A Point Mutation within Conserved Region VI of Herpes Simplex Virus Type 1 DNA Polymerase Confers Altered Drug Sensitivity and Enhances Replication Fidelity

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Herpes simplex virus type 1 (HSV-1) DNA polymerase contains several conserved regions within the polymerase domain. The conserved regions I, II, III, V, and VII have been shown to have functional roles in the interaction with deoxynucleoside triphosphates (dNTPs) and DNA. However, the role of conserved region VI in DNA replication has remained unclear due, in part, to the lack of a well-characterized region VI mutant. In this report, recombinant viruses containing a point mutation (L774F) within the conserved region VI were constructed. These recombinant viruses were more susceptible to aphidicolin and resistant to both foscarnet and acyclovir, compared to the wild-type KOS strain. Marker transfer experiments demonstrated that the L774F mutation conferred the altered drug sensitivities. Furthermore, mutagenesis assays demonstrated that L774F recombinant viruses containing the *supF* marker gene, which was integrated within the thymidine kinase locus (*tk*), exhibited increased fidelity of DNA replication. These data indicate that conserved region VI, together with other conserved regions, forms the polymerase active site, has a role in the interaction with deoxyribonucleotides, and regulates DNA replication fidelity. The possible effect of the L774F mutation in altering the polymerase structure and activity is discussed.

DNA polymerase (Pol) is the pivotal enzyme involved in DNA replication and plays a central role in controlling the accuracy of DNA replication. Amino acid sequence alignment of various Pols reveals that all Pols contain conserved residues distributed within several regions of the Pol domain. Previous studies demonstrated that these conserved residues play an important role in the polymerization reaction (reviewed in reference 38 and references therein). These conserved residues interact with incoming deoxyribonucleotides (deoxynucleoside triphosphates [dNTPs]) and the primer-template DNA. Therefore, mutations within these residues could result in altered polymerase activity and/or replication fidelity.

Herpes simplex virus (HSV) has proven to be a good model for genetic, molecular biological, and biochemical studies of Pol, since it can be manipulated to contain desired mutations for further characterization regardless of whether the mutated Pol is lethal for viral replication (reviewed in reference 11 and references therein). Examination of the HSV-1 Pol also reveals seven conserved regions within the carboxyl-terminal half of the Pol, which has been defined as the polymerase domain (Fig. 1). Indeed, mutations within most of these conserved regions confer altered sensitivities to certain nucleoside and pyrophosphate analog antiviral drugs, indicating that they are involved in the interactions with dNTPs and DNA (11).

Since only a few region VI mutants of HSV have been

described (4, 5, 34), and the effects of these mutations on replication fidelity have not been characterized, we constructed recombinant HSV-1 viruses containing a region VI mutation (L774F) for characterizing the effects of this mutation on drug sensitivity and, especially, replication fidelity. The results demonstrated that such a mutation confers altered drug sensitivities and improves DNA replication fidelity. Thus, conserved region VI, together with other regions within the polymerase domain, cooperatively interacts with dNTPs and DNA and plays an important role in DNA polymerization.

MATERIALS AND METHODS

Cells and viruses. Vero, Pol A5, and V658A cells were maintained as described previously (24, 27). Recombinant viruses—including the *pol*-null mutant HP66 (31) and its derivative, HPF11, which contains the pSupF1 amplicon integrated in the *tk* locus—were propagated in Pol A5 cells as described previously (25). YD12 recombinant viruses (24) that were constructed previously to contain Exo III mutations (Y577H/D581A) (Fig. 1A), and the newly constructed L3 and L3/F mutants that contain a Leu-to-Phe mutation at amino acid residue 774 (within the conserved region VI) and the L774F mutation together with pSupF1 amplicon sequences within the thymidine kinase locus (*tk*), respectively, were propagated in Vero cells. Recombinant viruses derived from marker transfer experiments also were propagated in Vero cells. Table 1 lists and describes the recombinant viruses used in this study.

Plasmids and DNA fragments. Table 1 also lists and describes the plasmids and DNA fragments used in this study. The 3.3-kbp *Bam*HI fragment containing the majority of the *pol* sequence was isolated from YD12 virus-infected cell DNA and cloned into pUC18. This plasmid, pH629, then was sequenced to confirm the presence of a C-to-T change at nucleotide position 2320, with the A residue of the first ATG codon of the *pol* gene defined as nucleotide position 1. A 750-bp *Mst*II-*Not*I fragment was isolated from pH629 (Fig. 1B) and used to replace the corresponding sequences within the pSVK-*pol* plasmid (24) that contains wild-type *pol* gene, to obtain the clone pSKV-B3. pSKV-B3 was sequenced to confirm the presence of the desired change.

Plasmids pH647, pH654, and pH655, -656, and -657 were constructed by isolating the 3.3-kbp *Bam*HI fragment from recombinants L3-B, L3-A1, and

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FIG. 1. (A). HSV-1 Pol open reading frame with regions of sequence similarity. Conserved amino acid regions I to VII and δ region C, which overlaps the Exo III motif, among diverse DNA polymerases are shown as empty boxes. The numbers refer to amino acid residues of HSV Pol. Amino acid residues of the Exo III motif and mutated residues of Y7 and YD12 mutants are shown below the line. Also shown is the sequence alignment of conserved region VI of HSV-1 Pol and several eukaryotic and viral DNA Pols. Numbers refer to amino acid residues. Highly conserved amino acids are boxed. The highly conserved residues are shown below. The following polymerases are presented: HSV-1, Epstein-Barr virus (EBV) (3), varicella-zoster virus (VZV) (12), cytomegalovirus (CMV) (9), human herpesvirus 6 (HHV6) (8), human herpesvirus 7 (HHV7) (33), bovine DNA Pol δ (BOV Pol δ) (41), DNA polymerase δ of *Saccharomyces cerevisiae* (Yeast Pol δ) (7), human DNA polymerase α (Hum Pol α) (39), vaccinia virus (Vac Pol) (15), 1GCX (20), and bacteriophage RB69 (RB69 Pol) (36). (B) Map of the 3.3-kbp fragment of HSV-1. The 750-bp *MstII-NotI* fragment containing the region VI mutation of L774F was isolated from pHC629 and used to replace the corresponding fragment of pSVK-pol for the construction of pSVK-B3. The relative positions of the oligonucleotide primers used for sequencing analyses are shown below the line. The numbers refer to the nucleotide position of the 3' end of the primer, and arrows denote the direction of sequencing. SP6 and T7 primers correspond to sequences located outside the multiple cloning sites of the vector pGEM3Zf(+). (C). Relative locations of DNA fragments (see Table 1) used for the marker transfer experiments. Numbers refer to amino acid (a.a.) residues corresponding to 5' and 3' residues of each DNA fragment. An arrowhead indicates the position of the L774F mutation. B, *Bam*HI; M, *Mst*II; N, *Not*I.

L3-A4 (A1 and A4 were different plaque isolates from transfection sample A), respectively, and cloning into pGEM3Zf(+). The altered nucleotide corresponding to the L774F mutation in each plasmid was confirmed by a sequencing reaction using oligonucleotide primer 3028L (corresponding to nucleotides 2448 to 2439). The entire *pol* insert in pHC647 also was sequenced by using the following primers: T7, SP6, 430R (nucleotides 431 to 450), 1038R (nucleotides 1011 to 1029), 2655R (nucleotides 2075 to 2095), and 2736L (nucleotides 2156 to 2136).

Oligonucleotide primers *pol*-5' (corresponding to nucleotides -18 to -2 of the *pol* gene) and 2736L (nucleotides 2156 to 2137) were used to amplify the 2.0-kbp 5' half of the *pol* gene; and primers 2564R (nucleotides 1984 to 2002) and 3776L (nucleotides 3776 to 3757) were used to amplify the 1.8-kbp 3' half of the *pol* gene. DNA fragments used to transfer the *pol* marker included the 1.6 kbp of the 5' sequences of the *pol* (designated fragment A; between nucleotides -18 and 1593), a 967-bp fragment B (between nucleotides 1190 and 2156), an 827-bp fragment C (nucleotides 1984 to 2810), and a 1,031-bp fragment D (nucleotides 2745 to 3776). PCR products were sequenced as described previously (25).

Construction of recombinant viruses and marker transfer experiments. Recombinant viruses L3-A, -B, and -C were constructed by independent cotransfection experiments as follows: 5×10^5 Pol A5 cells plated on 60-mm² tissue culture plates were cotransfected with HP66 infectious viral DNA (31) and pSVK-B3 using Lipofectamine (Invitrogen) as described previously (24). Recombinant

viruses containing the L774F mutation within the *pol* sequence were screened on Pol A5 cells from the transfection progeny and selected as white plaques after staining with X-Gal (5-bromo-4-chloro-3-indolyl- β -D-galactopyranoside). Recombinant viruses L3/F-A and -B also were constructed independently by cotransfection of HPF-11 infectious viral DNA and pSVK-B3 into Pol A5 cells as described above. To marker transfer the *pol* marker of the L3 recombinant virus, Vero cells were cotransfected with KOS infectious viral DNA, and different DNA fragments that were PCR amplified from L3-C-infected cell DNA by using Lipofectamine reagent. The resulting progeny obtained from independent transfection experiments were plaque assayed with or without fos-carnet (PFA). Plaques grown under 120 μ g of PFA per ml were selected and amplified in Vero cells for subsequent characterization. Recombinant viruses containing the L774F mutation were verified first by the loss of the *Dde*I restriction site corresponding to the nucleotides coding for amino acid residues 774 and 775. Sequencing of either the PCR-amplified *pol* sequences or the cloned 3.3-kbp *Bam*HI fragments was used to confirm that the C-to-T change at nucleotide 2320 resulting in the L774F mutation being the only change introduced. Each recombinant virus was plaque purified twice before further characterization.

Mutagenesis assay. The mutagenesis assay and data analyses were performed as described previously (23, 25). Briefly, 2×10^4 Vero cells were infected with recombinant virus at less than 200 PFU for 72 h. Total DNA, including that prepared from virions present in the supernatants, was extracted, purified, and

TABLE 1. Recombinant viruses, plasmids, and DNA fragments used in this study

Virus, plasmid or DNA	Description
Virus	
HP66	<i>pol</i> -null mutant derived from wild-type KOS strain (31)
HPF-11	<i>pol</i> -null mutant with integrated <i>pSupF-1</i> <i>E. coli</i> amplicon in the <i>tk</i> locus, constructed from HP66 (23)
Y7	Exonuclease-deficient Pol mutant containing Y577H mutation, constructed from HP66 (23–26)
YD12	Exonuclease-deficient Pol mutant containing Y577H/D581A/L774F, constructed from HP66 (24, 26)
L3-A, -B, and -C	Region VI Pol mutant containing L774F mutation, constructed from HP66
L3/F-A and -B	Region VI Pol mutant with integrated <i>pSupF-1</i> amplicon in the <i>tk</i> locus, constructed from HPF-11
K831-K843	Marker transfer recombinants derived from L3 virus
Plasmids	
pSVK-pol	Wild-type <i>pol</i> inserted in vector pSVK3 (24)
pSVK-B3	L774F mutated <i>pol</i> inserted in pSVK3
pHC629	3.3-kbp <i>Bam</i> HI fragment isolated from YD12 viruses and cloned into pUC18
pHC647	3.3-kbp <i>Bam</i> HI fragment isolated from L3-B viruses and cloned into pGEM3Zf(+)
pHC654 and -655	3.3-kbp <i>Bam</i> HI fragment isolated from L3-A1 viruses and cloned into pGEM3Zf(+)
pHC656 and -657	3.3-kbp <i>Bam</i> HI fragment isolated from L3-A4 viruses and cloned into pGEM3Zf(+)
PCR DNA fragments^a amplified from L3-C viruses DNA	
A (1.6 kbp)	Contains the <i>pol</i> sequences between nucleotides –18 and 1593
B (967 bp)	Contains the <i>pol</i> sequences between nucleotides 1190 and 2156
C (827 bp)	Contains the <i>pol</i> sequences between nucleotides 1984 and 2810, including the L774F mutation
D (1,031 bp)	Contains the <i>pol</i> sequences between nucleotides 2745 and 3776 (downstream of the stop codon)
PCR DNA fragments amplified from L774F viruses DNA for sequencing	
5'-half (2.0 kbp)	Contains the <i>pol</i> sequences between nucleotides –18 and 2137
3'-half (1.8 kbp)	Contains the <i>pol</i> sequences between nucleotides 1984 and 3757 (downstream of the stop codon)

^a Shown in Fig. 1C.

digested with *Bam*HI followed by self-ligation with T4 DNA ligase. After phenol-chloroform extraction and ethanol precipitation, aliquots of DNA were electroporated into *Escherichia coli* host MBM7070. Transformants were plated onto Luria-Bertani agar plates containing X-Gal, IPTG (isopropyl- β -D-thiogalactopyranoside), and ampicillin. White and light blue colonies were identified, and the mutation frequency was determined as the ratio of the number of white and light blue colonies to the total number of colonies recovered.

Plaque assays. Plaque reduction assays were performed to determine relative sensitivity of the virus to aphidicolin, acyclovir (ACV), and PFA (Sigma) as described previously (26) with either Vero or V658A cells as indicated.

DGGE. Denaturing gradient gel electrophoresis (DGGE) was performed as described previously (29, 30) with the following modification. Briefly, a 465-bp DNA fragment containing the conserved region VI and flanking sequences was PCR amplified with primers 2564R and 3028L, corresponding to nucleotides 1984 to 2002 and 2448 to 2429, respectively. The PCR fragments amplified from wild-type, YD12, and other control DNA were examined for their migration profiles of the homoduplex and heteroduplex bands by loading the samples on a DGGE gel containing 8% polyacrylamide with a linear gradient of 55 to 75% denaturants (100% denaturants equal to 7 M urea and 40% deionized formamide), followed by electrophoresis with 0.5× Tris-acetate EDTA (TAE) buffer at 150 V and 60°C for 8 h. The gel was stained with ethidium bromide and photographed under UV transillumination. Heteroduplexes were prepared by mixing both wild-type and experimental DNA fragments, heating at 95°C for 5 min, and gradual cooling to room temperature.

Sequence alignments. A position-specific iterated (PSI)-BLAST search (2) was performed with the National Center for Biotechnology Information server (<http://www.ncbi.nlm.nih.gov/blast>). This method has the advantage of detecting distantly related proteins. Three rounds of PSI-BLAST were done in order to

obtain an alignment between HSV Pol and the previously determined polymerases, whose structure has been determined, which includes the replicating complex of RB69 Pol (18).

Statistics. The statistical significance (*P* value) of the differences between the mutation frequencies induced by the viruses was examined by tests of differences between proportions (10). The chi-square values of goodness-of-fit tests also were used to compare the patterns of the types of mutations induced by different viruses (17).

RESULTS

We previously had constructed HSV-1 recombinants (Y7 and YD12) to contain mutations within the conserved Exo III motif of Pol. These mutant Pols exhibited defective or undetectable exonuclease activity, yet retained substantial polymerase activity. Furthermore, recombinant viruses containing the mutated Pol displayed only modestly defective replication in Vero cells and exhibited substantially increased mutation frequencies in the viral *tk* (23) and *supF* (25, 27) genes accompanying altered spectra of mutations (23, 29). Further characterization of these Pol mutants demonstrated that the Y7 exonuclease-deficient Pol mutant could generate heterogeneous viruses containing mutations in genes coding for DNA replicative proteins, including Pol and its accessory protein

UL42 (26). These suggested that the YD12 Pol mutant also could replicate additional mutations, including the *pol* gene.

Detection of L774F mutation. The DGGE was first developed by Fischer and Lerman (16) and has been applied widely for studies of DNA polymorphism and for detection of mutations in certain genes. This method is highly sensitive for detecting nearly 100% of single-base changes in a defined DNA fragment within a gradient gel containing increasing concentration of denaturants. Basically, it is based on the differential melting behavior of double-stranded DNA molecules containing as little as a single nucleotide difference (1). When DNA molecules are melted differentially due to differences in nucleotide sequences, the mobility of the molecule in a denaturing gel will be altered dramatically (i.e., because of a change of conformation). Therefore, examination of differences in the mobility of DNA samples in a DGGE gel will indicate whether the DNA molecule contains mutations.

Although this technique has not been applied to examine the HSV-1 *pol* gene, our previous experience with examining the mutation spectra of HSV-1 *tk* mutations (29, 30) suggested that mutations within the *pol* gene, despite its high G+C content, also could be detected by the DGGE technique. Our initial attempt at applying the DGGE technique to examine the *pol* gene revealed that a 445-bp DNA fragment spanning sequences between nucleotides 1984 and 2429 amplified by native primers (without the use of modified GC-clamping primers) exhibited a melting profile suitable for DGGE gel analysis (data not shown). Therefore, we performed experiments to examine whether Y7 and YD12 recombinant viruses could generate mutations within this region of the *pol* gene. Not to our surprise, the DGGE results demonstrated that the DNA fragment containing the conserved region VI amplified from YD12-infected cell DNA contained heteroduplex bands (Fig. 2, lane 5). This also indicated heterogeneity in the population of YD12 recombinants, including the wild-type sequences within this DNA fragment, during the early passages, as well as the change within this DNA fragment that evolved during or prior to the second passages of the virus. Subsequent passages of YD12, the 5th and 10th passages (Fig. 2, lanes 4 and 3, respectively), resulted in the loss of viruses containing the wild-type sequences of this DNA fragment. This was manifested by the presence of only the homoduplex band, which migrated at a position identical to the homoduplex band amplified from pHC629 (lane 6), the plasmid containing the 3.3-kbp *Bam*HI fragment of the *pol* gene isolated from YD12-infected cell DNA. Subsequent mixing experiments demonstrated this further. For example, the mixture of DNA fragments amplified from YD12 and pHC629 showed a homoduplex band (Fig. 2, lane 10), indicating that these DNA fragments contained identical sequences. The mixtures of DNA fragments amplified from a wild-type *pol* clone and YD12-infected cell DNA (lane 7), from KOS- and YD12-infected cell DNA (lane 8), and from wild-type *pol* and pHC629 plasmid DNA (lane 9), demonstrated the formation of heteroduplex bands, indicating that these DNA fragments contained different nucleotide sequences. Since these heteroduplex bands migrated with a pattern identical to those found in the second passage of the YD12 virus, these DNA fragments should contain the same change. Interestingly, Y7 recombinant virus, containing the Y577H mutation, did not evolve to include this additional change, since it showed a homoduplex band (lane

FIG. 2. DGGE analysis of *pol* mutations. PCR-amplified DNA fragments, spanning nucleotides 1984 to 2448 of the *pol* gene, of various *pol* mutants were examined by DGGE as described in Materials and Methods. The formation of heteroduplex bands indicates the presence of mutations within the samples, either a single sample or a mixture of two samples. Lanes: 1, KOS, wild-type HSV-1 strain; 2, Y7, an experimental stock (10th passage) of an exonuclease-deficient Pol mutant; 3, YD12, an experimental stock (10th passage) of the YD12 exonuclease-deficient mutant; 4, YD12a1v2, 5th passage of a plaque-purified YD12 recombinant; 5, YD12a1, 2nd passage of a plaque-purified YD12 recombinant; 6, pHC629, the 3.3-kb *Bam*HI clone of the YD12 *pol* gene; 7, wild-type full-length clone of the *pol* gene (*pol*) plus YD12; 8, KOS plus YD12; 9, Pol plus pHC629; 10, YD12 plus pHC629. *, homoduplex band of wild-type DNA; o, homoduplex band of mutant DNA; arrows, heteroduplex bands formed between wild-type and mutant DNA.

2) identical to the wild-type strain KOS (lane 1). Sequencing of the entire *pol* gene PCR amplified from the Y7 recombinant virus DNA (26), which does not contain mutation resulting in an altered amino acid sequence, confirmed that result. However, subsequent passages of Y7 recombinant viruses were found to contain different *pol* mutations (unpublished data).

Sequencing of pHC629 and the DNA fragments amplified from YD12-infected cell DNA, but not those from the wild-type *pol* clone or the infected cell DNA of KOS and Y7 viruses, identified that these samples contained a C-to-T mutation at nucleotide position 2320. Further, such a mutation resulted in a leucine (coded by CTC) to phenylalanine (TTC) mutation at amino acid residue 774. Sequence alignment revealed that L774 is highly conserved among α -like Pols, including other herpesvirus Pols, as well as yeast and human Pol α (Fig. 1). The L774F mutation was dissected from the YD12 mutated *pol* containing the triple mutations, as described in Materials and Methods, to obtain the plasmid pSVK-B3 containing only the L774F mutation. Of note is that an independent YD12 recombinant also was found to contain an identical mutation (data not shown), suggesting that this mutation may be advantageous to the growth of recombinants containing only the Y577H/

D581A double mutations. The effects of triple mutations on viral replication and Pol kinetics relative to the double mutations are under investigation and will be reported separately. However, it remains unclear why Y7 recombinant viruses do not evolve to have this mutation. It is possible that the residual exonuclease activity of Y7 Pol (24) could execute an activity comparable to that of the triple mutations of YD12 Pol. Further experiments are required to answer this question.

L774F recombinants conferred altered drug sensitivities. Three recombinants L3-A, -B, and -C were constructed from independent transfection experiments as described in Materials and Methods. The mutated nucleotide coding for the L774F mutation also was confirmed by sequence analysis. Plaque reduction assays were used to examine the sensitivity of the mutants to aphidicolin, ACV, and PFA (22, 26). Vero cells were used for the plaque reduction assays for measuring aphidicolin and PFA sensitivity, whereas an HSV-1 TK-expressing cell line, V658A (27), was used for examining the sensitivity to ACV. The latter cells offer the advantage of demonstrating that all ACV-resistant plaques are due to the altered Pol's phenotype, since wild-type TK is provided within the cells. The L774F recombinants were hypersensitive to aphidicolin (Fig. 3A) and resistant to both ACV (Fig. 3B) and PFA (Fig. 3C) compared to the wild-type KOS strain. The 50% effective dose (ED_{50}), the concentration of drug to reduce plaque formation by 50%, for the wild type and the L774F mutant were determined directly from the plaque reduction curves of these drugs with the results shown in Table 2. These data imply that the region VI sequences of HSV-1 Pol, together with other conserved regions, form the polymerase active site and play a role in the interaction with deoxyribonucleotides during the polymerization reaction.

Marker transfer experiment. To determine whether the altered drug sensitivities demonstrated by the L3 recombinant viruses were the result of the L774F mutation, marker transfer experiments were performed. Among transfectants derived from four different PCR-amplified DNA fragments cotransfected with wild-type strain KOS infectious DNA, only DNA fragment C, containing the mutated region VI sequences (Fig. 1C), was able to produce progeny viruses capable of replicating on Vero cells in the presence of 120 μ g of PFA per ml, the ED_{50} for L774F recombinant viruses, at a frequency of 0.14%. None of the samples derived from DNA fragments A, B, and D (with frequencies of less than 0.0022, 0.0025, and 0.002%, respectively) or the one without L3 *pol* DNA (<0.005%) formed any plaques in this experiment. Fourteen recombinant viruses resulting from the marker transfer experiment were selected randomly and plaque purified for further analyses.

Infected cell DNA was extracted from 14 different plaques and used for PCR amplification of the 827-bp sequence, corresponding to fragment C. Since the CTC (wild type) to TTC (L774F) change results in the loss of a *DdeI* restriction site, the PCR products were subjected to *DdeI* digestion. All 14 PCR products were resistant to cutting with *DdeI* at this position, whereas an upstream *DdeI* site was retained. This resulted in the cleavage of the 827-bp fragment into 228- and 599-bp fragments, whereas the 599-bp fragment of the wild-type sequences was further cleaved into 108- and 491-bp fragments (Fig. 4). To confirm that these recombinants contained the TTC sequences coding for phenylalanine at residue 774, four

FIG. 3. Effects of aphidicolin (A), PFA (B), and ACV (C) on plaque formation by the wild type and L774F recombinants. Plaque reduction assays were performed as described in Materials and Methods. KOS is the wild-type HSV-1 strain; L3A, L3B, and L3C are three independent recombinants containing the L774F mutation; and K830 and K834 are marker-transferred viruses containing the L774F mutation.

samples were randomly selected for sequencing analyses. The results demonstrated the altered nucleotide at the desired position. Moreover, sequencing of the entire 827-bp fragment of two samples (K-830 and K-834) revealed that this DNA fragment indeed contained only the CTC-to-TTC change, demon-

TABLE 2. ED₅₀ of various drugs on wild type and L774F recombinant

Strain	ED ₅₀ (fold difference) of ^a :		
	Aphidicolin (μg/ml)	ACV (μM)	PFA (μg/ml)
KOS	0.15 (1)	0.3 (1)	38 (1)
L774F	0.1 (0.67)	1.1 (3.7)	120 (3.2)
K830	0.11 (0.73)	1.2 (4)	125 (3.3)

^a The ED₅₀ of each drug for L774F and K830 was the average of those from three and two recombinants, respectively. Each was determined directly from the plaque reduction curves shown in Fig. 3. The difference in susceptibility to each drug for L774F and K830 relative to wild-type strain KOS is shown in parentheses. The ED₅₀ of each drug for KOS is defined as 1.

strating that the L774F mutation was responsible for the PFA resistance phenotype. Two recombinant viruses (K-830 and K-834) were examined for the drug sensitivity phenotype; these recombinants exhibited the same resistance to PFA and ACV and susceptibility to aphidicolin that were indistinguishable from those of the L3 viruses (Fig. 3 and Table 2). Therefore, the effect of L3 recombinants in altering the HSV-1 Pol phenotype was verified to be the result of the L774F mutation within the conserved region VI.

The L774F mutation exhibited a higher fidelity of DNA replication. Two independent recombinant viruses, L3/F-A and -B, were constructed to examine whether the L774F mutation affects replication fidelity. The *supF* mutagenesis assay was performed to examine the *supF* mutation frequency of viral stocks as described previously (23, 25). Both L3/F-A and -B viral stocks contained the *supF* mutants at frequencies of 0.0017 and 0.0014%, respectively, which were 15- to 20-fold less ($P < 0.01$) than those of the KOS/F recombinants (0.026%) (Table 3). The *supF* mutation frequency replicated by the L774F mutant Pol was determined further by inoculation of less than 200 PFU of recombinants (determined on Vero cells in parallel) into 2×10^4 Vero cells and incubation for 72 h. Total virions were collected from the supernatant by high-speed centrifugation and combined with the pellet of infected cells for DNA isolation and purification. Purified DNA then was subjected to the *supF* mutagenesis assay as described in Materials and Methods. Consistent with the observed lower mutation frequencies present in viral stocks, the L3/F-A and L3/F-B viruses replicated the *supF* genes with fivefold lower mutation frequencies ($P < 0.05$) than the KOS/F-B virus in Vero cells (Table 3). Thus, in addition to the altered sensitivities to aphidicolin, PFA, and ACV, compared to the wild type, HSV-1 Pol with a L774F mutation can replicate DNA more accurately.

DISCUSSION

Several observations indicated a need to examine the roles of conserved region VI of HSV-1 Pol in Pol's activity. First, only recently have a few region VI mutants of HSV-2 been isolated based on their resistance to PFA, a pyrophosphate analog (5, 34). Interestingly, two PFA-resistant mutants of HSV-2 were found to be susceptible to ACV, a nucleoside analog (34). In contrast, a recent study demonstrated that three engineered HSV-1 *pol* mutants, including two containing mutations equivalent to the two HSV-2 isolates, were resistant

FIG. 4. Loss of the *DdeI* restriction site at the sequence coding for F774 in region VI. (A). Map of *DdeI* sites within wild-type and L774F mutant *pol* sequences. The relative sizes of restriction fragments of wild-type (upper) and L774F (lower) DNA digested by *DdeI* are shown. (B). *DdeI* restriction patterns of DNA fragment C. DNA fragments amplified from wild-type KOS strain and marker-transferred recombinant virus-infected cell DNA were digested with *DdeI* and fractionated on a 0.8% agarose gel. Relative sizes of digested and marker (M) bands are shown on the left and right, respectively. Lanes 1 to 5 (K831, K833, K836, K837, and K843) contain five different recombinants derived from the marker transfer experiments; lane 6 contains wild-type strain KOS.

to PFA, ACV, adefovir, and, to a lesser extent, cidofovir (for those two recombinants) (4). However, the status of the *tk* gene within these mutants was uncertain. Similarly, studies of human cytomegalovirus (HCMV) had identified several PFA-resistant mutants containing region VI mutations that were either susceptible or resistant to ganciclovir (GCV) (32, 35, 37). However, it is not clear whether these mutations alone could confer resistance to GCV, since the GCV-resistant mutant also may contain a mutation in UL97 (35). Therefore, the role of conserved region VI of HSV-1 Pol remained unclear,

TABLE 3. SupF mutagenesis^a

Virus	Total no. of colonies	No. of mutants	% Mutation frequency (fold difference)
Stock			
KOS/F-B ^b	346,610	89	0.026 (1)
L3/F-A	71,273	1	0.0014 (0.054)
L3/F-B	57,810	1	0.0017 (0.065)
72 h of infection			
KOS/F-B	48,401	6	0.012 (1)
L3/F-A	125,580	3	0.0024 (0.2)
L3/F-B	140,058	3	0.0021 (0.175)

^a Shown is the ratio of the number of mutant colonies to the total number of colonies recovered. There were significant differences ($P < 0.05$) between the mutation frequencies induced by KOS/F and each L3/F recombinant, evaluated by tests for differences between proportions (10). The P values between the mutation frequencies induced by two L3/F recombinants and induced by L3/F recombinants in different cells were not statistically significant. The fold differences of the mutation frequencies of the SupF genes induced by L3/F recombinants relative to KOS/F-B are shown in parentheses with the mutation frequencies of KOS/F-B defined as 1.

^b Data from reference 25.

due in part to the lack of a well-characterized mutant. Second, a hypothetical model of HSV-1 Pol, based on the known structure of RB69, with sequences homologous between HSV-1 and RB69 Pols, and the kinetic study of certain HSV-1 mutants, was proposed to demonstrate the relative location of residues affecting the Pol's interaction with ACV-TP and dGTP (21). However, the relative configuration of region VI remained to be demonstrated. Third, the role of conserved region VI in the fidelity of DNA replication is not clear. Thus, studying mutations within region VI will be important for revealing its role in polymerase activity. The results from this study clearly demonstrate that the conserved region VI also is involved in the formation of the polymerase active site and the execution of polymerization.

Structural and functional roles of region VI. The conserved regions III and VI, which form the two long antiparallel α -helices N and P, respectively, of RB69 Pol, form the finger domain (37). The finger domain (N and P helices) in the binary structure of the RB69 Pol-DNA complex is probably in the open conformation (18). Upon nucleotide binding, the fingers would rotate to form the closed conformation of the Pol-DNA-dNTP ternary structure for the delivery of dNTP to the polymerase active site (18). A mutation in the finger residue could influence the conformational change. Consistent with this, the finger domain (both region III and VI) of other Pols has been demonstrated biochemically to have a role in dNTP binding and the rate of polymerization (6, 13, 14, 28, 40, 42).

Both phenotypic and fidelity assays with L774F, as well as studies of other region VI mutations, imply that the conserved region VI has a functional role in the HSV-1 Pol interaction with incoming dNTPs. The secondary structure analysis of α -like Pols suggests that L774 of HSV-1 is a consensus residue in the region VI sequence, presumably essential for structural integrity and accessibility to solvent (37). Based on the homologous sequences (Fig. 1A) and known structures of other Pols (18, 20, 37), the L774 is located at the beginning of one helical structure composed of the finger domain. The replacement of leucine with a hydrophobic phenylalanine at this position may

induce a local structural change in the finger domain. It may cause the fingers to adapt a different orientation relative to other polymerase domains, thereby influencing the Pol's interaction with incoming dNTPs. For example, the mutation may influence the interaction of other key residues involved in the orientation of the incoming nucleotide, such as the potential interaction of R785 from HSV-1 Pol with the γ phosphate of the incoming nucleotide. Alternatively, the fingers may become too stiff to close and thereby affect the polymerization reaction. Secondary structure analysis suggested, based on the method of Garnier et al. (19) via the PEPTIDESTRUCTURE and PLOTSTRUCTURE programs in GCG (Wisconsin Package; Genetic Computer Group, Madison, Wis.), that the L774F mutation induced increased hydrophobicity associated with reduced surface probability and chain flexibility of the seven nearby residues without altering the helical structure of these residues, including those critical for directly interacting with dNTP (18, 40, 42). Such a modification may slightly affect the movement of the fingers in forming a closed conformation. Thus, the L774F mutation in HSV-1 Pol may result in a structural change locally that subsequently affects the dNTP binding activity, leading to altered drug sensitivity and replication fidelity.

The finger residues R482 (R785 of HSV-1 Pol) and K486 (R789) of RB69 Pol (Fig. 1A) directly interact with the γ phosphate of the dNTP and are accompanied with the metal ion B for neutralizing the negative charge on the pyrophosphate product of the catalysis reaction (18). Optimal positioning of the substrates and the catalytic residues is required to catalyze the phosphoryl transfer of dNTP to the primer strand followed by the release of pyrophosphate. A slightly altered structure in the finger domain due to the L774F mutation may affect the relative position of the finger residues and subsequently lead to an altered catalytic reaction, binding affinity of dNTP and rate of polymerization, which may result in higher fidelity of replicating the *supF* gene.

Recently, a drug-resistant mutant of HCMV was isolated from viruses being replicated and selected with PFA (32), an analog of pyrophosphate. The mutant was found to contain a leucine-to-valine change at amino acid residue 773, the equivalent of L774 in HSV-1 Pol (Fig. 1). However, the HCMV mutant did not exhibit altered susceptibility to GCV, a nucleoside analog. Interestingly, sequence alignment showed that the RB69 DNA polymerase contains a valine at the corresponding residue (18, 37). It seems that a valine residue at the position corresponding to HSV-1 Pol residue 774 may not influence the binding of nucleotide, but could affect the polymerization step, especially the pyrophosphorylation reaction. It is possible that L774F will have a stronger effect on the pyrophosphorylation step than the nucleotide-binding step. Further biochemical and perhaps structural studies will be necessary to demonstrate these assumptions. Nevertheless, the conserved region VI of HSV-1 Pol, together with other conserved regions, plays an important role in DNA replication, especially with regard to the interaction with dNTP and polymerization.

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