Replication Fidelity of the supF Gene Integrated in the Thymidine Kinase Locus of Herpes Simplex Virus Type 1

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Recombinant viruses were constructed to have an Escherichia coli replicon containing a mutagenesis marker, the supF gene, integrated within the thymidine kinase locus (tk) of herpes simplex virus type 1. These viruses expressed either wild-type or mutant DNA polymerase (Pol) and were tested in a mutagenesis assay for the fidelity of their replication of the supF gene. A mutation frequency of approximately 10⁻⁴⁻ was observed for wild-type strain KOS-derived recombinants in their replication of the supF gene. However, recombinants derived from the PAA5 Pol mutant, which has been demonstrated to have an antimutator phenotype in replicating the tk gene, had three- to fourfold increases in supF mutation frequency (P < 0.01), a result similar to that exhibited when the supF gene was induced to replicate as episomal DNA (Y. T. Hwang, B.-Y. Liu, C.-Y. Hong, E. J. Shillitoe, and C. B. C. Hwang, J. Virol. 73:5326–5332, 1999). Thus, the PAA5 Pol mutant had an antimutator function in replicating the tk gene and was less accurate in replicating the supF gene than was the wild-type strain. The spectra of mutations and distributions of substituted bases within the supF genes that replicated as genomic DNA were different from those in the genes that replicated as episomal DNA. Therefore, the differences in sequence contents between the two target genes influenced the accuracy of the Pol during viral replication. Furthermore, the replication mode of the target gene also affected the mutational spectrum.

DNA replication in mammalian cells is a complicated process (reviewed in references 2 and 25). It requires intricate DNA replication machinery that both duplicates chromosomal DNA and maintains genomic integrity. As the pivotal enzymes, DNA polymerases (Pols) are critical for ensuring the accuracy of DNA synthesis via their roles in the selection of nucleotides to be incorporated during DNA replication (polymerization) and in the removal of misinserted nucleotides from the 3'-OH end of the primer strand (proofreading) (reviewed in references 20 and 23). Examination of mutant Pols in terms of their effects on polymerization or proofreading steps will provide valuable information towards understanding the mechanism by which these processes contribute to the fidelity of DNA replication. While mutant Pols can be engineered by molecular biological techniques to contain an altered residue(s) and can be characterized in vitro to illustrate the effects of a mutation(s) on their activities, the effects of mutant Pols at the molecular level may be difficult to study in vivo, because they can be lethal.

Herpes simplex virus (HSV) has proven to be a good model for studying DNA replication in mammalian cells, since it is amendable at the molecular, genetic, biochemical, and biological levels (reviewed in reference 5). For example, a recombinant virus can be constructed to harbor a mutated pol gene that includes lethal mutations and can be propagated in mammalian cells for genetic characterization as well as studied at the molecular biological level. Mutant Pol can also be expressed and purified for biochemical analysis to reveal whether its kinetic characteristics agree with the results observed in molecular biological studies. Previous studies demonstrated that an HSV type 1 (HSV-1) pol mutant, PAA5, exhibits the antimutator phenotype in the thymidine kinase gene (tk) of HSV-1 (11, 16), a characteristic that is associated with better selection of correct nucleotides during DNA replication (12). To our surprise, this mutant showed a modest mutator phenotype when a reporter supF gene was used for the mutagenesis assay (18). However, the supF genes analyzed were replicated extrachromosomally. This raised concerns about whether replication modes and sequence contents were involved in determining the fidelity of Pol in infected cells. This was a likely possibility, since the effects of sequence context on Pol fidelity have been documented for other Pols (reviewed in reference 10).

In this study, recombinant viruses were constructed to have the supF gene integrated into the tk locus of HSV-1. Mutagenesis assays were performed to examine the mutation frequencies and spectra of mutated supF genes mediated by wild-type, PAA5 mutant (6), and marker-rescued recombinant HSV-1 (6) strains. Results confirmed that PAA5 Pol exhibits a modest mutator phenotype in the supF gene, regardless of whether the replication mode is genomic or episomal. However, both the spectra and distributions of supF mutations isolated from genomic DNA were significantly different from those derived from episomal DNA (18). Thus, the effects of both sequence contents and replication modes must be considered in determining the Pol phenotype in terms of its DNA replication fidelity.

MATERIALS AND METHODS

Viruses and cells. Vero cells (African green monkey cells, American Type Culture Collection) were grown and maintained in Dulbecco modified Eagle medium supplemented with 5% newborn calf serum as described previously (17). Wild-type HSV-1 strain KOS, the pol mutant PAA5 (6), the recombinant strain

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12281 (P5Aph’K2) rescued from PAA r5 (6), and recombinant viruses constructed to harbor an integrated Escherichia coli replicon containing the supF gene marker were propagated in Vero cells as described previously (18). The TK-negative mutants, K5DG1 and PAA3.2 (16), each of which contains an inserted G nucleotide in the repeated 7 G’s of the tk genes derived from strains KOS and PAA r5, respectively, were propagated similarly.

Plasmids. The plasmid pSupF-tk-R (Fig. 1A) was constructed as follows. The plasmid ptkLTRZ1 (7) (kindly provided by D. M. Coen, Harvard Medical School), which contains the lacZ gene under the control of the Moloney murine leukemia virus long terminal repeat (LTR) promoter inserted at the PstI site of the HSV-1 tk locus, was partially digested with HindIII restriction enzyme, blunt ended by the Klenow fragment and deoxynucleoside triphosphate (dNTP), and then ligated to a SpeI linker. The resulting plasmid, ptkLTRZ1-S, contained two SpeI sites flanking both sides of the ampicillin resistance gene (amp) and colE1 sequences. Plasmid ptkLTRZ1-S was then digested with BamHI enzyme to remove the lacZ sequences and obtain the plasmid ptkLTR-S. The restriction enzyme SpeI was then used to remove the amp and colE1 sequences. After self-ligation, the resulting DNA contained the LTR and tk sequences, which were in a tail-to-head orientation separated by the SpeI restriction site. This DNA fragment was then linearized by BamHI and ligated to pSupF1 plasmid DNA (17), which contains the amp, supF, and colE1 sequences. The resulting plasmid, pSupF-tk-R (Fig. 1A), therefore contains amp, supF, colE1, LTR, and HSV-1 tk sequences. After SpeI digestion, the pSupF-tk-R becomes linearized and contains, from right to left, the 5’ portion of the tk sequences up to the PstI site, LTR, amp, supF, colE1, and the 3’ portion of the tk sequences (Fig. 1B, panel 3).

Plasmids containing the pol gene were constructed by isolation of the 3.3-kbp BamHI fragments from total DNA prepared from Vero cells that were infected with recombinant virus; total DNA was then cloned into the pUC18 vector, which was then digested into the pUC18 vector, which was digested with BamHI and dephosphorylated. The plasmids were then subjected to sequence analysis (BioResource Center, Cornell University) by using the M13 primer and the M13 reverse primer as well as the primers corresponding to nucleotides 431 to 450, 1011 to 1029, 1566 to 1585, and 1984 to

FIG. 1. (A) Map of the plasmid pSupF-tk-R; (B) restriction maps of sequences around the tk locus of the wild-type strain KOS (panel 1), the recombinant containing integrated pSupF1 sequences (panel 2), and the plasmid pSupF-tk-R (panel 3). Enzyme abbreviations: B, BamHI; P, PstI; R, EcoRI; S, SpeI. Parentheses indicate that the site is lost during cloning. Other abbreviations: Ori, colE1; amp, ampicillin resistance gene; F, supF gene; N, base pair.
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2002, with the A residue of the first ATG codon of the pol gene being defined as nucleotide 1.

Recombinant viruses. To construct recombinant viruses, Vero cells were co-transfected with infectious viral DNA and Spf1P-tk-R by using the Lipofectamine (Gibco/BRL) transfection reagent, as described previously (17). Transfected was harvested 4 days after transfection, and virus titers were determined by plaque assays with Vero cells. Recombinant viruses were selected as TK-negative plaques by plating the transfectant onto Vero cells in the presence of 10 μM ganciclovir (GCV) and screened for the presence of the pSpF1I sequence by PCR and/or Southern blot analysis as described below.

For the screening of potential recombinants, the EcoRI primer (5’-GTAT CACGAGGGCCT-3’), corresponding to the sequence immediately upstream of the supF gene in pSpF-tk-R, and the TK 12 primer (5’-AGGCAAACGTT TATACAGG-3’), corresponding to the noncoding strand of the tk sequences about 200 bases downstream of the PstI site, were used for PCR amplification. DNA fragments of 1.0 kb were amplified from recombinants that contained the integrated pSpF1I sequences, whereas GCV-resistant lacking the integrated pSpF1I sequences failed the PCR (Y. T. Hwang and C. B. C. Hwang, unpublished results). Southern blotting was also used to detect the presence of the pSpF1I sequences within the tk locus and to confirm the homogeneity of the recombinants. Each recombinant was plaque purified twice, or plaque purification was continued until the homogeneous viruses were obtained.

Mutagenesis assay. The titer of each recombinant was determined by plaque assay. Vero cells were infected with 2 × 10^4 Vero cells. Aliquots of the tissue culture supernatant were inoculated with an input virus containing less than 200 PFU of recombinant viruses that were simultaneously measured by plaque assay. At 72 h postinfection, infected cells were harvested by freeze-thawing three times, transferred to 1.5-mL Eppendorf tubes, and collected by brief centrifugation. Total DNA of infected cells was extracted and purified as described previously (18). About one-fifth of the DNA obtained was then digested with BamHI, ligated with T4 DNA ligase, purified by phenol-chloroform extractions, and precipitated with ethanol. Purified DNA was then electroporated into E. coli MBM70/0 host cells. Transformed E. coli was spread on Luria-Bertani agar plates containing 5-bromo-4-chloro-3-indolyl-β-D-galactopyranoside (X-Gal), isopropyl-β-D-thiogalactopyranoside (IPTG), and ampicillin. White and light blue colonies that contained the mutated supF1 gene in the pSpF1I replicon were isolated, and the mutation frequencies were determined as the ratios of white and light blue colonies to total colonies recovered (18).

The supF1 gene sequences induced by the transient-replication assay was performed as described previously (18) with the PHOS1 plasmid.

Characterization of mutated supF1 genes. Mutant pSpF1I or pHOS1 DNA was prepared from white and light blue colonies and characterized either by direct sequencing of the supF1 gene with the Jmol sequencing kit (Promega) and the EcoRI clockwise primer (New England BioLab), as described previously (18), or by being subjected to EcoRI digestion and fractionated on an 0.8% agarose gel. Mutagenic sequences were substituted base pairs, or a deletion or insertion of one or two bases were classified as point mutants. Other mutants were defined as complex mutants, including mutants with a deletion of a short stretch of the supF1 sequences, insertions of any sequences, or a lack of the EcoRI restriction site, which is located immediately downstream of the EcoRI clockwise primer binding site. DNA extracted from white colonies that failed to reveal any mutated sequence in the supF1 gene and formed blue colonies upon retransformation into E. coli MBM70/0 cells was defined as the wild-type plasmid DNA and excluded from the list of mutated clones.

Single-step growth and drug assays. To compare the abilities of the recombinant HSV-1 strains KOS/F-B and PAA5/F-A to replicate in Vero cells with those of their parental strains, about 10^5 Vero cells were infected with virus at a multiplicity of infection of 3 PFU per cell. One hour after adsorption, infected cells were washed with serum-free medium and refed with fresh Dulbecco modified Eagle medium supplemented with 2% newborn calf serum. At 5 and 24 h after infection, virus progeny were harvested and the titer was measured by a plaque assay with Vero cells.

Plaque reduction assays were performed to examine the drug sensitivity of each recombinant, as described previously (19). Since these recombinants are TK negative, only phosphonoacetic acid (PAA) and aminoglycin (APH) (Sigma) were used to analyze the Pol phenotype.

Semi-nested and dot blot. Aliquots of DNA prepared from virus-infected cells were digested with either BamHI or EcoRI, fractionated on 0.8% agarose gels, transferred to Zeta-Probe GT blotting membranes (Bio-Rad), and hybridized with the pSpF1I probe or a 300-bp probe of the 5’-PstI fragment of the tk gene (Fig. 1B, panel 3) labeled with [α-32P]dCTP by using the Ready to Go kit (Amersham Pharmacia Biotech). About 0.5 ng of pSpF-tk-R digested with BamHI was included as the size marker. Lambda DNA digested with HindIII and labeled with [α-32P]dCTP was used as the molecular mass marker.

Dot blotting was performed by diluting aliquots of virus-infected samples in Tris-EDTA to a final volume of 250 μL. An equal volume of denaturing buffer (0.8 M NaOH, 20 mM EDTA) was added to each sample and boiled at 100°C for 10 min. Samples were then transferred to Zeta-Probe GT membranes by using the dot blotting apparatus (Bio-Rad) according to the manufacturer’s recommended protocol. The blot was hybridized with the 300-bp tk probe. The intensities of hybridization signals were quantified with a phosphorimaget.

RESULTS

Based on the results of the tk mutagenesis assay, the wild-type strain KOS of HSV-1 replicates the tk gene with a mutagen frequency ranging from 5 × 10^-5 to 4 × 10^-4 among progeny viruses (11, 16, 17). A KOS derivative mutant, PAA5, which contains an arginine-to-serine mutation at amino acid residue 842 within the Pol conserved region III (9), demonstrates an antimutator phenotype with a mutation frequency in the tk gene ranging from 3 × 10^-6 to 9 × 10^-6 (11, 16). A previous study demonstrated that the KOS strain replicates the supF1 gene with a mutation frequency of 2 × 10^-4 to 7 × 10^-4 when it is inserted into an HSV-1-inducible replicon (18), a result which is consistent with the error frequency in the tk gene. To our surprise, PAA5 demonstrated a 1.6- to 3.25-fold increase in the mutation frequency in the supF1 gene (18), revealing a modest mutator phenotype that was in contrast to the antimutator phenotype observed for the tk mutation rates (11, 16). These results suggest that mutation contexts, including the sequence contexts and replication modes of the target gene, could influence the ability of a Pol to manage its accuracy in DNA synthesis. To examine whether replication modes contributed to replication fidelity, recombinant viruses were constructed to harbor an integrated E. coli replicon containing the target supF1 gene within the tk locus and were subjected to mutagenesis analysis.

Construction and characterization of recombinant viruses. A strategy was designed to construct the plasmid pSpF-tk-R (Fig. 1A). This plasmid contained an E. coli replicon with the mutagenesis target supF1 gene, the amp gene, and the colE1 sequences; this replicon was inserted into the PstI site of the tk gene of HSV-1. A SpeI linker was also inserted into the pSpF-tk-R to separate the 3’ and 5’ portions (with a tail-to-head orientation) of the tk sequence. Upon SpeI linearization of pSpF-tk-R, the pSpF1I sequence was flanked by the tk sequence at both ends with a 5’-to-3’ orientation, which allowed for homologous recombination into the HSV-1 genome. Recombinant viruses were then constructed by cotransfection of infectious virus DNA and SpeI-linearized pSpF-tk-R into Vero cells. Recombinants containing the integrated pSpF1I sequences in the tk locus were isolated as GCV-resistant mutants in Vero cells. The recombinants were plaque purified to homogeneity, as verified by Southern blotting (see below; Fig. 2). Homologous recombination occurred at a relatively high frequency; approximately 0.1 to 0.7% of the progeny viruses obtained from the transfection were recombinants (Hwang and Hwang, unpublished). Two independent recombinants were isolated from the wild-type strain KOS, namely KOS/F-A and KOS/F-B. Figure 1B, panel 1, depicts the relative BamHI and EcoRI restriction site around the tk locus of HSV-1. Recombinants with integrated pSpF1I sequences within the tk locus

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have altered restriction DNA fragments created by BamHI and EcoRI digestions (Fig. 1B, panel 2). A linearized restriction map of pSupF-tk-R and the probes used for Southern blots is shown in Fig. 1B, panel 3. The Southern blots demonstrated the presence and locations of the pSupF1 sequences within these recombinants as well as the homogeneity of each recombinant. The pSupF1 probe (Fig. 1B, panel 3) hybridizes only to DNA samples prepared from recombinants containing the integrated pSupF1 sequences. This probe hybridizes to BamHI- and EcoRI-digested samples containing the integrated pSupF1 sequences, and the plasmid pSupF-tk-R is depicted in Fig. 1B. (A and B) Lanes 1 and 5, KOS; lanes 2 and 6, KOS/F-A; lanes 3 and 7, KOS/F-B; lane 4, pSupF-tk-R. (C and D) Lanes 1 and 5, KOS; lanes 2 and 6, PAA5/F-A; lanes 3 and 7, 12281/F-A; lane 4, pSupF-tk-R. Samples in lanes 1 to 4 were digested with BamHI, and samples in lanes 5 to 7 were digested with EcoRI. Lane M contains 32P-labeled lambda DNA digested with HindIII, with the molecular sizes (in kilobase pairs) indicated at the left. The sizes of bands hybridized with the probes are shown as indicated. The arrows shown to the right of panels B and D indicate the incomplete digestion of viral DNA by EcoRI.
ples of both recombinants (Fig. 2A, lanes 2 and 3) but not to the KOS DNA (Fig. 2A, lane 1). In agreement with the expected sizes of the EcoRI restriction fragments, this probe hybridized to the 2.9- and 0.8-kbp fragments of both samples (Fig. 2A, lanes 6 and 7). The use of a 300-bp Sr1-PstI DNA fragment of the tk gene as a probe (Fig. 1B, panel 3) further demonstrated the presence of integrated pSupF1 sequences within the tk locus. This probe hybridized to the 3.6-kbp BamHI fragment (Fig. 2B, lane 1) and the 2.4-kbp EcoRI fragment (Fig. 2B, lane 5) of KOS DNA as well as to the 2.0-kbp BamHI (Fig. 2B, lanes 2 and 3) and 2.9-kbp EcoRI (Fig. 2B, lanes 6 and 7) fragments of DNA samples containing the integrated pSupF1 sequences. Thus, the Southern blot shown in Fig. 2B demonstrates the expected results. (The high-molecular mass bands shown in Fig. 2B were due to incomplete digestion by EcoRI [lanes 5, 6, and 7]). Therefore, both KOS/F-A and KOS/F-B recombinants contain integrated pSupF1 sequences in the tk locus and are homogeneous.

Similar approaches were used to construct and isolate the recombinants PAAr5/F-A, PAAr5/F-B, and 12281/F-A. The 12281/F-A recombinant was constructed from the HSV-1 strain 12281, which is derived from PAA5 and has the mutation in the PAAr5 pol sequence restored to the wild-type sequence. Southern blotting of BamHI- and EcoRI-digested DNA samples prepared from KOS-, PAAr5/F-A-, and 12281/F-A-infected cells was performed to demonstrate the presence of integrated pSupF1 sequences within the tk locus. Results with both the pSupF1 and 300-bp tk probes (Fig. 2C and D) were identical to those shown in Fig. 2A and B, respectively, and demonstrated that these recombinants were homogeneous. Identical results of Southern blotting were obtained for recombinant PAAr5/F-B (Hwang and Hwang, unpublished). Therefore, these recombinants contained the integrated supF sequences in the tk locus.

Genotypes and phenotypes of recombinants. To examine whether the recombinant viruses might contain other mutations in the pol gene, the 3.3-kbp BamHI fragment of the pol gene from each strain was subcloned into pUC18 and sequenced. As expected, the results demonstrated that recombinants PAAr5/F-A and PAAr5/F-B contained the sole change of C to A at nucleotide 2524, corresponding to the mutation in PAA5 (9), while KOS/F-A and KOS/F-B contained wild-type sequences within this DNA fragment (Hwang and Hwang, unpublished).

A single-step growth curve, the plaque reduction assay, and dot blotting were used to examine whether there was any phenotypic difference between recombinants and their parental strains. The results demonstrated that KOS/F recombinants exhibited sensitivities to PAA and APH that were identical to those of strain KOS and that PAAr5/F recombinants retained their resistance to PAA and hypersensitivity to APH, like the parental PAA5 strain (Hwang and Hwang, unpublished). The single-step growth curve also demonstrated that these recombinants exhibited growth kinetics in Vero cells that were identical to those of their parental strains, although PAA5 and its derivatives yielded approximately twofold fewer viral progeny at 24 h postinfection (Table 1). Similarly, PAA5 and its derivatives produced (approximately threefold) lower amounts of viral DNA than KOS and its derivatives. Nevertheless, recombinants produced amounts of DNA that were equal to those produced by their parental strains (Fig. 3). Therefore, these recombinants did not exhibit altered genotypes or phenotypes of the pol genes, despite their TK-negative phenotype.

Mutation frequency of the integrated supF gene. The mutagenesis assay was performed to measure the mutation frequency of the supF gene, which was replicated as part of the viral genomic DNA, as mediated by the wild-type or PAA5 mutant Pol. The amount of input virus was kept below 200 PFU to prevent the introduction of preexisting supF mutants. In addition, all recombinants were analyzed simultaneously under the same conditions for each experiment, with the exception that recombinant PAAr5/F-B was examined by inoculating two different plaques into Vero cells in different experiments. The results presented in Table 2 show that Pol from the wild-type virus replicated the supF gene with a mutation frequency ranging from 0.011 to 0.016%, whereas Pol from the PAA5 recombinants had an error frequency of 0.036 to 0.058%, which was two- to fourfold higher than that of wild-type Pol (P < 0.01; evaluated by tests for differences between proportions [4]). The recombinant 12281/F-A, constructed from the marker rescue virus P5Aph’K2 (6), exhibited a mutation frequency of 0.012%, which was consistent with those of the KOS recombinants. Thus, the increased frequency of supF mutations mediated by the PAA5/F recombinants resulted from the mutated pol gene in PAA5 or from the effect of an
arginine-to-serine mutation at amino acid 842 of the Pol enzyme.

The frequencies of supF mutation that occurred in viral stocks of the recombinants KOS/F-A, KOS/F-B, and PAA5/F-A, which were prepared by inoculating 2,000, 2,000, and 1,500 PFU, respectively, into 5 × 10^6 Vero cells (Table 2), were also examined. Table 2 shows that KOS/F-A and KOS/F-B stocks contained supF mutations at frequencies of 0.027 and 0.036%, respectively, whereas PAA5/F-A had a supF mutation frequency of 0.060%. These were about 1.5- to 2.5-fold higher than those observed with the assays for which a low input of viruses was used. Furthermore, these mutants also exhibited distributions and types of substitutions (Hwang and Hwang, unpublished) that were similar to those isolated by the mutagenesis assay (Table 3 and see Table 5; Fig. 4) with the exception that more consecutive sequence changes were found among these mutants, which were presumably derived from preexisting mutants that underwent a subsequent mutagenic event. Thus, it is critical to use a low input of viruses for the mutagenesis assay.

**Mutation frequency of the supF gene replicated transiently.**

Since these recombinants contain integrated sequences within the tk locus and are TK negative, it is important to examine whether the lack of TK activity affects the fidelity of DNA replication. To address this, we examined the mutation frequencies of the supF gene induced by KOS and PAA5 and by their TK-negative derivatives, K5DG1 and PAA3.2 (16), respectively, by using the pHOS1 plasmid, which contains the supF gene and the HSV-1 oriS sequences, and the transient-replication mutagenesis assay as described previously (18). In this assay, KOS and PAA5 generated supF mutation frequencies of 0.040 and 0.060%, respectively (Table 4), in agreement with previous results (18). K5DG1 and PAA3.2 induced supF mutations at frequencies of 0.042 and 0.062%, respectively (Table 4). Thus, the TK-negative phenotype did not affect the fidelity of Pol in replicating the supF gene, whether it was in the plasmid or genomic form, contradicting a previous report that showed that the tk gene was mutagenic in replicating the lacZ gene (22). Furthermore, the majority of supF mutations (>80%) were complex changes (Hwang and Hwang, unpublished), including deletions and rearrangements, a finding consistent with previous results (18).

**Types of supF mutants replicated as genomic DNA.**

Totals of 104 and 111 supF mutants isolated from KOS/F and PAA5/F recombinants, respectively, were characterized by restriction enzyme digestion and sequence analysis. Among these mutants, the majority of supF mutants contained point mutations, including deletions of either one or two nucleotides, that accounted for the changes in 86 and 83% of mutants isolated from KOS/F and PAA5/F, respectively (Table 3). The remaining mutants contained complex changes, including rearranged sequences and deletions of longer sequences of the supF gene. Although both Pols generated point mutations and complex changes with similar frequencies, they differed in the frequency with which they generated deletions of one or two nucleotides. Twenty-eight percent (24 of 85) of point mutations produced by KOS/F were single-nucleotide deletions, whereas 49% (45 of 92) of PAA5/F were single-nucleotide deletions. Nevertheless, the deletions were found in regions of supF containing either 4 repeated G’s or 5 repeated C’s, with higher mutation frequencies in the 5-C regions for both the wild-type and PAA5 Pols.

Table 3 summarizes the types of base substitution in the supF genes generated by both wild-type and PAA5 Pols. The majority of base substitutions generated by both Pols were G:C-to-A:T transitions, with 81% by wild-type Pol and 69% by PAA5 Pol. Although PAA5 Pol generated a relatively higher

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**TABLE 2. Mutation frequencies of the supF gene replicated in the HSV-1 genome**

<table>
<thead>
<tr>
<th>Virus strain and source</th>
<th>Amt of input virus (PFU)</th>
<th>No. of mutant colonies/total no. recovered</th>
<th>Mutation frequency (%)</th>
</tr>
</thead>
<tbody>
<tr>
<td>KOS/F-A</td>
<td>Stock 2,000</td>
<td>52/194,896</td>
<td>0.027</td>
</tr>
<tr>
<td></td>
<td>Expt A 91</td>
<td>27/177,112</td>
<td>0.016</td>
</tr>
<tr>
<td></td>
<td>Expt B ND</td>
<td>22/202,950</td>
<td>0.011</td>
</tr>
<tr>
<td>KOS/F-B</td>
<td>Stock 2,000</td>
<td>89/348,610</td>
<td>0.026</td>
</tr>
<tr>
<td></td>
<td>Expt A 75</td>
<td>26/219,725</td>
<td>0.012</td>
</tr>
<tr>
<td></td>
<td>Expt B ND</td>
<td>29/206,092</td>
<td>0.014</td>
</tr>
<tr>
<td>PAA5/F-A</td>
<td>Stock 1,500</td>
<td>345/359,260</td>
<td>0.088</td>
</tr>
<tr>
<td></td>
<td>Expt A 23</td>
<td>10/17,248</td>
<td>0.058</td>
</tr>
<tr>
<td></td>
<td>Expt B ND</td>
<td>48/94,197</td>
<td>0.051</td>
</tr>
<tr>
<td>PAA5/F-B-1⁣&lt;sup&gt;d&lt;/sup&gt;</td>
<td>Expt C 61</td>
<td>45/123,593</td>
<td>0.036</td>
</tr>
<tr>
<td>PAA5/F-B-2⁣&lt;sup&gt;d&lt;/sup&gt;</td>
<td>Expt C 121</td>
<td>51/95,568</td>
<td>0.053</td>
</tr>
<tr>
<td>12281/F-A</td>
<td>Expt A 152</td>
<td>31/254,800</td>
<td>0.012</td>
</tr>
</tbody>
</table>

* Stock indicates that the experiment was performed with DNA prepared from infected cells for making virus stock. Experiments designated A or B for each recombinant were performed simultaneously. Expt C was performed at the same time and under the same conditions as experiments A and B.

<table>
<thead>
<tr>
<th>Virus strain (no. of samples)</th>
<th>No. of mutants (% of total) with: Chi-square value</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>Simple change&lt;sup&gt;a&lt;/sup&gt; Complex change&lt;sup&gt;b&lt;/sup&gt;</td>
</tr>
<tr>
<td>KOS/F (104)</td>
<td>89 (86) 15 (14)</td>
</tr>
<tr>
<td>PAA5/F (111)</td>
<td>92 (83) 19 (17)</td>
</tr>
</tbody>
</table>

* Includes base substitutions and deletions of one or two nucleotides in the supF gene.

**TABLE 3. Classification of supF mutants replicated as genomic DNA**

<table>
<thead>
<tr>
<th>Virus strain</th>
<th>No. of mutants (% of total) with: Chi-square value</th>
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<sup>a</sup> Includes base substitutions and deletions of one or two nucleotides in the supF gene.

<sup>b</sup> The significance (P value) of the differences between the types of mutations induced by wild-type and PAA5 Pols was examined by the chi-square test (8).

<sup>c</sup> All mutants isolated from both experiments with recombinants KOS/F-A and KOS/F-B were analyzed.

<sup>d</sup> Mutants analyzed included all mutants isolated from both experiments with recombinant PAA5/F-A and experiment C with PAA5/F-B-1 and eight mutants isolated from experiment C with recombinant PAA5/F-B-2.
frequency of G:C-to-T:A transversion (25%) than the wild-type Pol did (12%), the difference between the frequencies of transition and transversion induced by these Pols was not statistically significant (Table 5). However, there was a difference in the distribution patterns of those transversions. While the wild-type Pol generated five A:T-to-C:G transversions (7%) scattered throughout different positions of the supF gene, PAAr5 Pol generated only one such mutation (2%).

Mutational hot spots. The distribution of altered bases created by these two Pols is shown in Fig. 4. It is clear that multiple runs of G’s and C’s were mutational hot spots, especially with regard to the deletion of a single nucleotide within these regions. The nucleotide positions 156, 160, and 168 were found to be hot spots of G:C-to-A:T transitions induced by both Pols. Furthermore, wild-type Pol caused G:C-to-A:T transitions at nucleotides 159 and 169 with a relatively high frequency, whereas PAA5 Pol caused replication of only G-to-T transversions at nucleotide 159 and more C-to-A transversions than C-to-T transitions at nucleotide 169. While G-to-T transversions generated by both Pols were commonly found at nucleotide position 123, which is within the 3 reiterated G’s, the wild-type Pol also caused replication of G-to-A transitions at this position. Interestingly, such G-to-A changes were part of GG-to-AA double mutations. In addition to these major differences in the distribution of mutated bases generated by wild-type and PAA5 Pols, minor differences were also observed at nucleotides 106 to 109, 115, 116, 129, 130, 135, and 140 (Fig. 4). Therefore, although the major changes induced by these Pols were similar, differences in the type and distribution of base substitutions existed. This suggests that amino acid mutation in PAA5 Pol has resulted in alterations in its replication fidelity.

DISCUSSION

The PAA5 Pol contains an arginine-to-serine mutation at amino acid residue 842 that is located within the conserved region III of α-like Pols (9). This mutation results in altered sensitivity to certain antiviral drugs that are nucleoside or pyrophosphate analogues (9). In agreement with the altered drug sensitivity, this mutant Pol also exhibits altered binding affinities to nucleosides (12, 15) and acyclovir triphosphate (15). This mutant was suggested to play a role in maintaining the higher replication fidelity for the tk gene of HSV-1 (12). However, a previous study (18) demonstrated that the PAA5 mutant induced a modest increase in the supF mutation frequency when the supF gene was replicated in a plasmid form. These conflicting results led to the hypothesis that the target gene,
the mode of DNA replication, and the assay method could be important in influencing the outcomes of the replication accuracy of HSV-1 Pol.

**Sequence contents and the identity of Pol affect fidelity.** To test whether the replication mode could play a role in determining replication fidelity, we constructed recombinant viruses harboring an integrated supF gene within the tk locus for mutagenesis study. In agreement with the results of a previous study (18), two independent recombinants derived from PAA′5 exhibited a modest mutator phenotype, replicating two to four times as many supF mutants (Table 2) as did recombinants derived from the wild-type strain KOS. This demonstrated that PAA′5 Pol induced an increase in supF mutations regardless of whether the supF gene was present as genomic DNA or episomal DNA. It also showed that PAA′5 Pol had a modest mutator phenotype of the supF gene, in contrast to its antimutator phenotype demonstrated by the tk mutagenesis assay (11, 16). Thus, the effect of the sequence content alongside that of the identity of the Pol could generate these conflicting results. A possible explanation is that PAA′5 Pol generated certain tk mutations that were silent changes or mutations without altered TK activity, thereby escaping detection in the tk mutagenesis assay.

Different mutational hot spots targeted by the wild-type and PAA′5 Pols (illustrated in Fig. 4) demonstrate the effect of the type of the Pol on replication fidelity. For example, G-to-A transitions at nucleotide position 159 were found at a relatively high frequency among supF mutants induced by wild-type Pol. In contrast, PAA′5 Pol induced only two mutants containing G-to-T transversions at this position. Similarly, C-to-A transversions at nucleotide position 169 were found only in mutants induced by PAA′5 Pol while C-to-T transitions at this nucleotide were observed in mutants induced by both Pols. Furthermore, only G-to-T transversions were found at nucleotide 159 in PAA′5 Pol-induced mutants, while only G-to-A transitions were found at this nucleotide in wild-type-Pol-induced mutants. Interestingly, these mutations occurred at nucleotides adjacent to the mutational hot spots of nucleotides 160 and 168 at which exclusively transitional changes were induced by both Pols. Thus, PAA′5 Pol replicated the supF gene with mutations that were either not induced or induced as different types of mutations by the wild-type Pol at certain nucleotides.

The fact that the mutational hot spots targeted by the wild-type and PAA′5 Pols are distinct from one another also supports the possibility that PAA′5 Pol replicates certain mutations at high frequencies in the tk gene without altering TK activity or drug sensitivity, a possibility that sheds light on the issue of differential drug sensitivities among tk mutants. Moreover, the increase in single-base deletions in the supF gene generated by PAA′5 also suggests that this Pol could replicate increased numbers of potentially lethal frameshift mutations in viral DNA. Supporting this hypothesis is experimental evidence demonstrating that PAA′5 Pol replicates less DNA and fewer progeny viruses than does KOS (Fig. 3 and Table 1), thereby suggesting that PAA′5 Pol has a lower replication rate than that of KOS and that fewer rounds of DNA replication mediated by PAA′5 would generate fewer mutations. Although Hall et al. (11) contested this possibility, experimental evidence strongly corroborates the observed antimutator phenotype of tk gene replication by PAA′5 and links it to the relatively lower numbers of viral progeny and DNA produced. In contrast, the supF mutagenesis assay impartially analyzes replicated DNA, regardless of the sensitivities of phenotypes or the yields of viral progeny. Nonetheless, the conflicting mutagenic results for the tk and supF genes generated by PAA′5 Pol reflect the combined effects of the identity and sequence contents of the Pol.

**Advantage of supF mutagenesis.** We estimate that more than 56% of tk mutations could escape the tk mutagenesis screening. This figure is based on an examination of the possible silent and missense mutations within 33 nucleotides coding for amino acid residues 165 to 175 of the TK enzyme, residues which form a portion of a nucleoside binding site (1). A random mutagenesis study demonstrated that most residues within this region can be replaced by amino acids with similar physical properties and still retain TK activity (21). A total of 56 (56%) of the 99 (3 × 33) possible changes within these 11 residues can be silent changes or missense mutations without losing TK activity. Given that other regions of the TK enzyme may have a much higher degree of plasticity of substituted residues, the overall ratio of undetectable tk mutants may be higher than 56%. On the other hand, about 96% of the 255 (3 × 85) possible changes within the 85 bases of the supF gene have been isolated (3). This indicates that the supF mutagenesis system is more sensitive in detecting mutations than the tk system.

The supF mutagenesis system also offers the advantage that the available information about supF mutants generated by cellular Pols can be compared to that about supF mutants generated by HSV-1 Pol. Interestingly, the supF substitutions induced by cellular Pols, presumably Pol δ and Pol α, and the supF mutations that occur in the context of viral genomic DNA have quite different distribution patterns. The former include a large proportion of substitutions located in noniterated bases (13, 14, 24), whereas the latter are found almost exclusively in repeated di- or trinucleotides of G or C bases, with the follow-
ing exceptions: the mutation hot spot at the noniterated nucleotide 156 is a target for both cellular and HSV-1 Pols, and four other types of substitution are generated by viral Pol at noniterated bases between nucleotides 125 and 155. Of note is the fact that cellular Pols replicated a large number of substitutions between nucleotides 125 and 155, while HSV-1 Pol induced only four types of substitution within this sequence. These differences imply that the HSV-1 and cellular Pols induce different spectra of supF mutations.

Replication modes influence the spectra of mutations. In addition to the difference in the distributions of the substitutions generated by cellular and viral Pols, we also observed more substitutions between nucleotides 125 and 155 induced by HSV-1 Pol when the supF gene replicated as a plasmid form, the same replication form used to characterize spontaneous mutations in mammalian cells (13, 14, 24). Furthermore, HSV-1 Pol also generated different types of supF mutations (Table 3) and substitutions (Table 5) in the two replication modes. While more than 50% of mutated supF genes isolated from the transient-replication assay (Table 2 in reference 18) contained complex changes, including the deletion or rearrangement of large stretches of sequences, fewer than 20% of mutants isolated from supF recombinants contained complex changes. Furthermore, approximately 30% of base substitutions isolated from the transient-replication assay (Table 3 in reference 18) were transitions, whereas the majority of the base substitutions in supF mutants that replicated as genomic DNA were transitions (Table 5).

Perhaps the most striking difference observed between these two replication modes was in the distributions of altered nucleotides within mutated supF genes. For example, deletions of either G or C in repeated G’s or C’s, respectively, represent the major types of simple mutations found in supF mutants isolated from supF-containing recombinants, whereas only a few supF mutants contained such deletions when they were replicated transiently by either Pol (18). The C-to-A transversion at nucleotide 109 was the hot spot for mutations among supF mutants isolated from the transient-replication assay, whereas there was no such mutation found at this position in genomically replicated supF mutants (Fig. 4). The major change at nucleotide 125 of the supF genes that replicated transiently was the G-to-A transition, which differed from the G-to-T transitions found in genomic supF mutants, although a few G-to-A transitions were also observed in genomic supF mutants. Other major differences among supF mutants isolated from these two studies included the presence of certain hot spots found in one study but not in the other. Double mutants containing spatial changes of 2 or 3 nucleotides were found in the previous study, while double mutations in supF genes isolated from this study contained altered nucleotides within repeated sequences. Some mutants isolated from the previous study contained altered bases scattered between nucleotide positions 125 and 155, yet only a few mutants analyzed in this study contained altered bases at three positions within this region. Thus, not only the spectra but also the distributions of altered bases within mutated supF genes were dramatically different among mutants isolated from the two replication modes.

These results were surprising, since we expected similar spectra of mutations within the same target gene. It is possible that the mode of DNA replication had a more significant effect in creating these differences than the use of the same gene for analysis had in maintaining similarity of results. Evidence further supporting this hypothesis includes the finding that transition is the major type of base substitution found in tk mutants replicated by the wild-type Pol (Q. Lu, Y. T. Hwang, and C. B. C. Hwang, unpublished data) and in supF mutants observed in this study but that transversions predominate in the oriS-based assay for supF mutants (18) and in episomally replicated supF mutants induced by cellular Pols (24). It is possible that the timing of the replication of supF genes in the shuttle plasmid containing the oriS sequence (18) may be different from that in recombinant viruses containing the supF gene integrated in the tk locus that is within the U1 sequences and located closer to the oriS sequences. It is also possible that the form of DNA replication starting from oriS is different from that starting from oriL (theta versus rolling circle replication form) and that these replication forms may be initiated at different phases during virus infection associated with different mutagenic mechanisms.

Is the tk gene mutagenic? Since recombinants constructed in this study are TK negative, this raises the question of whether TK affects supF mutagenesis and causes a difference in the selection of tk and supF mutants. This concern is unfounded for the following reasons. First, PAA’5 Pol exhibits higher Km’s for all dNTPs than does wild-type Pol (12, 15). Our dot blot demonstrates that PAA’5 also synthesizes threefold less DNA in infected cells, suggesting that it does not consume as many dNTPs as KOS does. Second, TK-negative recombinants used in this study do not alter their phenotypes, including those involving the replication of DNA (Fig. 3), virus yield (Table 1), and drug sensitivity (Hwang and Hwang, unpublished). Third, neither KOS- nor PAA’5-derived TK-negative mutants induce mutation frequencies of the supF gene different from those of their parental TK-positive strains in the transient-replication assay (Table 4). Taken together, these results suggest that TK is not responsible for the observed differences in selection between tk and supF mutants.

Our results also demonstrate that both TK-positive and -negative viruses (Table 4) induce similar supF mutation frequencies and spectra. This implies that the tk gene is not mutagenic, in contrast to the results of Pyles and Thompson (22), who showed that the tk gene was mutagenic by examining the mutation frequency of the lacZ gene in infected cells. This may be another example of an assay-dependent difference in DNA replication fidelity. Nevertheless, it is important to reexamine the role of tk in mutagenesis under different conditions. For example, it is necessary to isolate more base substitutions of supF mutants induced by TK-negative mutants in the plasmid form. Analysis of genomically replicated supF mutants under TK-positive conditions is also required to provide a complete picture of the role of the tk gene in mutagenesis.

We compared the outcomes of replication fidelity testing for the supF gene when it was replicated in different replication modes by a wild-type strain and a pol mutant of HSV-1. Our results demonstrated that although the frequencies of supF mutation associated with the two different replication modes were similar, the spectra and distributions of altered bases within the supF genes were significantly different. Therefore, the sequence contexts of genes to be analyzed, including the
sequence contents of the target genes (the tk and supF genes) and the modes of replication (episomal versus genomic DNA replication as well as oriV- versus oriV-directed DNA replication), play critical roles in determining replication fidelity. Further studies will be necessary to detail how these factors contribute to these differences.

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