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Effects of Acyclovir, Foscarnet and Ribonucleotides on Herpes Simplex Virus-1 DNA Polymerase: Mechanistic Insights and a Novel Mechanism for Preventing Stable Incorporation of Ribonucleotides into DNA[†]

Ashwani Kumar Vashishtha and Robert D. Kuchta*

Department of Chemistry and Biochemistry, University of Colorado, Boulder, Colorado 80309-0215

Abstract

We examined the impact of two clinically approved anti-herpes drugs, acyclovir and Foscarnet (phosphonoformate) on the exonuclease activity of the herpes simplex virus-1 DNA polymerase, UL30. Acyclovir triphosphate and Foscarnet, along with the closely related phosphonoacetic acid, did not affect exonuclease activity on single-stranded DNA. Furthermore, blocking the polymerase active site due to either binding of Foscarnet or phosphonoacetic acid to the E-DNA complex or polymerization of acyclovir onto the DNA also had minimal effect on exonuclease activity. The inability of the exonuclease to excise acyclovir from the primer 3'-terminus results from the altered sugar structure directly impeding phosphodiester bond hydrolysis as opposed to inhibiting binding, unwinding of the DNA by the exonuclease or transfer of the DNA from the polymerase to the exonuclease. Removing the 3'-hydroxyl or the 2'-carbon from the nucleotide at the 3'-terminus of the primer strongly inhibited exonuclease activity, although addition of a 2'-hydroxyl did not affect exonuclease activity. The biological consequences of these results are two-fold. First, the ability of acyclovir and Foscarnet to block dNTP polymerization without impacting exonuclease activity raises the possibility that their effects on herpes replication may involve both direct inhibition of dNTP polymerization along with exonuclease mediated destruction of herpes DNA. Second, the ability of the exonuclease to rapidly remove a ribonucleotide at the primer 3'-terminus in combination with the polymerase not efficiently adding dNTPs onto this primer provides a novel mechanism by which the herpes replication machinery can prevent incorporation of ribonucleotides into newly synthesized DNA.

TOC image

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*Corresponding author; Mailing address: Department of Chemistry and Biochemistry, University of Colorado, Boulder, CO 80309-0215, Phone: (303) 492-7027. Fax: (303) 492-5894. Kuchta@colorado.edu.

Supporting Information

Contains additional data showing: (1) Effects of foscarnet on exonuclease activity using single-stranded DNA as substrate; (2) Effects of PAA on exonuclease activity using single-stranded DNA as substrate; (3) Effects of foscarnet on exonuclease activity using double-stranded DNA as substrate; (4) Effects of PAA on simultaneous polymerase and exonuclease activity, and; (5) Effects of foscarnet on simultaneous polymerase and exonuclease activity.

Herpes viruses are complex DNA viruses that are responsible for a variety of indications including oral and genital herpes sores, chickenpox, viral encephalitis, etc. (7). Herpes simplex virus 1 (HSV¹) encodes seven proteins essential for viral DNA replication: a) the heterodimeric DNA polymerase-processivity factor complex (UL30-UL42), b) the heterotrimeric helicase-primase (UL5-UL8-UL52), c), an origin-binding protein (UL9), and a single-stranded DNA binding protein (UL29/ICP8) (8–10). In addition to polymerase activity, UL30 also possesses 3′–5′ exonuclease activity that proofreads the just-incorporated nucleotide (11–12).

Acyclovir, gancyclovir, and phosphonoformic acid are clinically useful anti-herpes drugs (13–14). Acyclovir is a remarkably powerful treatment for α -herpes virus infections due to its having minimal side effects and problems with resistance (15–18). Once converted to the triphosphate by cellular and viral kinases, acyclovir triphosphate (ACVTP) acts as a chain terminator of HSV polymerase (13–14, 19–23). The formation of acyclovir-terminated DNA followed by binding of the next required dNTP results in the formation of an extremely stable E–DNA–dNTP dead-end complex in the polymerase active site (24). Similarly, ganciclovir (as the biologically active GCVTP) primarily functions as a chain terminator during cytomegalovirus DNA replication (25). On the other hand, phosphonoformic acid (Foscarnet) is a pyrophosphate analogue that functions by directly binding to the pyrophosphate binding site in the polymerase active site (26–28).

Derse *et al.* observed that the exonuclease activity of herpes polymerase (UL30/UL42) did not efficiently excise acyclovir monophosphate that the polymerase activity had incorporated (20). Similarly, Hanes *et al.* used transient kinetic methods to directly show that the exonuclease does not efficiently hydrolyze acyclovir-terminated DNA as compared to DNA containing deoxyguanine at the primer terminus ($k_{\text{exo}} = 12 \text{ s}^{-1}$ for deoxyguanine terminated DNA versus $5 \times 10^{-3} \text{ s}^{-1}$ for acyclovir terminated DNA) (29). These authors also proposed

¹Abbreviations Used

ACV, acyclovir; ACVTP, acyclovir triphosphate; BSA, bovine serum albumin; ddNTP, 2′, 3′-di-deoxyribonucleoside-5′-triphosphate; DNA_{ACV}, DNA containing acyclovir at the primer 3′-terminus; DNA_{GCV}, DNA containing ganciclovir at the primer 3′-terminus; DTT, dithiothreitol; EDTA, ethylenediamine tetraacetic acid; GCV, ganciclovir; GCVTP, gancyclovir triphosphate; Hepes, 4-(2-hydroxyethyl)-1-piperazine-ethanesulfonic acid; HSV, herpes simplex virus; PAA, phosphonoacetic acid; PFA, phosphonoformic acid (Foscarnet); P/T, primer/template.

that the presence of acyclovir at the 3'-terminus of the primer interferes with movement of the DNA from the polymerase to the exonuclease active sites.

We recently showed that blocking the polymerase active site via formation of an UL30-DNA-Aphidicolin dead-end complex has little or no effect on the exonuclease activity, indicating that the polymerase and exonuclease active sites have independent DNA binding domains (30). This result also raises the possibility that other inhibitors may block polymerase activity but leave the exonuclease unbothered.

To better understand how acyclovir and Foscarnet can impact herpes replication, we employed synthetic oligonucleotides of defined sequence to examine how forming E-DNA_{ACV}-dNTP or E-DNA-PFA complexes in the polymerase active site affect exonuclease activity. In both cases, forming these complexes did not affect exonuclease activity. The poor ability of the exonuclease to hydrolyze acyclovir terminated DNA results from the modified sugar directly interfering with the hydrolysis reaction. Lastly, we observed that while the presence of a 2'-hydroxyl at the primer terminus does not affect exonuclease activity, it potently inhibits polymerase activity. The biochemical and medical significance of these results are discussed.

MATERIALS AND METHODS

Chemicals

All chemicals were of the highest grade available and were used as purchased. T4 polynucleotide kinase was from New England Biolabs, dNTPs and ddNTPs were from Invitrogen, and [γ -³²P]ATP was from Perkin-Elmer. T4 DNA polymerase, T7 DNA polymerase, and Klenow Fragment were obtained from New England Biolabs. Phosphonoformic acid and phosphonoacetic acid were from Sigma. Acyclovir triphosphate and gancyclovir triphosphate were obtained from Wayne Miller (Burroughs-Wellcome Corporation, Research Triangle Park, NC).

Enzymes

His-tagged UL30 and UL30/42 were purified from SF9 insect cells infected with recombinant baculoviruses that harbor the genes encoding these proteins as described previously (31).

Oligonucleotides

Oligonucleotides were obtained from Integrated DNA Technologies (Coralville, IA). Table 1 lists the sequences of all primers and templates used in this study. All DNAs were gel purified using 20% denaturing polyacrylamide gel electrophoresis. Primers were radiolabeled at the 5'-end using [γ -³²P]ATP and T4 polynucleotide kinase by standard procedures (32). DNA duplexes were formed by heating the primer-templates in a molar ratio of 1:1.4 to 95 °C followed by slow cooling to room temperature.

Preparation of ACV, GCV, dideoxyguanosine terminated duplex and single stranded DNAs

ACV terminated primer-template was prepared by incubating 40 μ M ACVTP with 5 μ M 5'-[³²P]-DNA_{15C} in 50 mM Hepes (pH 7.6), 5% glycerol, 0.1 mg/mL BSA, 1 mM DTT and 10 mM MgCl₂ at 37 °C. The reaction was initiated with 500 nM Klenow Fragment and allowed to proceed for 2 hours and then terminated by heating to 90 °C for 15 minutes. Excess ACVTP was removed from the reaction mixture by passing it through a G25 spin column. Denaturing polyacrylamide gel electrophoresis of the reaction products showed that >95% of the starting DNA_{15C} had been converted to DNA_{16ACV}. To prepare ACV terminated single-stranded DNA, the radiolabeled acyclovir terminated primer-template (DNA_{16ACV}) was heated to 95 °C for 5 min. and the ACV-terminated primer strand purified using 20% denaturing acrylamide gel electrophoresis. GCV and dideoxyguanine terminated single-stranded and duplex DNAs were prepared analogously.

Simultaneous Polymerase/Exonuclease Assays

All experiments were performed under steady-state conditions at 37 °C. Assays typically contained 1 μ M 5'-[³²P]-primer/template, 50 mM Hepes (pH 7.6), 5% glycerol, 0.1 mg/mL BSA, 1 mM DTT, 10 mM MgCl₂, 10 μ M dNTPs, and varying concentrations of inhibitor (ACVTP, PFA, or PAA). Reactions were initiated by adding enzyme (typically 50 nM) and quenched at various times by adding 5 volumes of 90% formamide, 10 mM EDTA, 1X Tris/Borate/EDTA buffer, and 0.1% bromophenol blue. Samples were heated for 2 min. at 90 °C and products separated by denaturing gel electrophoresis (20% acrylamide, 8 M urea) and analyzed by phosphorimager (Molecular Dynamics).

Exonuclease Assays

All exonuclease assays were performed under conditions of excess substrate as described above except that dNTPs were omitted from the assays. 5'-[³²P]-DNA (1 μ M) was incubated with reaction buffer in the presence of varying concentrations of ACVTP (0–200 μ M). Reactions were initiated by adding enzyme (typically 5 nM for single stranded DNA and 50 nM for duplex DNA) and aliquots quenched at designated time intervals.

Measurement of IC₅₀ values for various DNAs

Assays contained fixed concentration of either 5'-[³²P]-DNA_{35ss} or 5'-[³²P]-DNA_{35C} (1 μ M) and varying concentrations of an unlabeled DNA containing G, ddG, ACV or GCV at the primer 3' terminus. The reciprocal of the fraction of DNA_{35ss} or DNA_{35C} hydrolyzed was plotted against DNA concentration to obtain the IC₅₀ of the unlabeled DNA to inhibit exonuclease activity on the [³²P]-labeled DNA.

RESULTS

We previously showed that formation of an E-DNA-aphidicolin ternary complex where the DNA was bound in the polymerase active site had no effect on the exonuclease activity (30), raising the possibility that other inhibitors of herpes polymerase might likewise generate complexes with inhibited polymerase activity but active exonuclease. To test this possibility, we examined the effects of two clinically useful anti-herpes drugs, acyclovir and

phosphonoformic acid, on exonuclease activity using synthetic oligonucleotides of defined sequence.

We initially examined the effects of ACVTP, PFA and PAA on exonuclease activity using a single-stranded DNA as substrate (DNA_{35ss}). Figure 1 shows the time course for exonuclease activity in the presence of increasing concentrations of ACVTP. Even at saturating concentrations, ACVTP did not inhibit the exonuclease activity on DNA_{35ss} (Figure 1 Panel B). Similar results were obtained with PFA and PAA (Figures S1 and S2 in Supplementary Material). Using long, partially double-stranded DNAs as substrate (oligo(dG).poly(dC) (33), radiolabeled activated calf thymus DNA (34), and radiolabeled E. coli DNA (35)), previous work reported that both PFA and PAA inhibited exonuclease activity. However, we found that PFA only mildly inhibited exonuclease activity on 5'-[³²P]-DNA_{15C} (Figure S3A). To ensure that the lack of inhibition was not a consequence of the short length of DNA_{15C} and/or its sequence, we tested a somewhat longer DNA (DNA_{30C}). Again, adding PFA did not inhibit exonuclease activity (Figure S3B).

We next determined how these compounds impact exonuclease activity under conditions where the polymerase can simultaneously elongate a primer:template. We previously showed that under conditions of excess DNA over UL30 (or the UL30/UL42 complex), the enzyme processes some of the DNA via dNTP polymerization and some via exonuclease activity (30). Thus, these conditions allow simultaneous monitoring of both polymerase and exonuclease activity. Assays contained 1 μ M 5'-[³²P]-DNA_{15C}, all 4 dNTPs and increasing concentrations of ACVTP, PFA or PAA (Figures 2 (ACVTP), 3 (PFA) and S4 (PAA, Supplementary Material)). The DNA concentration is much greater than its K_D (Ca. 10–50 nM) such that all of the polymerase active sites should contain bound DNA. In the absence of any inhibitor, the polymerase elongates some of the DNA to the end of the template while the exonuclease hydrolyzes a fraction of the DNA. Adding increasing concentrations of ACVTP, PFA or PAA inhibited dNTP polymerization but had at most small effects on exonuclease activity. Polymerase inhibition without significantly impacting the exonuclease is consistent with the idea that the two active sites have independent DNA binding domains. Similar results were obtained with the UL30/UL42 complex, indicating that UL42 does not affect the independence of the polymerase and exonuclease DNA binding domains (Figure S5 in Supplementary Material).

PFA inhibits herpes replication by forming a UL30-DNA-PFA ternary complex while acyclovir can inhibit polymerase activity by forming either an UL30-DNA_{ACV} binary or UL30-DNA_{ACV}-dNTP ternary complex. To explicitly measure the effects of these complexes on exonuclease activity, we generated the UL30-DNA-($\pm$ dNTP) complexes using a 5'-[³²P] labeled primer-template while simultaneously measuring exonuclease activity on a separate 5'-[³²P]-ssDNA (Figure 4). The two 5'-[³²P] labeled DNAs were of different lengths, hence exonuclease activity on each DNA can be independently monitored. First, the effect of just a primer-template containing a normal nucleotide at the primer terminus was measured to control for the ability of a primer-template to directly bind in the exonuclease site. Figure 4, Panel A shows that whereas significant exonuclease activity occurred on the single-stranded DNA, virtually no degradation occurred on the primer-template (<5%), as expected in light of the exonucleases preference for single stranded DNA. Adding either

PFA or PAA to assays containing a primer-template and 5'-[³²P]-ssDNA had no effect on the rate at which UL30 hydrolyzed the ssDNA (Table 2 and Figures 4A, 4B and 4C), indicating that formation of either a E-DNA-PFA or E-DNA-PAA ternary complex in the polymerase active site did not affect the exonuclease. Likewise, formation of either an E-DNA_{ACV} or an E-DNA_{ACV}-dNTP complex in the polymerase active site did not inhibit exonuclease activity (Figure 4D and 4E). Thus, even under conditions where the polymerase active site is blocked due to binding and/or polymerization of various inhibitors, the exonuclease remains completely active.

Why is DNA containing ACV at the primer 3'-terminus a poor substrate for the exonuclease?

Previous work showed that double-stranded DNA containing ACV at the primer 3'-terminus is a very poor exonuclease substrate for the UL30/UL42 complex (20, 24, 29). Likewise, we observed that DNA containing ACV as the terminal nucleotide was a very poor substrate for the UL30 exonuclease (Table 3). The presence of ACV at the primer terminus (DNA_{16ACV}) decreased the rate of the exonuclease by 23-fold compared to the identical DNA containing dG at the 3'-terminus. This much slower rate clearly indicates that the sugar of the excised nucleotide greatly impacts phosphodiester bond cleavage. We therefore endeavored to determine what features of the sugar are needed for efficient exonuclease activity and the mechanistic consequences of eliminating these features.

Compared to the canonical 2'-deoxyribose, the sugar in ACV lacks a 3'-hydroxyl, lacks the hydrophobic 2'-methylene and is conformationally much less constrained due to its acyclic nature. To determine which of these features contributes to the decreased exonuclease efficiency, we synthesized primer templates that contained either a 2', 3'-dideoxynucleotide (to test the importance of a 3'-hydroxyl) or ganciclovir (to test the importance of an intact furan (Figure 5)) at the 3'-terminus. Just as the presence of acyclovir at the primer 3'-terminus greatly decreased exonuclease activity, the presence of either ganciclovir or a dideoxynucleotide at the primer 3'-terminus also greatly decreased exonuclease activity (Table 3). UL30 hydrolyzed a primer-template containing ganciclovir or 2', 3'-dideoxyG at the primer 3'-terminus 20- and 2900-fold less efficiently, respectively, than if it contained a normal nucleotide (Table 3). UL42 does not impact the effects of the altered sugars as UL30 and the UL30/UL42 complexes gave similar results. The rates of DNA degradation obtained with DNA_{16ACV}, DNA_{16GCV}, and DNA_{16dd} using UL30/UL42 were 90-, 90-, and 2200-fold lower than DNA_{15G}, respectively. Thus, the exonuclease activity of UL30 appears exquisitely sensitive to modifications in the sugar of the nucleotide at the 3'-terminus of the DNA.

To determine if modifying the sugar at the primer 3'-terminus affects the exonuclease associated with other DNA polymerases, we tested these modified DNAs with several other proofreading exonucleases. Table 3 shows that just as with UL30, modifying the structure of the 3'-terminal nucleotide greatly impacts the exonuclease of T4 DNA polymerase. Similar results were obtained with T7 DNA polymerase and Klenow Fragment when ACV terminated DNA was used. These data suggest that the exonucleases associated with at least some A and B family polymerases are quite sensitive to modification of the sugar moiety.

Mechanistically, three potential explanations could account for the decreased exonuclease rates of UL30 upon removing the 3'-hydroxyl from the 3'-terminal nucleotide or converting it into an acyclic sugar: (1) it greatly weakens binding to the exonuclease; (2) since the enzyme needs to transfer the DNA from the polymerase active site to the exonuclease active site and unwind two base-pairs of the double stranded DNA for the exonuclease to hydrolyze the substrate (30), the modified sugar might inhibit unwinding and/or DNA transfer, and; (3) the altered sugar structure interferes with hydrolysis of the phosphodiester linkage. We initially tested the hypothesis that the altered sugar structure interfered with binding to the exonuclease site. Exonuclease activity was measured on a 5'-[³²P]-labeled single-stranded DNA (DNA_{35ss}), and the effects of adding increasing amounts of a primer-template containing either ddG, ACV, GCV or a normal dG at the primer 3'-terminus measured. If altering the sugar structure interfered with binding to the exonuclease site, then the DNAs with altered sugars should have inhibited exonuclease activity on the 5'-[³²P]-labeled single-stranded DNA much less potently than the DNA with a normal sugar. However, all four DNAs inhibited exonuclease activity with only small differences in potency, indicating that modifying the sugar did not significantly interfere with binding of a primer-template to the exonuclease active site (Table 4). Similarly, all four DNAs also inhibited exonuclease activity of an exogenous 5'-[³²P]-labeled primer-template (DNA_{35C}) to the same extent (Table 4). To test the possibility that the modified sugars interfered with the unwinding and/or active site transfer reactions as well as explicitly determine if the modified sugar interferes with the hydrolysis reaction, we synthesized 5'-[³²P]-labeled single-stranded DNAs containing dG, ACV or ddG at the 3'-terminus and measured exonuclease activity. Unlike with double-stranded DNA, neither unwinding nor transfer between polymerase and exonuclease sites are needed for hydrolysis of single-stranded DNA. Whereas the exonuclease rapidly hydrolyzed the dG terminated single-stranded DNA, it did not detectably hydrolyze either the ACV or ddG terminated DNA (Figure 6). Moreover, these single stranded DNAs containing dG, ACV or ddG at the 3'-terminus had very similar binding affinities towards the exonuclease active site as measured by their ability to inhibit exonuclease activity on a second, 5'-[³²P]-ssDNA (Table 5). Thus, just as with the primer-templates, a modified nucleotide at the primer 3'-terminus does not affect the ability of single-stranded DNAs to bind to the exonuclease site. Additionally, since a modified nucleotide in single stranded DNAs blocked activity even though hydrolysis requires neither unwinding of any duplex DNA nor transfer between the polymerase and exonuclease sites, these data indicate that the modified sugar inhibits exonuclease activity by interfering with phosphodiester bond cleavage.

Substitution of the 2'-H with an OH group inhibits polymerase but not exonuclease activity

In order to determine the effect of replacing a 2'-H with an OH group, we prepared DNA containing a single ribonucleotide at the 3'-terminus and measured both exonuclease and polymerase activity. Figures 7A and 7B show the time courses for degradation of DNA_{16riboss} (a single-stranded DNA with a NMP at the 3'-terminus) and DNA_{16ribo} (duplex DNA with a NMP at the primer 3'-terminus), respectively. The polymerase degraded DNA_{16riboss} and DNA_{15ss} at similar rates, ($k_{\text{cat}} = 49 \pm 3 \text{ min}^{-1}$ and $67 \pm 2 \text{ min}^{-1}$ respectively) while it degraded DNA_{16ribo} slightly faster than DNA_{15G} ($k_{\text{cat}} = 10 \pm 2 \text{ min}^{-1}$

and $4 \pm 1 \text{ min}^{-1}$, respectively). Thus, unlike the other modifications, the presence or absence of a 2'-hydroxyl does not significantly impact the exonuclease.

In contrast to the minimal effect on exonuclease activity, the presence of even a single ribonucleotide at the primer 3'-terminus strongly inhibited dNTP polymerization. Adding a single NMP to the 3'-end of a DNA primer (DNA_{16ribo}) decreased k_{cat}/K_m for polymerization of the next correct dNTP by 3200-fold as compared to DNA containing a dNMP at the 3'-terminus (Figure 8). Similarly, we previously observed that herpes polymerase does not efficiently elongate RNA primers (36). To further test these results, we incubated UL30 (exonuclease proficient) with excess primer-template that contained a ribonucleotide at the primer 3'-terminus and the next correct dNTP. This resulted in the vast majority of the DNA being processed in the exonuclease site and only small amounts of elongated product (<5%, Figure 9).

DISCUSSION

Two clinically useful inhibitors of herpes polymerase, ACVTP and PFA, result in E-DNA complexes where the DNA is tightly bound in the polymerase active site. In the case of ACVTP, the DNA resulting from incorporation (E-DNA_{ACV}) binds with moderate affinity. However, the next correct dNTP binds very tightly ($K_D = 76 \text{ nM}$) to generate a very stable E-DNA_{ACV}-dNTP ternary complex (24). Importantly, the presence of this bound DNA in the polymerase active site had no effect on 3'-5' exonuclease activity. In the case of acyclovir, this occurred for both the E-DNA_{ACV} binary complex generated immediately after ACVTP polymerization as well as the E-DNA_{ACV}-dNTP ternary complex generated by ACVTP polymerization followed by binding of the next correct dNTP. Mechanistically, these data provide further support for UL30 containing at least two independent and non-overlapping DNA binding domains, one for the exonuclease and one for the polymerase.

The exonuclease likely remains active throughout the polymerase catalytic cycle (i.e., when the polymerase is in the open and closed complexes). We previously showed that the exonuclease retains activity upon formation of either an E-DNA binary complex or an E-DNA-aphidicolin ternary complex (30). Based on the structures of other B-family polymerases with just DNA or DNA and aphidicolin bound, the polymerase active site is likely in the open complex in both cases (37). In contrast, formation of the herpes polymerase-DNA_{ACV}-dNTP complex likely generates the closed complex. While the actual structure of this complex is not known, two data suggest it will resemble the closed complex. (1) The next correct dNTP binds very tightly to the E-DNA_{ACV} complex (24). (2) Other B-family polymerases form the closed complex upon generating the E-DNA-dNTP ternary complexes where the DNA cannot be elongated (e.g., it contains a 2', 3'-dideoxynucleotide at the 3' terminus) (38–44). Likewise, binding of PFA to the E-DNA binary complex to generate the E-DNA-PFA ternary complex probably converts UL30 into the closed conformational state, albeit the complex present after phosphodiester bond formation but prior to release of pyrophosphate. While the structure of this ternary complex has not been solved for UL30, the structure of a chimeric version of the B-family polymerase RB69-DNA-PFA ternary complex closely resembles the closed E-DNA-dNTP ternary complex

(45). Importantly, these observations suggest that the exonuclease remains active regardless of whether or not the polymerase is actively polymerizing dNTPs.²

Depending upon assay conditions, we found that PFA and PAA either did not inhibit exonuclease activity or gave very mild inhibition. This, however, contrasts with previous studies that reported significant exonuclease inhibition by PFA and/or PAA (33–35). The likely cause of these differences is the DNAs used. Whereas previous work used long DNAs whose structure is not well defined (oligo(dG).poly(dC) (33), activated calf thymus DNA (34) and *E. coli* DNA (35)), we used two shorter DNAs of defined sequence (DNA_{15C} and DNA_{30C}). The latter DNA was designed to be able to completely fill the DNA binding domain of the UL30/UL42 complex as defined by the nuclease protection studies of Challberg (46). This begets the question, however, of which DNA more closely resembles the DNA found *in vivo*? On one hand the length of calf thymus DNA or poly(dC) is certainly more similar to that found *in vivo*. However, the structure/sequence of these DNAs will be very different than is found *in vivo*. For example, the activated calf thymus DNA will contain loops, nicks, variable lengths of single- and double-stranded DNA, etc., depending upon how it was handled. The DNAs we used will closely resemble a typical primer-template in terms of structure, but they are certainly much shorter than *in vivo* DNA.

The observation that both acyclovir and Foscarnet mediated polymerase inhibition minimally impact exonuclease activity may have significant implications for how these compounds impact herpes replication. The potent polymerase inhibition exhibited by both compounds is certainly critical for their biological effects as evidenced by the mechanisms by which herpes can become resistant to these drugs (13–14). With both drugs, mutations that affect ACVTP polymerization and PFA binding can give high level resistance³. In the case of drug sensitive virus, however, if these inhibitors simply stopped the polymerase from synthesizing new DNA, herpes could potentially continue replicating its DNA once the drug concentration dropped below the value needed for effective polymerase inhibition. Inhibiting the polymerase but leaving the exonuclease active could reduce this possibility since upon encountering a 3' end of a DNA molecule, the exonuclease could degrade this already synthesized DNA. This destruction of preexisting DNA would be expected to negatively impact the potential for herpes to resume DNA replication upon diminution of the intracellular drug concentration.

Similar to previous work, we found that the exonuclease very poorly excises acyclovir from the 3'-terminus of DNA (20, 29). This was true for single-stranded DNA, a double-stranded primer-template and if the polymerase active site was occupied by a separate piece of DNA. Additionally, the presence of acyclovir in the DNA did not significantly impact binding of the DNA to the exonuclease active site. Together, these data indicate that the acyclovir inhibits exonuclease activity by directly blocking the hydrolytic reaction. This contrasts with the conclusions of Hanes *et al.* who inferred that acyclovir terminated DNA is a poor

²While unlikely, an alternative mechanism that we cannot absolutely rule out is the presence of two populations of polymerase, one with an active polymerase and one with an active exonuclease. As far as we are aware this has never been observed with a polymerase.

³The primary mechanism by which HSV-1 becomes resistant to acyclovir is via mutations in the viral thymidine kinase gene such that HSV infected cells no longer accumulate the biologically active ACVTP when treated with acyclovir (1–4). Secondly, resistance is also mediated by mutations in the UL30 gene such that the polymerase does not readily incorporate ACVTP (5–6).

substrate for the exonuclease because of inefficient transfer of the DNA from the polymerase to the exonuclease based on transient kinetic methods (29). Three observations from our studies indicate that this conclusion is incorrect. First, the presence of acyclovir at the 3'-terminus of both single-stranded DNA and a primer-template does not affect binding of the DNA to the exonuclease active site. Second, the exonuclease inefficiently hydrolyzes acyclovir terminated single-stranded DNA, a DNA that never has to be transferred between the two active sites. Third, blocking the polymerase active site with another DNA still results in inefficient excision of acyclovir even though the blocked polymerase site eliminates the possibility of DNA transfer between the polymerase and exonuclease. Thus, inefficient hydrolysis of acyclovir results from the altered sugar structure directly impacting the exonuclease, not from an effect on transfer.

The herpes exonuclease exhibits surprising specificity with respect to the sugar but not the base. As would be expected for a proofreading exonuclease, the enzyme readily excises any of the four natural dNMPs. In contrast, removing the 3'-hydroxyl and/or the 2'-methylene greatly inhibits exonuclease activity. While interactions of the exonuclease active site with the sugar of the 3'-terminal nucleotide are not important for binding of the nucleic acid, these interactions likely either directly stabilize the transition state for phosphodiester bond cleavage and/or properly align the phosphodiester bond for attack by H₂O.

Unlike the inhibitory effects of removing the 3'-hydroxyl or the 2'-methylene from the terminal nucleotide, the presence of a 2'-hydroxyl had no effect on exonuclease activity such that the enzyme removed NMPs and 2'-dNMPs at similar rates. Conversely, the presence of a single NMP at the 3'-end of the primer reduced the efficiency of addition of the next correct dNTP by 3200-fold as compared to a primer containing a dNMP at the 3'-terminus. Similarly, previous studies showed that herpes polymerase elongates RNA primers much less efficiently than DNA primers (700- to 26,000-fold). The comparable effects indicate that the 2'-hydroxyl at the primer 3'-terminus causes much, if not all, of the slow elongation of RNA primers (36).

The greatly reduced rate of dNTP polymerization onto a ribonucleotide in combination with an active exonuclease also suggests a simple and novel "proofreading" mechanism by which the herpes replication machinery may minimize the stable incorporation of NMPs into DNA. Upon incorporation of a ribonucleotide, the greatly reduced rate of further dNTP polymerization will provide time for the exonuclease to remove the NMP prior to the polymerase eventually incorporating the next correct dNTP. This ribonucleotide proofreading is analogous to how UL30 can proofread incorrectly polymerized dNTPs, although it should be noted that the efficiency of NTP proofreading will likely be less efficient than that for incorrect dNTP proofreading. Whereas UL30 discriminated against ribonucleotide elongation by 3,200-fold, UL30 discriminates against mismatch elongation by 10⁴- to 10⁵-fold (47).

As far as we are aware, this ability of UL30 to efficiently remove incorporated ribonucleotides via proofreading distinguishes it from all other replicative DNA polymerases. DNA pol α , δ and ϵ replicate eukaryotic nuclear DNA and none of them efficiently proofread incorporated ribonucleotides. Pol α efficiently elongates primers

containing one (or more) ribonucleotides at the primer 3'-terminus and lacks a proofreading exonuclease (48). The 3'-5' exonuclease of pol δ does not proofread ribonucleotides incorporated by pol δ 's polymerase activity while the 3'-5' exonuclease of pol ϵ only weakly proofreads ribonucleotides incorporated by pol ϵ 's polymerase activity (49–50). This inability of pol α , δ and ϵ to efficiently remove just incorporated ribonucleotides from newly synthesized DNAs results in eukaryotic cells employing RNase H2 to perform this essential function (49). Likewise, bacterial pol III and Φ 29 polymerase incorporate NTPs and then continue dNTP polymerization (51–52). In the case of herpes, UL30 can use its proofreading function to minimize stable ribonucleotide incorporation into DNA and potentially eliminate the need for RNaseH2 or other post-replicative mechanisms for excising ribonucleotides.

The apparently complete independence of the polymerase and 3'-5' exonuclease active sites of UL30 has two important implications. First, it suggests a novel mechanism by which current herpes treatments may fatally disrupt herpes replication, the destruction of already synthesized herpes DNA. Second, it raises the questions of where on UL30 the DNA binding domain for the exonuclease resides and how the exonuclease and polymerase activities are coordinated at the replication fork. Studies to answer these questions are in progress.

Supplementary Material

Refer to Web version on PubMed Central for supplementary material.

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Figure 1. ACVTP does not affect exonuclease activity on DNA_{35ss}. UL30 was incubated with DNA (1 μM) and aliquots were taken out at various times. **(A)** Phosphorimages of the products of DNA_{35ss} degradation using UL30 at varying concentrations of ACVTP including 0, 1, 5, 20, and 200 μM at various time intervals: 0, 0.5, 1, 5, 10, and 20 min. **(B)** Plot of exonuclease products as a function of ACVTP concentration at various time intervals. Note: The gels shown are representative of experiments that were performed multiple times.

Figure 2.

Effect of ACVTP on polymerase and exonuclease activities under processive conditions. UL30 was incubated with DNA_{15C} (1 μM) in the presence of 0 μM or 10 μM dNTPs and varying concentrations of acyclovir triphosphate (0–80 μM). Aliquots of each reaction were analyzed at various times after initiating the reaction. **(A)** Phosphorimages of the products of DNA_{15C} full extension and degradation using UL30. **(B)** Plot of exonuclease products as a function of ACVTP concentration at 6 min.

Figure 3. Effect of Foscarnet on polymerase and exonuclease activities under processive conditions. UL30 was incubated with DNA_{15C} (1 μM) in the presence of 0 μM or 10 μM dNTPs, and 0–80 μM Foscarnet Aliquots were analyzed at 6 min. **(A)** Phosphorimages of the products of DNA_{15C} full extension and degradation using UL30. **(B)** Plot of exonuclease products as a function of PFA concentration.

Figure 4.

Formation of UL30-DNA_{15C}-PFA, UL30-DNA_{15C}-PAA, UL30-DNA_{16ACV}, and UL30-DNA_{16ACV}-dTTP complexes do not affect the exonuclease activity on a second DNA. Assays contained DNA_{35ss} and the additional DNAs and compounds as noted. All DNAs were present at 1 μ M. (A) DNA_{15C}. (B) DNA_{15C} and 50 μ M PFA. (C) DNA_{15C} and 50 μ M PAA. (D) DNA_{16ACV}. (E) DNA_{16ACV} + 50 μ M dTTP. The time points for legends A-C were: 0, 0.25, 0.75, 1, 2, 3, 5 and 7 min. The time points for legends D-E were: 0, 0.25, 0.5, 0.75, 1, 2 and 5 min. In Panels B-E, DNA_{15C} is omitted for clarity.

Figure 5.
Nucleoside structures.

Figure 6.

The UL30 exonuclease does not efficiently remove acyclovir or dideoxyguanosine from single-stranded DNA. UL30 was incubated with various DNAs and aliquots were taken out at the noted times. All DNAs were present at 1 μ M. (A) DNA_{15ss}. (B) DNA_{16ACVss}. (C) DNA_{16ddss}. (D) Amount of exonuclease products generated for each DNA.

Figure 7.

Time course for degradation of DNA_{16riboss} and DNA_{16ribo}. UL30 was incubated with DNA (1 μ M) and aliquots were taken out at various times. **(A)** Phosphorimages of the products of DNA_{16riboss} degradation using UL30 (1 nM) after 0, 0.25, 0.5, 1, 2, 5, 7, 10, 15, 20, 25, and 30 min. **(B)** Phosphorimages of the products of DNA_{16ribo} degradation using UL30 (25 nM) after 0, 0.25, 0.5, 1, 2, 5, 7, 10, 15, 20, 25, and 30 min.

5'-GCTCTGTTCCGGTTT**rG***
 3'-CGAGACAAGGCCAAACACAAGTAGATTAGTAC

Figure 8.

HSV pol does not efficiently polymerize dNTPs onto DNA containing a ribonucleotide at the 3'-terminus. UL30/UL42 (exo-) (50 nM) was incubated with DNA_{ribo} (1 μM) in the presence of 100 μM TTP and aliquots were taken out after 15 min. **(A)** Phosphorimages of the products. **(B)** Plot of products as a function of TTP concentration. Data were fit to the Michaelis-Menten equation and gave $V_{\max} = 4.7 \pm 0.4$ (nM/min.) and $K_{\text{mdTTP}} = 163 \pm 53$ μM.

Figure 9.

UL30 preferentially degrades DNA containing a ribonucleotide at the primer 3'-terminus rather than adding the next correct dNTP. UL30 (exo+) (70 nM) was incubated with DNA_{ribo} (1 μ M) in the presence of 100 μ M dTTP and aliquots were taken at various time intervals. Phosphorimages of the products of DNA extension and degradation using 100 μ M dTTP which is the next correct incoming dNTP.

Table 1

DNA substrates used. Templating bases are underlined and **A***, **G***, **D***, and **rG*** indicate acyclovir, ganciclovir, dideoxy, and ribose-terminated DNAs respectively.

DNA _{15ss}	5'-GCTCTGTTCCGGTTG
DNA _{15C}	5'-GCTCTGTTCCGGTTT
	3'-CGAGACAAGGCCAAAC <u>A</u> CAAAGTAGATTAGTAC
DNA _{15G}	5'-GCTCTGTTCCGGTTG
	3'-CGAGACAAGGCCAAC <u>C</u> CAAAGTAGATTAGTAC
DNA _{16ACVss}	5'-GCTCTGTTCCGGTTTA*
DNA _{16ddss}	5'-GCTCTGTTCCGGTTT D *
DNA _{16ribo}	5'-GCTCTGTTCCGGTTT rG *
DNA _{16ACV}	5'-GCTCTGTTCCGGTTTA*
	3'-CGAGACAAGGCCAAAC <u>A</u> CAAAGTAGATTAGTAC
DNA _{16GCV}	5'-GCTCTGTTCCGGTTT G *
	3'-CGAGACAAGGCCAAAC <u>A</u> CAAAGTAGATTAGTAC
DNA _{16dd}	5'-GCTCTGTTCCGGTTT D *
	3'-CGAGACAAGGCCAAAC <u>A</u> CAAAGTAGATTAGTAC
DNA _{16ribo}	5'-GCTCTGTTCCGGTTT rG *
	3'-CGAGACAAGGCCAAAC <u>A</u> CAAAGTAGATTAGTAC
DNA _{30C}	5'-ATCGTATGTTTATTTGCTCTGTTCCGGTTT
	3'-TAGCATACAAATAAACGAGACAAGGCCAAAC <u>A</u> TAAAGTAGATTAGTAC
DNA _{35ss}	5'-ATGTAATCGTATGTTTATTTGCTCTGTTCCGGTTT
DNA _{35C}	5'-ATGTAATCGTATGTTTATTTGCTCTGTTCCGGTTT
	3'-TACATTAGCATACAAATAAACGAGACAAGGCCAAAC <u>A</u> TAAAGTAGATTAGTAC

Table 2

Effects of forming E-DNA-PFA, E-DNA-PAA, E-DNA_{ACV}-dTTP, and E-DNA_{ACV} complexes on the 3'–5' exonuclease activity of UL30 on DNA_{15C}.

Inhibitor	Rate (nM/min.)
No Inhibitor	162 ± 4
50 μM PFA	156 ± 4
50 μM PAA	162 ± 3
1 μM DNA _{16ACV} + 50 μM dTTP	160 ± 15
1 μM DNA _{16ACV}	130 ± 20

Table 3

Summary of rates of exonucleolytic removal of the 3'-terminal nucleotide of DNA_{15G}, DNA_{16ACV}, DNA_{16GCV}, and DNA_{16dd} using UL30, T4 DNA polymerase, T7 DNA polymerase, and Klenow Fragment. In each case, the -fold decrease in rate is also noted.

Enzyme	Rate (nM/min.)			
	DNA _{15G}	DNA _{16ACV}	DNA _{16GCV}	DNA _{16dd}
UL30	183 ± 5	8 ± 1 (23-Fold)	9 ± 1 (20-Fold)	0.063 ± 0.002 (2900-Fold)
T4	253 ± 4	11 ± 1 (23-Fold)	13 ± 1 (20-Fold)	0.50 ± 0.02 (500-Fold)
T7	297 ± 15	9.0 ± 0.2 (33-Fold)	ND ¹	ND
KF	99 ± 13	2.0 ± 0.1 (50-Fold)	ND	ND

¹ND = Not Done.

Table 4

The ability of double-stranded DNAs containing dG (DNA_{15G}), acyclovir (DNA_{16ACV}), ganciclovir (DNA_{16GCV}), and dideoxyG (DNA_{16dd}) to inhibit exonuclease activity on 1 μ M 5'-[³²P]-labeled single-stranded DNA (DNA_{35ss}) or 1 μ M 5'-[³²P]-labeled double-stranded DNA (DNA_{35C}).

Inhibitor DNA	Substrate DNA	IC ₅₀ (μ M)
DNA _{16GCV}	DNA _{35C}	1.0
DNA _{16ACV}	DNA _{35C}	1.7
DNA _{16dd}	DNA _{35C}	2.5
DNA _{15G}	DNA _{35C}	1.3
DNA _{16GCV}	DNA _{35ss}	0.6
DNA _{16ACV}	DNA _{35ss}	1.0
DNA _{16dd}	DNA _{35ss}	0.3
DNA _{15G}	DNA _{35ss}	0.3

Table 5

Effects of varying the 3'-terminal nucleotide of single stranded DNA on their ability to inhibit exonuclease activity on a second, 5'-[³²P]-labeled single-stranded DNA.

Inhibitor DNA	Substrate DNA	IC ₅₀ (nM)
DNA _{35ss}	DNA _{15ss}	55
DNA _{16ACVss}	DNA _{35ss}	35
DNA _{16ddss}	DNA _{35ss}	70