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Effects of Mutations in the Exo III Motif of the Herpes Simplex Virus DNA Polymerase Gene on Enzyme Activities, Viral Replication, and Replication Fidelity

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The herpes simplex virus DNA polymerase catalytic subunit, which has intrinsic polymerase and 3′-5′ exonuclease activities, contains sequence motifs that are homologous to those important for 3′-5′ exonuclease activity in other polymerases. The role of one such motif, Exo III, was examined in this study. Mutated polymerases containing either a single tyrosine-to-histidine change at residue 577 or this change plus an aspartic acid-to-alanine at residue 581 in the Exo III motif exhibited defective or undetectable exonuclease activity, respectively, yet retained substantial polymerase activity. Despite the defects in exonuclease activity, the mutant polymerases were able to support viral replication in transient complementation assays, albeit inefficiently. Viruses replicated via the action of these mutant polymerases exhibited substantially increased frequencies of mutants resistant to ganciclovir. Furthermore, when the Exo III mutations were incorporated into the viral genome, the resulting mutant viruses displayed only modestly defect in replication in Vero cells and exhibited substantially increased mutation frequencies. The results suggest that herpes simplex virus can replicate despite severely impaired exonuclease activity and that the 3′-5′ exonuclease contributes substantially to the fidelity of viral DNA replication.

Replicative DNA polymerases, which are the central enzymes for DNA replication, play an important role in the fidelity of DNA replication. Aside from selecting the correct nucleotides for incorporation during DNA synthesis, most replicative DNA polymerases are associated with 3′-5′ exonuclease activities that proofread misincorporated nucleotides. DNA replication by mutant polymerases with either impaired or defective 3′-5′ exonuclease is less accurate by 1 to 3 orders of magnitude in many in vivo and in vitro systems (reviewed in references 18 and 42).

The replicative polymerase of herpes simplex virus (HSV) consists of a catalytic subunit (Pol) and a processivity subunit, UL42 (27, 31). Because of the variety of molecular, genetic, pharmacological, and biochemical tools that can be applied to its study, this enzyme is an excellent model system for studying polymerase functions in mammalian cells. The Pol subunit of HSV polymerase contains intrinsic 3′-5′ exonuclease activity (39, 40, 48), which maps to the N-terminal half of the polypeptide (58). Within this region, HSV Pol contains three segments that align with conserved, homologous segments in other polymerases called Exo motifs (2) (Fig. 1). In a variety of other polymerases, residues within these motifs are known to be critical for 3′-5′ exonuclease activities (2, 5, 13, 14, 19–21, 25, 30, 41, 50–54). For example, ^{368}Asp (Exo I), ^{471}Asp (Exo II), ^{581}Asp (Exo III), and ^{577}Tyr (Exo III) in HSV Pol correspond, respectively, to residues ^{355}Asp, ^{424}Asp, ^{501}Asp, and ^{497}Tyr of the 3′-5′ exonuclease domain of *Escherichia coli* DNA polymerase I (Pol I). In Pol I, these residues are critical for exonuclease activity due to effects on metal ion binding (the Asp residues) or catalysis per se (^{497}Tyr) (13, 14, 20). In another example, mutation of the residue in bacteriophage T4 DNA polymerase that corresponds to ^{424}Asp of Pol I results in an enzyme that retains polymerase activity yet lacks detectable exonuclease activity. Bacteriophage containing this mutation exhibit markedly increased mutation frequencies (21).

For HSV Pol, there is only limited information regarding the importance of the Exo motifs for various *pol* functions, including replication fidelity. Gibbs et al. (25) demonstrated that certain Exo II mutations were lethal to the virus and abolished polymerase activity. The effects of the mutations on exonuclease activity were not reported; however, recently, an Exo II mutation was shown to cause defects in both exonuclease and polymerase activities in vitro (41). It has been suggested that exonuclease activity is essential for viral replication, based on the failure to obtain recombinant viruses containing certain Exo mutations, including one that had no detectable effect on polymerase activity (30).

In this study, we constructed two mutant Pols containing alterations within the Exo III motif to examine their effects on the enzyme activities, viral replication, and the fidelity of DNA replication. These mutations were not lethal in vivo, despite severely impaired 3′-5′ exonucleases. Furthermore, the mutants exhibited drastic increases in mutation frequencies which could be the result of altered exonuclease activity.

MATERIALS AND METHODS

Cells and viruses. Vero (American Type Culture Collection), DP6 (49), and Pol A5 (see below) cells were grown and maintained as described (35). HSV type 1 (HSV-1) strain KOS, the *pol* null mutant HP66, and the mutant viruses generated in this study (see below) were propagated as described previously (49).

Plasmids. To construct eukaryotic expression plasmids of the HSV-1 *pol* gene, a 3.8-kbp *Bcl*I-*Kpn*I (partial digestion) fragment which contains the entire HSV-1 *pol* open reading frame was isolated from plasmid pDP4 (49) and cloned into the *Bam*HI and *Kpn*I sites of pGEM3Zf(+) (Promega) to obtain pGEM3-pol. A 3.8-kbp *Xba*I fragment was then isolated from pGEM3-pol and cloned into the *Xba*I site of the eukaryotic expression vector pSVK3 (Pharmacia) and the *Nhe*I site of the baculovirus expression vector pBlueBac (Invitrogen) to obtain pSVK-

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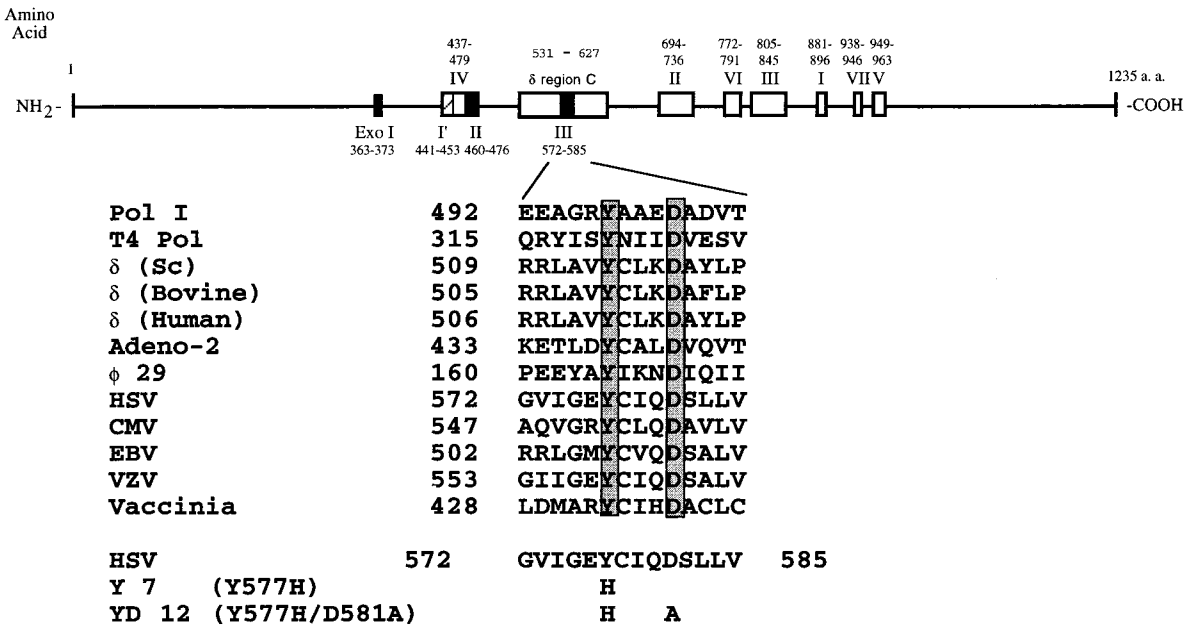

FIG. 1. Homology sequences of the Exo III motif shared among diverse DNA polymerases. The top line is a schematic of the HSV Pol polypeptide with the locations of regions of sequence similarity shared among other DNA polymerases. Regions I to VII (25, 35, 57, 59) and δ region C (10, 61), which overlaps the Exo III motif, are shown as empty boxes; the Exo I, II, and III motifs (2–5) are shown as the dark boxes; the Exo I′ segment, which was originally assigned as the Exo I motif (2, 3), is also shown as a dark box. The numbers above the line refer to amino acid (a.a.) residues of HSV Pol. Sequence alignment of the Exo III motif of HSV Pol and several other DNA polymerases is shown below the line. Numbers refers to amino acid residues. Highly conserved amino acids are shaded. The mutated amino acids of the two Exo III mutants in this study, Y7 and YD12, are indicated at the bottom. The following polymerases are presented: *E. coli* DNA Pol I (Pol I) (38), bacteriophage T4 Pol (55), DNA polymerase δ of *Saccharomyces cerevisiae* [δ (Sc)] (6), human DNA Pol δ [δ (Human)] (9), bovine DNA Pol δ [δ (Bovine)] (61), and Pols of adenovirus type 2 (Adeno-2) (26), bacteriophage ϕ29 (60), HSV (24), human cytomegalovirus (CMV) (7), Epstein-Barr (EBV) (1), varicella-zoster virus (VZV) (12), and vaccinia virus (17).

pol and pBlue-pol, respectively. The inserted *pol* gene in plasmid pSVK-pol was under the control of the simian virus 40 (SV40) promoter/enhancer and could be transcribed by T7 RNA polymerase in vitro (37). The *pol* gene inserted in pBlue-pol was under the control of the polyhedrin promoter (56). The orientation of the inserted *pol* gene in these two clones was confirmed by restriction digestion.

The protocol of transformer site-directed mutagenesis (Clontech Laboratories, Inc.) and the recipient double-stranded DNA of pGEM3-pol were used for obtaining specific mutations within the *pol* gene. The following oligonucleotides were used. G3HM (5′-GCATGCACGCGTGAGTATTCT-3′), which converts the *Hin*dIII site within the multiple cloning site to a *Mlu*I site, was used as the selective primer to identify mutated clones; 577/581 (5′-CAGGGAAT[G/C]CC TGTATGCAGTA[G/C]CTCGCC-3′), which contains degenerate nucleotide(s) at codons 577 and 581, and 441/443 (5′-CATCTCGAAT[G/A/T/C]CGCTG[G /A/T/C]CGAATTC-3′), which contains degenerate nucleotides at codons 441 and 443, were used as targeted primers for site-directed mutagenesis. Clones resistant to *Hin*dIII digestion were selected and sequenced to identify mutated sequences within the corresponding region.

The 2.3-kbp *Bgl*II-*Not*I fragment containing each mutated *pol* gene was then cloned into pSVK-pol and pBlue-pol to replace the corresponding wild-type 2.3-kbp fragment. Each clone was then sequenced by using the fmol DNA sequencing system (Promega) to confirm that only the desired mutation was found in the corresponding 2.3-kbp fragment. The primers from the *pol* gene corresponding to nucleotides 546 to 562 (546R), 754 to 770 (754R), 1010 to 1027 (1010R), 1190 to 1209 (1190R), 1566 to 1585 (1566R), 1593 to 1577 (1593L), 1643 to 1665 (1643R), 1984 to 2002 (1984R), 2156 to 2138 (2156L), 2429 to 2446 (2429R), 2745 to 2765 (2745R), 2810 to 2791 (2810L), and 3109 to 2990 (3109L), where the A nucleotide of the first ATG codon of the *pol* gene is defined as nucleotide 1, were used for sequencing analyses.

Recombinant baculovirus, protein expression, and purification. Baculovirus expression plasmids containing recombinant *pol* genes were cotransfected with linearized viral *Autographa californica* nuclear polyhedrosis virus DNA by using a transfection module kit (Invitrogen). Recombinant viruses were selected by their ability to form blue plaques in the presence of 5-bromo-4-chloro-3-indolyl-β-D-galactopyranoside (X-Gal) and purified by the manufacturer's protocols. Recombinant virus was used to infect *Spodoptera frugiperda* Sf9 cells, and expressed proteins were then purified as described previously (58), with modifications. Briefly, 2×10^9 Sf9 cells were infected with recombinant virus for 72 h. Infected cells were harvested, washed with phosphate-buffered saline, and resuspended in 30 ml of hypotonic buffer (20 mM HEPES-NaOH [pH 7.6], 0.5 mM $MgCl_2$) containing a protease inhibitor (0.5 mM phenylmethylsulfonyl fluoride). After incubation on ice for 10 min, infected cells were disrupted by Dounce homogenization. Cellular debris was removed by low-speed centrifugation, and the resulting supernatant was clarified by centrifugation at 15,000 rpm for 30 min. About 10 ml of buffer A (20 mM HEPES-NaOH [pH 7.6], 0.5 mM dithiothreitol, 0.5 mM phenylmethylsulfonyl fluoride, 5 μg of leupeptin per ml, 4 μg of pepstatin A per ml, 10% glycerol) containing 0.05 M NaCl was added to the clarified supernatant, which was then batch absorbed in approximately 25 ml of phosphocellulose P11 (Whatman) for 1 h at 4°C. The absorbed P11 was loaded into a column. After being washed with 50 ml of buffer A containing 0.05 M NaCl, the column was eluted with a linear 160-ml gradient of 0.05 to 1 M NaCl in buffer A. Pol-containing fractions were identified by Western blotting by using Pol-specific antiserum, pooled, and batch absorbed in 5 ml of preequilibrated hydroxylapatite resin for 30 min at 4°C. The absorbed hydroxylapatite resin was loaded into a column and washed with 25 ml of buffer A containing 1 M NaCl followed by 25 ml of buffer A containing 0.05 M NaCl. Pol was eluted with 25 ml of buffer A containing 0.05 M NaCl and 50 mM potassium phosphate. The Pol-enriched fractions were then absorbed to 2 ml of preequilibrated single-stranded DNA agarose for 30 min at 4°C. The absorbed agarose was loaded into a column and washed with 15 ml of buffer A containing 0.05 M NaCl. Pol was eluted with a linear 25-ml gradient of 0.05 to 1 M NaCl in buffer A. Fractions of purified Pol, which were determined to be apparent homogeneity as indicated by analysis of a sodium dodecyl sulfate (SDS)-polyacrylamide gel stained with Coomassie blue (Fig. 2), were then concentrated in a Centricon 100 (Amicon), diluted with buffer A to remove excess salt, and stored at −80°C.

Polymerase and exonuclease activity assays. Polymerase activity was analyzed by the ability of Pol to incorporate [α-^{32}P]dCTP into activated calf thymus DNA as described previously (48). The 50-μl reaction mixture included approximately 1 or 10 pmol of Pol, 25 μg of activated DNA, 60 μM each dATP, dTTP, and dGTP, 50 μM dCTP, and 5 μCi of [α-^{32}P]dCTP (3,000 Ci/mmol) in reaction buffer P [20 mM Tris-HCl, 0.1 mM EDTA, 40 μg of bovine serum albumin per ml, 4% glycerol, 3 mM $MgCl_2$, 5 mM dithiothreitol, 150 mM $(NH_4)_2SO_4$]. The reaction mixture was incubated at 37°C for 30 min, and the reaction was terminated by adding 200 μl of 0.1 M sodium pyrophosphate–10 mM EDTA; 500 μl of 10% trichloroactic acid was then added, and the mixture was incubated on ice for 15 min. Precipitated DNA was filtered through a Whatman GF/C glass filter, washed with 5% trichloroacetic acid containing 0.1 M sodium pyrophosphate, and then washed with ethanol. The filters were dried, and the radioactivities were measured in a liquid scintillation counter.

3′-5′ exonuclease activity was analyzed by using a 5′-^{32}P-labeled 16-mer

(5′-CCGGGGGGGAGGCGCC-3′) oligonucleotide (5,000 cpm/0.14 pmol/1.25 ng) hybridized to a 25-mer (5′-GGAAGCTTGGGCGCCTCCCCCCCGG-3′) oligonucleotides to form a primer-template. The primer-templates were incubated with different amounts of the wild-type, Y7, and YD12 HSV-1 Pols in buffer P, Klenow fragment of Pol I (New England Biolabs, Beverly, Mass.), and Sequenase version 2 (a T7 DNA polymerase deficient in 3′-5′ exonuclease activity; U.S. Biochemical) in reaction buffer supplied by the manufacturer in a total volume of 10 μl for 5 min at 37°C. The reaction was stopped by quenching with 4 μl of loading buffer and analyzed on a 20% denatured acrylamide gel containing 8 M urea. The 3′-5′ exonucleolytic activity, indicated by the reduction in size of the labeled oligonucleotide, was detected by autoradiography. The integrated band intensity was quantified with a PhosphorImager, and the relative activity of each mutant was compared to that of the wild-type Pol.

Construction of new HSV DNA polymerase-expressing cell lines. The polymerase-expressing cell line DP6 (49) was used for experiments at the beginning of this study. For unknown reasons, however, this cell line diminished in its ability to support the replication of *pol* null mutants. Therefore, new polymerase-expressing cell lines were constructed by using pSVK-pol and the method of Marcy et al. (49). A total of about 150 G418-resistant clones were obtained from two independent experiments, and 11 independent clones were able to complement the growth of the HSV-1 *pol* null mutant, HP66 (49). Five were further analyzed by Southern blot, and each cell line was found to contain integrated HSV-1 *pol* DNA (44). The Pol A5 cell line, which contains two copies of the integrated *pol* gene, was used in this study.

Complementation assay. Complementation assays were performed by transfection of either the wild-type or mutated *pol* expression plasmids into Vero cells by the DEAE-dextran transfection method and superinfection with HP66 *pol* null mutants as described previously (16). In some experiments, LipofectAmine transfection reagent (Life Technologies) was also used to transfect DNA into Vero cells according to the manufacturer's protocol. The progeny viruses harvested from each sample were titered on DP6 or Pol A5 cells (permissive for HP66 replication) and also on nonpermissive Vero cells, in which any plaques formed should represent recombinants between HP66 and the *pol* gene in complementing cells. Complementation efficiencies were calculated as the ratio [(titer on DP6 or Pol A5 cells − titer on Vero cells)$_{mutant}$]/[titer on DP6 or Pol A5 cells − titer on Vero cells)$_{wild\ type}$] × 100.

Construction of Exo III recombinant viruses. Marker transfer experiments were performed as described previously (8, 46), using infectious HP66 DNA, individual Exo III mutant plasmid DNAs, and Pol A5 cells to obtain recombinant viruses. Recombinant viruses were purified twice as white plaques on Pol A5 cells in the presence of X-Gal. Recombinant viruses were amplified in Pol A5 cells and examined by sequencing as described below. Those recombinants containing desired mutations were plaque purified twice further on Vero cells, and the existence of the desired mutations was confirmed by sequencing.

To examine whether recombinant viruses contained the Y7 or YD12 mutation, DNA fragments containing the Exo III region were PCR amplified from crude virion DNAs of recombinant viruses by using primers 1190R and 2156L and the method described previously (34). The resulting DNAs were sequenced by using primer 1643R.

Single-step growth analysis. To compare the abilities of recombinant Y7 and YD12 viruses to replicate in Vero cells with that of the wild-type KOS strain, about 2 × 10^5 Vero cells were infected with each virus with a multiplicity of infection of 5 PFU per cell. At 24 h postinfection, virus progeny was harvested and titered on Vero cells.

Measurement of mutagenesis frequency. The *tk* mutagenesis method originally developed by Hall et al. (29) was used to measure the frequency of acyclovir (ACV)- or ganciclovir (GCV)-resistant mutants with the following modifications. For analysis of mutants in an HP66 background, the polymerase protein required for the growth of HP66 viruses in Vero cells was transiently provided by the transfection of either wild-type or mutant *pol* expression plasmids. HP66 progeny viruses were then titered on Pol A5 cells. The relative mutation frequency of each virus stock was determined by the ratio of titers with and without 20 μM GCV. Each GCV resistant mutant was isolated, amplified, and further confirmed by its ability to grow on Pol A5 cells in the presence of GCV. When recombinant viruses were used, three independent plaques of Y7 and YD12 recombinant virus were each amplified in 5 × 10^4 Vero cells. The mutation frequencies of these independent samples were determined by the ratio of titers of viruses in the presence and absence of 50 μM ACV as described previously (33). Ten independent virus stocks for each recombinant were then prepared by the inoculation of only 5 PFU of a virus stock which exhibited the lowest mutation frequency (Table 5) into approximately 5 × 10^4 Vero cells. The mutation frequency of each independent virus stock was then determined by the ratio of titers in the presence and absence of 50 μM ACV.

RESULTS

Previous studies (25, 30, 41) of HSV mutant Pols with alterations in Exo motifs were inconclusive regarding the importance of exonuclease activity for viral replication and replication fidelity. To address these questions, we used site-directed mutagenesis to construct three mutated *pol* genes, Y7, YD12, and DE35. Mutant Y7 (Y577H) contains a single change at codon 577 (a change from TAC to CAC), and YD12 (Y577H/D581A) contains mutations at codons 577 and 581 (the same change at Y7 plus a change from GAC to GCT at codon 581), both of which are highly conserved amino acids within the Exo III motif, with ^{577}Tyr and ^{581}Asp of HSV Pol corresponding to ^{497}Tyr and ^{501}Asp of Pol I (Fig. 1). Mutant DE35 (D441A/E443A), which contains mutations at codons 441 (change from GAC to GGC) and 443 (change from GAA to GCA), was also constructed, because residues 441 and 443 resembled residues 368 and 370 of the Exo I motif and indeed were originally proposed as the conserved residues of the Exo I motif (3, 4) (Fig. 1). However, this mutant was not able to complement the replication of HP66 virus in Vero cells (see below). Attempts to purify the mutant protein from recombinant baculovirus-infected Sf9 cells were not successful because of its insolubility (37). This mutant, therefore, served as a negative control for this study.

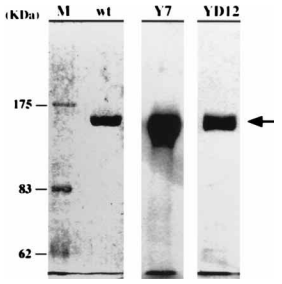

FIG. 2. Coomassie blue-stained gel of purified Pols. Wild-type (wt), Y7, and YD12 Pols expressed in Sf9 cells infected with recombinant baculovirus were purified as described in Materials and Methods. For each Pol, 10 μl of the peak fraction from the single-stranded DNA agarose column was analyzed separately on SDS–7.5% polyacrylamide gels which were stained with Coomassie blue. Positions of molecular weight markers (lane M) are shown at the left.

Effects of Exo III mutations on polymerase and 3′-5′ exonuclease activities. To examine the effects of these mutations on polymerase and exonuclease activities, recombinant baculoviruses harboring the mutated Y7 and YD12 *pol* genes were constructed and plaque purified. Recombinant viruses BV-Y7 and BV-YD12 and the virus containing the wild-type HSV-1 *pol* gene, BBP-3, were used to infect Sf9 cells. Proteins expressed from these recombinant baculoviruses were then purified to apparent homogeneity, as indicated by analysis on SDS-polyacrylamide gels (Fig. 2). When the polymerase activities were examined by their abilities to incorporate nucleotides into activated calf thymus DNA, Y7 and YD12 mutant Pols exhibited polymerase activity indistinguishable from and only about 20% less than that of the wild-type Pol, respectively (Table 1). Therefore, single or double mutations at amino acids 577 and 581 in Y7 and YD12 had at most modest effects on polymerase activity as measured by this assay.

Mutant Pols were also examined for 3′-5′ exonuclease activity by using primer-templates containing 5′-end-labeled primers as substrates. Exonucleolytic products were resolved by denaturing polyacrylamide gel electrophoresis and quantified as described in Materials and Methods. Note that some material (~2%) migrated on the gels at a position ~1 base smaller than full-length primer even with no enzyme added (Fig. 3, lane 1). This value was subtracted for the purposes of quantification.

The 3′-5′ exonuclease-proficient Klenow fragment of Pol I was included as a positive control, and its ability to degrade the 3′-end nucleotides and form a ladder of bands is shown in Fig.

TABLE 1. Polymerase and exonuclease activities of the wild-type and mutants Y7 and YD12 Pols

Pol	Polymerase activity[a] (cpm)		Exonuclease activity,[b] 1 pmol (%)
	1 pmol	10 pmol	
Wild type	4.9×10^5 (100)	4.1×10^6 (100)	100
Y7	4.8×10^5 (97)	4.0×10^6 (99)	2
YD12	4.2×10^5 (84)	3.3×10^6 (79)	0
None	3.2×10^3 (0)	3.2×10^3 (0)	0

[a] Analyzed by using 25 μg of activated calf thymus DNA. One or 10 pmol of HSV Pol was used to determine the polymerase activity. The amounts of ^{32}P-labeled dCTP incorporated were measured in a liquid scintillation counter. The counts per minute incorporated by the wild-type Pol minus the counts per minute found with no enzyme added is defined as 100%. The relative activities of the different polymerases (percentages) are shown in parentheses.

[b] Assayed by using a 5′-labeled 16-mer primer and a 25-mer template as described in Materials and Methods. Two microliters of each reaction mixture was loaded on a 20% denaturing acrylamide gel containing 8 M urea. The 3′-5′ exonuclease activity, indicated by a reduction in size of the labeled oligonucleotide, was detected by autoradiography (Fig. 2). The integrated band intensity of smaller species was quantified with a PhosphorImager, the band intensity found in the absence of enzyme was subtracted (~2% of the intensity of full-length primer), and the relative activity of each mutant Pol was compared to that of the wild-type Pol (defined as 100% for 1 pmol of Pol used).

3 (lanes 2 and 3). Similarly, wild-type HSV Pol exhibited substantial 3′-5′ exonuclease activity (lanes 4 and 5). In contrast, the Y7 mutant Pol exhibited very little exonuclease activity (Fig. 3, lanes 6 and 7), which when quantified corresponded to only 2% that of wild-type Pol (Table 1). The YD12 mutant Pol was even more impaired and exhibited no detectable activity above the background of the assay (Fig. 3, lanes 8 and 9; Table 1). Thus, this mutant Pol is at least 50-fold impaired for exonuclease activity. Results with YD12 were comparable to the results with the 3′-5′ exonuclease-deficient version of T7 DNA polymerase (Sequenase), which also failed to detectably cleave

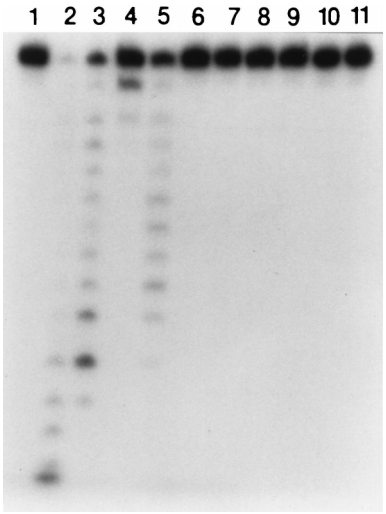

FIG. 3. Autoradiograph of an exonuclease activity assay. The 16- and 25-mer double-stranded primer-templates were used as substrates for the exonuclease assay as described in Materials and Methods. 3′-5′ exonuclease activity was demonstrated by its ability to cleave the 3′-end base from the 5′-end-labeled primers and form the ladder of bands on the gel. Lane 1, no enzyme added (primer-template alone); lanes 2 and 3, Klenow fragment, 0.5 and 0.05 U, respectively; lanes 4 and 5, wild-type HSV Pol, 0.1 and 1 pmol, respectively; lanes 6 and 7, mutant Y7 Pol, 1 and 0.1 pmol, respectively; lanes 8 and 9, mutant YD12 Pol, 1 and 0.1 pmol, respectively; lanes 10 and 11, Sequenase, 1.3 and 0.13 U, respectively. The relative activity of each mutant was compared to that of the wild-type Pol (Table 1).

the labeled primers (Fig. 3, lanes 10 and 11). Similar results were obtained for each enzyme when the same primer was used as a substrate in the absence of template or when an oligonucleotide with a stable hairpin structure was used as the substrate (37). Therefore, the Y7 and especially the YD12 mutant were severely impaired for 3′-5′ exonuclease activity.

Characterization of Exo III mutants for *pol* function in transient transfection assays. Complementation assays were applied to examine the ability of mutant Y7 and YD12 *pol* genes to support viral replication. A wild-type *pol* gene and a *pol* gene containing mutations at codons 441 and 443 (DE35) were included as positive and negative controls, respectively. The various *pol* genes were cloned into expression vector pSVK3, which contains an SV40 promoter for transient expression of Pol upon transfection into Vero cells. Results of six independent experiments using two different transfection methods and two different complementing cell lines for analyzing progeny viruses recovered from Vero cells are shown in Table 2. When no plasmid was used, little or no progeny virus was recovered (Table 2, experiments I and III). In all assays, both mutants Y7 and YD12 increased the yield of *pol* null mutant, HP66, substantially more than did the no-plasmid control (greater than 50-fold), albeit less efficiently than the wild-type Pol. In contrast, mutant DE35 failed to complement HP66 in nonpermissive Vero cells. Similar efficiencies were obtained when different batches of plasmid DNAs and HP66 stocks were used. Interestingly, relatively higher complementation efficiencies of the Exo III mutants were obtained when progeny viruses were titered on DP6 cells (Table 2); this may reflect a decrease of the ability of Pol A5 cells to support mutant virus replication.

Replication fidelity of the Exo III mutant Pols in transient experiments. To examine the effects of Exo III mutants on the fidelity of DNA replication, we performed a modified *tk* mutagenesis assay to examine the frequency of GCV-resistant mutants, which are likely to be *tk* mutants (29, 33). The established *tk* mutagenesis assay (29, 33) requires the use of recombinant viral stocks. At the earlier stages of this study, such recombinant viruses were not available. Therefore, Pol required for DNA replication was transiently provided by transfection of each mutant Pol expression plasmid in order to complement the growth of HP66 in Vero cells. By this approach, the frequencies of drug-resistant mutants transiently induced by these Exo III mutants and the wild-type Pol were determined by the ratio of the titers of the HP66 progeny viruses in the presence or absence of GCV (33).

Using this modified mutagenesis assay, the mutation frequency of HP66 was about 3.5×10^{-5} if the wild-type Pol was provided in *trans* to induce the replication of HP66 virus (Table 2, experiments V and VI). This mutation frequency induced by the wild-type Pol is in the lower end of the range reported when the wild-type KOS virus was used (29, 33). Mutants Y7 and YD12 induced 20- to 80-fold-higher mutation frequencies (Table 2, experiments V and VI). Therefore, in this assay, both Y7 and YD12 mutants exhibited mutator phenotypes (29).

Isolation of Exo III recombinant viruses. To construct recombinant viruses containing Exo III mutations, infectious HP66 DNA was cotransfected with Y7 or YD12 plasmid DNA. Recombinant viruses were isolated from the ensuing progeny by screening for white plaques on Pol A5 cells in the presence of X-Gal (49). About 0.6% of progeny viruses from each transfection experiment formed white plaques. Two of 18 Y7 white plaques derived from two independent transfections of Y7 DNA and 2 of 20 YD12 white plaques derived from two independent transfections of YD12 DNA contained the desired Y7 or YD12 mutation. This result implied a marker transfer fre-

TABLE 2. Complementation assays and mutation frequencies transiently induced by Exo III mutants

Expt[a]	Titer (PFU/ml)		Total PFU	Complementation[b] (%)	No. of GCV-resistant mutants	Mutation frequency	Relative ratio[c]
	DP6 and Pol A5[d]	Vero					
I							
pSVK-pol	4.8×10^4	3.0×10^0	1.2×10^5	100	ND[e]	ND	ND
pSVKY7	1.4×10^4	7.2×10^1	3.4×10^4	28	ND	ND	ND
pSVKYD12	3.1×10^3	$<1 \times 10^0$	7.4×10^3	6.2	ND	ND	ND
pSVKDE35	4.0×10^0	3.0×10^0	3.0×10^0	2.5×10^{-3}	ND	ND	ND
No plasmid	2.0×10^0	1.0×10^0	3.0×10^0	2.5×10^{-3}	ND	ND	ND
II							
pSVK-pol	4.5×10^4	$<1 \times 10^0$	1.1×10^5	100	ND	ND	ND
pSVKY7	1.2×10^4	$<1 \times 10^0$	2.9×10^4	26	ND	ND	ND
pSVKYD12	3.5×10^3	$<1 \times 10^0$	8.4×10^3	7.6	ND	ND	ND
III							
pSVK-pol	3.0×10^4	$<1 \times 10^0$	7.2×10^4	100	0		
pSVKY7	5.0×10^2	$<1 \times 10^0$	1.7×10^3	2.3	1	6×10^{-4}	
pSVKYD12	9.5×10^2	$<1 \times 10^0$	1.7×10^3	2.3	8	5×10^{-3}	
No plasmid	$<1 \times 10^0$	$<1 \times 10^0$	$<1 \times 10^0$	$<1.4 \times 10^{-3}$	0		
IV							
pSVK-pol	1.2×10^4	$<1 \times 10^0$	4.2×10^4	100	0		
pSVKY7	1.6×10^2	$<1 \times 10^0$	4.8×10^2	1.1	1	2×10^{-3}	
pSVKYD12	2.3×10^2	$<1 \times 10^0$	8.5×10^2	2.0	6	7×10^{-3}	
V							
pSVK-pol	6.5×10^4	3.8×10^1	1.6×10^5	100	6	4×10^{-5}	1
pSVKY7	1.7×10^3	2.0×10^0	4.3×10^3	2.7	3	7×10^{-4}	20
pSVKYD12	1.2×10^3	$<1 \times 10^0$	3.0×10^3	1.9	9	3×10^{-3}	80
VI							
pSVK-pol	5.0×10^4	3.4×10^1	1.3×10^5	100	4	3×10^{-5}	1
pSVKY7	2.3×10^3	4.6×10^1	5.8×10^3	4.5	6	1×10^{-3}	30
pSVKYD12	2.2×10^3	$<1 \times 10^0$	5.5×10^3	4.2	8	2×10^{-3}	70

[a] The DEAE-dextran-DNA transfection technique was applied in experiments I to IV, and LipofectAmine transfection reagent was used in experiments V and VI.
[b] The complementation efficiency of the wild-type Pol for the growth of HP66 in Vero cells was defined as 100% in each experiment. The relative ability of each mutant *pol* to complement the growth of HP66 in nonpermissive Vero cells was compared to that of the wild-type *pol* (pSVK-pol) and expressed as the percentage.
[c] Mutants were selected by their resistance to 20 μM GCV, and mutation frequencies were determined as described in Materials and Methods. The increased rates of the mutation frequencies were determined as the ratio of the mutation frequency of each mutant Pol to that of the wild-type Pol from the same experiment.
[d] HSV-1 polymerase-expressing cell lines DP6 and Pol A5 were used in experiments I and II and experiments III to VI, respectively, for complementation assays.
[e] ND, not determined.

quency comparable to those obtained with plasmid containing wild-type DNA or nonlethal mutation (22, 47). The mutation containing viruses were plaque purified once further on Pol A5 cells. However, 4 of 10 plaques derived from a Y7 plaque that contained the desired mutation now contained wild-type *pol* sequences, which could be the result of recombination between mutant viruses and the *pol* gene in Pol A5 cells.

Because of these results, we were concerned that further plaque purification and propagation on Pol A5 cells might give rise to wild-type virus that could confound further experiments. We therefore tested the abilities of the Y7b and YD12a recombinants that contained the desired Exo III mutations to form plaques on Vero cells. As shown in Table 3, both mutants, which were previously propagated only on Pol A5 cells, readily formed plaques on Vero cells, albeit with efficiencies two- to threefold less than that of KOS. Therefore, these Y7b and YD12a recombinants were plaque purified twice further on Vero cells to obtain Y7b.v and YD12a.v, respectively. Again, the mutated Exo III sequence within these purified recombinants were confirmed by sequencing. The plating efficiencies of these four-time-plaque-purified recombinants were not meaningfully different from those of the original recombinants that had been isolated from Pol A5 cells. This result indicated that there was little or no selective pressure on the Exo III mutants during passage in Vero cells. We also measured the yields of Y7b.v and YD12a.v viruses on Vero cells in a single-step growth experiment. As shown in Table 4, the virus yields of these two mutants were only ~3-fold less than that of KOS. These results suggest that the Exo III mutations are not lethal to viral replication, although they do appear to reduce plaque formation and yield modestly.

Replication fidelity of the Exo III recombinant viruses. With these recombinant viruses available, a *tk* mutagenesis assay (29, 33) was performed to examine the fidelity of DNA replication. In an initial experiment, we tested two independent Y7 recombinant viruses and two independent YD12 recombinants from the first round of plaque purification (see above). Remarkably, one isolate of each mutant was resistant to 100 μM GCV due to the mutation in the *tk* gene (37). Based on this

TABLE 3. Plating efficiencies of recombinant Y7 and YD12 viruses

Virus[a]	Titer (PFU/ml) on:		Plating efficiency (%)[b]
	Pol A5 Cells	Vero cells	
Y7b	1.5×10^6	7.5×10^5	50
YD12a	2.2×10^6	8.5×10^5	39
Y7b.v	4.0×10^6	2.1×10^6	53
YD12a.v	2.2×10^6	1.0×10^6	45
KOS	5.7×10^7	5.5×10^7	97

[a] Recombinant viruses Y7b and YD12a were plaque purified twice on Pol A5 cells and confirmed by sequencing to contain the expected mutation(s). These recombinants were then further purified twice on Vero cells to obtain Y7b.v and YD12a.v. These isolates were confirmed by sequencing to contain the expected mutation(s).
[b] Determined as the ratio of the titers on Vero cells and Pol A5 cells.

TABLE 4. Single-step growth analysis

Virus	Virus yield in PFU/ml (% of wild-type yield)
KOS	2.3×10^7 (100)
Y7b.v	7.0×10^6 (30)
YD12a.v	7.0×10^6 (30)

finding and the relatively high mutation rates obtained from transient experiments (Table 2), we modified the mutagenesis assay as described in Materials and Methods. Three plaques of GCV-sensitive Y7b.v virus and three plaques of GCV-sensitive YD12a.v virus were isolated and amplified in Vero cells. Each stock was examined for the mutation frequency (Table 5). Ten independent virus stocks were then prepared from one of three parental stocks which had the lowest *tk* mutation rate (Table 5). Only ~5 PFU was inoculated into Vero cells to avoid preexisting *tk* mutants (29). Both the Y7 and YD12 Exo III mutants exhibited an average mutation rate of 4.1×10^{-2} (range from 2.6×10^{-3} to 24%) and 1.7×10^{-2} (range from 1.4×10^{-3} to 8.4%), respectively. In contrast, on average the mutation frequencies of three wild-type KOS stocks analyzed in this study were only 5×10^{-5}. These results correspond to an ~800- and ~300-fold increases of mutation rates for Y7 and YD12 mutant Pols, respectively, in comparison to that of the wild-type Pol.

DISCUSSION

In this study, we examined the effects of mutations within the Exo III motif of the HSV-1 DNA polymerase on polymerase and exonuclease activities, viral replication, and replication fidelity. The Exo III mutations were not lethal, despite their severe effects on exonuclease activities. This represents the first report, to our knowledge, of HSV *pol* mutants with severely impaired 3′-5′ exonuclease activity and of the effects of altered exonuclease activity on the fidelity of DNA replication in virus-infected mammalian cells.

Effects of Exo III mutations on HSV Pol enzyme activities. Our results demonstrated that two Exo III mutants are severely impaired for exonuclease activity yet retain substantial polymerase activity in vitro (Table 1). Consistent with these results, Kühn and Knopf (41) found that a mutation of ^{577}Tyr to phenylalanine (similar to the Y577H mutation in Y7) and a mutation of ^{581}Asp to alanine (identical to one of the mutations in YD12) decreased exonuclease activity 6- and 16-fold, respectively, while decreasing polymerase activity less than 30%. Our results are also consistent with those obtained with Exo III mutants in other systems. In the case of Pol I, ^{497}Tyr and ^{501}Asp are critical for catalytic activity and metal ion binding of the 3′-5′ exonuclease (13, 14, 20), respectively; our results and those of Kühn and Knopf (41) are consistent with similar roles for the corresponding residues ^{577}Tyr and ^{581}Asp of HSV-1 Pol.

A continuing issue in studies of polymerases is the relative independence of different enzymatic activities. The results reported here and elsewhere (30, 41) in which various Exo mutations severely impair the exonuclease activity of HSV Pol with little effect on polymerase activity can be simply interpreted to mean that the two enzyme activities are functionally independent. However, the validity of this interpretation depends on whether the in vitro assays of polymerase activity reflect all aspects of that activity. The in vitro polymerase assays used in this and other (30, 41) studies of HSV Exo mutants that appear to retain polymerase activity have mainly entailed short extensions across gapped or single-stranded templates. Even with this assay, Exo III mutants had displayed modest reduction in polymerase activity (reference 41 and this report). It also remains possible that these HSV Exo mutations have effects on other aspects of polymerase activity such as processivity. Along these lines, an Exo III mutation caused a defect in the strand displacement activity of ϕ29 DNA polymerase (54).

Interestingly, the Exo III motif is contained within δ region C, a region that is found in DNA polymerase δ and ε and certain viral polymerases (Fig. 1). Mutations both upstream and downstream of Exo III in δ region C can confer drug resistance in vitro and/or in infected cells (23, 41, 43) are thus presumed to affect the binding of deoxynucleoside triphosphate and PP_i, which the drugs mimic. For at least some of the mutants, enzyme kinetic studies support this presumption (15, 32). Similarly, preliminary data indicate that our two Exo III mutant Pols are resistant to phosphonoacetic acid (37) in vitro. This finding suggests that Exo III mutations not only alter the exonuclease activity but also influence the interactions between polymerase and PP_i. Regardless, given that certain Exo

TABLE 5. Mutation frequencies of the *tk* genes induced by the Exo III recombinant viruses

Virus stock	ACV^r mutation frequency
Y7 parental stock[a]	1.7×10^{-3}
Y7.A	4.3×10^{-3}
Y7.B	2.6×10^{-3}
Y7.C	8.0×10^{-2}
Y7.D	5.9×10^{-3}
Y7.E	4.4×10^{-3}
Y7.F	3.4×10^{-3}
Y7.G	2.4×10^{-1}
Y7.H	4.5×10^{-3}
Y7.I	5.3×10^{-2}
Y7.J	9.1×10^{-3}
Avg	4.1×10^{-2}
YD12 parental stock[b]	1.1×10^{-3}
YD12.A	5.6×10^{-2}
YD12.B	1.4×10^{-2}
YD12.C	1.4×10^{-3}
YD12.D	1.4×10^{-3}
YD12.E	6.7×10^{-3}
YD12.F	1.9×10^{-3}
YD12.G	3.0×10^{-3}
YD12.H	8.4×10^{-2}
YD12.I	1.7×10^{-3}
YD12.J	3.0×10^{-2}
Avg	1.7×10^{-2}
KOS[c]	
KOS.A	4.9×10^{-5}
KOS.B	5.3×10^{-5}
KOS.C	5.0×10^{-5}
Avg	5.1×10^{-5}

[a] The Y7 isolate having the lowest mutation frequency of three independent isolates (the other two isolates had frequencies of 3.1×10^{-3} and 3.2×10^{-3}) was used to prepare 10 independent stocks and assayed as described in Materials and Methods.

[b] The YD12 isolate having the lowest mutation frequency of three independent isolate (the other two isolates had frequencies of 1.9×10^{-3} and 6.7×10^{-3}) was used to prepare 10 independent virus stocks and assayed as described in Materials and Methods.

[c] Three independent KOS stocks were prepared and assayed for mutation frequency. The mutation frequency of the wild-type KOS strain is within the range observed in previous studies (28, 32).

II mutations can affect polymerase activity, even when measured using activated DNA templates (25, 41), caution should be exercised in ascribing the importance of specific conserved motifs to one enzymatic function, but not another.

Is the exonuclease activity of HSV Pol essential for viral replication? Although the Exo III mutations reduced exonuclease activity at least 50-fold, they were not lethal. They did reduce the ability of *pol* plasmids to complement the replication of a *pol* null mutant (Table 2) and resulted in a modest reduction in plating efficiency (Table 3) and virus yield (Table 4) in Vero cells. (The greater effect of mutations on replication in transient complementation assay may be due to incompletely appropriate expression of the *pol* gene under the control of an SV40 promoter.) Thus, one interpretation of our results is that the exonuclease activity of HSV Pol is not essential for polymerase function in vivo. However, it is possible that the YD12 Pol contains a small amount of exonuclease activity, which is beyond the sensitivity of our assays, and/or that the exonuclease activity may function to at least a limited extent in infected cells, for example, in the presence of other replication proteins. Nevertheless, the mutator phenotypes of Y7 and YD12 strongly suggest that exonuclease activity is substantially impaired in infected cells. Thus, our results argue that HSV can replicate even when exonuclease activity is severely impaired.

Our results contrast with those of Hall et al. (30), who were unable to recover HSV mutants containing various Exo mutations including Y581A, which is included in YD12 and which evidently exerts a more modest effect on exonuclease activity than does YD12 (41). Hall et al. (30) raised the possibility, among several, that 3′-5′ exonuclease activity was required for HSV replication to prevent intolerable increases in mutation frequency. One possible explanation for the previous failure to recover Exo mutants is the necessity of using a complementing cell line. The Exo mutations studied here did reduce the replication of HSV and would likely have been difficult to recover without the use of complementing cells. It could be argued that these mutants may have acquired additional mutation within the *pol* gene or the other replicative genes which may suppress the effects of the Exo III mutations. Although we cannot rule out this possibility completely, we know that these mutants are recovered in complementing cells at a frequency comparable to those of other nonlethal mutations (22, 47) and are able to form plaque on Vero cells without previously having been passaged on them. Moreover, we have not found any other change at the amino acid level within the entire open reading frame of the *pol* gene of the Y7b.v mutant (37). Further study is required to determine whether there is a specific requirement for 3′-5′ exonuclease activity for HSV replication.

Effects of exonuclease activity on the fidelity of DNA replication. 3′-5′ exonuclease activities associated with replicative polymerases have proofreading functions to improve replication fidelity. Mutant polymerases with defective exonuclease activity can increase the mutation frequency up to 3 orders of magnitude (18, 42). We found that Y7 and YD12 Pols increased mutation frequencies 20- to 80-fold in transient replication assays (Table 2) and 300- to 800-fold in assays of recombinant viruses. The difference in the magnitudes of the increases between the two assays is probably due to the transient assay accumulating mutations during one replication cycle, whereas the recombinant virus assay accumulates mutations over many replication cycles. The increases in mutation frequency induced by the Exo III mutant Pols can be readily explained by their altered exonuclease activities. To our knowledge, we have provided the first evidence of a contribution of exonuclease proofreading activity to replication fidelity in virus-infected mammalian cells. It will be interesting to determine the effects of the mutations on the removal and extension of mispaired primer termini. Kühn and Knopf (41) found that the D581A mutation permitted extension of mispaired primer termini.

Fifteen years ago, Hall and Almy (28) found that a mutation closely linked to the HSV *ts*C7 *pol* mutation in a ts^+ derivative of *ts*C7 confers a mutator phenotype. The *ts*C7 mutation maps within the 5′ portion of the *pol* gene (11) that encodes the N-terminal half of Pol that is sufficient for exonuclease activity. It is therefore tempting to speculate that the mutator phenotype observed by Hall and Almy (28) was due to an exonuclease defect.

Several virus stocks of Y7 and YD12 exhibited extremely high mutation frequencies, up to 24%, for both recombinants (Table 5). (Fluctuations in mutation frequencies were observed, as expected [45].) It is possible that these high frequencies were due to preexisting drug-resistant mutants that might have been inoculated in the assay; however, this seems unlikely as only 5 PFU was inoculated from stocks containing only ~1 mutant per 1,000 PFU. It is also conceivable that such high frequencies might reflect the evolution of even more mutagenic viruses from the Y7 and YD12 mutants. Regardless, we speculate that high mutation frequencies could have clinical significance. Although the mutants reported here exhibit some defects in replication, it is possible that mutator strains of HSV can cause disease, particularly in immunocompromised patients. Such strains might be especially likely to give rise to drug-resistant mutants upon antiviral therapy. It may be of interest to examine HSV strains associated with clinical drug resistance for their mutation frequencies.

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