Nucleotide incorporation by the herpes simplex virus type 1 DNA polymerase catalytic subunit (pol) is less faithful than for most replicative DNA polymerases, despite the presence of an associated 3'- to 5'-exonuclease (exo) activity. To determine the aspects of fidelity affected by the exo activity, nucleotide incorporation and mismatch extension frequency for purified wild-type and an exo-deficient mutant (D368A) pol were compared using primer/templates that varied at only a single position. For both enzymes, nucleotide discrimination during incorporation occurred predominantly at the level of $K_m$ for nucleotide and was the major contributor to fidelity. The contribution of the exo activity to reducing the efficiency of formation of half of all possible mispairs was 6-fold or less, and 30-fold when averaged for the formation of all possible mispairs. In steady-state reactions, mismatches imposed a significant kinetic barrier to extension independent of exo activity. However, during processive DNA synthesis in the presence of only three nucleotides, misincorporation and mismatch extension were efficient for both exo-deficient and wild-type pol catalytic subunits, although slower kinetics of mismatch extension by the exo-deficient pol were observed. The UL42 processivity factor decreased the extent of misincorporation by both the wild-type and the exo-deficient pol to similar levels, but mismatch extension by the wild-type pol-UL42 complex was much less efficient than by the mutant pol-UL42. Thus, despite relatively frequent (1 in 300) misincorporation events catalyzed by wild-type herpes simplex virus pol-UL42 holoenzyme, mismatch extension occurs only rarely, prevented in part by the kinetic barrier to extending a mismatch. The kinetic barrier also increases the probability that a mismatched primer terminus will be transferred to the exo site where it can be excised by the associated exo activity and subsequently extended with correct nucleotide.

Herpes simplex virus type 1 (HSV-1)$^1$ is the best characterized member of the large family of Herpesviridae pathogenic to humans, which also includes Epstein-Barr virus, varicella-zoster virus, human cytomegalovirus, and Kaposi sarcoma-associated herpes virus (reviewed in Ref. 1). Viruses in this family encode most of the proteins essential for and directly involved in DNA replication (2–4), including a well conserved DNA polymerase catalytic subunit (pol), which is a member of the polymerase B family (5, 6). HSV-1 pol possesses 5'- to 3'- polymerizing and 3'- to 5'-exonuclease (exo) activities (7, 8), the latter of which is involved in the removal of incorrectly incorporated deoxyribonucleoside triphosphates (9–12). The importance of this proofreading activity for maintaining fidelity of DNA replication was suggested by studies from our laboratory that demonstrated the relatively poor ability of HSV-1 pol to discriminate between the correct and incorrect nucleotide for incorporation in single turnover experiments (13). That study (13) reported that selectivity of correct over incorrect dNTP was ~300, whereas bacteriophage T4 and T7 DNA polymerases discriminate among dNTPs by a factor of >1000 and >100,000, respectively (14–16). Previous studies suggested that the exo activity of HSV-1 pol contributes substantially to the fidelity of HSV replication in vivo, because mutation of the conserved exo site III of the HSV-1 pol catalytic subunit was associated with a strong mutator phenotype (10, 17, 18). Moreover, such pol mutants are genetically unstable and revert or recombine with high frequency (10), suggesting strong in vivo selection against an HSV-1 pol lacking exo activity.

To gain a better understanding of the contribution of the associated exo activity of HSV-1 pol in maintaining fidelity of DNA replication, we compared misincorporation and mismatch extension by the wild-type pol with that of a mutant enzyme, which lacks exo function. We selected for study the D368A mutant pol, which converts an asparagine to alanine in the conserved exo site I domain. This site is thought to be involved in the coordination of two divalent metal ions responsible for the exo catalytic activity (19), and previous studies demonstrated that the D368A mutant pol lacked any detectable exo activity (9, 20). A previous study with this mutant pol used a limited set of primer/templates (P/Ts) and concluded that the enzyme possessed a defect in mismatch extension (11). Because polymerization, misincorporation, and mismatch extension frequency are dependent upon sequence context (21, 22), the contribution of the exo activity for maintaining fidelity of DNA synthesis was assessed by varying the P/T at only one position at a time to reduce or eliminate contextual differences. Our results demonstrate that nucleotide selectivity is the major contributor to fidelity and is controlled predominantly at the level of increased affinity for correct versus incorrect nucleotide. Exo activity enhanced nucleotide selectivity by roughly an order of magnitude in multiple turnover reactions. However, the primary effect of exo activity on fidelity was manifest during processive DNA synthesis because mismatches impeded
the extension ability of pol, particularly in the presence of UL24 processivity factor, and increased the probability for excision and repair of misincorporated nucleotides.

**EXPERIMENTAL PROCEDURES**

**Cells and Viruses**—Recombinant baculoviruses were used to express the HSV-1 wild-type (gift of Dr. Robert Lehman, Stanford University) or exo-deficient (D386A) pol genes with or without the UL24 processivity factor gene and have been described elsewhere (9, 25). Recombinant viruses were propagated in S99 insect cells at 27 °C as described previously (24).

**Protein Purification**—Cells infected with recombinant baculovirus (10 plaque-forming units/cell) were harvested at 40-h postinfection and lysed, and soluble extracts were prepared as described previously (24).

**Flash-Freeze**—Flash-freezing in liquid nitrogen, and stored at −80 °C.

Protein fractionation was conducted with the aid of a fast protein liquid chromatography system (Amersham Biosciences) at room temperature as described below, and fractions were collected on ice. The presence of HSV-1 wild-type or exo-deficient pol in fractions was monitored by enzyme assay using activated calf thymus DNA as template (25) with the exception that salt concentration in reactions was reduced to 50 mM KCl. The identity of the pol activity was confirmed by the presence of a polypeptide of 136 kDa following denaturing gel electrophoresis. Cellular extracts were dialyzed against B-2 buffer (20 mM Tris·HCl, pH 8.2, 50 mM NaCl, 5 mM 2-mercaptoethanol, 1 mM EDTA, and 60% glycerol) and slowly cooling it to room temperature over several hours. Annealed P/T was stored at 4 °C.

**Exonuclease Assays**—Exo activity of each enzyme was evaluated by monitoring the removal of nucleotides from the 3′ end of the 5′-end-labeled 45-mer single-stranded (ss) DNA corresponding to the primer strand used in standard nucleotide incorporation experiments (44A in Table I). The concentration of each enzyme was determined by active-site (for polymerization) titration as described previously (13). For the pre-steady-state reactions, enzyme and ssDNA (5:1 molar ratio) were preincubated in buffer containing 20 mM Tris·HCl, pH 8.0, 50 mM KCl, 2 mM dithiothreitol, 800 μg of bovine serum albumin/ml, and 2 mM EDTA. Reactions were initiated with MgCl₂ to a final concentration of 6 mM and were terminated at the molar ratios were (from 10 mM to 50 μM) with a large volume of 0.3 M EDTA with the aid of an RQ-3 rapid quench apparatus (Kintek, Claremont, CA). Reaction products were analyzed by electrophoresis through 7% urea, 12% polyacrylamide gels, and the amount of 45-mer remaining was quantified by phosphorimaging analysis using a Molecular Dynamics PhosphorImage (Amersham Biosciences) and ImageQuant software (Sunnyvale, CA) and normalized for variations in loading as described previously (13). Autoradiograms were obtained from gels exposed to x-ray film at −80 °C using intensifying screens.

The pre-steady-state rate constant for excision (kₚₑₓ) was determined by plotting the concentration of 45-mer remaining ([45-mer]) as a function of time (t). The data were fit to a double exponential function as shown in Equation 1,

\[
[45\text{-mer}] = a e^{-bt} + ce^{-dt}
\]

where a is the amplitude of the first exponential, b is the rate constant of the rapid phase, c is the amplitude of the second exponential, and d is the rate constant of the slower phase.

**Steady-State Reactions**—Steady-state kinetics of extension of primer strand was monitored by using the 45-mer single-stranded DNA template (Table I) at 37 °C under multiple turnover conditions (no DNA trap) as described previously (36–38). A time course for extension of primer strand with maximum concentrations of correct dNTP indicated that reactions with wild-type or mutant pol were steady-state for at least 2 min. Therefore, reactions were quenched with an equal volume of 0.3 M EDTA 2 min after initiation with enzyme and the observed rate (Vₒₜₙ) was calculated by dividing the concentration of product formed by the reaction time. Vₒₜₙ was plotted as a function of each dNTP concentration, and the apparent Kₘ values for incorporation of each dNTP into each P/T were estimated for those which approached saturation by fitting the data to the Michaelis-Menten function as shown in Equation 3.

\[
V_{obs} = \left( V_{max}[dNTP]/(dNTP) + K_{m} \right)
\]

**Steady-state Extension Assays**—Steady-state kinetics of extension of all 16 base pairs were measured by incorporation of increasing concent...
...the next correct nucleotide (dTTP) into alternative P/Ts (44NNA in Table I) at 37 °C for 2 min under multiple turnover conditions as described previously (26, 27, 29). The P/Ts differed only in the base pairs (matched and mismatched) at the 45th position with respect to primer and were extended by incubation with increasing concentrations of dTTP. In some cases, the high dTTP concentrations used for extension of mismatches by the exo-deficient pol promoted utilization of >50% of the substrate during a 2-min incubation. In those cases, reactions were performed for shorter periods (30 s) to ensure steady-state kinetics. The observed rates of extension ($V_{max}$) were obtained by dividing the concentration of 46-mer by the reaction time and plotting these rates as a function of dTTP concentration to estimate the apparent $K_m$ and $V_{max}$ as described above. The $V_{max}/K_m$ ratio indicated the efficiency of extension of each base pair.

**Processive Elongation in the Presence of Three dNTPs**—The 45-mer primer strand (44A) was labeled at the 5' end with [$^{32}$P] as indicated above and annealed to template 4 (Table I). The P'T (100 nM) was incubated with 50 nM wild-type or exo-deficient pol in the presence of 2 mM EDTA for 10 min, and reactions were initiated by the addition of (final concentrations) MgCl$_2$ (6 mM), dTTP, dCTP, and dGTP (250 μM each) and activated calf thymus DNA trap (500 μg/ml). Reactions were terminated with excess 0.3 M EDTA at various intervals from 0 to 2 s with the aid of a rapid quench instrument, and reaction products were analyzed by electrophoresis through denaturing gels. The efficacy of the DNA trap was demonstrated by the lack of primer extension in control reactions, which the enzyme and DNA trap were added together prior to initiation of reactions and product formation was monitored over the 2-s time course (results not shown). The concentration of products of various sizes that were formed was calculated from the proportion of all of the primers extended to greater than or equal to that size and normalized to total radioactivity in the lanes. The plots of the concentration of each product ([product]) versus time (t) were fit to the exponential burst equation as shown in Equation 4.

$$[\text{Product}] = A(1 - e^{-kt}) \quad (\text{Eq. 4})$$

to estimate the amplitude (A) and observed burst rate constant (k) except as indicated under "Results." For each experiment, the concentration of enzyme productively engaged with the P'T was the amplitude of the curve for the plot of products extended by at least one nucleotide (to ≥46 nt in length) as a function of time. The concentration of primer extended to an indicated length at 2 s was calculated from the curve fits and divided by the concentration of productively engaged enzyme to estimate the proportion of enzyme capable of correct extension (≥45 nt), misincorporation (≥49 nt), or mismatch extension with correct nucleotide (≥50 nt).

**RESULTS**

**Nucleotide Excision Activity**—A mutation in the conserved exo site I domain of the HSV-1 pol (D368A) had been reported previously to lack significant exo activity (9, 20). To confirm the complete absence of exo activity in D368A pol and to ensure that the mutation did not compromise the polymerizing activity of the enzyme, we purified the mutant and wild-type enzymes from Sf9 insect cells infected with recombinant baculoviruses, which expressed these genes as described under "Experimental Procedures." Comparable purities were achieved for the D368A and wild-type enzymes, which were estimated to be >95% by comparison of Coomassie Blue-stained bands to bovine serum albumin controls (13) and by the absence of other polypeptides on silver-stained gels (data not shown). Active site titration of D368A and wild-type pol preparations indicated that each contained a comparable proportion of active enzyme (~40 and 50% active, respectively).

The exo activities of the purified pols were analyzed under sensitive pre-steady-state conditions using 5' end-labeled 45-mer ssDNA (10 nt) and 50 nM enzyme. Reactions were initiated by the addition of MgCl$_2$, and the products were separated by denaturing gel electrophoresis. Concentration of 45-mer remaining as a function of time was fit to a double exponential function (Equation 1), and the results demonstrated rapid removal of nucleotide from the 3' end of 20% ssDNA (rate constant of 21.5 ± 6.6 s$^{-1}$) by the wild-type pol (Fig. 1A). This was followed by slower excision (rate constant of 0.065 ± 0.006 s$^{-1}$) of the remainder of the DNA, most probably reflecting multiple turnovers of enzyme with DNA substrate. Under the same conditions, no excision of the 45-mer by D368A mutant pol was observed over a period of 50 s (Fig. 1A). To better assess whether the D368A mutant was capable of digesting the ssDNA after extensive periods of incubation, wild-type or mutant enzymes were incubated with the ssDNA at a 1:100 ratio to allow for sustained multiple turnovers. The steady-state rate constant for the removal of the 3'-nucleotide from the ssDNA by the wild-type enzyme was estimated at 0.047 ± 0.003 s$^{-1}$ by fitting the data to Equation 2 (Fig. 1B) in close agreement with that observed for slow excision with high enzyme:DNA ratios (Fig. 1A). However, no loss of 45-mer was detected even when incubated with D368A pol for up to 2 h (Fig. 1B). The D368A pol also failed to excise nucleotide from mismatched P'T under pre-steady-state or steady-state conditions (results not shown but see Fig. 4A, below). Thus, D368A pol is completely deficient in 3'- to 5'-exo activity.

We next determined the pre-steady-state kinetic constants for correct nucleotide (dTTP) incorporation by D368A pol as described previously (13) and found that D368A pol bound P'T and dTTP in productive complex with virtually the same affin-
the concentration, and if nucleotide concentrations approached saturation, the data were fit to the Michaelis-Menten function (Equation 3) to estimate and C.

The observed rates for correct (\( \text{dTTP} \)) of correct (\( \text{dTTP} \)) or incorrect (\( \text{dCTP}, \text{dGTP}, \text{and dATP} \)) dNTP, and the amount of extended primer following a 2-min incubation period was quantified. A and B, representative autoradiograms of gels of products formed with the indicated dNTP. P denotes the migration of the 45-mer primer strand. Positions of extension products are marked as +1, +2, or +3, whereas positions of degradation products are marked as –1 and –2. The observed rates for correct (\( \text{C} \)) and incorrect (\( \text{D} \)) incorporations were determined and plotted as a function of nucleotide concentration, and if nucleotide concentrations approached saturation, the data were fit to the Michaelis-Menten function (Equation 3) to estimate the \( V_{\text{max}} \) and apparent \( K_m \).

Steady-state Correct and Incorrect dNTP Incorporation—Because the D368A mutation did not alter the polymerization function of HSV-1 pol, we reasoned that the impact of the pol-associated exo activity on nucleotide selectivity could be determined by comparing the efficiency of incorporation of correct and incorrect dNTPs by the wild-type and D368A pols. The D368A or wild-type pol was incubated at 37 °C with a 100-fold excess of 5′ end-labeled P/T (444A/TA) and increasing concentrations of each incorrect nucleotide for this P/T (Fig. 2). The absence of exo activity resulted in an increased efficiency, ranging from 1.6- to 170-fold, for all of the nucleotides as did the wild-type pol (\( K_d \) (DNA) = 44 and 31 nM and \( K_d \) (dTTP) = 1.6 and 1.7 μM for the wild-type and D368A pols, respectively). However, the rate constant (\( k_{\text{pol}} \)) for polymerization by the D368A mutant pol was 190 ± 26 s\(^{-1}\), 20% faster than the 157 ± 31 s\(^{-1}\) reported for dTTP incorporation by the wild-type pol (13). The slower rate constant for the wild-type enzyme most probably reflects competition of the forward polymerization reaction with the competing excision reaction (9).

Taken together, these results demonstrate that the D368A mutation destroys exo activity without significantly altering the polymerization domain or its inherent function.

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Taken together, these results demonstrate that the D368A mutation destroys exo activity without significantly altering the polymerization domain or its inherent function.

The efficiencies (\( V_{\text{max}}/K_m \)) with which all of the 12 possible mispairs were formed with the D368A and wild-type pols are shown in Fig. 3. The absence of exo activity resulted in an increased efficiency, ranging from 16- to 170-fold, for all of the quantifiable misincorporation events. On average, the exo activity of the wild-type pol reduced the efficiency of misincorporation <30-fold, although differences were not significant for the formation of four mispairs. There was no detectable misincorporation of dCTP opposite a C residue on the template (C:C mispair) for either the wild-type or mutant pol, a result that was observed on other P/Ts with a different sequence context.3

Incorporation of dGTP opposite an A residue was also among the least favored misincorporation events for both enzymes, although formation of G:G mispairs was even less efficient for

\[ ^3 \text{R. Strick and C. W. Knopf, unpublished observations.} \]
Table II

<table>
<thead>
<tr>
<th>Primer/template</th>
<th>Base pair formed</th>
<th>D368A *</th>
<th>Wild-type pol *</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>( V_{\text{max}} ) &amp; ( K_m )</td>
<td>( V_{\text{max}} ) &amp; ( K_m )</td>
<td></td>
</tr>
<tr>
<td>44A/TA</td>
<td>A:A</td>
<td>36.4 ± 3.5 &amp; 79.2 ± 21.9</td>
<td>16.1 ± 2.1 &amp; 90.8 ± 43.0</td>
</tr>
<tr>
<td></td>
<td>C:A</td>
<td>31.6 ± 2.1 &amp; 25.1 ± 32.7</td>
<td>29.3 ± 3.5 &amp; 348 ± 100</td>
</tr>
<tr>
<td></td>
<td>G:A</td>
<td>NS &amp; NS</td>
<td>NS &amp; NS</td>
</tr>
<tr>
<td></td>
<td>T:A</td>
<td>25.0 ± 1.0 &amp; 0.09 ± 0.03</td>
<td>18.2 ± 1.2 &amp; 0.52 ± 0.24</td>
</tr>
<tr>
<td>44A/TC</td>
<td>A:C</td>
<td>26.2 ± 1.0 &amp; 159 ± 14.1</td>
<td>13.8 ± 3.0 &amp; 175 ± 82</td>
</tr>
<tr>
<td></td>
<td>C:C</td>
<td>I &amp; I</td>
<td>I &amp; I</td>
</tr>
<tr>
<td></td>
<td>G:C</td>
<td>23.6 ± 0.8 &amp; 0.04 ± 0.01</td>
<td>17.3 ± 0.4 &amp; 0.18 ± 0.05</td>
</tr>
<tr>
<td></td>
<td>T:C</td>
<td>26.3 ± 0.8 &amp; 15.4 ± 2.7</td>
<td>9.2 ± 0.6 &amp; 80.7 ± 16</td>
</tr>
<tr>
<td>44A/TG</td>
<td>A:G</td>
<td>NS &amp; NS</td>
<td>NS &amp; NS</td>
</tr>
<tr>
<td></td>
<td>C:G</td>
<td>40.6 ± 0.7 &amp; 0.08 ± 0.01</td>
<td>32.7 ± 0.9 &amp; 0.30 ± 0.07</td>
</tr>
<tr>
<td></td>
<td>G:G</td>
<td>34.6 ± 0.8 &amp; 121 ± 6.9</td>
<td>NS &amp; NS</td>
</tr>
<tr>
<td></td>
<td>T:G</td>
<td>46.5 ± 15.6 &amp; 27.1 ± 3.9</td>
<td>15.2 ± 1.0 &amp; 160 ± 32</td>
</tr>
<tr>
<td>44A/TT</td>
<td>A:T</td>
<td>27.7 ± 0.4 &amp; 0.05 ± 0.01</td>
<td>15.8 ± 0.4 &amp; 0.33 ± 0.07</td>
</tr>
<tr>
<td></td>
<td>C:T</td>
<td>42.9 ± 1.1 &amp; 92.1 ± 6.4</td>
<td>25.6 ± 2.0 &amp; 309 ± 61</td>
</tr>
<tr>
<td></td>
<td>G:T</td>
<td>48.5 ± 1.2 &amp; 28.3 ± 2.9</td>
<td>6.0 ± 0.4 &amp; 505 ± 67</td>
</tr>
<tr>
<td></td>
<td>T:T</td>
<td>43.6 ± 0.4 &amp; 61.1 ± 1.8</td>
<td>9.1 ± 1.1 &amp; 390 ± 110</td>
</tr>
</tbody>
</table>

* The indicated primer-templates (125 nM) were incubated for 2 min with 1.25 nM wild-type or D368A mutant pol in the presence of increasing concentrations of correct or incorrect nucleotide. The rates of incorporation were calculated as the concentration of primer extended divided by 2 min and plotted as a function of dNTP concentration. The data were fit to Equation 3 to estimate the apparent maximum velocity at infinite dNTP concentration \( V_{\text{max}} \) and the concentration of nucleotide required for half-maximal velocity \( K_m \). NS, not saturated over the concentration range tested. I, indeterminate due to absence of detectable incorporation.

**Fig. 3.** Efficiencies for incorporation of incorrect dNTPs into various P:T. Steady-state assays were performed as indicated in the legend to Fig. 2 using four different P:T and monitoring incorporation of each incorrect dNTP to form the various mispairs shown (incorporated dNTP:template base). The efficiencies of incorporation \( V_{\text{max}}/K_m \) of incorrect dNTPs from Table II were calculated and are displayed as mean ± S.D. for the exo-deficient pol (open bars) and for the wild-type pol (hatched bars). In the cases in which concentrations of nucleotide used did not approach saturation, the efficiency was reported as the slope of the data fit to a linear function by regression analysis.

the wild-type pol (Fig. 3). For D368A pol, the most efficient misincorporation events included dTTP opposite G- or C-complementing residues. Whereas efficiency of misincorporation of dTTP opposite G or C was also among the highest for the wild-type pol, similar efficiencies were observed for the formation of four other mispairs.

Steady-state Mismatch Extension Efficiency—The ability of the 44A/TA P:T to be extended by three nucleotides in the presence of dCTP indicated that both the wild-type and exo-deficient pols were capable of extending mismatched primer termini, at least at high concentrations of dNTP (Fig. 2, A and B). To determine the efficiency by which each enzyme could extend different mismatched P:T, we used P:T with termini composed of each possible base pair and measured their respective abilities to be extended with increasing concentrations of the next correct dNTP (dTTP). An example of the results of this assay for the four different 44N/TA primer/templates is shown in Fig. 4, and the results for all of the P:T are summarized in Fig. 5. As observed for misincorporation, mismatch extension by the exo-deficient pol varied most prominently at the level of \( K_m \) of the nucleotide, although more variation in the \( V_{\text{max}} \) for extension of mismatches was observed than that for misincorporation (data not shown). The exo-deficient pol displayed a wide range of efficiencies for extending different mismatched base pairs, whereas the wild-type pol exhibited uniformly low efficiencies with the exception of the 44N/A A P:T. For this P:T, mismatches were removed by the exo activity of the wild-type pol and then repaired and extended with the single nucleotide added (dTTP) (Fig. 5). This resulted in extension efficiencies by the wild-type enzyme similar to those that were matched. By contrast, the D368A pol was inefficient at extending all of the mismatched termini, confirming the absence of editing function.
The low efficiencies for mismatch extension of the other P/Ts by the wild-type pol were attributable in large part to the loss of the mismatched primer terminus (see for example, Fig. 4B). Therefore, mismatch extension per se can be measured accurately in steady-state reactions for only the exo-deficient pol. The efficiencies for extension of a mismatched primer terminus by the exo-deficient pol ranged from $-10^{-7}$ min$^{-1}$ for extension of a G:T mispair to $10^{-8}$ min$^{-1}$ for extension of a G:A mispair, whereas the efficiencies for extension of matched termini averaged 0.2 min$^{-1}$. Thus, it is clear that mismatches represent a significant kinetic barrier to extension by the HSV-1 pol independent of exo activity.

Mismatch Extension during Processive DNA Synthesis—Because removal of a mismatch by the wild-type pol would be favored in multiple turnover reactions if initial binding of the P/T was to the pol exo site, we examined the ability of the

**Fig. 4.Mismatch extension assays.** The D368A exo-deficient mutant (A, C, and D) or wild-type pol (B, E, and F) (1.25 nM) was incubated with primer/templates (125 nM) as shown (44N/TA in Table I) where N is the 3'-nucleotide on the primer strand. Reactions contained increasing concentrations (long open triangle) of next correct nucleotide dTTP, and the amount of primer extended following a 30-s or 2-min incubation period (for D368A or wild-type pol, respectively) was quantified. A and B, representative autoradiograms of the products of extension are shown. The positions the primer (P) and other products are as indicated in the legend to Fig. 2. For the 44G/TA P/T, extension by D368A approached saturation with the lower concentration range (0–250 $\mu$M) of dTTP shown on the autoradiogram compared with that shown (0–1000 $\mu$M) with the other mismatched P/Ts. The rate of extension at each concentration was plotted as a function of time, and if saturation was approached, the data were fit to Equation 3 to estimate the apparent V$_{\text{max}}$ and K$_{m}$, For those plots in which dTTP concentrations did not approach saturation, the slope of a linear fit of the data was used to estimate extension efficiency ($V_{\text{max}}/K_{m}$). The plots for extension of matched (C and E) and mismatched (D and F) primer termini are shown.

**Fig. 5. Efficiencies for extension of mismatched primer termini with next correct nucleotide.** Steady-state reactions were performed as described in the legend to Fig. 4 using increasing concentrations of the next correct nucleotide, dTTP, to extend each of the P/Ts indicated. The apparent V$_{\text{max}}$ and K$_{m}$ for dTTP incorporation into each P/T were determined as described in the legend to Fig. 4. The efficiency of extension (min$^{-1}$) with dTTP ($V_{\text{max}}/K_{m}$) by the exo-deficient pol (open bars) and by the wild-type pol (hatched bars) is displayed as mean ± S.D. for each set of mismatched P/Ts.
wild-type and mutant pol to extend mismatches under conditions that did not permit enzyme cycling to new P/Ts. Running start reactions were performed in the presence of only three dNTPs (250 \( \mu M \) each) using a P/T designed to allow correct incorporation of several nucleotides before a templating residue for which no matched nucleotide would be encountered by the pol (Fig. 6A). The pol was allowed to bind stably to the P/T prior to initiation of reactions with MgCl\(_2\), and excess activated calf thymus DNA as a trap. In the absence of dATP, processive extension with correct dNTP was possible only up to 48 nt in length. Extension of the primer to 49 nt required a misincorporation, whereas extension to 50 nt required extension of a mismatch (Fig. 6C). Fig. 6, D and E, shows plots for formation of these products as a function of time (from 10 ms to 2 s) for the wild-type and D368A pol, respectively. The data for extension of the primer by both enzymes to form products of \( \geq 48 \) or \( \geq 49 \) nt in length fit well to the exponential burst function (Equation 4). Nearly all of the P/T productively engaged with either pol was extended from 3 to 48 nt in length without dissociation (Fig. 6, D and E). However, only 76 and 49% P/T productively engaged by the wild-type and exo-deficient pol, respectively, was extended at least 1 nt further by 2 s, presumably with incorrect dNTP. Table III demonstrates that the apparent burst rate constants for extension with correct dNTP to form 46- or 48-nt products did not vary significantly with respect to the pol, although the apparent burst rate constants for the formation of the 49-mer mispaired product was -4 times slower for D368A compared with wild-type pol. Surprisingly, the plot for extension of mismatched P/T by the exo-deficient pol to form a 50-nt product did not fit an exponential function but did fit well to a linear function, suggesting a different and/or substantially slower rate-limiting step than observed with the wild-type pol (Fig. 6, D and E, and Table III). Similar results were observed with lower dNTP concentrations (100 \( \mu M \)), although the proportion of P/T extended to 49 and 50 nt was somewhat reduced (results not shown).

Because the UL42 protein increases the processivity of the pol catalytic subunit, we tested its effect on the abilities of the wild-type and mutant pols to misincorporate and extend mismatches in similar experiments. A stable purified 1:1 complex of wild-type or D368A pol with UL42 was prebound to P/T and extended in the presence of 250 \( \mu M \) of the three dNTPs (Fig. 7, A and B, respectively). Complexes of UL42 with the wild-type or D368A pol showed a reduced capacity to misincorporate and to extend mismatches compared with the catalytic subunit (compare Figs. 7, C and D, with 6, D and E). Significantly, only 5.5% P/T productively engaged with wild-type pol-UL42 could extend the mismatched termini in 2 s without dissociating, whereas 18% was extended to \( \geq 50 \) nt by the mutant pol-UL42 complex. UL42 enhanced the apparent burst rate constant by which the wild-type pol formed these limited products but had little or no effect on the rate constants for misincorporation or mismatch extension by the exo-deficient pol (Table III). As observed in the absence of UL42, mismatch extension by the exo-deficient pol-UL42 complex displayed linear kinetics (Fig. 7D).
The data were fit to Equation 4 or a linear function as detailed in the legend to Fig. 6.

Exonuclease Activity and HSV-1 DNA Polymerase Fidelity

Table III

<table>
<thead>
<tr>
<th>Enzyme</th>
<th>46 nt*</th>
<th>48 nt</th>
<th>49 nt</th>
<th>50 nt</th>
</tr>
</thead>
<tbody>
<tr>
<td>Wild-type pol</td>
<td>290 ± 22</td>
<td>15.5 ± 1.8</td>
<td>2.7 ± 0.2</td>
<td>1.8 ± 0.2</td>
</tr>
<tr>
<td>Wild-type pol-UL42</td>
<td>201 ± 23</td>
<td>28.1 ± 3.2</td>
<td>12.0 ± 1.7</td>
<td>8.6 ± 1.1</td>
</tr>
<tr>
<td>D368A pol</td>
<td>243 ± 16.2</td>
<td>22.9 ± 3.1</td>
<td>0.60 ± 0.08</td>
<td>0.24 ± 0.02b</td>
</tr>
<tr>
<td>D368A pol-UL42</td>
<td>182 ± 14.4</td>
<td>24.0 ± 3.5</td>
<td>0.87 ± 0.22</td>
<td>0.25 ± 0.03b</td>
</tr>
</tbody>
</table>

*a* Burst rate constants ± S.D. were determined from the plots of data shown in Figs. 6 and 7 fit to the burst equation (Equation 4) except as noted.

*b* Rate constants ± S.D. were estimated by dividing the slope of a linear fit of the data for formation of 50 nt by the concentration of enzyme predicted to be productively engaged prior to extension. The latter was estimated to be the amplitude of the functions fit to the data for formation of the 49-nt products.

**DISCUSSION**

Comparison of the wild-type HSV-1 pol and a mutant pol that destroys the exo function without significantly altering the polymerizing function allowed us to determine the contribution of the exo function to misincorporation and mismatch extension frequencies. We used steady-state kinetics as a means to measure the ability of each enzyme to selectively incorporate correct versus incorrect nucleotide. The validity of this approach is supported by the fact that we observed comparable selectivities using pre-steady-state or steady-state analysis. For example, the selectivity of the wild-type pol for correct (dTTP) versus incorrect (dATP) nucleotide into the 44A/TA P/T in pre-steady-state reactions was 265 (13), whereas relative efficiencies for steady-state incorporation of the same correct and incorrect nucleotides into an identical P/T was 194 (Table II and results not shown). Because the ability for a polymerase to distinguish correct from incorrect dNTP for incorporation is influenced by sequence context (21, 22), we compared misincorporation frequencies for wild-type and D368A pol using P/Ts, which varied at only a single position on the template. The efficiencies for formation of half of the 12-possible mispairs were <10-fold higher for the exo-deficient compared with the wild-type pol. Averaged across all of the possible mispairs, the contribution of the exo activity in reducing the efficiency for formation of mispairs was <30-fold (Fig. 3). Using a lacZ reversion assay, Baker and Hall (11) showed that in the presence of 1 mM dNTP, the average base substitution frequency of D368A mutant pol was up to 18-times higher than that of the wild-type pol, in general agreement with the results reported herein. This overall exo effect also is of the same order of magnitude as the 2- to 40-fold increase with respect to the wild type in single base substitution frequency with exo-deficient T4 DNA polymerase (30, 31) and the 20-fold increase in base substitutions reported for exo-deficient T7 DNA polymerase (32). Thus, as observed for T4 and T7 DNA polymerases, the exo activity of HSV-1 pol contributes much less to the overall fidelity of nucleotide incorporation in steady-state reactions than does the inherent ability of the polymerase domain to distinguish among nucleotides during incorporation.

The relatively high misincorporation frequency of the wild-type HSV-1 pol (−1 in 300 incorporation events, Ref. 13) predicts that >500 mismatches could be introduced per genome replicated, surely to be lethal if fixed in the genome. However, failure of the pol to extend mismatches would reduce misincorporation frequency in vivo. Steady-state kinetic analysis of the D368A exo-deficient pol demonstrates that mismatches do impose a kinetic barrier to the polymerizing function of the pol (Figs. 4 and 5), independent of the presence or absence of exo activity. For an exo-deficient pol, this barrier would be expected to promote stalling and/or dissociation of the enzyme prior to extension. For the wild-type pol with an associated exo activity, the kinetic barrier to mismatch extension increases the probability that the mismatch will be switched to the exo site and then removed. That this switching occurs is demonstrated by...
the fact that the low efficiency for mismatch extension by the wild-type pol in steady-state reactions is due in large part to excision of the mismatch (Fig. 4). Because the wild-type HSV-1 pol preferentially excises mismatched compared with matched primer termini (13), these steady-state reactions do not accurately measure the relative probability for extending a mismatched compared with a matched primer terminus for that enzyme. 

To more directly determine the ability of each pol to extend mismatches during processive synthesis, we forced misincorporation and prevented repair of excised primer termini by the wild-type pol by limiting the nucleotide pool to three dNTPs. Under these conditions, both the wild-type and exo-deficient pol catalytic subunits not only readily incorporated an incorrect primer terminus (13), these steady-state reactions do not accurately reflect the rate by which the D368A pol switches the mismatched P/T eventually terminates versus the absence of UL42, thereby reducing the substrate for mismatch extension. The kinetic barrier imposed by the mismatch that survives switching from the pol to exo site, enhancement of the rate of switching by UL42 perhaps in conjunction with reduced dissociation, and the presence of an active exo function would favor excision and repair when all four dNTPs are present. Together, these factors suggest the means by which overall mutation frequency observed in vivo is significantly lower than that predicted by the poor nucleotide selectivity of the HSV-1 pol.

UL42 may also promote a more rapid transfer of the primer terminus from the pol to the exo site. If so, a reduced proportion of mismatched P/T would be predicted to accumulate following extension by the wild-type pol in the presence versus the absence of UL42, thereby reducing the substrate for mismatch extension.

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Contribution of the 3′- to 5′-Exonuclease Activity of Herpes Simplex Virus Type 1 DNA Polymerase to the Fidelity of DNA Synthesis
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