have been described (8, 11, 31–33, 41). Because hairpin-like products resulting from this reaction must be deleterious for viral replication, back-priming is unlikely to be a genuine initiation mechanism used in vivo. Our results indicate that the shift from de novo initiation mechanism of wild-type \( \phi6 \)pol to the back-priming mechanism is caused by the modifications at the C-terminal initiation platform. In the case of HCVpol, the bias toward back-priming mode might be a consequence of using soluble forms of the enzyme in in vitro experiments, whereas in vivo HCVpol is associated with intracellular membranes using its C terminus as an anchor (9).

In addition to the back-primed RNA synthesis, the \( \phi6 \)pol(GSG) mutant can extend a complementary oligonucleotide primer annealed to the template (Fig. 6). At least in the case of anti s-117 primer, the primer-dependent initiation of \( \phi6 \)pol(GSG) is even more efficient than de novo initiation of the wild-type enzyme as shown in Fig. 7B. PRimer-dependent initiation of the \( \phi6 \)pol(GSG) is consistent with the results on the
HCVpol C-terminal -hairpin mutant (30). However, it is not clear how mutated polymerases with completely encircled active sites access an internally bound primer. It is unlikely that 6pol(GSG) conformation is more open than that of the wild-type enzyme, because mobility of the two proteins on a sizing column (Superdex 200) under native conditions is indistinguishable (data not shown). Two possibilities appear plausible: (i) the template threads through the enzyme and the polymerase pauses at the annealed primer, or (ii) occasional fraying of the 3′-ends that disfavor the back-primed conformation. (iii) The de novo initiation complex is further stabilized by high concentration of initiation nucleotides.

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