On the fidelity of DNA replication: herpes DNA polymerase and its associated exonuclease

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ABSTRACT

Procaryotic DNA polymerases contain an associated 3'→5' exonuclease activity which provides a proofreading function and contributes substantially to replication fidelity. DNA polymerases of the eucaryotic herpes-type viruses contain similar associated exonuclease activities. We have investigated the fidelity of polymerases purified from wild type herpes simplex virus, as well as from mutator and antimutator strains. On synthetic templates, the herpes enzymes show greater relative exonuclease activities, and greater ability to excise a terminal mismatched base, than procaryotic DNA polymerases which proofread. On a ØX174 natural DNA template, the herpes enzymes are more accurate than purified eucaryotic DNA polymerases; the error rate is similar to *E. coli* polymerase I. However, conditions which abnegate proofreading by *E. coli* polymerase I have little effect on the herpes enzymes. We conclude that either these viral polymerases are accurate in the absence of proofreading, or the conditions examined have little effect on proofreading by the herpes DNA polymerases.

INTRODUCTION

A biological issue of interest is the question of how cells are able to replicate their DNA with very high fidelity. Measurements of spontaneous mutation frequencies suggest that the average rate of basepair substitutions is in the range of 10^{-8} to 10^{-11} misincorporations per basepair replicated (1). Procaryotic DNA polymerases exhibit high fidelity in vitro, in part due to an integral 3'→5' proofreading exonuclease that excises noncomplementary nucleotides after addition to the primer terminus. With progressive inhibition of this proofreading activity, procaryotic polymerase error rates increase by one to two orders of magnitude (2). Purified eucaryotic DNA polymerases α and β are relatively inaccurate, exhibiting error rates of 10^{-3} to 10^{-4} (2); these enzymes generally exhibit no associated exonuclease activity (3). In addition, several groups have reported the purification of a high molecular weight eucaryotic enzyme, generally designated DNA polymerase δ , which contains an associated 3'→5' exonuclease (4,5,6).

DNA polymerases of some eucaryotic viruses also contain an associated 3'→5'

exonuclease activity (7,8); it is of interest to examine such an enzyme for proofreading activity. We have investigated the fidelity of DNA polymerase purified from wild type herpes simplex virus type 2 (HSV-2), and from mutator and antimutator viral strains. We find that these polymerases exhibit exonuclease/polymerase ratios even greater than those of proofreading procaryotic DNA polymerases. The HSV polymerases will also preferentially excise a terminal mismatched base on hybridized polynucleotides. With investigations using a ØX174 natural DNA template, we find that the HSV polymerases are more accurate than any purified eucaryotic DNA polymerase so far investigated. This high accuracy is maintained under conditions which are used to inhibit proofreading in procaryotic DNA polymerases.

MATERIALS AND METHODS

Materials

Unlabeled dNTPs, dCMP, and GMP were obtained from Sigma. Synthetic oligodeoxynucleotides and other dNMPs and NMPs were obtained from P-L Biochemicals, and labeled dNTPs and NTPs from New England Nuclear. Sephadex G-25 was obtained from Pharmacia.

Enzymes

Viral DNA polymerases from wild type herpes simplex virus type 2 (HSV-2) and from virus resistant to phosphonoacetic acid (PAA^r) or to aphidicolin (Aph^r) were purified from infected cells, as described by Nishiyama et al. (9), as have the mutator (Aph^r) and antimutator (PAA^r) viral phenotypes (10). Specific activities of the herpes enzymes were 875, 725, and 850 units/mg protein for PAA^r, wild type (wt), and Aph^r polymerases, respectively. The characterization of the HSV wt and Aph^r enzymes has been reported (6). The 10 S DNA polymerase- α from calf thymus was purified through the poly(I)-Sephadex-4B step as previously described (11,12); specific activity was 1250 units/mg protein. *E. coli* DNA polymerase I (Pol I) was purified to homogeneity by the method of Jovin et al. (13) as modified by Slater et al. (14). T4 DNA polymerase was obtained from Bethesda Research Laboratories.

DNA Polymerase Assay

Assays were carried out as previously described (15). Reactions contained 20 mM Tris-HCl (pH 8.0), 2 mM dithiothreitol (DiTT), 10 mM MgCl₂, 50 μ M each of dATP, dCTP, dGTP, and dTTP (one dNTP was labeled), 200 μ g/ml activated calf thymus DNA, and the indicated amount of DNA polymerase. One unit of DNA polymerase activity represents the incorporation of 1 nmol of labeled nucleotide into activated DNA in 60 min at 37°C.

DNA Substrates

Activated calf thymus DNA was labeled at 3' termini and isolated as described by Derse et al. (16,17). 100 µg activated DNA was labeled in a DNA polymerase reaction (0.5 ml) containing the components described above, except that the labeled substrate was 20 µM [³H]dGTP (4000 dpm/pmol). 0.4 units of Pol I were included in the reaction mixture and incubation was carried out for 20 min at 37°C. The reaction was stopped by heating at 65°C for 15 min, and DNA was separated from unincorporated nucleotides by Sephadex G-25 chromatography. DNA was precipitated with ethanol, centrifuged, and resuspended in 100 µl of 20 mM Tris-HCl, pH 8.0. Specific activity was 2.2×10^5 dpm/µg DNA.

The polynucleotides dT₄₆-[³H]dT (21 cpm/pmol terminal nucleotide) and dT₄₆-[³H]dC (20 cpm/pmol terminal nucleotide) were synthesized with terminal deoxynucleotidyl transferase and were a generous gift from Dr. F.J. Bollum (Uniformed Services University). These primers were mixed separately with poly dA in a dA:dT ratio of 5:1 (18) and hybridized by heating to 100°C for 5 min, incubating at 65°C for 30 min, and cooling slowly to room temperature.

Exonuclease Assays

3'→5' exonuclease activity was examined in a 25 µl reaction mixture containing the same components as a DNA polymerase assay, except that MgCl₂ was 8 mM, dNTPs were absent, and activated DNA was replaced by 3' terminally labeled, double-stranded, activated calf thymus DNA. Exonuclease activity was monitored by the loss of acid-precipitable radioactivity.

Excision of a terminal mismatch was examined as described by Brutlag and Kornberg (19). Reaction mixtures (40 µl) contained 50 mM Tris-HCl, 2 mM DiTT, 8 mM MgCl₂, 200 mM KCl, and 2 µg of template-primer, which was either poly dA·dT₄₆-[³H]dT or poly dA·dT₄₆-[³H]dC, hybridized as described above. Exonuclease activity was measured by the reduction in acid-precipitable radioactivity. In parallel reactions, polymerase activity was measured with the same components plus 50 µM [α-³²P]dTTP, which allowed incorporation to be followed. Reactions were carried out at 30°C to reduce partial denaturation of the primer-template. Incubation times were up to 60 min with the correctly paired substrate, and up to 15 min for the substrate with terminal mismatch.

Hybridization

ØX174 am3 viral DNA was hybridized at a primer:template molar ratio of 10:1 with synthetic oligodeoxynucleotides, each 15 nucleotides long, which had their 3'-hydroxyl groups 1 or 3 nucleotides from position 587 (designated oligo-1 or oligo-3, respectively). Hybridizations were carried out as previously

described; DNA sequencing has shown that these oligomers hybridize with ØX viral DNA at the expected regions (15).

In Vitro Copying of ØX DNA

Reaction mixtures (25 µl) contained 0.05 µg of primed ØX DNA, 20 mM Tris-HCl (pH 8.0), 2 mM DTT, 20 µM to 1 mM concentrations each of dATP, dCTP, dGTP, and [α - ^{32} P]dTTP (500-1250 dpm/pmol), MgCl₂ as divalent metal activator, and polymerase. MgCl₂ concentrations were 5 mM for DNA polymerase- α , 8 mM for all HSV polymerases and Pol I. Enzyme amounts in each reaction mixture were 0.01-0.02 units of the HSV polymerases, or a 25:1 molar ratio of enzyme:template for homogeneous Pol I. Incubation was for 20 min at 30°C, and incorporation was calculated from acid-precipitable radioactivity (20). Unless otherwise indicated, in all experiments average incorporation was more than sufficient to proceed past the amber site. Copied DNA was transfected and reversion frequency determined by the progeny phage method, as previously described (21,22). Background reversion frequency was determined with primed ØX DNA which was not incubated with DNA polymerase ("uncopied DNA"). The ØX system using synthetic oligodeoxynucleotides as primers has been characterized (23).

Analysis of Oligomer Primer Extension

The percentage of primers extended was determined as described by Silber et al. (24). In this protocol, the synthetic oligomer is labeled with ^{32} P at the 5' terminus, and hybridized at a 2:1 molar ratio with ØX DNA. In vitro copying of ØX DNA is then carried out as described above. The copying reaction contained 0.2 µg of DNA primed with oligo-1. After an incubation time of 20 min, DNA is precipitated with ethanol, resuspended in loading buffer, and loaded onto a 12% polyacrylamide gel containing 7 M urea. After electrophoresis, the gel is transferred to a sheet of Whatman 3 MM paper, dried under vacuum, and bands visualized by autoradiography. The percentage of primers extended is assessed by cutting the gel sample into 2 cm strips, determining ^{32} P by liquid scintillation counting, and correcting for ^{32} P counts from an uncopied control. Other control samples indicated that the HSV enzymes degraded essentially all unhybridized primers during a 20 min incubation. For these enzymes only, it was therefore assumed that all corrected ^{32} P counts represented hybridized primers.

Markers 16 and 17 nucleotides in length were produced by an in vitro ØX copying reaction using Pol I and 20 µM of dTTP and dATP only, which are the complementary nucleotides for the next two positions after the 3' end of the oligomer. Unextended oligomer provided a marker 15 nucleotides in length.

Table 1
Exonuclease and DNA Polymerase Activities

<u>Polymerase</u>	<u>Incubation Time (min)</u>	<u>Exonuclease: Excision (pmol)</u>	<u>Polymerase: Incorporation (pmol)</u>	<u>Ratio, Excision/Incorporation</u>
HSV PAA ^r	5	5.81	1.25	4.6
HSV wt	5	6.22	1.79	3.5
HSV Aph ^r	5	4.53	0.46	9.8
Pol I	5	1.03	2.75	0.37

For each enzyme, equal volumes of sample were analyzed separately for exonuclease and polymerase activity, with incubation times as indicated. The reaction mixture for measuring 3'→5' exonuclease activity contained 0.01 µg activated DNA labeled with [³H]dGMP. Exonuclease activity is calculated as pmol of dGMP excised. Polymerase activity is calculated as pmol of the labeled nucleotide, [α-³²P]dTTP, incorporated. Exonuclease and polymerase assays, and the procedure for labeling activated DNA, were as described in Materials and Methods.

Calculation of Error Rate

With the ØX system, the error rate for any DNA polymerase is calculated from the following expression (21,22):

$$\text{Error rate} = \frac{\text{reversion frequency}}{\text{fraction copied}} \times \frac{1}{\text{penetrance}}$$

Penetrance is the frequency with which the newly synthesized DNA strand is expressed in the ØX system. Penetrance with the synthetic oligomer primers is 0.16 (23).

RESULTS

Exonuclease/Polymerase Activity

Table 1 compares the exonuclease and polymerase activities of the wt herpes and mutant enzymes with the same activities of Pol I. In this experiment, exonuclease activity was measured in the absence of polymerization. Each of the herpes enzymes showed a higher exonuclease/polymerase ratio than Pol I, and also showed a higher ratio than T4 DNA polymerase (data not shown). Small differences were observed between the herpes enzymes.

Excision of a Terminal Mismatch

It was of interest to determine if this enhanced exonuclease activity of the herpes enzymes extended to the removal of a terminal mismatched nucleotide, the classical criterion by which Brutlag and Kornberg (19) demonstrated the

Figure 1. Exonuclease activity on terminally labeled nucleotides. Excision was measured as described in Materials and Methods. Acid-precipitable counts are plotted against time of incubation. Reaction conditions for each panel were as follows: A) The reaction contained poly dA·dT₄₆-dT*. B) The reaction contained the same components as in Panel A, with the addition of 50 μM [α -³²P]dTTP. C) The reaction contained poly dA·dT₄₆-dC*. D) The reaction contained the same components as in Panel C, with the addition of 50 μM [α -³²P]dTTP. Reaction volumes were 40 μl , and 10 μl aliquots were processed for acid-precipitable radioactivity at the times indicated. Values on the ordinate represent equivalent radioactivity remaining in the entire reaction mixture. Note that Panels A and B have different time scales from Panels C and D. DNA polymerases are represented in each panel by the following symbols: no enzyme (open circles), Pol I (solid circles), HSV wt (squares), HSV PAA^r (open triangles), HSV Aph^r (solid triangles).

proofreading activity of Pol I. Experiments to examine this question are presented in Figure 1 and Table 2. In Figure 1, all of the herpes enzymes showed preferential excision of the mismatched terminal nucleotide compared to the correctly paired substrate. In the absence of dTTP, there is some excision of the correctly paired terminus, but this is essentially eliminated when dTTP

Table 2
Mismatch Excision and DNA Polymerase Activities

<u>Polymerase</u>	<u>Mismatch Excision (pmol)</u>	<u>Incorporation (pmol)</u>	<u>Ratio, Excision/ Incorporation</u>
HSV PAA ^r	22.2	3.12	7.1
HSV wt	12.2	1.84	6.6
HSV Aph ^r	11.2	3.28	3.4
Pol I	5.4	11.6	0.46

Values for excision are taken from the 1 min incubation time point in Panel C of Figure 1, and represent pmol of terminal nucleotide excised. For each enzyme, polymerase activity is reported as pmol of the labeled nucleotide, [α -³²P]dTTP, incorporated in 1 min on the poly dA-oligo dT template under the reaction conditions of Figure 1, Panel B.

is present. This implies that under these conditions, the herpes enzymes more readily elongate the primer than excise the terminus. In contrast, even in the presence of dTTP, rapid excision of the mismatched terminus still occurs, suggesting that excision of the terminal mismatch is necessary before elongation can occur. For all enzymes, incorporation of dTTP was demonstrated by the increase of acid-precipitable ³²P counts with time (data not shown).

In Table 2, each of the viral enzymes shows a markedly higher excision:incorporation ratio than Pol I. There are small differences between the viral enzymes, and the PAA^r polymerase shows a relative excision capability about twice that of the Aph^r enzyme.

Next Nucleotide Effect on ØX DNA

It was of further interest to determine if proofreading activity of the herpes enzymes could be detected on a natural DNA system. We have previously developed a system to examine the replication fidelity on a ØX174 am3 DNA template (21-23). With this system, a DNA polymerase copies the ØX viral template in vitro. The copied DNA is transfected into spheroplasts, which complete replication and produce progeny phage. Fidelity is assessed by plating phage on E. coli strains permissive or nonpermissive for the amber codon, and measuring reversion frequency to wt. We have identified several conditions with the ØX system which inhibit the exonuclease proofreading activity of procaryotic DNA polymerases, or defeat this activity through kinetic mechanisms (2).

One such condition is the "next nucleotide effect." Previous work, including sequencing data, indicates that viable revertants from the am3

Table 3
Effect of Next Nucleotide

Polymerase	Condition	Reversion Frequency (x 10 ⁻⁶)
HSV PAA ^r	1 mM dATP	1.89
	1 mM dCTP	1.48
	1 mM dATP + dCTP	1.13
HSV wt	1 mM dATP	2.46
	1 mM dCTP	2.18
	1 mM dATP + dCTP	4.94
HSV Aph ^r	1 mM dATP	1.02
	1 mM dCTP	1.86
	1 mM dATP + dCTP	1.10
Pol I	1 mM dATP	1.60
	1 mM dCTP	3.74
	1 mM dATP + dCTP	29.00
Uncopied DNA		1.30

ØX DNA was primed with the synthetic oligonucleotide designated oligo-3, and in vitro copying reactions were carried out as described in Materials and Methods. Baseline substrate concentrations were 20 µM dATP and dTTP, and 50 µM dCTP and dGTP. Concentrations of individual substrates were increased to the values indicated for each experiment. Copying reactions also contained 200 mM KCl. Reversion frequencies were the average of duplicate samples.

phenotype are produced only through misincorporation at position 587, opposite the viral template A in the middle of the amber codon (25). Exonucleolytic proofreading by procaryotic enzymes involves competition between excision and polymerization. When a mistake is inserted, it can be removed by excision or locked into the growing DNA chain by the subsequent incorporation of the next nucleotide (2,26). The sequence of the am3 template, in order of synthesis from the synthetic oligomer primer, is -G-A-T. Thus an incorrect substitution at position 587 would be locked in by the next correct nucleotide, an A, opposite the template T at position 586.

Table 3 indicates this next nucleotide effect on the fidelity of Pol I. An increased concentration of dATP, which itself is incorrect for position 587, produces a reversion frequency which is only slightly above background. With increased concentrations of both dCTP and dATP, the reversion frequency using Pol I is greater than the additive effects of either individual incorrect substrate. This enhanced reversion frequency has been shown to be due to dCTP misincorporation, and has been attributed to the effect of the next nucleotide

Table 4
Effect of Monophosphates on Fidelity

<u>Polymerase</u>	<u>Monophosphate, Concentration</u>	<u>Reversion Frequency (x 10⁻⁶)</u>	<u>Reversion Freq. Above Background (x 10⁻⁶)</u>
<u>HSV PAA^r</u>	none	2.19	
	11 mM dTMP	2.71	
<u>HSV wt</u>	none	2.60	
	9 mM dAMP	2.78	
	10 mM dCMP	2.03	
	11 mM dTMP	1.50	
	10 mM AMP	2.81	
	8 mM GMP	2.80	
	10 mM IMP	2.03	
<u>HSV Aph^r</u>	none	1.46	
	9 mM dAMP	2.04	
	10 mM dCMP	1.35	
	10 mM dGMP	0.91	
	11 mM dTMP	2.46	
	10 mM AMP	1.62	
	8 mM GMP	3.58	
	10 mM IMP	2.12	
<u>Pol I</u>	none	1.96	0.68
	9 mM dAMP	7.60	6.32
	10 mM dCMP	6.40	5.12
	10 mM dGMP	9.11	7.83
	11 mM dTMP	11.70	10.40
	10 mM AMP	5.47	4.19
	8 mM GMP	5.31	4.03
	10 mM IMP	12.40	11.10

In vitro copying reactions with ØX DNA were carried out as described in Table 3. For all reactions, dNTP concentrations were 20 µM dATP and dTTP, 50 µM dGTP, and 1 mM dCTP, and KCl was present at 200 mM. Reversion frequencies represent the average of duplicate samples in the absence of monophosphates, and single samples with monophosphates. Conditions not shown were those where average incorporation per template was insufficient to proceed past the amber site. Reversion frequency of uncopied ØX DNA was 1.28×10^{-6} . This was subtracted from the samples for Pol I only, since almost all samples with the HSV enzymes showed reversion frequencies close to background.

substrate, dATP (25). With the HSV enzymes, however, the reversion frequency with elevated levels of both dATP and dCTP is a value no greater than the additive effects of individual biased dNTPs. Table 3 thus provides no evidence for the next nucleotide effect with the herpes enzymes.

Effect of Nucleoside Monophosphates

Both nucleoside monophosphates and deoxynucleoside monophosphates inhibit

the 3'→5' exonuclease activity of Pol I (27). We have previously shown that dNMPs also increase reversion frequency of this enzyme in the ØX system (25). With HSV DNA polymerases, dNMPs do not significantly inhibit the associated exonuclease activity, but purine NMPs do (16,28). In addition, some hypothetical mechanisms of fidelity allow for the rejection of incorrect substrates as monophosphates either before or after incorporation (29-31). Thus an effect of monophosphates on the fidelity of the herpes enzymes might provide some insight into the mechanisms by which these enzymes avoid errors.

As Table 4 indicates, monophosphates had no significant effect on the fidelity of any of the HSV enzymes. In contrast, reversion frequency of Pol I was increased by dNMPs, consistent with earlier observations (25), as well as by NMPs. We presume that the effect of NMPs also occurs through the nonspecific inhibition of the 3'→5' exonuclease activity. We believe this is the first report of the mutagenic effect of NMPs on Pol I with a natural DNA template.

Pyrophosphate also increases the error rate of Pol I in copying ØX DNA. This effect is seen in reactions containing saturating amounts of the next nucleotide or high concentrations of dNMPs. Furthermore, pyrophosphate does not enhance misincorporations with AMV DNA polymerase, an enzyme which carries out pyrophospholysis but which has no 3'→5' exonuclease. These results suggest that pyrophosphate-induced misincorporation occurs by inhibition of a proofreading pathway which is distinct from exonuclease excision (32). We have also found that pyrophosphate modestly improves the fidelity of DNA polymerase-α in the ØX system (33), and others have reported that pyrophosphate enhances fidelity of the same enzyme on synthetic templates (31). With the herpes enzymes, however, we found that pyrophosphate neither increased reversion frequency in the ØX system nor consistently reduced it as with polymerase-α (data not shown).

Error Rates of Herpes Polymerases

Since the HSV polymerases showed reversion frequencies close to background under all conditions examined, it was important to confirm that these enzymes extended the oligonucleotide primers past the amber codon. DNA synthesis at other sites can occur on the ØX DNA template, presumably due to random primer contamination. Table 5 indicates the percentage of primers elongated by each herpes enzyme and by 10 S polymerase-α which was run for comparison. The herpes enzymes did elongate a relatively small but significant portion of primer molecules; the low reversion frequencies seen with these enzymes cannot be attributed to the trivial explanation that all synthesis was unprimed.

Information on reversion frequencies and the extent of primer elongation allowed the calculation of error rates for each of the herpes polymerases, as

Table 5
Polymerase Error Rates

<u>Polymerase</u>	<u>Molecules Copied (%)</u>	<u>Reversion Frequency ($\times 10^{-6}$)</u>	<u>Reversion Freq. Above Background ($\times 10^{-6}$)</u>	<u>Uncorrected Error Rate</u>
HSV PAA ^r	18.8	3.97	2.05	1/14,700
HSV wt	15.2	3.89	1.97	1/12,300
HSV Aph ^r	25.7	4.95	3.03	1/13,600
10 S Pol- α	53.0	22.60	20.30	1/4,200
	70.0	35.60	33.70	1/3,300

The synthetic oligodeoxynucleotide designated oligo-1 was labeled on its 5' end with ^{32}P , hybridized to ϕX viral DNA, and extended as described in Materials Methods. The in vitro copying reaction contained 0.2 μg of primer-template, 1 mM each of dATP, dCTP, and dGTP, and 20 μM of dTTP. DNA polymerase amounts were 0.06 units of HSV PAA^r, 0.09 units of HSV wt, 0.08 units of HSV Aph^r, and 0.08 and 0.15 units, respectively, of 10 S calf thymus DNA polymerase- α . Half of each sample was analyzed for primer extension by electrophoresis on a 12% polyacrylamide gel, as described in Materials and Methods. The remaining half was transfected and plated, and error rates were calculated from reversion frequencies, as described in Materials and Methods. Reversion frequency of uncopied DNA was 1.92×10^{-6} . The error rate is not corrected for the bias in incorrect nucleotide concentrations, since we have not demonstrated the proportionality of error rate to the ratio of incorrect to correct dNTPs for each of the DNA polymerases used in these studies (37).

reported in Table 5. Under the conditions examined, the herpes enzymes all showed similar error rates, and were more accurate than DNA polymerase- α . Thus the herpes enzymes appear to be relatively accurate even under conditions which inhibit both exonucleolytic and kinetic proofreading in procaryotic enzymes.

DISCUSSION

Contributors to fidelity in eucaryotic cells are not well-established. Purified eucaryotic DNA polymerases show error rates of 10^{-3} to 10^{-4} (2), much greater than expected for the cell, which suggests that other cellular factors must enhance fidelity. No significant eucaryotic enhancing factors have been identified, however (33). Some procaryotic DNA polymerases contain an integral proofreading 3'→5' exonuclease activity. With *E. coli* DNA polymerase III holoenzyme, the 3'→5' exonuclease is provided by a subunit different from the one containing polymerase activity (34). Since the procaryotic proofreading activity contributes substantially to fidelity, one can hypothesize that

proofreading in eucaryotes could be provided by the exonuclease of DNA polymerase- δ , or by a separate excision enzyme. Alternatively, DNA polymerase- α might exhibit an exonuclease activity in the presence of chromosomal factors that are not included in in vitro fidelity assays. DNA polymerases of the eucaryotic herpes-type viruses contain an associated 3'→5' exonuclease activity (7,8). Investigations of these enzymes might provide insights into mechanisms of eucaryotic fidelity.

The clearest evidence for proofreading by the HSV-2 polymerases comes from experiments with synthetic templates. The HSV enzymes show a greater exonuclease/polymerase ratio than procaryotic polymerases which proofread. In addition, the HSV enzymes show a clear ability to preferentially excise a terminal nucleotide mispair, the classical criterion by which proofreading was demonstrated for procaryotic polymerases (18,19). Moreover, the HSV polymerases show a greater mismatch excision activity, relative to polymerase activity, than a procaryotic DNA polymerase which proofreads.

It is not clear whether the small differences in relative excision activities of the herpes enzymes (Table 2) are sufficient to account for the antimutator and mutator characteristics of the PAA^r and Aph^r viral strains, respectively. Alternative explanations for the mutation frequencies of these strains may lie with altered basepairing selectivity of the mutant enzymes, or altered interaction with accessory replication proteins. Hall et al. (35) found that DNA polymerases from wt HSV-1 and an antimutator PAA^r mutant showed similar exonuclease/polymerase ratios, although the antimutator enzyme showed lower error rates on synthetic templates, suggesting an altered base selectivity. It should be noted that this group assessed fidelity by examining incorporation of incorrect nucleotides into synthetic templates. Limitations of such a system include a lack of sensitivity and difficulty in detecting proofreading with procaryotic enzymes (2,36).

Only by using a biological assay has it been possible to establish the relatively high accuracy of the HSV-2 DNA polymerases. With a ϕ X174 natural DNA template, these enzymes produced mutation frequencies at an amber locus that were only slightly greater than background. This observation was a consequence of the fact that the HSV enzymes copied a relatively smaller percentage of templates, and showed lower error rates, than DNA polymerase- α , the replicative eucaryotic enzyme. Conditions which inhibit the proofreading of procaryotic DNA polymerases had little effect on the fidelity of the HSV enzymes. We conclude that either the HSV enzymes are relatively accurate even

in the absence of proofreading, or the conditions examined are insufficient to inhibit proofreading by these enzymes.

We also find on the ϕ X template no significant difference in error rates of the PAA^r, HSV wt, or Aph^r enzymes. The Aph^r enzyme shows a lower K_m for dCTP than the wild type (9), but this does not produce higher mutation frequencies with the ϕ X experiments performed. This might be explained, however, by the lower K_m of the Aph^r enzyme as well for dTTP (9), which is the correct substrate for position 587 of the amber site. The K_m differences thus may balance with this locus. An alternative explanation may lie with the observation that in order to produce am3 mutation frequencies above background, it was necessary to conduct copying reactions with the HSV enzymes, using dNTP substrate concentrations that were far above K_m values for the polymerases. Differences in enzyme K_m values may have a much greater effect in producing or preventing mutations in the cell.

DNA polymerases tested with the ϕ X system show different specificities of misinsertions at the am3 locus (2). If one assumes that HSV polymerase misinserts one substrate predominantly, rather than all three incorrect substrates equally; and further assumes that error rate is proportional to the concentration ratio of incorrect:correct substrate (37), then from Table 5 one can estimate an error rate at equal dNTP concentrations for HSV polymerase of about 1/650,000. We recognize that both assumptions have yet to be demonstrated for the HSV polymerases, so this estimate probably represents an upper bound of accuracy. Under the conditions examined, the HSV polymerases are moderately more accurate than the homopolymeric form of DNA polymerase- α , the replicative eucaryotic polymerase.

Abbreviations used are:

DiTT	dithiothreitol
HSV	herpes simplex virus
dNMP	deoxynucleoside monophosphate
dNTP	deoxynucleoside triphosphate
NMP	nucleoside monophosphate
NTP	nucleoside triphosphate
Pol I	<u>E. coli</u> DNA polymerase I

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