3' to 5' Exonuclease Activity of Herpes Simplex Virus Type 1 DNA Polymerase Modulates Its Strand Displacement Activity

Yali Zhu,1 Kelly S. Trego,2 Liping Song,1 and Deborah S. Parris1,2*

Department of Molecular Virology, Immunology, and Medical Genetics,1 and Department of Molecular Genetics,2 Ohio State University, Columbus, Ohio 43210

Received 10 March 2003/Accepted 17 June 2003

Using a minicircle DNA primer-template, the wild-type catalytic subunit of herpes simplex virus type 1 (HSV-1) DNA polymerase (pol) was shown to lack significant strand displacement activity with or without its processivity factor, UL42. However, an exonuclease-deficient (exo−) pol (D368A) was capable of slow strand displacement. Although UL42 increased the rate (2/s) and processivity of strand displacement by exo− pol, the rate was slower than that for gap-filling synthesis. High inherent excision rates on matched primer-templates and rapid idling-turnover (successive rounds of excision and polymerization) of exo-proficient polymerases correlated with poor strand displacement activity. The results suggest that the exo activity of HSV-1 pol modulates its ability to engage in strand displacement, a function that may be important to the viability and genome stability of the virus.

The herpes simplex virus type 1 (HSV-1) DNA polymerase is a stable heterodimer composed of a large catalytic subunit (pol) and a processivity factor (UL42), both of which are essential for viral DNA synthesis in vivo and for long-chain DNA synthesis in vitro (for a review, see reference 6). Similar to the catalytic subunits of many other replicative polymerases, HSV-1 pol possesses not only a 5' to 3' polymerizing activity but also a 3' to 5' exonuclease (exo) activity (2, 4, 13, 20, 23, 25, 30). An important role for the 3' to 5' exo activity of polymerases is in the coupled removal of misincorporated nucleotides during polymerization, resulting in increased fidelity of DNA synthesis (10, 15, 21, 25). However, the 3' to 5' exo activity influences other activities of polymerases, such as strand displacement synthesis. Indeed, mutations that destroy the associated 3' to 5' exo activity of pol 8 or bacteriophage T4 or T7 DNA polymerases lead to the acquisition or enhancement of strand displacement synthesis (17, 24, 26, 29).

The biological importance of the 3' to 5' exo activities of polymerases, aside from their role in proofreading, has been demonstrated for the budding yeast Saccharomyces cerevisiae. Mutations resulting in loss of the exo function of pol 8, while well tolerated alone, were synthetically lethal when combined with flap endonuclease 1 (Fen-1) null mutations (17). Fen-1 has been shown to be important in the processing of Okazaki fragments during lagging-strand synthesis (3), and in vitro biochemical analysis demonstrated that the increased strand displacement activity of exo-deficient S. cerevisiae pol 8 leads to an uncoupling of 5' flap production and cleavage of flaps by Fen-1 (1, 16). Thus, modulation of strand displacement by the associated exo activity of polymerases may be generally important for an organism's long-term viability and genome stability.

We were intrigued by reports that suggested that several Exo domain mutations in the HSV-1 pol gene (UL30) might be lethal in the context of virus. These included individual substitutions of alanine for aspartate residues 368, 471, or 581, representing mutations in conserved Exo sites I, II, or III, respectively (2, 14). Nevertheless, a virus containing a single mutation in the pol gene within the conserved Exo site III (Y577H), or a virus containing both the Y577H and D581A mutations, had mutator phenotypes (15) but was fully viable. These results suggest that increased mutation frequency alone cannot account for replication failure of most of the Exo domain mutations.

We wished to examine the role of the 3' to 5' exo activity on the ability of the HSV-1 pol to modulate strand displacement synthesis in order to identify additional functions for this activity. Few studies have examined the inherent ability of herpesvirus DNA polymerases to engage in strand displacement synthesis, and none have examined strand displacement by an exo-deficient (i.e., exo−) polymerase. In a rolling-circle model for HSV DNA replication, the pol and UL42 proteins failed to produce long strand displacement products in the absence of other replication proteins (11). However, the highly conserved polymerase from another human herpesvirus, Epstein-Barr virus, was reported to possess strong strand displacement activity (31). In this report, we describe a sensitive assay to characterize the ability of wild-type and exo-deficient HSV-1 pol and pol/UL42 to engage in strand displacement synthesis. We discuss the possibility that the lack of modulation of the strand displacement activity by the exo-deficient pol mutant may have catastrophic consequences for replication apart from a high spontaneous mutation frequency.

Each of the HSV-1 genes encoding proteins used in these studies was cloned into a baculovirus genome downstream from the polyhedrin promoter and expressed in Sf-9 insect cells as previously described (7). The wild-type HSV-1 pol, UL42, and ICP8 baculovirus recombinants were gifts from Robert Lehman (Stanford University), Mark Challberg (National Institutes of Health), and Nigel Stow (MRC Virology Unit, Glasgow, Scotland, United Kingdom), respectively. The exo− pol gene encoded a D368A mutation in the conserved Exo site I.
domain. The D368A mutant pol has been reported to possess no detectable 3’ to 5’ exo activity (2, 19) and is thought to be defective in the ability to coordinate divalent cations required for cleavage. A baculovirus recombinant that expresses the D368A exo− mutant pol was a kind gift of Charles Knopf (Heidelberg, Germany). The wild-type and exo− pol catalytic subunits were purified alone or as a stable complex with the UL42 processivity factor as detailed previously (7). We confirmed that the purified D368A catalytic subunit alone or in complex with UL42 lacked any detectable exo activity (results not shown). The HSV-1 ICP8 was purified using the chromatography protocol described by Boehmer and Lehman (5). All proteins used in the studies described herein were >95% pure as judged by the failure to detect other polypeptides in silver-stained sodium dodecyl sulfate-polyacrylamide gels loaded with at least 2 μg of protein (7, 8, and data not shown).

Wild-type pol and pol/UL42 possess only limited strand displacement activity. The ability of the wild-type pol and pol/UL42 complex to carry out strand displacement synthesis was assessed using a 70-mer single-stranded (ss) minicircle template (27) annealed to a complementary 50-mer primer strand. All oligonucleotides (Table 1) were purchased in gel-purified form from Integrated DNA Technologies (Corvalle, Iowa). Oligonucleotide A, phosphorylated on the 5’ end, was circularized with a bridging oligonucleotide (oligonucleotide D), complementary to 10 nucleotides on each end of oligonucleotide A, and the ends were ligated with T4 DNA ligase (Invitrogen, Carlsbad, Calif.) essentially as described previously (12). The circularized 70-mer ss template was purified by denaturing gel electrophoresis and annealed (1:1) to a 50-mer primer (oligonucleotide B) labeled at the 5’ end using [γ-32P]ATP according to standard procedures. The 5’ end label allowed precise determination of the proportion of primer that was extended to a particular size, because all labeled products have the same specific activity. Extension of the primer by polymerase across the ss gap would yield a product 70 nucleotides (nt) in length or shorter, while strand displacement synthesis would result in a product of >70 nt (Fig. 1A) in length.

Purified HSV-1 wild-type pol or pol/UL42 complex (57 and 20 nM, respectively) was incubated for 10 min at 37°C with 0.5 nM minicircle primer-template (P/T) in buffer containing 50 mM EPPS (pH 8.6), 25 mM NaCl, 5 mM dithiothreitol, bovine serum albumin (0.1 mg/ml), 10% glycerol, and 250 μM each of dATP, dCTP, dGTP, and dTTP (Amersham Biosciences, Piscataway, NJ.). EDTA (2.5 mM) was also included to prevent premature initiation and degradation of the primer by the 3’ to 5’ exo activity of the polymerase. Reactions were initiated by the addition of MgCl2 to a final concentration of 6 mM, and portions were removed and terminated at intervals (ranging from 20 s to 20 min) by the addition of EDTA to a final concentration of 50 mM. Products were separated by electrophoresis through 10% polyacrylamide (7 M urea gels) and exposed with an intensifying screen to X-ray film (Kodak T-Mat) at ~80°C. The length of reaction products was compared to that of the labeled 50-mer oligonucleotide B and a 70-nt marker with the same content as the predicted gap-filled product. The 70-nt marker was prepared by annealing a 5’ end-labeled 20-mer (oligonucleotide C, Table 1) to the 3’ end of the linear 70-mer template strand, followed by extension with

<table>
<thead>
<tr>
<th>Table 1. Oligonucleotides used for construction of DNA primer-templates and markers</th>
<th>Sequence (5’ to 3’)</th>
</tr>
</thead>
<tbody>
<tr>
<td>A 70 Template</td>
<td>CACCATAACCTCCACCCTCCCCAATATTCACCATCAACCCTTCACCTCACTTCACTCCACTATACCACTC</td>
</tr>
<tr>
<td>B 50 Primer for circular template</td>
<td>GTGGAGTGGTGCAAAAGGAGGAGGGTTGATGGTGAATATTGGGGAGGGTGGA</td>
</tr>
<tr>
<td>C 20 Primer for linear template</td>
<td>GAGTGGTATAGTGGAGTGGA</td>
</tr>
<tr>
<td>D 20 Circularization of template</td>
<td>GGTTATGGTGGAGTGGTATA</td>
</tr>
<tr>
<td>E 45 Primer for idling-turnover</td>
<td>GCCACTACGACACCTTGATCGCCTCGCAGCCGTCCAACCAACTCA</td>
</tr>
<tr>
<td>F 67 Template for idling-turnover</td>
<td>ATTTGCTGACCTTTGTTCTGGTTGAGTTGGTTGGACGGCTGCGAGGCGATCAAGGTGTCGTAGTGGC</td>
</tr>
</tbody>
</table>

10148 NOTES J. VIROL.
Klenow fragment (Invitrogen) for 15 min at 37°C in buffer containing 50 mM Tris-HCl (pH 8.0), 10 mM MgCl$_2$, 50 mM NaCl, and 500 μM each of all four dNTPs. Figure 1B demonstrates that the 50-mer primer was readily extended by the wild-type pol and pol/UL42 holoenzyme to form a prominent 70-mer band after 20 s of incubation, corresponding to gap-filling synthesis. A less abundant extension product corresponding to strand displacement synthesis through 2 nt was observed for both the catalytic subunit and the pol/UL42 complex, but significant accumulation of products greater than 82 nt in length did not occur with wild-type pol after 20 min of incubation. Strand displacement synthesis by the pol/UL42 complex was even more restricted, as indicated by the smaller size distribution of extension products compared to those that accumulated with pol in the absence of UL42. Thus, the wild-type HSV-1 pol, with or without its processivity factor, stalls upon encountering a 5' DNA end, although a small fraction of bound enzyme can engage in limited strand displacement synthesis through very short stretches of DNA.

**Strand displacement activity of exonuclease-deficient HSV-1 pol and pol/UL42.** We tested the ability of the D368A pol mutant protein to carry out strand displacement synthesis with and without the UL42 processivity factor. To ensure the same level of sensitivity for detecting strand displacement synthesis by the nonprocessive (exo– pol) and processive (exo– pol/UL42 complex) polymerase, dilutions of enzyme preparations were incubated with 0.5 nM minicircle P/T and dNTPs in the presence of EDTA, and reactions were initiated by the addition of MgCl$_2$. The products were incubated at 37°C, and portions were removed 0.33, 1, 5, 10, and 20 min later and terminated with EDTA. Product formation was analyzed by electrophoresis of samples through a denaturing polyacrylamide gel and exposure of the gel to X-ray film or phosphor storage screen. Lanes 1 and 12, the 70- and 50-nt markers; lane 13, incubation of P/T in reaction buffer for 20 min at 37°C without enzyme.

FIG. 1. Gap-filling and strand displacement synthesis on a minicircle DNA primer-template. (A) A 70-mer circularized template was annealed to a 5' end-labeled 50-mer primer strand. Extension across the ss gap would yield a reaction product 70 nt in length, while strand displacement would be characterized by products of >70 nt in length. (B) Purified wild-type pol (lanes 2 to 6) or pol/UL42 complex (lanes 7 to 11) was preincubated with 0.5 nM minicircle P/T and dNTPs in the presence of EDTA, and reactions were initiated by the addition of MgCl$_2$. The samples were incubated at 37°C, and portions were removed 0.33, 1, 5, 10, and 20 min later and terminated with EDTA. Product formation was analyzed by electrophoresis of samples through a denaturing polyacrylamide gel and exposure of the gel to X-ray film or phosphor storage screen. Lanes 1 and 12, the 70- and 50-nt markers; lane 13, incubation of P/T in reaction buffer for 20 min at 37°C without enzyme.

FIG. 2. Comparison of strand displacement synthesis by HSV-1 wild-type and exo– polymerases. Strand displacement assays were performed with the catalytic subunit of wild-type (wt, lanes 3 to 6) or exo– (lanes 7 to 10) polymerase in the absence of processivity factor or when complexed with UL42 (wt, lanes 11 to 14; exo–, lanes 15 to 18) as described in the legend of Fig. 1. The products were analyzed by electrophoresis through a 1.2% alkaline agarose gel and exposed to X-ray film. The triangles for each enzyme set indicate reaction times of 0.33, 1, 5, and 10 min. Lanes 1 and 2 contain the 70- and 50-nt markers, respectively, and the migration positions of precision DNA markers are shown to the right.
Biolabs, Beverly, Mass.) or an exonuclease-deficient mutant T7 DNA polymerase holoenzyme (Sequenase version 2.0; USB, Inc., Cleveland, Ohio). Although the wild-type T7 polymerase engaged in only limited strand displacement synthesis, similar to observations with the wild-type HSV-1 pol/UL42, strand displacement synthesis by Sequenase occurred at a faster initial rate than that observed for the HSV-1 exo-pol/UL42 complex (Fig. 3B). The strand displacement rates for the exonuclease-deficient HSV-1 and T7 enzymes are substantially slower than the pre-steady-state catalytic rates of extension (150 and 300/s, respectively) determined in single-turnover experiments (8, 32).

The results in Fig. 3A indicate that the rate of extension by the exo-pol/UL42 complex was linear through 5 min, but strand displacement synthesis slowed thereafter. The slowing of strand displacement synthesis could have been due to the accumulation of long ss DNA on the replicating template. If so, it was possible that the ss DNA binding protein, ICP8, might enhance the strand displacement ability of the polymerase. The exo-pol, with or without the UL42 processivity factor, was incubated with the minicircle P/T, and reactions were initiated by the addition of MgCl₂ and increasing concentrations of purified ICP8 (3.1 to 100 nM). Figure 4 indicates that the addition of ICP8 to a final concentration of 25 nM had little or no effect on the length of extension products observed after 20 s or 10 min of incubation at 37°C. Moreover, the addition of ICP8 to concentrations up to 100 nM failed to increase the strand displacement activity of either exo-pol or the exo-pol/UL42 complex and also did not promote strand displacement synthesis by the wild-type pol or pol/UL42 complex (data not shown).

**Sensitivity of strand displacement to DNA challenge.** One possible reason for the slower rate of strand displacement compared to gap-filling synthesis was continuous dissociation and reassociation of enzyme with the P/T. To distinguish between processive strand displacement synthesis and synthesis

---

**Fig. 3.** Steady-state rates of strand displacement synthesis. (A) The HSV-1 wt pol catalytic subunit ( ), the exo⁺ pol ( ), and the exo⁺ pol/UL42 complex ( ) were incubated with minicircle P/T for various times as described in the legend of Fig. 1, and the products were analyzed on alkaline agarose or denaturing polyacrylamide gels as appropriate. The maximum length of extension products was estimated from size markers and plotted as a function of reaction time. The results shown are the mean values ± standard deviations from three independent experiments. (B) Wild-type T7 DNA polymerase holoenzyme ( ) or an exo-deficient derivative, Sequenase version 2 ( ), was incubated with minicircle P/T as described, and the maximum size of extension products was plotted as a function of time.

**Fig. 4.** Effect of ICP8 on strand displacement synthesis by HSV-1 exo-deficient pol or pol/UL42. The HSV-1 exo⁺ pol (lanes 1 to 4) or exo⁺ pol/UL42 (lanes 5 to 8) was assembled with minicircle P/T as described in the legend of Fig. 1, except that increasing concentrations of purified ICP8 (3.13, 6.25, 12.5, and 25 nM in sequential lanes) were added with MgCl₂ to initiate reactions. In lanes 9 and 10, the exo⁺ pol and exo⁺ pol/UL42 complex were preincubated with P/T as above but without ICP8. Reactions were terminated after 20 s (lanes marked “a”) or after 10 min (lanes marked “b”) for each enzyme. The 70-nt marker is shown in lanes marked “M,” and the arrows show the positions of markers in adjacent lanes.
that occurs as a result of multiple turnovers of enzyme with DNA, we included an excess amount of activated calf thymus DNA (500 μg/ml) in the initiation buffer to trap polymerase molecules that dissociate from the prebound P/T (8). The resistance of strand displacement synthesis to DNA challenge by the HSV exo- pol or pol/UL42 complex was also compared to that of other well-characterized polymerases, including wild-type and exo-deficient (Sequenase) T7 DNA polymerase holoenzymes and Klenow fragment (Fig. 5). The results demonstrate that strand displacement synthesis by the exo- pol/UL42 complex was resistant to challenge by the DNA trap. By contrast, the exo- pol catalytic subunit did not engage in significant strand displacement synthesis in the presence of the DNA trap, although gap-filling synthesis was observed. As previously reported (29), T7 polymerase does not engage in significant strand displacement synthesis in the presence or absence of the DNA trap (Fig. 5). The presence of extension products less than 70 nt in length in the absence, but not in the presence, of the DNA trap indicates that a significant proportion of this commercial enzyme preparation is distributive and likely contains excess T7 polymerase catalytic subunit. Indeed, extension of 70% of the minicircle DNA template to form a 70-mer in the presence of the DNA trap required 7.5-fold more of the commercial preparation of T7 holoenzyme than that required to achieve 80% extension in the absence of the DNA trap (data not shown). Nevertheless, an exo-deficient T7 polymerase (Sequenase) carries out efficient and processive strand displacement (Fig. 5). The Klenow fragment, which possesses 3' to 5' exo activity but lacks the 5' to 3' nick translation activity of *Escherichia coli* DNA polymerase I, catalyzes strand displacement synthesis as previously noted (9), but the distributive nature of this activity is evident in the presence of competitor DNA (Fig. 5). Thus, UL42 stabilizes the HSV-1 exo- pol on the DNA template, and the exo- pol/UL42 engages in processive strand displacement synthesis, similar to the T7 exo- holoenzyme (Sequenase).

The ability of the wild-type pol with intrinsic exo activity to limit strand displacement synthesis when it encounters a 5' DNA end is likely to be caused by stalling, which could lead to dissociation of the enzyme from the P/T. However, rapid dissociation of enzyme cannot account for lack of strand displacement activity, although it does slow the rate, as observed for HSV-1 exo- pol and Klenow fragment (Fig. 3A and 5). Alternatively, the ability of a polymerase to engage in successive rounds of excision followed by polymerization (i.e., idling-turnover reactions) at the stall site could compete for its strand displacement ability. Elimination of the exo function of a polymerase would be expected to prevent this competition, favoring strand displacement. However, strand displacement synthesis does occur with the Klenow fragment, despite the presence of a 3' to 5' exo activity (Fig. 5). In keeping with our model for competition between the idling-turnover reaction and strand displacement, we hypothesized that polymerases with poor strand displacement activities would have high idling-turnover rates, whereas those that engaged in strand displacement...
displacement synthesis would have poorer inherent idling-turnover rates.

We measured the idling-turnover rates of several DNA polymerases using an unlabeled linear P/T composed of a 45-mer (oligonucleotide E) primer strand annealed to a 67-mer (oligonucleotide F) template strand (Table 1). This P/T allows the incorporation of a single nucleotide ([α-32P]dATP), but further extension is prevented or inhibited by the absence of appropriate complementary nucleotides (8). However, a polymerase with exo activity may excise the freshly incorporated nucleotide as [α-32P]dAMP. Idling-turnover reactions (100 μl) were performed at 37°C and contained 50 mM Tris-HCl (pH 7.5), 50 or 125 mM KCl as indicated, 4 mM MgCl2, 1 mM dithiothreitol, bovine serum albumin (400 μg/ml), 1 μM P/T, 300 μM dATP, and a quantity of [α-32P]dATP (specific activity of approximately 500 cpm/pmol) to yield a specific activity of approximately 500 cpm/pmol as described in the text. All reaction buffers contained 50 mM KCl except that for HSV-1 pol/UL42, which contained 125 mM KCl—the optimum salt concentration for that enzyme (7). The accumulation of radioabeled dAMP was monitored by thin-layer chromatography, normalized to enzyme concentration, and plotted as a function of time. Apparent rate constants were estimated from the linear portion of each plot (—) by least-squares regression analysis.

Idling-turnover rates are affected by the inherent exo and polymerizing rates of an enzyme as well as by the dissociation rate constant from DNA. For example, wild-type HSV-1 pol and pol/UL42 possess the same inherent exo and polymerizing rates (8). However, the pol dissociation rate constant is approximately three times that of the pol/UL42 complex (8), which is consistent with the threefold-higher idling-turnover rate that was observed (Fig. 6). Likewise, for enzymes with similar dissociation rate constants, higher idling-turnover rates would be expected for those possessing the most potent exo activities. The dissociation rate constants for the least processive enzymes, Klenow, HSV-1 pol catalytic subunit, and T7 pol catalytic subunit, are similar (0.17, 0.09, and 0.4/s, respectively), whereas their inherent exo activities on matched P/Ts differ (8, 10, 18). Figure 6 clearly demonstrates higher idling-turnover rates for the T7 and HSV-1 pol DNA polymerase catalytic subunits, which reflect their potent exo activities (7.9 and 5.9/s, respectively; see references 8 and 10). In contrast, the exo activity of Klenow is weak (0.002/s) and results in the slowest idling-turnover rate among the exo-proficient DNA polymerases tested (18). Thus, strand displacement synthesis occurs when the relative polymerizing activity is greatly favored over the exo activity in a given enzyme-DNA association event and negatively correlates with high idling-turnover rates.

It is noteworthy that Exo site I and site III mutations in the S. cerevisiae pol gene both produce polymerases with significantly enhanced strand displacement activities that lead to defects in Okazaki fragment maturation in the complete absence of Fen-1 (16). However, only the site III mutant is viable when combined with a mild Fen-1 mutant that cannot interact with the pol β processivity factor, PCNA (16, 17). It is not clear why the yeast Exo site III mutation has a milder phenotype than Exo site I mutants. However, a similar pattern has been observed for HSV-1, in that only viable Exo site III mutants have been isolated to date (14, 15).

The manner in which Okazaki fragments are processed during HSV-1 DNA replication has not been elucidated, but control of the process is likely to involve HSV-1 pol. During lagging-strand synthesis, it is important that the extending pol displace the 5’ RNA end of the Okazaki fragment previously synthesized and that the pol be released in order to engage another primer. The limited ability of the wild-type enzyme to displace a 5’ downstream DNA strand could be envisaged as a means to not only modulate the amount of strand displace-
ment synthesis but also as a means to promote dissociation and recycling of the enzyme during lagging-strand synthesis. It is interesting to speculate that excessive strand displacement by the D368A Exo site I HSV-1 mutant pol, particularly in the presence of the essential processivity factor UL42, would lead to longer 5' flap structures. These long flaps might be more difficult to cleave than those expected to accumulate during normal Okazaki fragment maturation, as suggested from previous studies with yeast (1). Moreover, the ability of an essential component of the HSV-1 replisome, ICP8, to promote strand invasion by such flaps (22) could lead to excessive cross-linking of genomes and failure to properly resolve replication intermediates and/or to package the viral DNA into capsids. Work is currently under way to investigate whether the HSV-1 Exo site I mutant pol is defective in lagging-strand synthesis or in the processing of replication intermediates in vitro and in vivo.

We thank Murari Chaudhuri for providing purified wild-type HSV-1 pol/UL42 and workers in the Parrish laboratory for helpful discussions during the course of these studies.

This work was supported in part by grants GM34930 and GM58809 from the National Institutes of Health and by services supplied by the Ohio State University Comprehensive Cancer Center Core Grant (P30 CA16058) and the Department of Molecular Virology, Immunology, and Medical Genetics.

REFERENCES


