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Mechanisms by which Herpes Simplex Virus DNA Polymerase Limits Translesion Synthesis through Abasic Sites

Yali Zhu[‡], Liping Song[‡], Jason Stroud[§], and Deborah S. Parris^{‡,§,*}

[‡] Department of Molecular Virology, Immunology, and Medical Genetics Ohio State University Columbus, OH 43210

[§] Department of Molecular Genetics Ohio State University Columbus, OH 43210

Abstract

Results suggest a high probability that abasic (AP) sites occur at least once per herpes simplex virus type 1 (HSV-1) genome. The parameters that control the ability of HSV-1 DNA polymerase (pol) to engage in AP translesion synthesis (TLS) were examined because AP lesions could influence the completion and fidelity of viral DNA synthesis. Pre-steady-state kinetic experiments demonstrated that wild-type (WT) and exonuclease-deficient (exo⁻) pol could incorporate opposite an AP lesion, but full TLS required absence of exo function. Virtually all of the WT pol was bound at the exo site to AP-containing P/Ts at equilibrium, and the pre-steady-state rate of excision by WT pol was higher on AP-containing than on matched DNA. However, several factors influencing polymerization work synergistically with exo activity to prevent HSV-1 pol from engaging in TLS. Although the pre-steady-state catalytic rate constant for insertion of dATP opposite a T or AP site was similar, ground-state binding affinity of dATP for insertion opposite an AP site was reduced 3–9-fold. Single-turnover running-start experiments demonstrated a reduced proportion of P/Ts extended to the AP site compared to the preceding site during processive synthesis by WT or exo⁻ pol. Only the exo⁻ pol engaged in TLS, though inefficiently and without burst kinetics, suggesting a much slower rate-limiting step for extension beyond the AP site.

1. INTRODUCTION

Human herpesvirus 1, otherwise known as herpes simplex virus type 1 (HSV-1), encodes a DNA-dependent DNA polymerase required for the replication of its 152 kilobase-pair genome [reviewed in reference 1]. The 1235-amino-acid HSV-1 DNA polymerase catalytic subunit (pol), a B family polymerase, is encoded by the UL30 gene and contains conserved 5' to 3' polymerizing and 3' to 5' exonuclease (exo) domains [2–6]. Functional analysis of proteins containing mutations in the exo domain indicates that the polymerizing and exo activities can function independently [7–9]. The region encoding the C-terminal domain of HSV-1 pol is poorly conserved. However, this domain has been shown to be important for

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* Corresponding Author and Contact Information: Department of Molecular Virology, Immunology, and Medical Genetics Ohio State University 2198 Graves Hall 333 West Tenth Ave. Columbus, OH 43210 Phone: 614–292–0735 Fax: 614–292–9805 Email: parris.1@osu.edu.

The first two authors contributed equally to this work

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physical interaction with the processivity factor, UL42, to form a stable heterodimer that constitutes the processive polymerase holoenzyme [10-13].

Members of the diverse family of viruses known as *Herpesviridae* cause substantial morbidity in the human population, particularly among those that are immunosuppressed [1, 14]. Given that all members of this virus family encode a DNA polymerase that is highly conserved with that encoded by HSV-1 [1, 4, 5], the mechanisms by which these polymerases maintain faithful replication are likely to be similar. Because HSV-1 pol is the ultimate target of the antiviral agents currently used to block replication and reduce spread of the virus in humans [15, 16], a detailed knowledge of the mechanisms that enhance (or reduce) the fidelity of the viral DNA polymerase could lead to a better understanding of acquisition of resistance of HSV to these agents. For example, due to the rapid and exponential nature of viral DNA replication in infected cells, the ability of the HSV-1 pol to bypass or to stall at lesions in DNA is likely to have an impact on overall mutation frequency of viral genomes as well as the ability to complete genome replication in a timely manner [17, 18]. Whereas a high mutational frequency in mammalian cells may be lethal or contribute to genetic instability, a high mutation frequency of viral DNA may be advantageous to the virus in that it contributes to genetic diversity and the potential to evade natural and therapeutic antiviral defenses. It is also possible that the rapid replication of the virus may not permit sufficient time to repair genetic lesions, making it possible to devise strategies for intervention that could selectively target viral genomes.

To date very little is known regarding the ability of any of the herpesvirus DNA polymerases to bypass genetic lesions. In one of the only studies that have examined the ability of the HSV-1 pol to bypass a lesion—in this case an intrastrand G-G linkage, such as that formed by cisplatin treatment—it was found that the HSV-1 pol could not completely bypass the lesion in the presence of $MgCl_2$, even in the absence of the proofreading exo activity [19]. However a detailed analysis of the factors affecting nucleotide addition at and beyond the lesion was not reported.

More common, and potentially more relevant, genetic lesions are apurinic/apyrimidinic (abasic or AP) sites. AP sites are produced spontaneously by exposure to ionizing radiation and some carcinogens or by the action of DNA glycosylases in response to deamination of nucleotide bases [20, 21]. We were interested to know whether or how the HSV-1 pol could bypass AP sites. Although mechanisms of AP lesion bypass have been studied for several other replicative polymerases [22, 23], to our knowledge none have described the behavior of a eukaryotic viral B type DNA polymerase upon encounter with an AP site. It is interesting to note that HSV-1 encodes a uracil DNA glycosylase that removes uracil residues that have been incorporated directly into DNA from a dUTP precursor or that are present as a result of cytosine deamination [24, 25]. Thus, the viral DNA glycosylase activity might enhance the formation of AP sites in HSV-1 DNA during replication. Although most AP sites in mammalian cells are efficiently repaired by the base excision repair system [26], it is unclear whether or how such sites would be repaired during HSV-1 DNA replication.

In the present study, we demonstrate that there is a high probability that HSV-1 pol will encounter at least one AP site on average per replicating genome. Thus, the ability or inability of the HSV-1 pol to bypass an AP lesion is likely to have an impact on the means by which HSV DNA replication can proceed. Using predominantly pre-steady-state kinetic analysis of wild-type (WT) and exo-deficient (exo⁻) HSV-1 pol, we have examined in detail the parameters that impact the ability of the polymerase of this important human pathogen to engage in translesion synthesis (TLS) through AP sites. Our findings demonstrate not only that exo-proficient HSV-1 pol fails to bypass AP sites, but defines precisely why it cannot.

Given the apparent abundance of AP sites within HSV DNA, we discuss how the mechanisms used by the HSV-1 pol to control TLS through an AP site may contribute to overall fidelity of HSV-1 DNA replication and/or the use of alternative pathways, such as recombination, to enable completion of DNA synthesis in the absence of direct TLS.

2. MATERIALS AND METHODS

2.1. AP site detection

DNA was prepared from Vero cells that were infected with WT HSV-1 (strain KOS) at an input multiplicity of infection of 5 plaque forming units per cell or were mock-infected and the cells harvested at various times postinfection. Total DNA was purified from cultured cells using the DNeasy kit (Qiagen, Inc., Valencia, CA) according to the instructions of the manufacturer. In some cases as indicated, virions were purified from HSV-1 infected Vero cells by sedimentation through sucrose gradients and the DNA was extracted using phenol and chloroform as described previously [27, 28]. DNA concentrations were determined using the Quant-IT DNA broad range assay kit (Molecular Probes, Inc., Eugene, OR) and adjusted to a final concentration of 100 µg/ml. The number of AP sites present in various DNA samples was estimated using a commercially available DNA damage quantification kit (FR-09, Oxford Biomedical Research, Oxford, MI) according to the instructions of the manufacturer. The kit employs a biotin-labeled aldehyde reactive probe (ARP) to tag AP sites on DNA samples coupled with an ELISA assay to colorimetrically quantify the bound probe with horseradish peroxidase-conjugated streptavidin. AP site quantification of unknown samples was based on linear regression analysis of a plot of absorbance at 650 nm against known standards supplied by the manufacturer. All assays were performed in triplicate.

2.2. Preparation of enzymes

The WT and *exo⁻* mutant (D368A) HSV-1 pol genes (UL30) were expressed by infecting *Spodoptera frugiperda* 9 (Sf9) insect cells with recombinant baculoviruses containing the respective genes [9, 29]. The baculoviruses containing the WT and D368A HSV-1 pol genes were gifts from Bob Lehman (Stanford University) and Charles Knopf (University of Heidelberg), respectively. Enzymes were purified to >95% purity as detailed previously [9, 29] except that whole cell extracts were used.

2.3. Preparation of synthetic primer-templates (P/Ts)

Oligonucleotides were purchased in gel-purified form from Integrated DNA Technologies, Inc. (Coralville, IA). The template sequences used were 67 nucleotides (nt) in length and differed only at the 46th position (X) from the 3' end (3'-CGGTGATGCTGTGGAAGTAGCGGAGCGTCGGCAGGTTGGTTGAGTXGGTCTTGT TTCCAGTCGTTTA-5'). The normal template (67T) contained a thymidine residue at the 46th position. Because a true AP site is unstable, a 1', 2'-dideoxyribose (dSpacer) was added at the 46th position during synthesis of the damaged template (67AP) to produce a tetrahydrofuran residue, which mimics an AP site [30-33]. To create a mismatched P/T, the template strand (67A) contained an adenosine residue at the 46th position. Primers of varying length were completely complementary to the 67T template strand and when annealed to template, resulted in a P/T that was duplex on one end and contained a 5' single-stranded template overhang on the other end. The sequence of the first 42 nt of all primers was 5'-GCCACTACGACACCTTGATCGCCTCGCAGCCGTCACCAAC-3'. To the 3' end of this sequence, 5'-TCA was added to generate the 45 nt primer and 5'-TCAA was added to generate the 46 nt primer. Primers were labeled at the 5' end using γ -[³²P]-ATP and T4 polynucleotide kinase by standard procedures. Labeled primers were purified using ProbeQuant™ G-50 Micro columns (Amersham Biosciences, Piscataway, NJ), heated to

65°C for 15 min with a 20% molar excess of template strand in buffer containing 20 mM Tris-HCl, pH 8.0, 100 mM KCl, 1 mM EDTA, and cooled slowly to room temperature to form annealed P/Ts. Labeled and unlabeled P/Ts were mixed to yield a final specific activity of 150–300 $\mu\text{Ci/nmol}$.

2.4. Efficiency of single dNTP incorporation into AP-containing P/Ts

The 45/67AP P/T (125 nM) was incubated with 1.25 nM WT or D368A exo^- HSV-1 pol catalytic subunit at 37°C in pol reaction buffer (50 mM Tris-HCl, pH 7.5, 1 mM dithiothreitol, 1 mM EDTA, 50 mM KCl, and 400 μg bovine serum albumin/ml) and reactions were initiated by the addition of MgCl_2 (6 mM final concentration) and single dNTP at the concentration indicated. Samples were removed at various times and terminated by the addition of excess EDTA. The products were separated by electrophoresis through 12% polyacrylamide gels containing 7 M urea, and quantified using a Molecular Dynamics Phosphorimager (Sunnyvale, CA) or Typhoon 9200 Variable Mode Imager (Amersham Biosciences, Piscataway, NJ) and ImageQuant software to measure incorporation opposite the AP site (formation of 46 nt product). The radioactivity in the product of interest was normalized to total radioactivity in the lane to minimize loading error [34] and was plotted as a function of time. The steady-state rate of incorporation of each dNTP was determined as the slope of the linear portion of the plot by regression analysis.

2.5. Pre-steady-state single-turnover extension reactions

All pre-steady-state reactions were performed at 37°C using the RQF-3 rapid quench flow apparatus (KinTek Corporation, State College, PA). All concentrations of enzyme reported are active concentrations determined as described previously [35]. Enzyme and P/T, at the respective concentrations indicated for each experiment, were incubated for at least 10 min in pol reaction buffer at 0°C. In most cases, the experiments were carried out by loading the enzyme-DNA complex (15 μl) in one sample loop. A mixture containing 12 mM MgCl_2 , activated calf thymus DNA trap (1 mg/ml), and nucleotide, as indicated for each experiment, in reaction buffer was loaded into the second sample loop (15 μl) and all reactants were equilibrated at 37°C. Reactions were initiated by mixing the contents of the two loops and were terminated by the addition of excess 0.3 M EDTA, pH 8.0, at the times indicated. For extension reactions, the concentration of product was plotted as a function of time (t) and the data were fit to the exponential burst equation (eq. 1) to give the amplitude of the burst (A) and the observed rate constant (k) for the burst:

$$[\text{Extension product}] = A (1 - e^{-kt}) \quad (\text{eq. 1})$$

To determine the true equilibrium dissociation constant (K_d) for productive binding of the HSV-1 pol to P/T, pre-steady-state kinetics for incorporation of next correct dNTP were determined at or near saturating dNTP concentrations using a fixed concentration of enzyme and a range of P/T concentrations. The amplitudes of the burst at each P/T concentration were plotted as a function of DNA concentration and the data were fit to the quadratic equation (eq. 2):

$$[E \cdot D] = 0.5 (K_d + E_0 + D_0) - \left[0.25 (K_d + E_0 + D_0)^2 - (E_0 D_0) \right]^{0.5} \quad (\text{eq. 2})$$

where $[E \cdot D]$ is the concentration of enzyme and DNA in productive complex, and E_0 and D_0 are the initial enzyme and P/T concentrations, respectively.

Experiments to measure the K_d for dATP in single-turnover reactions were conducted as described above except that a fixed concentration of enzyme (50 nM) and P/T (100 nM)

were used and the kinetics for extension of the primer in the presence of different concentrations of dATP were measured. For each dATP concentration tested, the observed rate constant (k_{obs}) was determined from the burst equation (eq. 1) and plotted as a function of dATP concentration. The data were fit to a hyperbolic function (eq. 3) to estimate the pre-steady-state rate constant for polymerization (k_{pol}) at nonlimiting concentrations of dATP and the concentration of dATP at one-half k_{pol} (K_d):

$$k_{\text{obs}} = \frac{k_{\text{pol}} [\text{dNTP}]}{([\text{dNTP}] + K_d)} \quad (\text{eq. 3})$$

2.6. Pre-steady-state excision reactions

Single-turnover reactions with WT pol (25 nM) and labeled P/Ts (10 nM) were performed with the aid of a rapid quench apparatus as described for pre-steady-state extension assays, except that reactions contained no dNTP. Reactions were terminated at various times with excess EDTA and products were quantified as described above. The concentration of intact primer remaining was plotted as a function of time and fit to a five-parameter double exponential decay function:

$$[\text{intact primer}] = ae^{-bt} + ce^{-dt} + f \quad (\text{eq. 4})$$

where a and b represent the amplitude and burst rate constant of the fast phase, respectively, c and d represent the amplitude and burst rate constant of the slower phase and f is a constant that describes the amount of DNA left unbound at equilibrium to the pol. In some cases, the fast and slow phases were equivalent such that the data fit best to a three parameter single exponential decay function:

$$[\text{intact primer}] = ae^{-bt} + f \quad (\text{eq. 5})$$

3. RESULTS

3.1. Estimate of number of AP sites per HSV-1 genome equivalent

Before undertaking studies to examine the ability of HSV-1 DNA polymerase to engage in TLS through AP sites, it was important to understand the probability that the viral-encoded polymerase would encounter such a lesion in the course of viral DNA replication. Several techniques have been used to estimate the frequency of AP sites in genomic DNA, the most sensitive of which utilize a tagged ARP. In a recent study, it was estimated that up to 50,000–200,000 AP lesions are formed per cell each day which would correspond to a range of approximately 1.5 to 6 AP lesions per 10^5 bp (base pairs) of mammalian cell DNA [36]. However, the number of AP sites formed can differ based on cell type and whether or not cultured cells are used. Moreover, the steady-state number of AP sites detected at a given time is likely to be influenced by the repair functions present in those cells. It is not known whether or to what extent repair functions are available to replicating HSV-1 genomes, and HSV-1-encoded uracil DNA glycosylase activity is induced in infected cells, which could contribute to the number of AP sites present [37]. Therefore, we wished to examine the steady-state number of AP sites detected in infected *vs.* mock-infected cells. DNA was gently extracted from Vero cells at various times after mock-infection or infection with HSV-1 (5 plaque forming units/cell) and the number of AP sites present in DNA was determined using a biotin-labeled ARP. The number of AP sites in mock- and HSV-1-infected cells ranged from 1.9 to 3.4 sites per 10^5 bp of DNA (Table 1), in excellent agreement with the range estimated in liver cells [36]. Most notably, the number of AP sites

did not differ significantly in infected compared to mock-infected cells, suggesting either that HSV-1 infection did not substantially alter the overall rate of formation and repair of AP sites, or virus infection altered DNA damage and repair in a compensatory manner. Because the samples contained both cellular and viral DNA such that the specific effects on viral DNA could not be deduced, we also tested two independently isolated samples of DNA from purified virions. Our results indicate that DNA packaged into virions contained a slightly greater number of AP sites (3.44 and 3.88 per 10^5 bp) than present in cultured cells. Since conditions for extraction of virion DNA differed somewhat from those used for cell culture samples, this difference may not be significant. Given an HSV-1 genome size of 1.52×10^5 bp, and a steady-state number of AP sites ranging from 1.9 to 3.9 per 10^5 bp, the number of AP sites present per HSV-1 genome equivalent ranges from 2.8 to 5.9. These results suggest a high probability that the HSV-1 pol will encounter at least one AP lesion as it replicates a single viral genome.

3.2. Efficiency of nucleotide insertion opposite an AP site

Because an AP lesion is non-instructional, the preference by which HSV-1 pol incorporates a particular nucleotide opposite an AP site will influence mutation frequency as well as mutation spectrum. For example, an AP lesion that is formed as a result of cytosine deamination is likely to produce a higher mutation frequency if dATP is the preferred dNTP for incorporation opposite the AP site than if dGTP is preferred. To gain insight into selectivity of nucleotide for incorporation opposite a non-instructional site, we utilized primers 45 nt in length annealed to 67AP templates. The abilities of the WT and *exo⁻* HSV-1 pol to incorporate a single dNTP opposite the AP site were monitored in multiple enzyme turnover reactions as a function of time using a 1:100 ratio of enzyme to P/T. For each dNTP, a linear rate for extending the primer by 1 nt was observed during short periods of incubation of the AP-containing P/T with WT pol, provided that the single dNTP was present at high concentration (500 μ M; Fig. 1A-D). These linear rates are reported in Table 2 as a measure of relative efficiencies for incorporation. The highest rate for incorporation by WT pol opposite the AP site was observed with dATP (54.8 ± 7.9 nM/min for 1.25 nM pol). A similar rate (48.1 ± 3.7 nM/min) was observed for dATP incorporation into the undamaged 45/67T P/T, even in the presence of 10-fold lower concentration (50 μ M) of dATP (Fig. 2A). However, this dATP concentration was clearly limiting for incorporation opposite an AP site, since it reduced the concentration of P/T extended approximately 3-fold, and the rate of incorporation observed (27.7 ± 6.7 nM/min) was reduced by 40% compared to that observed on matched P/Ts (Fig. 2B). When dGTP, dTTP, or dCTP were used at 500 μ M concentration for incorporation by the WT pol into the AP-containing P/T, significant degradation of the primer occurred within the first 90 sec, resulting in a loss of linear kinetics for incorporation (Fig. 1B-D).

Since the 3' nucleotide of the primer was an A residue, dATP, but not the other nucleotides, could both repair and extend a primer subjected to excision by the associated *exo* activity of the WT pol. Thus, to ensure that nucleotide incorporation efficiency was independent of the identity of the terminal nucleotide in the primer, we measured incorporation rates of each dNTP by the *exo⁻* (D368A) pol under multiple enzyme-P/T turnover conditions (Table 2). Consistent with the absence of *exo* activity in the D368A mutant pol, no degradation of primer was observed and the rate of incorporation for dCTP was linear throughout the 10 min reaction period (Fig. 1H). For dATP, dGTP, and dTTP, the incorporation rate was linear until approximately 50% of the primer was extended (Fig. 1E-G). Even in the absence of pol *exo* activity, dATP was incorporated at the highest rate which did not differ significantly from that observed with the WT pol (Table 2). The rate for incorporation of dGTP or dTTP opposite the AP site by the *exo⁻* pol was only 50–60% of that for incorporation of dATP. The least efficiently incorporated dNTP opposite the AP site by both WT and *exo⁻* pols was

dCTP, such that the relative efficiencies for dNTP incorporation by the HSV-1 pol opposite an AP site is dATP>dGTP, dTTP>dCTP. Taken together the results suggest that the HSV-1 pol follows the “A-rule” for incorporation opposite an AP site as observed for many polymerases [31, 32, 38-40].

3.3. Productive binding of HSV-1 pol to damaged vs. undamaged P/Ts

The rates of nucleotide incorporation in the multiple turnover experiments described above are more likely to reflect the rate of dissociation of the pol from the P/T rather than the true rate of extension by the pol. Therefore, we performed single-turnover pre-steady-state experiments to gain insight into the effect of the AP lesion on the productive binding affinity of the HSV-1 pol for P/T. Because dATP was the most efficiently incorporated dNTP opposite an AP site, we selected a concentration of dATP (250 μ M) expected to be saturating, based upon an apparent K_m of 34.4 μ M for steady-state incorporation of dATP into the 45/67AP P/T (results not shown). Activated calf thymus DNA (500 μ g/ml, final concentration) was added at the time of initiation, but following equilibrium binding of enzyme to DNA, in order to trap any enzyme that dissociated, ensuring single-turnover conditions. Product (46 nt) formation using a fixed concentration of WT pol (50 nM) with different concentrations (12.5–400 nM) of end-labeled AP-containing P/T was monitored as a function of time. An example of the time-course (5–300 msec) for extension in the presence of 50 nM DNA P/T is shown in Fig. 3A. The amplitude of the burst at each DNA concentration was determined according to eq. 1 (Fig. 3B) and plotted as a function of P/T concentration (Fig. 3C). The data were fit to the quadratic equation (eq. 2) to predict the true K_d (42.1 ± 7.4 nM) for productive binding of the WT pol to the 45/67AP P/T. An indistinguishable value (44 ± 3.0 nM) was obtained for productive binding of the WT pol catalytic subunit (in the presence of dTTP) with a P/T that differed from the 45/67AP P/T only at the 46th position of the template [35]. Thus the WT pol binds productively to the AP-containing P/T for incorporation opposite the lesion with approximately the same affinity as for correct nucleotide incorporation to a P/T with essentially the same sequence context.

To ascertain whether dATP binding affinity was impacted by the AP site, single-turnover reactions were performed as described above, except that fixed concentrations of WT pol (50 nM) and 45/67AP P/T (100 nM) were incubated with different concentrations of dATP. Cycling of the pol following dissociation was prevented by the use of a calf thymus DNA trap. The kinetics for incorporation were monitored to determine the burst rate constant for incorporation at each dATP concentration according to eq. 1, and these values were plotted as a function of dATP concentration (Fig. 3D). The data in the plot were fit to a hyperbolic function (eq. 3) and estimated a k_{pol} of 209 ± 33 sec⁻¹ for dATP insertion opposite the AP site. The k_{pol} for dATP insertion opposite an AP site by the WT HSV-1 pol is consistent with values obtained for correct nucleotide incorporation. For example, the k_{pol} for dATP incorporation opposite a T residue was 258 ± 38 sec⁻¹ (results not shown) and 157 ± 31 sec⁻¹ for dTTP incorporation opposite an A residue on comparable P/Ts differing only in the 46th position of the template [35]. The ground state equilibrium binding constant [K_d (dATP)] for dATP insertion opposite an AP site was also estimated to be 75.7 ± 37.4 μ M (Fig. 3D), somewhat higher (3–9-fold) than that similarly calculated for pre-steady-state correct nucleotide insertion (12.2 ± 5.7 μ M) [35]. Thus under pre-steady-state conditions, nucleotide insertion efficiency opposite a non-instructional site depends at least in part on the binding affinity of the enzyme-P/T complex for dATP.

3.4. AP lesion bypass during processive synthesis

We compared the efficiency with which the WT and *exo*⁻ pols could extend beyond an AP site during processive DNA synthesis in single-enzyme turnover running-start experiments. To ensure that the pol was engaged in active elongation prior to encountering an AP site, we

employed a P/T in which a 42 nt primer was annealed to the 67AP template. The location of the 3' end of the primer 4 nt upstream of the AP lesion permitted the polymerase a “running start” for extension before encountering the target site. Each polymerase (50 nM) was pre-incubated with P/T (100 nM) in pol reaction buffer in the presence of 250 μ M each of dATP, dCTP, dGTP, and dTTP, and reactions were initiated by the addition of MgCl₂ (6 mM final concentration) and calf thymus DNA trap (500 μ g/ml, final concentration) and were terminated at various times (5–2000 msec) with excess EDTA. The gel profiles for formation of products with the wild-type enzyme and *exo*⁻ pols are shown in Fig. 4A and 4C, respectively (upper panels) and demonstrate that full-length extension of the P/T (67 nt) was observed only for the *exo*⁻ pol. Lack of significant extension of primer when the DNA trap was added to enzyme prior to initiation with labeled P/T and MgCl₂ (Fig. 4A and 4C, lower panels) demonstrated the overall effectiveness of the trap in precluding multiple turnovers of enzyme and labeled P/T.

The single-turnover kinetics for extension by the wild-type and *exo*⁻ pols are shown in Fig. 4B and 4D, respectively). The formation of product 43 nt in length was used to indicate the concentration of P/T productively engaged by each enzyme. Extension to 1 nt prior to the AP lesion (n-1) was measured as the formation of products 45 nt in length, extension to opposite the AP site was indicated by formation of products 46 nt in length, and extension beyond the AP site was indicated by formation of products 47 nt in length, all in a single association of enzyme with P/T. The amplitudes and burst rate constants for product formation are reported in Table 3. Nearly all (99%) of the WT pol that productively engaged the AP-containing P/T extended the primer to the n-1 site to form products at least 45 nt in length (Fig. 4B). However, only 30% of the WT pol that began extension of the P/T was able to incorporate dNTP opposite the AP lesion in a single turnover, and no product > 46 nt was observed in reactions with the WT pol, indicating no TLS. By contrast, essentially 100% of the WT pol productively engaged to an undamaged P/T extended the primer to 47 nt with burst kinetics (Table 3). Because of the presence of a DNA trap, the reduction in the percentage of primers extended to the AP site most likely reflects dissociation of a large proportion (~70%) of the pol prior to extension opposite the AP site. Extension of the primer by the WT pol to opposite the AP lesion displayed burst kinetics and a burst rate constant for formation of 46 nt product that was reduced by only 2-fold (from 56 to 23 per sec) compared to that observed for formation of 45 nt product (Fig. 4, Table 3). A somewhat smaller reduction in burst rate constant was observed for extension of the primer from 45 nt to 46 nt opposite an undamaged template (Table 3). These results are consistent with those shown in Fig. 3D that indicate the rate constant for addition of dATP opposite an AP site is not substantially different from that for formation of a correctly matched base-pair.

As observed for the WT pol, the vast majority (93%) of the *exo*⁻ pol that productively engaged the P/T, extended it to 1 nt prior to the AP site (Fig. 4D). Much more primer (66%) was extended to opposite the AP site by the *exo*⁻ pol compared to the WT enzyme, although extension from the n-1 to the target site was slowed more than 5 times (Table 3). The *exo*⁻ pol did permit a small proportion (19%) of the actively engaged enzyme to extend beyond the AP site by 2 sec in a single turnover of enzyme with P/T. However the data did not fit the burst equation, but instead fit a linear function (Fig. 4D). The effectiveness of the DNA trap in preventing significant extension when added to enzyme prior to the addition of labeled P/T (Fig. 4D, lower panel), argues that the linear kinetics for TLS by the *exo*⁻ pol do not reflect multiple turnovers of enzyme with P/T, but likely result from the switching of the P/T between the *exo* and pol sites on the enzyme. Thus, the rate-limiting step for TLS past the AP site is different from and/or considerably slower than that for either correct nucleotide incorporation or for incorporation opposite the AP site.

3.5. Role of exo activity in preventing AP bypass synthesis

Because extension beyond the AP site was observed only in the absence of a functional exo activity, we hypothesized that competition between the exo and polymerizing activities of the WT pol was enhanced by the AP lesion. To gain an understanding of the effect of the AP lesion on the exo activity of the WT pol, pre-steady-state kinetic analysis was performed on P/Ts that differed only in the nucleotide pair at the primer-template interface. To this end, we used a 5' end-labeled 46 nt primer with an A residue at the 3' end annealed to templates that differed only in the 46th position to produce a matched (A:T) pair, an A:A mispair, or an A:AP pair at the primer-template interface. WT pol (25 nM) and P/T (10 nM) were preincubated together in the absence of nucleotide, and activated calf thymus DNA was added at the time of initiation to prevent multiple turnovers of enzyme with P/T. Removal of a matched primer terminus followed biphasic kinetics as previously demonstrated [35], although at the ratio of enzyme to P/T used, not all of the P/T was bound initially to the pol. Therefore the data were fit to a five parameter double exponential function (eq. 4) to estimate the fraction of unbound (or rapidly dissociating) P/T. The amplitude and burst rate constants for the fast and slow phases of excision over a 300 msec period are reported in Table 4 and did not vary significantly from those derived from plots of excision over a 2000 msec period (results not shown). The fast phase most likely corresponds to the rate at which the primer terminus is removed when the P/T is bound initially at the exo site [41] whereas the slow phase reflects excision by pol following switching of the P/T from the exo to the pol site. Thus, the smaller percentage (6%) of matched P/T excised at the fast rate compared to that excised at the slow rate (68%) reflects the lower affinity of a matched P/T for the exo site compared to the pol site. The slow-phase burst rate constant for excision of matched primer termini with WT pol is consistent with a rate-limiting step associated with melting of the terminal residues followed by a switch from the pol to the exo domain [41].

When the data for excision of the A:AP or A:A mispaired termini were fit to eq. 4, the fast and slow phases were equivalent, demonstrating that the data fit best to a three parameter single-exponential decay function (eq. 5). Thus, for both mismatched and AP-containing P/Ts, essentially all of the pol in complex with P/T at equilibrium (29% and 39%, respectively) was bound at the exo site (Fig. 5, Table 4). The mismatched (A:A) P/T terminus was cleaved with a burst rate constant of 113 ± 7 per sec and the A:AP P/T was cleaved with a burst rate constant of 130 per sec (Table 4), values that approached that observed for excision of single-stranded DNA [35, 41, and results not shown]. Moreover, these rate constants were approximately 2 times higher than the fast rate constant for excision of the matched P/T. The substantially larger percentage of mismatched and AP-containing P/Ts, compared to matched P/T, that remained intact following incubation with pol most likely reflects the more rapid dissociation of pol from P/Ts that do not contain matched base-pairs at the primer-template interface and/or a smaller proportion of damaged or unmatched P/T bound initially by the enzyme (Fig. 5, Table 4).

4. DISCUSSION

In this report we demonstrate that the WT HSV-1 DNA polymerase is unable to engage in TLS through AP sites in vitro, although it can incorporate nucleotide opposite the AP site. The preferred nucleotide for incorporation opposite the AP site is dATP (Table 2), suggesting that HSV-1 pol follows the “A-rule” as observed for many polymerases [31, 32, 38-40]. The associated 3' to 5' exo activity of HSV-1 pol has been shown to play a major role in preventing TLS since low frequency bypass of AP lesions was observed by an exo⁻ mutant of HSV-1 pol but not by WT pol (Fig. 4). However, we have found that other parameters, inherent to the HSV-1 pol domain, contribute to the failure of the WT pol to synthesize past AP sites. As described below, we have shed some insight into how these parameters may work synergistically with the exo activity to prevent TLS.

4.1. Mechanisms by which parameters other than *exo* activity impede synthesis past AP sites

Using pre-steady-state kinetic analysis and excess dNTP concentrations, we determined that WT HSV-1 pol bound P/T with approximately the same affinity for productive incorporation of nucleotide (dATP) opposite an AP site as for correct nucleotide incorporation (Fig. 3B-C). In addition, the rate constant at unlimiting dATP concentration (k_{pol}) for incorporation opposite the AP site ($209 \pm 33 \text{ sec}^{-1}$; Fig. 3D) was not significantly different from the k_{pol} for dATP incorporation opposite a T residue ($258 \pm 38 \text{ sec}^{-1}$, results not shown). However, the AP lesion had a moderate effect on the ground state binding affinity of dATP for a complex of WT pol bound to P/T. The K_d for dATP for incorporation into the 45/67AP P/T was increased 3- to 9-fold compared to the K_d for correct dNTP incorporation into undamaged P/T. Given the fact that concentrations of dATP that are truly saturating for incorporation opposite the AP site (5 times K_d , or $\sim 375 \mu\text{M}$) are unlikely to be present in mammalian cells permissive for HSV-1 replication [42, 43], the reduced affinity of the enzyme:AP-containing DNA complex for dATP would be expected to lower the probability for incorporation of nucleotide opposite an AP site compared to opposite a matched nucleotide. This prediction was confirmed in single-turnover running-start experiments in which we observed many fewer primers extended to the target AP site than to the site immediately prior to the AP lesion (Fig. 4) even in the presence of moderately high concentrations of dNTPs that approached saturation ($250 \mu\text{M}$).

Extension beyond the AP lesion was completely inhibited by the presence of a functional *exo* activity during processive synthesis in single enzyme turnover reactions (Fig. 4A-B, Table 3). However, extension beyond the AP site was also greatly impeded even in the absence of *exo* activity (Fig. 4C-D), demonstrating that *exo*-independent parameters are important in preventing or limiting TLS. The absence of a burst for TLS past the AP site by the *exo*⁻ HSV-1 pol suggests a slower rate-limiting step for extension beyond the AP site compared to that for incorporation opposite the AP site. Further experiments will be required to identify the actual rate-limiting step(s) for extension by the *exo*⁻ pol beyond the AP site. However, it is interesting to note that there also was an absence of a burst and a very slow rate for extension of mismatches during processive synthesis by the *exo*⁻ pol [9]. As suggested in that study, we think it likely that the P/T is held in the *exo* site for an extended period of time due to the inability of the pol to cleave the last nucleotide from the primer. Thus, the slow (and linear) kinetics for extension by the *exo*⁻ pol past the AP site likely reflects the slow rate of switching from the *exo* to the *pol* site.

4.2. Role of the *exo* activity in preventing TLS through AP lesions

In previous results from our group and those described above, pre-steady-state excision of the terminal residue on a primer located opposite a matched nucleotide by the WT HSV-1 pol was shown to display biphasic kinetics (Fig. 5 and ref. 35). In the study described herein, excision of a mismatched P/T was shown to follow single exponential decay kinetics, equivalent to the fast phase for excision. Although biphasic kinetics for excision of a mismatched P:T were also reported in the earlier study [35], the second slower phase reported actually represented P/T that was not bound to the pol at the time of initiation of reactions. The difference was due to the fact that the previous data, which used a higher enzyme:P/T ratio than the studies presented in the current report, were fit to a double exponential function without a constant to account for the small proportion of uncleaved P/T [35].

When biphasic excision is observed, the proportion of P/T cleaved in the fast phase is generally considered to be the proportion bound at equilibrium to the *exo* site of the polymerase [41]. This represents only a small proportion of the pol bound to a matched P/T,

but essentially all of the pol bound to the mismatched or AP-containing DNA. Indeed, in the current study, we found that five- to six-times more of the A:A or A:AP-containing P/Ts, respectively, compared to matched (A:T) P/Ts were cleaved by the pol bound to their respective P/Ts at the exo-site (Table 4). The fast rate of cleavage at mismatched or AP-containing terminal pairs approaches that for cleavage of the undamaged ssDNA template by the WT pol (results not shown). As demonstrated by the crystal structure of HSV-1 pol and the similar pol γ -like bacteriophage RB69 polymerase, the exo catalytic site is bound to a ssDNA primer that is separated from the template because of mismatches or unwinding by the polymerase [6, 44]. Thus for HSV-1 pol, the increased relative affinity of the exo site compared to the pol site for the mismatched or AP-containing P/T may be due to thermal instability of the A:AP and A:A primer-template interfaces, to a reduced affinity of the P/Ts for the pol site, or both. The relative affinity of AP-containing templates for the exo site on the WT pol together with the rapid cleavage of the terminal residue from A:AP P/Ts are likely to operate synergistically with polymerization-specific parameters (observed even in the absence of exo activity) that impede extension to and beyond the AP site to prevent TLS by WT HSV-1 pol.

This synergism between the exo and polymerizing functions does not significantly reduce the extension ability of the WT pol on undamaged P/Ts. Indeed, even for the small proportion of matched P/T that is bound at equilibrium to the enzyme at the exo site (~6%), the matched primer is cleaved at a rate approximately 2-times slower than from the AP-containing or mismatched pair in the absence of dNTP (Table 4). Cleavage could be slowed on a “matched” P/T by transient reannealing of frayed ends and/or by switching the primer terminus between the exo and pol sites prior to cleavage.

4.3. Biological implications

Abasic sites are frequently occurring lesions that contribute to mutations. In this report we have demonstrated that AP lesions occur at least as frequently in HSV-1 genomes as they do in the cells in which they replicate in culture (Table 1). Based on a steady-state number of AP sites that range from 1.9 to 3.9 per 10^5 bp, the results suggest that from 2.8 to 5.9 AP lesions are present per HSV genome equivalent. We cannot rule out the possibility that some of these lesions were introduced during the process of DNA isolation, although we obtained similar results for two different methods of DNA preparation and the number of lesions found in mock-infected cells was similar to that previously reported by others [36]. The results suggest a high probability that AP sites will be encountered by the HSV-1 pol during replication of a single genome. Nevertheless, the results reported herein demonstrate that the WT pol is unable to synthesize past an AP lesion in vitro, suggesting that 1) these lesions are repaired during replication; 2) other factors present in the replication complex promote the ability of the pol to engage in TLS through AP sites; and/or 3) AP lesions are bypassed indirectly by recombination.

It has been shown that many proteins associated with DNA repair or low fidelity bypass synthesis are induced by HSV-1 infection [45-47], though their precise roles in HSV DNA replication have not been elucidated. Thus, it remains possible that AP lesions in HSV-1 genomes are repaired prior to or during viral genome replication.

Preliminary results from our laboratory indicate that no extension past AP sites occurs with the WT HSV-1 pol during processive synthesis in single turnover experiments even in the presence of UL42, the pol processivity factor (results not shown). These results are in agreement with the failure of accessory proteins to promote TLS by the wild-type T4 DNA polymerase, although the clamp and clamp-loading complex do facilitate AP TLS by the exo $^-$ T4 DNA polymerase [48]. It is possible that other enzymes known to be present in the HSV-1 DNA replication complex could facilitate TLS by the WT pol through AP sites.

Experiments are in progress to test the effect of other essential HSV-1 DNA replication proteins, such as the helicase-primase or single-stranded DNA binding protein, on the ability of the HSV-1 pol to bypass AP lesions.

HSV-1 has a high rate of recombination that is associated with the replication machinery encoded by the virus [17, 18]. Like HSV-1 pol, exo-proficient bacteriophage T4 DNA polymerase fails to bypass AP sites [23, 49]. Genetic and biochemical evidence suggests that T4 virus-encoded products, rather than host proteins, promote rescue of stalled replication forks by recombination-repair or by replication-repair mechanisms [50-54]. It is interesting to speculate that as shown for bacteriophage T4, stalling and/or dissociation of HSV-1 pol at AP sites promotes recombination that would in effect bypass these sites during replication. Thus, a more thorough understanding of the behavior of the HSV-1 DNA polymerase when it encounters a frequent lesion such as an AP site in vivo, is likely to lead to a more comprehensive understanding of the mechanism by which HSV-1 DNA replicates in infected cells.

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Figure 1. Incorporation of single nucleotide into AP-containing P/Ts

HSV-1 WT pol (A-D) or D368A exo⁻ pol (E-H) at a concentration of 1.25 nM was incubated with 125 nM 45/67AP P/T, labeled at the 5' end with ³²P, in pol reaction buffer containing 500 μM of a single nucleotide as follows: A and E (dATP); B and F (dGTP); C and G (dTTP); D and H (dCTP). Reaction conditions permitted multiple turnovers of enzyme with P/T and samples were removed at various times and terminated by the addition of excess EDTA. Extension products (46 nt in length) were separated by denaturing gel electrophoresis and quantified as described in Materials and Methods by storage phosphor analysis using ImageQuant software. Rates of extension were determined by regression analysis of the portion of each reaction that displayed linear kinetics for incorporation as shown. Rates are reported in Table 2.

Figure 2. Incorporation of dATP into undamaged and AP-containing P/Ts at limiting nucleotide concentration

WT pol (1.25 nM) was incubated with undamaged (A) or AP-containing (B) P/Ts (125 nM) which differed only in the presence of a T residue or AP site, respectively, at the 46th position from the 3' end of the template. Reactions contained 50 μ M dATP, which was close to saturating for incorporation into the 45/67T P/T, but limiting for incorporation into the 45/67AP P/T. Reactions were terminated and quantified as described in the legend to Fig. 1. The maximum rate for incorporation of dATP into the 45/67T P/T was 48.1 ± 3.7 nM min⁻¹, but 27.7 ± 6.7 nM min⁻¹ for incorporation of dATP into the 45/67AP under these reaction conditions.

Figure 3. Pre-steady-state incorporation of dATP by WT pol. A and B

A fixed concentration of WT pol (50 nM) was incubated to equilibrium with the indicated concentrations of 5' end-labeled 45/67AP P/T and 250 μM dATP in the presence of EDTA. Reactions were initiated by the addition of MgCl₂ and calf thymus DNA trap as described in Materials and Methods to prevent multiple turnovers of enzyme with P/T. Reactions were terminated after 5, 8, 10, 20, 30, 40, 50, 80, 100, 200, and 300 msec with excess EDTA with the aid of a rapid quench apparatus. **A.** A representative example of extension products produced at increasing times of incubation with 50 nM P/T and separated by denaturing gel electrophoresis. **B.** Extension products were quantified as indicated in the legend to Fig. 1, plotted as a function of time, and fit to the burst equation (eq. 1). Concentrations of P/T used were 12.5 nM (●), 25 nM (○), 50 nM (□); 100 nM (△); 200 nM (◇); and 400 nM (▽). **C.** The burst amplitudes estimated by each plot in panel B represent the concentration of enzyme in productive complex with the P/T and were plotted as a function of P/T. The data were fit to the quadratic equation (eq. 2) to yield a true K_d for productive binding of the WT HSV-1 pol to the AP-containing P/T of 42.1 ± 7.4 nM. **D.** Single-turnover pre-steady-state reactions were conducted using a fixed concentration of WT pol (50 nM) and 45/67AP primer-template (100 nM) and varying the concentrations of dATP. The kinetics for extension of the P/T at each dATP concentration were determined and the data were fit to eq. 1 to obtain the burst rate constants (k_{obs}) that were plotted as a function of final dATP concentration.

Figure 4. Single enzyme-turnover running-start processive synthesis on AP-containing P/Ts
 A fully complementary 42 nt primer was annealed to a 67 nt template that possessed an AP site at the 46th position (target position n) from the 3' end of the template (67AP). The HSV-1 WT pol (**A and B**) or exo⁻ pol (**C and D**) at a concentration of 50 nM was incubated to equilibrium with the P/T (100 nM) in pol reaction buffer containing EDTA and 250 μ M each of dATP, dCTP, dGTP, and dTTP. Reactions were initiated by the addition of MgCl₂ and calf thymus DNA trap as described in Materials and Methods to prevent multiple turnovers of enzyme with P/T. Reactions were terminated with EDTA using a rapid quench apparatus and extension products were separated and quantified. Gels of products formed by incubation of HSV-1 WT or exo⁻ pol are shown in the upper panels of **A and C**, respectively. The efficiency of the DNA trap in precluding multiple turnovers of enzyme with P/T is shown in the lower panels of **A and C** for control reactions in which the order of addition of the DNA trap and labeled P/T to enzyme was reversed. Products representing primers extended to 1 nucleotide prior to the AP site (n-1) and beyond (45 nt; •), those extended to the AP site and beyond (46 nt; ◻), and those extended beyond the AP site (47 nt; ◴) are shown. Products were quantified as described in Materials and Methods. The numbers in parenthesis indicate the proportion of primers extended to that length compared to those productively engaged by the polymerase to form 43 nt products. All of the plots were fit to the burst equation (eq. 1), except for the plot for products 47 nt formed by the exo⁻ pol, which was fit to a linear function.

Figure 5. Pre-steady-state kinetics of excision by WT pol-associated exo activity

A. WT pol (25 nM) was incubated to equilibrium with 10 nM 5' end-labeled 46/67 P/T containing a matched A:T base pair (●), a mismatched A:A pair (○), or an A:AP pair (◻) at the 3' primer terminus as described in Materials and Methods. Reaction mixtures contained no dNTPs, were initiated by the addition of MgCl_2 and activated calf thymus DNA trap to prevent multiple turnovers of enzyme, and were terminated with excess EDTA from 5–300 msec with the aid of a rapid quench apparatus. Concentrations of remaining full-length primer were plotted as a function of time. The data for the matched P/T demonstrated biphasic kinetics for excision and fit best to a five-parameter double exponential decay function (eq. 4) as shown. For the mismatched and AP-containing P/Ts, the fast and slow rates for fits to eq. 4 were equivalent and the data were fit to a three-parameter, single exponential decay curve (eq. 5). The apparent kinetic constants derived from the plots are reported in Table 4. **B.** Products formed in control reactions to demonstrate the efficiency of the DNA trap in preventing multiple turnovers of enzyme with P/T. The DNA trap was mixed with the enzyme prior to initiation of reactions with labeled P/T and degradation of the 46 nt primer was monitored as a function of time through 300 msec.

Table 1

Abasic site detection in DNA from cultured cells and from purified virions

Sample	Time postinfection ^a	AP sites/10 ⁵ bp $\pm$ S.D. ^b
Mock-infected Vero	0 hr	1.86 $\pm$ 0.08
Mock-infected Vero	4 hr	2.41 $\pm$ 0.46
Mock-infected Vero	11 hr	2.23 $\pm$ 0.68
Mock-infected Vero	19 hr	2.42 $\pm$ 0.17
HSV-1-infected Vero	4 hr	3.46 $\pm$ 2.04
HSV-1-infected Vero	11 hr	1.99 $\pm$ 0.16
HSV-1-infected Vero	19 hr	1.75 $\pm$ 0.13
HSV-1 virions #1	72 hr	3.44 $\pm$ 0.17
HSV-1 virions #2	72 hr	3.88 $\pm$ 0.10

^aTime in which cells were harvested after the addition of 0.1 ml medium without (mock) or with HSV-1 wild-type strain KOS (5 plaque forming units/cell) and total DNA was extracted as described in Materials and Methods. Inoculum was removed 1 hr later and medium was added. Cells harvested at 0 hr were not inoculated. For virion DNA preparations, Vero cells were inoculated with HSV-1 at low input multiplicity (0.1–0.5 plaque forming units/cell) and virions were purified from cells 72 hr later and DNA extracted as described previously [27, 28]. Two independent virion preparations that had been stored at -80°C were tested.

^bDetermined using a DNA Damage Quantification kit (Oxford Biomedical, Oxford, MI). The assay uses a biotin-tagged aldehyde reactive probe to tag AP sites, followed by ELISA detection of biotin with horse radish peroxidase-conjugated streptavidin probe against a standard curve of DNA containing known numbers of AP sites according to the instructions of the manufacturer. The data were fit to a linear function by least squares regression analysis. Assays for standards and unknowns were performed in triplicate and the mean values $\pm$ standard deviation (S.D.) are shown.

Table 2

Nucleotide incorporation into AP-containing primer-templates.

Enzyme ^a	Rate of dATP incorporation (nM/min) ^b	Rate of dGTP incorporation (nM/min) ^b	Rate of dTTP incorporation (nM/min) ^b	Rate of dCTP incorporation (nM/min) ^b
WT pol	54.8 ± 7.9	7.9 ± 1.0 ^c	13.3 ± 2.3 ^c	3.2 ± 0.8 ^c
D368A (exo ⁻) pol	59.1 ± 5.7	33.2 ± 2.0	35.1 ± 2.1	9.1 ± 0.2

^aEnzyme (1.25 nM) and 45/67AP primer-template (125 nM) were incubated in the presence of high concentrations of a single dNTP (500 μM) in bulk reactions as described in Materials and Methods. Samples were removed and placed into excess EDTA prior to the addition of enzyme and 0.33, 0.67, 1, 1.5, 2, 3, 4, 6, 8, and 10 min thereafter.

^bProduct formation (46 nt) was quantified following denaturing gel electrophoresis, normalized to total radioactivity in the lane, and plotted as a function of time. Rates were determined by regression analysis of the linear portion of the plot.

^cExtensive degradation of full-length primer was observed within a 1.5 min reaction time (see Fig. 1).

Table 3
Running start processive DNA synthesis on undamaged and AP-containing primer-templates

Enzyme ^a	P/T ^b	Products 45 nt ^c		Products 46 nt		Products 47 nt	
		Burst rate constant (sec ⁻¹)	Burst amplitude (nM)	Burst rate constant (sec ⁻¹)	Burst amplitude (nM)	Burst rate constant (sec ⁻¹)	Burst amplitude (nM)
WT pol	42/67T	55.2 ± 7.1	35.3 ± 1.6	45.8 ± 7.1	35.3 ± 2.1	23.7 ± 4.1	35.7 ± 3.0
WT pol	42/67AP	55.7 ± 7.5	26.3 ± 0.7	23.4 ± 2.0	8.1 ± 0.2	nd ^d	nd
Exo ⁻ pol	42/67AP	17.3 ± 2.0	20.7 ± 0.7	3.3 ± 0.7	14.7 ± 1.3	0.16 ^e	4.3 ^e

^a Polymerase (50 nM) was incubated to equilibrium with P/T (100 nM) in the presence of EDTA and initiated by the addition of MgCl₂ (6 mM), dNTPs (250 μM of each), and activated calf-thymus DNA trap (500 μg/ml) to achieve the indicated final concentrations of each. The DNA trap ensured a single turnover of enzyme with P/T. Reactions were terminated with excess EDTA using a rapid quench apparatus as described in Materials and Methods.

^b P/Ts were composed of identical 42 nt sequences annealed to 67 nt templates which differed only in the 46th position from the 3' end. The 67T template contained a T residue at that position while the 67AP template contained an abasic site at that position.

^c Burst rate constants and amplitudes (± standard deviation) were determined from the plots of product formation as a function of time (Fig. 4) based on fit to the burst equation (eq. 1) except as noted. All kinetic constants are apparent.

^d None detected

^e There was no burst so the data were fit to a linear function and the rate constant was estimated as the slope of the function (2.3 ± 0.1) divided by the concentration of enzyme productively engaged with the P/T prior to extension (i.e. the amplitude of the burst for formation of 46 nt products). The amplitude indicated was the concentration of primer extended past the AP lesion during the 2 sec reaction period.

Pre-steady-state burst rate constants for excision of a matched, mismatched, and AP-containing primer-template

Table 4

P:T Interface ^a	Fast burst rate constant (sec ⁻¹) ^b	Primer-template excised at fast rate (%) ^b	Slow burst rate constant (sec ⁻¹) ^b	Primer-template excised at slow rate (%) ^b	Primer-template not excised (%) ^c
A:T	59 ± 18	6	2.2 ± 0.7	68	26%
A:A	113 ± 7	29	NA ^d	NA	71%
A:AP	130 ± 0	39	NA	NA	61%

^aWild-type pol catalytic subunit (25 nM) was incubated to equilibrium with 10 nM of primer-template containing the indicated base-pairs. Activated calf thymus DNA (500 µg/ml final concentration) was added at the time of initiation to trap enzyme that dissociated.

^bPlots (see Fig. 5) for the amount of intact primer remaining as a function of time were fit to a five-parameter double exponential decay function for the A:T P/T (eq. 4). Data for the A:A and A:AP P/Ts were fit to a three-parameter single exponential decay function (eq. 5). Kinetic constants are apparent.

^cEstimated from the constant (y0) for the fit to eq. 4 or 5.

^dNot applicable because data fit a three parameter single exponential decay function (eq. 5).