Impaired Mismatch Extension by a Herpes Simplex DNA Polymerase Mutant with an Editing Nuclease Defect*

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The D368A mutation within the 3′-5′-exonuclease domain of the herpes simplex type 1 DNA polymerase inactivates this nuclease and severely interferes with virus viability. Compared with the wild type enzyme, the D368A mutant exhibits substantially elevated rates of incorrect nucleotide incorporation, as measured in a LacZ reversion assay. This high rate occurs in the presence of high levels of dNTPs, a condition that forces the enzyme to extend mismatched primers. Hence, the mutant fails to correct many misincorporations that are removed in the wild type. In addition, the mutant shows a much reduced ability to replicate DNA templates primed with a 3′-mismatch as compared with wild type. This extension defect also appears more severe than observed for replicas which naturally lack editing nucleases. Based on these findings, we suggest that the inability of the D368A herpes simplex mutant polymerase to replicate beyond a mismatched base pair severely inhibits viral replication.

A major mechanism for controlling the accuracy of chromosomal replication involves 3′-5′-exonucleases associated with DNA polymerases. Although DNA polymerases actively control fidelity by selecting correctly base-paired dNTPs during the insertion step of polymerization (1, 2), misincorporation does occur. In polymerases with editing functions, the polymerase responds to a mismatched primer by pausing, thus facilitating removal of the misincorporated nucleotide (1). However, conditions, such as high concentrations of dNTPs, may favor extension of the mismatched primer rather than editing (1). Misincorporations that escape editing can later be repaired by mismatch repair mechanisms, which act on DNA containing mispaired bases (3).

This study examines the 3′-5′-exonuclease activity associated with the herpes simplex virus type 1 (HSV-1) DNA polymerase (6). The exonuclease domain within this enzyme is indicated by the presence of three highly conserved Exo motifs (Exo I, II, and III) (Fig. 1) and the retention of nuclease activity by an N-terminal proteolytic fragment (7, 8). Mutations within the motifs inactivate the exonuclease activity (9–11). Based on the structural model provided by DNA polymerase I (Escherichia coli), these motifs encompass sites that bind two divalent Mg²⁺ ions and coordinate the binding of reaction components. Aspartate residues within these motifs are critical for ion binding and catalytic activity (4, 5). The HSV-1 D368A mutation described here inactivates the aspartate residue in the Exo I motif.

Mutations that inactivate DNA polymerase-associated 3′-5′-exonucleases frequently result in high mutation rates (1, 11–17). These effects presumably result from fixation of replication errors by extension of mismatched primers after the editing function fails to act. In some systems, exonuclease deficiencies impair viability. This response has often been attributed to production of a high level of deleterious mutations. For example, certain mutations in the exonuclease subunit of DNA polymerase III (E. coli) are lethal, unless suppressed by a second mutation that lowers the high mutation rate by increasing insertion accuracy or overproducing mismatch repair enzymes (13). Reduced viability occurs in yeast (Saccharomyces cerevisiae) carrying exonuclease deficiencies in the mitochondrial polymerase or in both the δ and ε nuclear DNA polymerases (17, 18). The nuclear mutations become lethal when post-replication mismatch repair is also inactivated (19).

However, another factor that may contribute to low viability in editing-deficient mutants is the pausing of the polymerase after misincorporation. Failure to edit might prevent extension of a mismatched primer, thus impairing replication. In support of this possibility, exonuclease deficiencies in both T4 bacteriophage and HSV-1 DNA polymerases show reduced abilities to extend mismatched primers (10, 23). In addition, several exonuclease-deficient DNA polymerases exhibit lower processivity that might be attributable to a mismatch extension deficiency. For example, mutants from bacteriophages ø29 and prd1 fail to replicate duplex templates that require strand displacement for fork movement (20, 21). Mutants from yeast mitochondria (Saccharomyces cerevisiae), prd1, and E. coli (DNA polymerase III) produce short replication products during DNA synthesis on primed single-strand templates (17, 21, 22). Finally, mutants from T4 exhibit low polymerization activity when mis-incorporation can occur, as compared with activity on homopolymer templates (23).

Certain mutations within the Exo I in motif 3′-5′-exonuclease domains produce more severe phenotypes than other alleles. For example, the lethal exonuclease alleles associated with DNA polymerase III (E. coli) are within Exo I. In addition, HSV-1 viruses carrying mutations in this region cannot be isolated and appear to be inviable (9). In contrast, HSV-1 mutants with changes in the Exo III motif are viable (11). Since the Exo I motif affects binding of both the metal ions required for catalysis (4, 5), mutations within this motif may be especially inhibitory to catalysis.

The present study examines several biochemical properties of an HSV-1 DNA polymerase carrying a mutation in the Exo I motif (D368A). Since this mutation appears to be lethal (9), we were interested in uncovering mechanisms to explain the se-
verity of this defect. We find that the D368A mutation dramatically elevates the mutation frequency during replication but only under conditions that force the extension of mismatched primers. In addition, this mutation causes an exceptional deficiency in extension of mismatched primers. These abnormalities may drastically impair viral replication, thus resulting in the lethality of this mutant.

**Experimental Procedures**

**Polymerase Purification**—SBF cells were infected with recombinant baculovirus carrying the appropriate HSV-1 polymerase gene, and the cell lysate was used to transform 50 ml of NH4SO4 was added to the clarified cell lysates to a final concentration of 20%. Lysates were subjected to low pressure liquid chromatography using phenyl-Sepharose columns (Amersham Pharmacia Biotech) equilibrated in Buffer C (9) supplemented with 20% glycerol, 50 mM NaCl, and 30% (NH4)2SO4. Fractions were eluted with a decreasing (NH4)2SO4 gradient from 20% to 0% and then with a step gradient of Buffer C supplemented with 20% glycerol, 50 mM NaCl, and either 0.1, 0.5, 1, or 2% Triton X-100. Both wild type and D368A mutant polymerase peaks eluted between 1 and 2 Triton X-100. Fractions containing peak activity were pooled, dialyzed against Buffer C containing 20% glycerol and 50 mM NaCl, and further purified by ion exchange chromatography (phosphocellulose), using a linear NaCl gradient in Buffer C containing 20% glycerol. Wild type and D368A mutant polymerase fractions were pooled, dialyzed against Buffer C containing 20% glycerol, 50 mM NaCl, and either 0.1, 0.5, 1, or 2% Triton X-100. Both wild type and D368A mutant polymerase peaks eluted between 1 and 2 Triton X-100. Fractions containing peak activity were pooled, dialedyzed against Buffer C containing 20% glycerol and 50 mM NaCl, and further purified by affinity chromatography on either single-stranded DNA agarose (wild type) or heparin-Sepharose (D368A mutant) (Amersham Pharmacia Biotech). Columns were eluted with a linear gradient (50–400 mM NaCl) in Buffer C containing 20% glycerol, 50 mM NaCl and 60% glycerol, and stored at 4°C.

**Exonuclease of HSV-1 DNA Polymerase**—stranded phage DNA. The oligos contained a terminal 3'-end, 4 of which were labeled (\(5'-\mathrm{dTTP}\)) of heat-denatured substrate. Polymerase assays were performed as described for the mutagenesis assays (above) for 15 min at 37 °C, using 0.05 units of wild type or 0.06 units of D368A mutant polymerase, 0.1 pmol of primer/template, and one or more dNTPs. Reactions were stopped by rapid cooling on ice and addition of EDTA (pH 8.0) to 30 mM followed by ethanol precipitation. Pellets were resuspended in 20 μl of H2O and 10 μl of loading buffer (95% formamide, 0.25% bromophenol blue, and 0.25% xylene cyanol FF). Samples were run on denaturing 20% polyacrylamide gels containing 8 M urea. Gels were scanned on a Molecular Dynamics PhosphorImager, bands quantified using the program ImageQuant (Molecular Dynamics), and Kcat and Vmax values calculated by fitting the data to rectangular hyperbola by nonlinear regression. Values for Vmax corresponded to the extrapolated velocity at infinite substrate concentration, and values for Kcat corresponded to the substrate concentration at 1/2Vmax.

For determination of kinetics of addition of individual nucleotides during primer extension, the percent of substrate extended was calculated using Equations 1–3.

\[
P \rightarrow P + 1 \text{ reaction, } (P + 1)/(P + (P + 1) \times 100) \quad (1) 
\]

\[
P \rightarrow P + 5 \text{ reaction, } (P + 5)/(P + (P + 1) + (P + 5)) \times 100 \quad (2) 
\]

\[
P \rightarrow P + 5 \text{ reaction, } (P + 5)/(P + (P + 1) + (P + 5)) \times 100 \quad (3) 
\]

where \(P\) indicates the amount of unextended primer, and \(P + 1\) and \(P + 5\) indicate the amount of reaction product extended by one and five bases, respectively.

**dTMP Turnover Assays**—Reactions were performed as described above for primer extension assays, except that the reactions contained 0.06 nmol of unlabeled primer/template with the T-G mismatched oligo, wild type HSV-1 polymerase, and a mixture of non-radioactive dTTP and \(\alpha\)-[32P]dTTP (800 Ci/mmol, 10 μCi/μl) at the concentrations indicated. Reactions were incubated 30 min at 37 °C, stopped by the addition of EDTA (pH 8.0) to 30 mM, and subjected to thin layer chromatography analysis on polyethyleneimine cellulose plates of 100-μm thickness (Aldrich) using 1.2 M LiCl. Plates were dried and subjected to autoradiography on a PhosphorImager. Individual spots were quantified using the program ImageQuant (Molecular Dynamics). Total dTMP production was calculated based on the observed rate of \([\alpha\text{-}32P]dTTP\) production.

**RESULTS**

To gain insight into the mechanism of the 3'→5' exonuclease associated with the HSV-1 DNA polymerase, we have compared the biochemical characteristics of the wild type polymerase and a mutant carrying the D368A mutation. As shown in Fig. 1, this mutation lies within the Exo I motif that is highly conserved between the HSV-1 polymerase and DNA polymerases (1). Structural studies with DNA polymerases indicate that the corresponding residue, Asp-356, is essential for exonuclease function because it binds two Mg2+ ions required for catalysis (42, 43). We previously reported that the D368A mutation in HSV-1 DNA polymerase reduces exonuclease activity to undetectable levels (9, 24), demonstrating that this residue is also required for enzyme function.
3’–5’-Exonuclease of HSV-1 DNA Polymerase

**Purification of the HSV-1 DNA Polymerase**—To obtain enzymes for these experiments, wild type and D368A mutant polymerases were overexpressed using baculovirus vectors (9) and purified as described under “Experimental Procedures” and Table I. Although we had previously purified the wild type HSV-1 DNA polymerase to near-homogeneity (24), the D368A mutant polymerase failed to survive the glycerol gradient step in this original procedure (not shown). Hence, we devised a new purification scheme using a three-step procedure (Table I) consisting of hydrophobic interaction chromatography on phenyl-Sepharose, anion exchange chromatography on phosphocellulose, and affinity chromatography. In the affinity chromatography step, the wild type polymerase was purified using single-stranded DNA agarose. The D368A mutant enzyme failed to bind to this matrix (not shown) and was consequently purified on heparin-Sepharose. Gel analyses (not shown) reveal no large differences between these two preparations in the levels of minor contaminants. Hence, the difference in specific activities between these polymerases is most likely due to differences in the relative fractions of active enzyme molecules.

The identity of the purified enzymes was confirmed by two biochemical tests. First, the enzymes were assayed for polymerase activity in the presence of phosphonacetic acid, a specific inhibitor of the HSV-1 polymerase. As shown in Fig. 2A, both the wild type and D368A mutant enzymes were severely inhibited by phosphonacetic acid at concentrations at or above 10 μM. In contrast, DNA polymerase I (Klenow fragment) from E. coli (25) remained relatively active at concentrations up to 100 μM. Second, enzymes were subjected to exonuclease measurements using a single-stranded DNA substrate. As shown in Fig. 2B, the wild type enzyme contains exonuclease activity, and the D368A mutant is completely deficient. This observation confirms our earlier finding that the D368A mutant lacks detectable exonuclease activity (9) and indicates a lack of contaminating exonucleases in the mutant preparation.

**Reversion Frequencies**—Studies from several systems, including HSV-1 (11), have revealed that DNA polymerases with inactive editing nucleases show increased mutation frequencies compared with wild type (1). The D368A mutation in HSV-1 inactivates the polymerase-associated exonuclease but also appears to be lethal to the virus (9). Hence, we suspected that an editing deficiency might increase the viral mutation frequency, which favors mismatch extension at higher concentrations, thus producing higher reversion rates but at dNTP concentrations of 103 M but increased 6.7-fold at 104 M dNTPs), the mutant showed an 18-fold higher activity compared with wild type (1).

Results from this assay (Table II) showed that the D368A mutation caused significantly elevated reversion frequencies compared with wild type but that the reversion rate was dependent on dNTP concentrations. We conducted additional analyses to understand the substantial variation observed between reactions carried out under different conditions and to identify conditions most appropriate for comparing the activities of the wild type and D368A mutant polymerases. Based on these analyses, we conclude that the wild type enzyme edits many of its replication errors at low dNTP concentrations but favors mismatch extension at higher concentrations, thus producing higher reversion rates. In contrast, the D368A mutant polymerase fails to replicate the template efficiently at low dNTP concentrations, possibly due to stalling at mismatches, but is able to extend mismatches at higher concentrations.

Under conditions where the wild type exonuclease is fully functional and both enzymes completely replicate the gapped template (i.e. 103 μM dNTPs), the mutant showed an 18-fold higher reversion rate than wild type.

To test this idea, we compared the reversion frequencies of purified wild type and D368A mutant HSV-1 DNA polymerases in vitro. A partially duplex, gapped M13 bacteriophage DNA molecule was used as a primer/template for these polymerase reactions. The gap contained a mutant lacZa gene (from E. coli) carrying a single base change that produces a nonsense codon. The polymerase reaction allowed filling of the gap by DNA synthesis and production of LacZ revertants by replication errors at the nonsense codon site. These revertants were detected by transfecting the reaction products into E. coli and scoring for blue M13 plaques in agar containing 5-bromo-4-chloro-3-indolyl-β-D-galactopyranoside.

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We first examined the effect of dNTP concentrations on the wild type polymerase. In this case, the reversion frequency remained low at 10 to 103 μM but increased 6.7-fold at 104 μM. Experiments with DNA polymerase II from E. coli (25), human, and calf DNA polymerase δ (26, 27) and DNA polymerase I (Klenow fragment) from E. coli (27) have shown similar increases in reversion rates but at dNTP concentrations of 103 μM.
TABLE II
Reversion of a LacZα mutation by HSV-1 DNA polymerases and reverse transcriptases in the presence of varying dNTP concentrations

| Polymerase | μM dNTP | Plaques scored | Reversion frequency |
|------------|---------|----------------|---------------------|
| None       | 10³     | 3.8 × 10⁶      | 13                  |
| HSV-1      | 10³     | 3.4 × 10⁶      | 9.7                 |
| Wild type  | 10³     | 2.9 × 10⁶      | 12⁷                |
| 10⁻⁴       | 1.0 × 10⁶ | 8              | 50⁷                |
| 10⁻³       | 6.5 × 10⁵ | 12       |                     |
| HSV-1      | 10³     | 7.6 × 10⁶      | 20                  |
| 10⁻³       | 1.9 × 10⁶ | 39          | 211 (18⁷)          |
| 10⁻⁴       | 1.0 × 10⁵ | 14          | 140 (ND)           |
| 10⁻⁵       | 1.0 × 10⁵ | 13          | 130 (11)           |
| MLV-RT     | 10³     | 2.9 × 10⁵      | 10                  |
| 10⁻³       | 4.9 × 10⁶ | 12          | 24                 |
| AMV-RT     | 10³     | 1.0 × 10⁶      | 6                   |

* These experiments were also conducted under conditions where the Mg²⁺ concentration was varied to maintain a constant Mg²⁺/dNTP ratio to compensate for the chelating effects of the dNTPs. The results (not shown) were similar to those shown here at constant Mg²⁺ concentration.

The polymerase to replicate in the presence of 10 μM dNTPs and then increased the dNTP concentration to either 10³ or 10⁴ μM. If stalling had occurred at 10 μM, then the subsequent increase in dNTP concentration should force the polymerase past the mismatch, allowing complete replication. As shown in Table II, when the D368A mutant polymerase was subjected to the two-step protocol, the reversion rate was comparable to that seen when high dNTPs were present throughout the reaction and was substantially higher than when the reaction was conducted entirely at 10 μM dNTPs. In contrast, when the wild type HSV-1 polymerase was subjected to the two-step protocol (i.e., a shift from 10³ to 10⁴ μM), the reversion frequency was about the same as when 10 μM dNTPs were present throughout the reaction. These results suggest that whereas the wild type polymerase completely replicates the gap at 10 μM dNTPs without stalling, the D368A mutant stalls at low dNTP levels, leading to a lower observed reversion rate, but completes gap filling at higher dNTP levels (>10 μM). Hence, the true reversion frequency of the D368A mutant polymerase is represented by the values observed at high dNTP concentrations.

The data in Table II also show reversion assays using reverse transcriptases from Moloney murine leukemia and avian myeloblastosis viruses (MLV-RT, AMV-RT). These enzymes lack any editing function and are known to exhibit high mutation rates (20). These assays reveal that, in the presence of high dNTP levels (≥10³ μM), the D368A HSV-1 mutant reversion frequencies are significantly higher than those of the reverse transcriptases (7.2-fold for MLV-RT and 3.5-fold for AMV-RT). This comparison suggests that the D368A mutant HSV-1 polymerase exhibits an exceptionally high mutation rate, which is substantially greater than those of the strongly mutagenic reverse transcriptases.

From these reversion data, we can calculate a corresponding base substitution rate for the D368A mutant polymerase, using the method of Bebenek and Kunkel (28). This calculation corrects for spontaneous background revertants, for the extent of revertant expression during scoring, and for the fact that revertants can arise by any of several changes within the target nonsense codon. By using this method, we find that a reversion rate of 2.11 × 10⁻⁴ (D368A at 10³ μM dNTPs, Table II) corresponds to 1.2 × 10⁻⁴ errors per replicated base.

Reversion frequencies from Table II were used to generate bar graphs in Fig. 3 to evaluate the statistical significance of these data. Non-overlapping vertical error bars indicate statistically significant differences between values. All values for polymerase reactions are significantly greater than that of the control without enzyme (p = 0.005). In reactions with the wild type HSV-1 polymerase, the value at 10³ μM is significantly higher than those obtained under other conditions (p = 0.001), whereas these other values do not differ significantly from each other. This analysis supports our hypothesis that the wild type favors mismatch primer extension, rather than editing, at 10³ μM dNTPs. In reactions with the D368A mutant polymerase, the value at 10 μM dNTP concentration is significantly different from the other four values (p = 0.001), and these other values do not differ significantly. This result supports our contention that the reactions at 10 μM dNTPs do not represent true reversion frequencies but rather result from incomplete gap filling after misinsertions. Finally, the D368A mutant values at dNTP levels that allow complete gap filling were significantly higher than either the wild type values obtained at dNTP levels that allow editing (p = 0.001) or values for the MLV (p = 0.001) and AMV (p = 0.01) reverse transcriptases. These comparisons confirm our conclusion that the D368A mutant DNA polymerase exhibits exceptionally high mutation rates in vitro, significantly higher than those of the wild type HSV-1 polymerase...
and the MLV and AMV reverse transcriptases.

Mismatch Extension—The reversion experiments described above suggest that the D368A mutant polymerase stalls during replication at low dNTP levels, probably at sites of misinsertion. Since the D368A mutation also appears to be lethal (9), we suspected that inefficient extension of mismatched primers might severely impair viral replication in vivo. To obtain evidence for a stalling mechanism, we conducted experiments in vitro to compare the efficiency of primer extension by the wild type and the D368A mutant polymerases.

This analysis was conducted using primer/templates consisting of 5'-labeled oligonucleotides annealed to single-stranded M13 DNA. Primers contained a 3'-terminal nucleotide that was either matched or mismatched to the template. The HSV-1 polymerases were allowed to extend the primers by five nucleotides in the presence of one or two dNTPs. Because the D368A mutant polymerase cannot excise mismatches, full-length products with a mismatched primer should only be produced by this polymerase by extension of the primer. However, the wild type polymerase should be able to excise a mismatch and, depending on the dNTPs present, should be able to insert the correct nucleotide or should be forced to re-insert the mismatch prior to extension. Products from reactions at varying concentrations of dNTPs were analyzed by gel electrophoresis, and the percentages of fully extended primers were measured (Fig. 4). These data were used to calculate the kinetic values \( K_m \) and \( V_{max} \) to determine extension efficiencies (\( V_{max}/K_m \)) (Table III).

From these analyses, we observed that both the wild type and D368A polymerases extend a C-G matched terminus with approximately equal efficiency. As calculated in Table III, the extension efficiency (\( V_{max}/K_m \)) was \( 375 (\% \text{ min}^{-1} \mu \text{M}^{-1}) \) for the wild type polymerase and \( 509 (\% \text{ min}^{-1} \mu \text{M}^{-1}) \) for the D368A mutant. Hence, the D368A mutation does not have a substantial effect on the efficiency of polymerization.

In reactions with a mismatched primer, both the wild type and D368A mutant polymerases exhibit impaired extension efficiencies compared with the matched primer. In general, this response results from large increases in the \( K_m \) with relatively small changes in \( V_{max} \). These results suggest that a correctly matched primer is necessary for stable binding of the next nucleotide.

The efficiency of the extension of the mismatched primer depended dramatically on the potential for editing of the mismatch. In reactions with a T-G mismatched primer, the wild type polymerase showed an efficiency of \( 0.0088 (\% \text{ min}^{-1} \mu \text{M}^{-1}) \) when only dTTP was present, a condition that disallows productive editing. When the correct nucleotide (dCTP) was also added, the extension efficiency increased (180-fold) to 1.6. Thus, when productive editing is prevented, the wild type polymerase stalls for a substantially longer time than when editing is allowed. Similar but more dramatic results were obtained with an A-G mismatch, and a 4800-fold increase in extension efficiency was observed when productive editing was allowed. We believe that the larger increase in extension efficiency of the A-G mismatch as compared with the T-G mismatch results from the decreased stability of the A-G base pair, causing decreased editing efficiency and/or decreased extension efficiency. In contrast to the wild type, the D368A mutant polymerase failed to show substantial increases in extension efficiencies of either the T-G or A-G mismatch when two dNTPs were present. This result is consistent with the editing deficiency of the D368A mutant polymerase.

To compare extension efficiencies of the wild type and D368A mutant polymerases, we calculated standard extension efficiencies for each enzyme by dividing the mismatched extension efficiencies by the matched efficiency, as shown in Table III. This comparison revealed a defect in the D368A mutant in mismatch extension. For the T-G mismatch, when two dNTPs are present, allowing productive editing by the wild type polymerase, the wild type enzyme extended much more efficiently (380-fold) than the D368A mutant. In contrast, when only one dNTP was present, the wild type polymerase extended slightly less efficiently (0.27-fold) than the D368A mutant. Similar results were obtained with the A-G mismatch. In the presence of both dNTPs, the wild type extended 590-fold more efficiently than the mutant, whereas with only one dNTP it extended less efficiently (0.22-fold). These results suggest that the D368A mutant polymerase stalls substantially at mismatches compared with a productively editing wild type polymerase.

Evidence for Polymerase Cycling—In the primer extension experiments above, the wild type polymerase is less efficient at extending mismatched primers when editing is prevented. We
containing matched or mismatched
3'-5' exoribonucleases, using
Reactions with M13 templates were performed as described
above for each reaction is indicated
for the T-G and A/G mismatched
exonucleases (% per min) is plotted as a function of dNTP concentration. Addi-
tional experiments were performed for the T/G and A/G mismatched
mutants HSV-1 polymerase.

Effects of Internal Mismatches on Extension Efficiency—We
suspect that under these conditions, the wild type enzyme
becomes trapped in a cycle of removal and reinsertion of
the mismatch. To test this possibility, we conducted dNTP turnover assays. The polymerase was incubated with an unlabeled primer/template carrying a T-G mismatch at the primer 3'-end in the presence of [α-32P]dTTP. Reaction products were ana-
yzed by thin layer chromatography and autoradiography. As shown in Table IV, radioactive dTMP was generated in the
presence of [α-32P]dTTP. Reaction products were ana-
yzed by autoradiography, as shown. dNTP concentration (μM) for each reaction is indicated above the corresponding gel lane. Upper arrows indicate the position of fully extended primers, and lower arrows indicate the position of unextended primers. In each graph directly below the corresponding autoradiogram, the velocity of primer exten-
sion (% per min) is plotted as a function of dNTP concentration. Addi-
tional experiments were performed for the T/G and A/G mismatched
primers using two dNTPs (not shown). The results of these experiments are
summarized in Table III.

Effects of Internal Mismatches on Extension Efficiency—Because
our primer/templates allowed incorporation of multiple
nucleotides after a mismatch, we were able to evaluate the
effects of the mismatch on addition of both the next nucleotide
and other downstream nucleotides. This analysis revealed that
internal mismatches also promote stalling by the D368A mu-
ant HSV-1 polymerase.

In the mismatch extension reactions shown in Fig. 4B (T-G), a
band corresponding to the primer extended by one nucleotide
(P + 1) was typically observed, indicating a kinetically slow
step for the addition of a second nucleotide onto the mis-
matched primer. Although not as prominent, a similar band
was seen in reactions involving the extension of A-G mismatches. Kühn and Knopf (10) have observed a similar phe-
nomenon. Subsequent addition, up to the five allowed in these
reactions, occurred very rapidly, and no further intermediate
bands were observed. Hence, these later additions contribute
equally to the extension rates.

The effect of internal mismatches is quantified in Table V for
the T-G mismatched primer. Km and Vmax values were calcu-
lated using graphs of percent substrate extended versus dNTP concentration. Primer extension efficiencies for the addition of both the first (indicated as P → P + 1) and second (indicated as P + 1 → P + 5) nucleotides reveal that the second addition is
at least as slow as the first. This effect may act as an additional
pause in the elongation of a primer, amplifying the effective-
ness of proofreading by increasing the chance that a mismatch
will be detected. A similar effect has been noted for the T4
bacteriophage DNA polymerase (23).

**DISCUSSION**

This study examines the effect of an impaired editing nucle-
ase during DNA replication by the HSV-1 DNA polymerase. We
showed previously that the D368A mutation inactivates the
exonuclease and severely interferes with virus viability (9, 24).
In the present experiments, we find that this defect increases
the production of replication errors but only in the presence of
high levels of dNTPs to favor the extension of mismatched
primers. In addition, this mutation reduces the efficiency of
extension of 3'-mismatched primers. These abnormalities ap-
ppear to be exceptionally severe when compared with other
replicases. Hence, loss of editing activity in the HSV-1 DNA
polymerase may interfere with viral growth by enhancing the
production of deleterious mutations and/or by prematurely ter-
minal replication forks at sites of misinsertions.

**High Mutation Rates in the D368A Mutant—Inactivation of
editing exonucleases increases mutation rates in many systems
(1). Elevated mutation frequencies have been reported for
HSV-1 derivatives carrying mutations in the highly conserved
Exo III motif within the 3'-5'-exonuclease domain of the DNA
polymerase (11). However, viruses with mutations in the con-
served Exo I motif appear to be inviable (9). Therefore, we have
analyzed the mutation frequency of the Exo I D368A mutant
polymerase *in vitro* by measuring reversion of a nonsense
codon. This analysis reveals up to an 18-fold increase in the
reversion frequency of the D368A mutant polymerase com-
pared with wild type. Although the reversion levels vary with
the reaction conditions, we argue that this maximum increase
best reflects the intrinsic difference between these enzymes.
This difference was obtained using a dNTP concentration of 103
μM, a condition that allows maximum activity of the wild type
editing exonuclease and efficient replication of the template/
primer by both the wild type and mutant enzymes.

A comparison of our findings with other studies reveals that
reversion frequencies for the wild type HSV-1 DNA polymerase
resemble those of other mammalian polymerases with editing
functions. In contrast, the mutant HSV-1 polymerase exhibits
an exceptionally high reversion frequency, even higher than
enzymes that naturally lack an editing function. Frequencies
for the wild type HSV-1 polymerase at dNTP concentrations
that allow editing (<103 μM) are within 2-fold of those reported
for bovine DNA polymerases δ (27) and ε (28). Frequencies for
the D368A mutant HSV-1 polymerase at dNTP concentrations
that allow efficient primer extension (≥104 μM) are approxi-
mately 4-fold greater than those for bovine DNA polymerase α
(27) and 2–7-fold greater than those for reverse transcriptases
from human immunodeficiency virus (28), or MLV and AMV
DNA polymerase reactions were conducted and analyzed as shown in Fig. 4. Kinetic values are the means of two to four experiments and S.E. are indicated. Standard extension efficiency is defined as the ratio of the extension efficiency of a mismatched primer divided by the extension efficiency of the matched primer. Values in parentheses are the ratios of the standard efficiencies of the wild type compared with the D368A mutant. Units for extension efficiency are (% \cdot \text{min}^{-1} \cdot \mu\text{M}^{-1}).

| Base pair at primer 3’-end (primer/template) | dNTPs present | $K_m$ | $V_{max}$ | Extension efficiency | Standard extension efficiency |
|---------------------------------------------|----------------|--------|-----------|----------------------|-----------------------------|
| **Wild type**                               |                |        |           |                      |                             |
| C-G                                        | dGTP + dTTP    | 0.016 ± 0.003 | 6.0 ± 0.07 | 375                  | 1 (1)                       |
| T-G                                        | dTTP           | 205 ± 46 | 1.8 ± 0.1 | 0.0088               | 2.4 \times 10^{-3} (0.27)  |
| T-G                                        | dTTP + dCTP    | 3.5 ± 0.7 | 5.5 ± 0.4 | 1.0001               | 4.2 \times 10^{-3} (380)   |
| A-G                                        | dATP           | 2250 ± 520 | 4.0 ± 0.8 | 0.0018               | 4.8 \times 10^{-6} (0.22)  |
| A-G                                        | dATP + dCTP    | 0.21 ± 0.04 | 1.8 ± 0.5 | 8.6                  | 2.3 \times 10^{-2} (590)   |
| **D368A mutant**                           |                |        |           |                      |                             |
| C-G                                        | dGTP + dTTP    | 0.011 ± 0.0006 | 5.6 ± 0.01 | 509                  | 1                           |
| T-G                                        | dTTP           | 150 ± 2.6 | 6.9 ± 0.12 | 0.046               | 9.0 \times 10^{-5}         |
| T-G                                        | dTTP + dCTP    | 2180 ± 170 | 12.3 ± 2.8 | 0.0056               | 1.1 \times 10^{-5}        |
| A-G                                        | dATP           | 345 ± 90 | 3.9 ± 0.7 | 0.011                | 2.2 \times 10^{-5}        |
| A-G                                        | dATP + dCTP    | 78.1 ± 6.5 | 1.6 ± 0.1 | 0.020                | 3.9 \times 10^{-5}        |

### Table IV

**dTMP production by wild type HSV-1 polymerase at a T-G mismatched primer.**

Reactions were performed as described under “Experimental Procedures” using a T-G mismatched primer/template.

| Units of polymerase | dTTP | dTTP produced |
|--------------------|------|---------------|
|                    | nmol |               |
| 0                  | 100  | 0             |
| 0.2                | 30   | 3.0           |
| 0.2                | 100  | 3.3           |
| 0.2                | 300  | 3.4           |

### Table V

**Kinetics of addition of individual nucleotides after a T-G mismatch by D368A mutant HSV-1 DNA polymerase.**

DNA polymerase reactions were conducted as shown in Fig. 4, using conditions that allowed extension of the primer, of length P, by up to five nucleotides. The kinetically slow steps of addition of the first base past the mismatch (P → P + 1) and addition of the second and subsequent bases (P + 1 → P + 5) were measured independently from the same autoradiograms.

| Nucleotide extension past mismatched primer of length P | $K_m$ | $V_{max}$ | Extension efficiency |
|--------------------------------------------------------|------|-----------|----------------------|
| P → P + 1                                              | 40   | 6.4       | 0.16                 |
| P + 1 → P + 5                                          | 62   | 6.9       | 0.11                 |
| P → P + 5                                              | 150  | 6.9       | 0.046                |

Stalling of the D368A Mutant at Mismatches—Primers with mispaired 3’ termini slow primer extension by DNA polymerases (1, 36), providing an opportunity for editing. Processivity defects have been reported for exonuclease-deficient mutants in several organisms (17, 21, 22, 37), suggesting that misinsertion events might block replication or promote polymerase disassociation when the editing exonuclease is inactivated. Evidence supporting these stalling mechanisms occurs in T4 bacteriophage, where exonuclease-deficient polymerases are defective in extension of mismatched primers and exhibit poor replication of templates which allow misincorporation (23). By using a more quantitative approach, our studies indicate that the loss of exonuclease in the HSV-1 DNA polymerase results in an exceptionally severe defect in mismatch extension. This defect may contribute to the viability of the D368A mutant.

To study mismatch extension by the HSV-1 DNA polymerase, we measured extension efficiencies of primers annealed to M13 bacteriophage DNA. By controlling the types of dNTPs, we could determine whether the polymerase had the potential to correct the mismatch. The wild type polymerase rapidly extended the primer when editing was allowed. When editing was inhibited, extension was reduced, and the polymerase entered into repeated cycles of removing and re-inserting the mismatch, thus interfering with extension. In contrast, primer extension by the D368A mutant DNA polymerase was several hundred-fold less efficient than the wild type under conditions where the wild type could edit.

Comparison of our primer extension data with those of other studies reveals that the D368A mutant polymerase stalls at mismatches considerably longer than at least two other polymerases that naturally lack an editing nuclease. This comparison is possible since in each case the extension efficiency for mismatched primers is standardized to the efficiency for matched primers, thus eliminating intrinsic differences in efficiency of matched primer extension for different enzymes. This comparison reveals that the D368A mutant polymerase extends a T/G mismatch 40-fold less efficiently than *Drosophila melanogaster* DNA polymerase α and 110-fold less efficiently than AMV reverse transcriptase (36). For an A/G mismatch, although both polymerase α and reverse transcriptase extend this mismatch much less efficiently than a T/G mismatch, the HSV-1 D368A mutant is again more inefficient (30- and 2-fold less, respectively). Hence, the D368A mutant enzyme appears to be severely inhibited in its ability to extend mismatched primers.

**Editing Defects and Loss of Viability**—Editing defects have
been linked to lower viability in several systems (9, 11, 17–19). A model frequently proposed to explain this phenomenon is the insertion of the Exo I motif into the exonuclease site. The Exo I motifs appear to be exceptionally severe. This mutant carries an altered aspartic acid residue (16), and the defect in our D368A mutant appears to be exceptionally reduced by several thousand-fold. This effect, coupled with the accumulation of deleterious mutations, might explain the apparent lethality of this mutant. HSV-1 DNA polymerase stalls 300–600-fold longer than the wild type. At this level, the type of mismatch, the D368A mutant HSV-1 DNA polymerase suggests that high mutation rates may also contribute to the impaired viability of the D368A mutant may substantially reduce the extent of viral replication and may explain the apparent lethality of this mutation.

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