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# Development and use of an *in vitro* HSV-*tk* forward mutation assay to study eukaryotic DNA polymerase processing of DNA alkyl lesions

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## ABSTRACT

**We have developed an *in vitro* DNA polymerase forward mutation assay using damaged DNA templates that contain the herpes simplex virus type 1 thymidine kinase (HSV-*tk*) gene. The quantitative method uses complementary strand hybridization to gapped duplex DNA molecules and chloramphenicol selection. This design ensures exclusive analysis of mutations derived from the DNA strand produced during *in vitro* synthesis. We have examined the accuracy of DNA synthesis catalyzed by calf thymus polymerase α-primase, polymerase β and exonuclease-deficient Klenow polymerase. Using unmodified DNA templates, polymerase β displays a unique specificity for the loss of two bases in a dinucleotide repeat sequence within the HSV-*tk* locus. Treatment of the DNA template with *N*-ethyl-*N*-nitrosourea resulted in a dose-dependent inhibition of DNA synthesis concomitant with an increased mutation frequency. Similar dose–response curves were measured for the three polymerases examined; thus the identity of the DNA polymerase does not appear to affect the mutagenic potency of ethyl lesions. The HSV-*tk* system is unique in that damage-induced mutagenesis can be analyzed both quantitatively and qualitatively in human cells, in bacterial cells and in *in vitro* DNA synthesis reactions at a single target sequence.**

## INTRODUCTION

An early step in the mechanism of chemical carcinogenesis is the production of premutagenic DNA lesions in target tissues (1). The conversion of these lesions into mutations is modulated by the competing rates of DNA repair and DNA replication. Traditional approaches to understanding the mutagenic consequences of DNA damage have correlated specific DNA lesions and/or their repair with specific types of mutations (2,3). We are interested in examining damage-induced mutagenesis from the perspective of the DNA polymerase as a variable. DNA polymerases constitute a multi-membered family of enzymes that share a common structural motif for the basic catalytic mechanism of phosphodiester bond formation (4,5). Human cells contain four known nuclear DNA polymerases (α, β, δ and ε) that differ significantly in the kinetic parameters for DNA and dNTP substrate binding, catalysis, processivity and mechanisms of error discrimination (6,7). The recent description of a novel polymerase activity involved in DNA repair in yeast (8) illustrates that the complexities of eukaryotic cells with respect to mutagenesis have yet to be fully realized. Variable mutagenic responses dependent upon the DNA sequence context and identity of the polymerase have been observed for a limited number of DNA adducts (9–14). The extent to which such variability affects the mutational potency and specificity of various classes of carcinogens within the human genome is unknown.

*In vitro* DNA synthesis assays have shown that the frequency and specificity of polymerase-induced errors on undamaged DNA templates is unique for each enzyme (15). Forward mutation assays of polymerase error rates have the ability to analyze errors produced in many different DNA sequence contexts (16). Only a few forward mutagenesis studies have been carried out using damaged DNA templates (17–19). We have developed an *in vitro* forward mutation assay to examine how different eukaryotic DNA polymerases respond to the presence of DNA damage and process DNA lesions into mutations. Our mutagenic target is the herpes simplex virus type 1 thymidine kinase (HSV-*tk*) gene, which we have previously used to study damage-induced mutations in *Escherichia coli* (20,21) and human cells (22–24). The assay allows for rapid, yet sensitive selection and DNA sequence analysis of induced mutants by focusing on polymerase-mediated errors in the 5′-region of the HSV-*tk* target. Unlike previous forward mutational studies, which utilized uracil-substituted, damaged templates (17–19), the HSV-*tk* assay employs naturally occurring, damaged templates for DNA synthesis. Moreover, a method has been developed which does not introduce the modified DNA template into *E.coli* for selection of mutants, yet quantitatively recovers DNA synthesis fragments containing polymerase-mediated errors. The experimental design is versatile and can be applied to the study of any type of DNA damaging lesion (random or site-specific) and DNA polymerase combination. The HSV-*tk* system is unique in that mutagenesis at a single DNA target sequence can be analyzed both quantitatively and qualitatively in human cells, in bacterial cells or *in vitro* using purified DNA polymerases. Keeping the mutagenic target constant will eliminate biases due to DNA sequence context (damage distribution) and protein

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structure (mutant selection), thus allowing direct comparisons to be made among the different levels of biological complexity.

## MATERIALS AND METHODS

### Reagents

Calf thymus DNA polymerase α-primase complex was a gift from Dr Fred Perrino (Wake Forest University, Winston-Salem, NC). One unit is defined as the amount of protein incorporating 1 nmol [α-$^{32}$P]dTMP in 60 min at 37°C using activated calf thymus DNA. Recombinant DNA polymerase β was a gift from Dr Samuel Wilson (MIEHS, Research Triangle Park, NC). The 3′→5′ exonuclease-deficient form (D355A, E357A) of the Klenow fragment of *E.coli* DNA polymerase I (Exo$^-$ Klenow) was purchased from Amersham Life Science Inc. (Arlington Heights, IL). *N*-Ethyl-*N*-nitrosourea (ENU) and 5-fluoro-2′-deoxyuridine (FUdR) were purchased from Sigma Chemical Co. (St Louis, MO). The purity of the ENU lot used for these analyses was determined to be 75% by absorbance at 399 nm (ε =110 $M^{-1}cm^{-1}$). All restriction endonucleases were supplied by Gibco BRL Life Technologies (Gaithersburg, MD) and used according to manufacturer's instructions.

### Construction of recombinant HSV-*tk* molecules

To construct a DNA template for the *in vitro* synthesis reactions, we subcloned the 5′-end of the HSV-*tk* gene (*Eco*RI–*Sph*I fragment of pETK; 20) into the multiple cloning site of bacteriophage M13mp18. To make a unique *Eco*RV restriction site within the HSV-*tk* gene, the *Eco*RV site at position 577 of pETK was eliminated by oligonucleotide site-directed mutagenesis using uracil single-stranded (ss)DNA (25), forming M13TK3.5. The 630 bp *Eco*RI–*Sph*I fragment from M13TK3.5, which includes this mutated site, was reintroduced into plasmid pETK to form pSHTK. The entire HSV-*tk* gene (1978 bp *Eco*RI–*Bam*HI fragment) was subcloned from pSHTK into the multiple cloning site of the pGem3Zf(–) phagemid vector (Promega Corp., Madison, WI) along with the 958 bp *Bam*HI fragment from pND123 (23), which contains the chloramphenicol acetyltransferase gene (*cat*). The resulting vector, pGTK2 (Fig. 1), can be used to produce both double-stranded and single-stranded HSV-*tk*-containing DNA forms. Plasmid pGTK3 was derived by oligonucleotide-directed mutagenesis of pGTK2 to inactivate the chloramphenicol acetyltransferase enzyme (CAT) by a H195A change. A second oligonucleotide was used to eliminate the *Eco*RI site within the *cat* gene by a silent base substitution in order to physically differentiate plasmids pGTK2 and pGTK3. The reversion frequency of pGTK3 to confer chloramphenicol resistance to plasmid-bearing *E.coli* was measured as $<5 \times 10^{-8}$.

### *In vitro* polymerase reactions

DNA synthesis templates were created by hybridization of a 20mer oligonucleotide complementary to the unique *Eco*RV restriction site to M13TK3.5 ssDNA at a 1:1 molar ratio. Using this template, DNA synthesis is initiated at *tk* position 282, 34 nt downstream of the ATP binding site. The molar ratios of template DNA to polymerase were optimized for each enzyme such that at least a 203 nt strand is synthesized to include the *Mlu*I restriction site. The *in vitro* reactions contained 2 pmol template DNA at

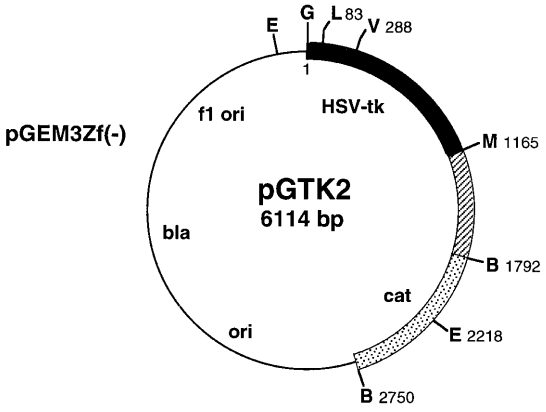


**Figure 1.** Plasmid pGTK2. The pGem3Zf(–) portion of the vector is shown as a thin line. The positions of the bacterial origin of replication and the phage f1 origin are indicated. The coding region of the HSV-*tk* gene is indicated by a filled box and the 3′-non-coding region is indicated by a hatched box. The positions of two antibiotic resistance markers, β-lactamase (*bla*) and chloramphenicol acetyltransferase (*cat*; stippled box) are also shown. E, *Eco*RI; G, *Bgl*II; L, *Mlu*I; V, *Eco*RV; M, *Sma*I; B, *Bam*HI.

40 nM concentration. Reaction conditions for Exo$^-$ Klenow polymerase and polymerase α-primase were 20 mM Tris–HCl, pH 7.5, 10 mM $MgCl_2$, 2 mM dithiothreitol, 1 mM dNTPs and 0.6 pmol Exo$^-$ Klenow polymerase per pmol template DNA or 1.2 U polymerase α-primase per pmol template DNA. Reaction conditions for polymerase β were 50 mM Tris–HCl, pH 8.5, 50 mM NaCl, 1 mM dithiothreitol, 1 mM dNTPs, 200 μg/ml bovine serum albumin and 10 pmol enzyme per pmol template DNA. All reactions were incubated at 37°C for 60 min and terminated with 15 mM EDTA. The extent of DNA synthesis was determined by parallel reactions (0.2 pmol DNA, same molar ratios of enzyme to substrate as above) supplemented with 5 μCi [α-$^{32}$P]dCTP (3000 Ci/mmol). The DNA products of these reactions were analyzed on an 0.8% agarose gel followed by autoradiography. Complete synthesis by T7 polymerase was used as a full-length marker, while hybridization of a $^{32}$P 5′-end- labeled *Mlu*I–*Eco*RV restriction fragment hybridized to the ssDNA template was used as a minimal synthesis marker. When necessary, an 8–12% denaturing polyacrylamide gel was used to ensure that DNA synthesis had proceeded past the *Mlu*I restriction site.

For mutant analyses, synthesis reactions were heated to 68°C for 3 min to inactivate the polymerase and washed with 9 vol. TE using a Microcon-30 ultrafiltration unit (Amicon Inc., Beverly, MA). The DNA products were then digested with *Eco*RV and *Mlu*I restriction enzymes at 37°C for 60 min. The 203 bp fragment (small fragment, SF) was separated from larger DNA fragments by selective polyethylene glycol (PEG) precipitation using 0.54 M NaCl, 6% PEG8000 and 20 nM DNA (26). After overnight incubation on ice, the large DNA fragments were pelleted by centrifugation and the SF precipitated from the supernatant with ethanol. The SF was resuspended in 100 μl purified $H_2O$ and concentrated to 10 μl using a Microcon-30 device. The concentration and purity of the SF was estimated by agarose gel electrophoresis using DNA mass ladder standards. Typically, polymerase reactions that had completed synthesis of the 7.8 kb M13TK3.5 DNA molecule yielded 50–100 ng purified SF (see Fig. 2).

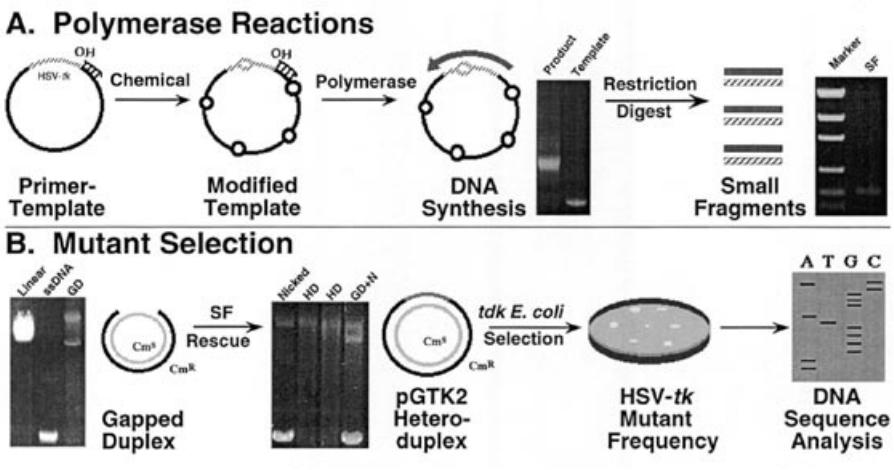


**Figure 2.** Schematic of the *in vitro* HSV-*tk* forward mutation assay. (**A**) Polymerase reactions. Single-stranded HSV-*tk*-containing DNA primed for DNA synthesis by specific oligonucleotides was treated with a DNA damaging agent (bubble structure denotes lesion). DNA synthesis was targeted to the ATP binding site of the HSV-*tk* gene (hatched). The 203 bp restriction fragments (SF) containing newly synthesized DNA were purified. (**B**) Mutant selection. A gapped duplex (GD) was constructed to contain one DNA strand from a plasmid containing a functional *cat* gene ($Cm^R$) and one strand from a plasmid containing a non-functional *cat* gene ($Cm^S$). The newly synthesized SF DNA strands were rescued by hybridization to the GD (N, nicked DNA marker). The heteroduplex (HD) pGTK2 plasmid molecules were used to electroporate *E.coli* strain FT334 (*rec*A$^-$, *tdk*). Selection with chloramphenicol killed bacteria harboring plasmids that were progeny of the $Cm^S$ strand. The bacteria were also plated in the presence of FUdR to determine the HSV-*tk* mutant frequency. The type and location of the damage-induced polymerase errors were determined by DNA sequence analysis.

### DNA modification

Alkylated DNA substrates were created by random DNA modification of oligonucleotide-primed ssDNA to avoid potential loss of thermally labile DNA adducts during hybridization. The DNA (final concentration 20 nM) was incubated in PEN buffer (10 mM sodium phosphate, pH 7.4, 1 mM EDTA, 0.1 mM NaCl) containing various doses of ENU (in dimethylsulfoxide, DMSO) at 37°C for 60 min. A 1 M ENU stock was freshly prepared for each experiment and used immediately in the DNA modification reaction. Modification was quenched by diluting the DNA in ice-cold PEN and washing with 20–40 vol. PEN using a Centricon-30 filtration device at 4°C, as previously described (20–22). Modified DNA was used immediately in the polymerase reactions.

### Mutational analysis of *in vitro* DNA reaction products

A gapped duplex (GD) molecule was constructed by hybridizing a linear DNA fragment from pGTK2 to ssDNA from pGTK3, thereby creating a single-stranded region complementary to the SF purified from the polymerase reactions. The linear DNA fragment was prepared by digestion of pGTK2 with *Eco*RV and *Mlu*I, followed by selective PEG precipitation (26) at 0.55 M NaCl, 5% PEG8000, 0.4 μg/μl DNA in a 37°C water bath overnight. The 5.9 kb fragment was recovered by centrifugation and purified by ethanol precipitation. Single-stranded pGTK3 DNA was prepared by R408 helper phage infection of plasmid-bearing *E.coli* strain DH5α-IQ. Uracil-containing ssDNA was isolated after helper phage infection of plasmid-bearing *dut,ung* CJ236 bacteria (25). UV-irradiated ssDNA was obtained by exposure of 1 μg/ml ssDNA in TE buffer to 254 nm light at a fluence of 800 J/m$^2$. The irradiated DNA was protected from exposure to fluorescent light by wrapping tubes in foil or working under yellow light. The full-length ssDNA was separated from other ssDNA forms by preparative agarose gel electrophoresis and purified using silica (Pierce, Rockford, IL), according to the manufacturer's instructions. GD DNA was formed by hybridization of linear DNA to ssDNA at a 1:1 molar ratio. Linear duplex DNA was diluted in water (final concentration in hybridization 250 ng/μl) and heated to 85°C for 10 min. During the last minute, ssDNA was added to the tube. The denatured DNA was placed on ice for 5 min before adding standard sodium citrate (SSC) to a 2× final concentration (300 mM NaCl, 30 mM sodium citrate). The tubes were incubated at 60°C for 30 min to promote hybridization and the DNA purified by ethanol precipitation. Under these conditions, nearly all of the ssDNA was converted to the GD form, with residual linear DNA as visualized by agarose gel electrophoresis (see Fig. 2).

To sample DNA synthesis fragments for mutations, SF prepared as described above were hybridized to the GD, thus forming pGTK2 heteroduplex plasmid molecules. The SF (35–150 ng, 0.25–1.2 pmol) was heated to 85°C for 5 min. After addition of SSC to 0.5× (75 mM NaCl, 7.5 mM sodium citrate), GD (200–225 ng, 0.05 pmol) was added to a final concentration of 15 ng/μl and hybridization allowed to proceed at 45°C for 60 min. An aliquot (30 ng) of the hybridization was removed for mutational analysis. The remaining hybridization mixture was analyzed by electrophoresis through a 0.8% agarose gel at 90 V for at least 10 h with GD and double-stranded nicked pGTK2 standards. Successful hybridization of SF to GD yielded DNA products that migrated coincident with the nicked standard, with no evidence of products the size of the GD (Fig. 2).

### HSV-*tk* mutational analysis

To select for SF containing HSV-*tk* mutations, the aliquoted DNA from the final hybridization was used to transform *rec*A13, *upp, tdk E.coli* strain FT334 by electroporation and the bacteria were

plated on VBA selective medium as previously described (20), replacing ampicillin with chloramphenicol as the antibiotic. The presence of 50 μg/ml chloramphenicol selects progeny of the 'rescued' pGTK2 DNA strand. To select for HSV-*tk* mutant plasmids, the bacteria were also plated in the presence of 40 μM FUdR. The HSV-*tk* mutant frequency is defined as the number of FUdR-resistant + chloramphenicol-resistant colonies divided by the total number of chloramphenicol-resistant colonies. Mutation frequency curves were analyzed statistically using one-way and two-way ANOVA tests (27).

The post-electroporation doubling time of FT334 in SOC medium at 37°C was measured as 45–50 min, while full FUdR resistance requires a 2 h recovery period (data not shown). To ensure independence of the mutants selected for DNA sequencing, bacteria were placed on ice immediately following electroporation and aliquoted into multiple tubes containing 1 ml VBA medium. Following a 2 h expression period at 37°C, the individual cultures were plated on selective medium and one FUdR-resistant mutant was isolated from each culture. The HSV-*tk* mutant phenotype was confirmed by replating plasmid-bearing bacteria in the presence of 2 μg/ml trimethoprim, a folate analog used to select for $TK^+$ activity (22). The DNA sequence of the HSV-*tk* gene in the *Mlu*I–*Eco*RV region of each mutant was determined by dideoxy DNA sequence analysis of plasmid DNA using Sequenase 2.0 according to the manufacturer's instructions (Amersham Corp.). Differences in proportions of specific types of mutations were analyzed statistically using Fisher's exact test (two-tailed) (27).

## RESULTS

### Development of the assay

In the HSV-*tk* forward mutation assay (Fig. 2), oligonucleotide-primed ssDNA is used as a template for DNA polymerase reactions. The template may be treated with mutagen and purified prior to the addition of polymerase. The DNA reaction products are digested with restriction enzymes and a 203 bp DNA fragment (SF) is purified (Fig. 2A). To recover and analyze these DNA fragments for the presence of mutations, a GD molecule is used. The GD molecule is formed by hybridization of a linear chloramphenicol-resistant ($Cm^R$) DNA fragment to a chloramphenicol-sensitive ($Cm^S$) ssDNA, thereby forming a molecule whose single-stranded region is complementary to the DNA fragments to be analyzed. DNA synthesis fragments containing potential mutations within the *tk* gene are rescued by hybridization to the GD to form pGTK2 heteroduplex plasmid molecules (Fig. 2B). These plasmid DNA molecules are used to transform *tdk E.coli* strain FT334. Incubation of the transformed bacteria in the presence of chloramphenicol kills bacteria harboring plasmids that are progeny of the $Cm^S$ strand of the hybridized GD, therefore, the only bacteria which survive selection are those that are progeny of the 'rescued' DNA strand. HSV-*tk* mutant plasmids are selected by plating the bacteria in the presence of FUdR. The resulting HSV-*tk* mutant frequency is a measure of the proportion of DNA fragments containing mutations.

*Quantitative rescue of DNA fragments.* We have quantitatively evaluated the effectiveness of our method for rescuing HSV-*tk* mutant DNA fragments (Fig. 3). Small fragments were isolated from wild-type and mutant (C→T transition at position 256) HSV-*tk* plasmids. The two DNA populations were combined in different proportions ranging from 0.1 to 100% mutant SF molecules. The fragments were hybridized to GD molecules containing $Cm^S$ ssDNA and the HSV-*tk* mutant frequency determined. Full recovery of input mutant DNA molecules was observed, where the $FUdR^RCm^R$ frequency was equal to the mutant SF fraction in the sampled DNA population (Fig. 3). We have compared our approach to two other methods which selectively cripple replication of one strand of the GD molecule: uracil and UV lesions (17–19,28). The use of uracil lesions in the GD resulted in recovery of only ~10% of the mutant SF (Fig. 3). Presumably, the uracil-containing DNA strand is not completely degraded by uracil DNA glycosylase in FT334 prior to replication of the plasmids. This results in a majority (~90%) of bacteria containing both mutant and wild-type plasmids, which die in the presence of FUdR. We observed results similar to the uracil GD when UV irradiated ssDNA was used in the GD (data not shown). In contrast, in the $Cm^S/Cm^R$ GD method, the observed HSV-*tk* mutant frequency is a direct quantitation of the proportion of SF DNA strands containing mutations.

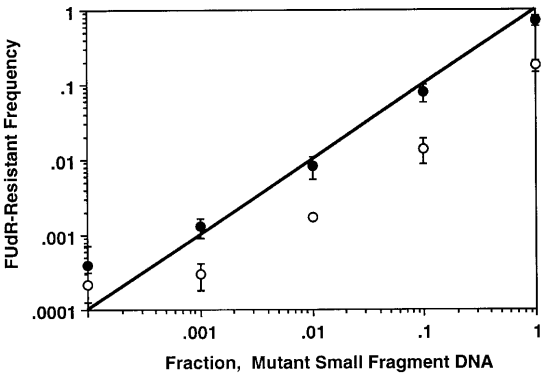


**Figure 3.** Quantitative evaluation of the effectiveness of HSV-*tk* mutant DNA synthesis fragment rescue. HSV-*tk* small fragments (SF) were isolated from both wild-type and mutant HSV-*tk* plasmids. The two DNA populations were combined in different proportions ranging from 100 to 0.1% mutant molecules. The fragments were analyzed for the presence of mutations as shown in Figure 2, using GD molecules made from either $Cm^S$ (filled circles) or uracil ssDNA (open circles). In the graph, the line represents a perfect recovery of input mutant DNA molecules, where the $FUdR^RCm^R$ frequency is equal to the mutant fraction in the sampled DNA population. Symbols are the mean (± SD) mutant frequencies from three independent experiments. Data points on the *y*-axis represent background frequencies (no added mutant DNA) for each type of GD.

*Heteroduplex mismatch correction.* Hybridization of $tk^-$ DNA synthesis fragments to the GD will result in mismatched bases at the site of the mutation. We tested whether the mismatch repair-competent *E.coli* strain used to select for HSV-*tk* mutant plasmids (FT334) preferentially repairs the SF portion of the heteroduplex DNA to the wild-type sequence. Various heteroduplexes between a $tk^-$ SF and a $tk^+$ GD were formed as described above to create A·C (SF·GD) mispairs at two different positions, a G·T mispair, a T·T mispair and an unpaired A base (Table 1). The heteroduplexes were introduced into strains FT334 and PP102, an isogenic *mut*L derivative of FT334 (kindly provided by Dr Raymond Monnat, University of Washington) and the HSV-*tk* mutant frequencies were determined for each DNA, relative to wild-type DNA. If preferential repair of the mutant DNA strand was occurring, we would expect that the HSV-*tk* mutant frequency measured in strain PP102 would be higher than that observed in FT334. As shown in Table 1, this was not observed for three of the five mispairs tested. For two

mispairs, the small difference observed between strains is within experimental error of the assay. To confirm that the mutants recovered in FT334 were due to retention of the input mutations, we took advantage of the fact that the C→T mutation used for the heteroduplex A·C mispair (position 256) creates a new *Bsa*AI restriction enzyme site. All colonies (15/15 for each strain) arising after electroporation of the A·C heteroduplex contained only plasmids derived from the A strand. All control colonies arising after electroporation of the wild-type (G·C) DNA contained only plasmids derived from the C strand. As a final test for the loss of input mutations by mismatch repair, the experiment depicted in Figure 3 was repeated using strain PP102 as recipient for the DNA populations and the same HSV-*tk* mutant frequencies were observed as are shown for strain FT334 (data not shown). Therefore, we conclude that strain FT334 does not repair the hybridized SF strand preferentially over the GD strand. This result is consistent with the fully methylated status of the DNA fragments used to make the heteroduplex molecule.

**Table 1.** Lack of preferential heteroduplex mismatch correction in the HSV-*tk* assay

| Mispair *tk*$^-$·*tk*$^+$ | HSV-*tk* mutant frequency[a] | |
|---|---|---|
| | FT334 (*mut*$^+$) | PP102 (*mut*L) |
| None (wild-type) | $4.2 \times 10^{-4}$ (5) | $4.4 \times 10^{-4}$ (5) |
| A·C at 256 | 0.70 (4) | 0.39 (4) |
| A·C at 227 | 0.42 (2) | 0.32 (2) |
| G·T at 203 | 0.85 (2) | 0.28 (2) |
| T·T at 216 | 0.13 (1) | 0.20 (1) |
| _·A at 241 | 0.30 (1) | 0.51 (1) |

[a]Average value; number of determinations shown in parentheses.

*Background HSV-*tk *frequencies.* The spontaneous frequency of HSV-*tk* mutants resulting from growth of pGTK2 in FT334 is $1.7 \pm 0.65 \times 10^{-5}$. The mutation frequency determined after electroporation of unfilled GD (4 ng) is $1.8 \times 10^{-5}$. To assess the frequency of mutations resulting from manipulations to rescue DNA fragments, we hybridized wild-type HSV-*tk* fragments to the GD and scored for mutations. The mutation frequency as determined in this manner was $2.9 \pm 2.1 \times 10^{-4}$ (mean of 10 independent determinations) and is referred to as the background mutation frequency from this point. This frequency reflects mutations arising not only in the *Mlu*I–*Eco*RV region, but throughout the 1350 bp HSV-*tk* promoter and coding sequences. Due to the 10-fold difference between the background mutation frequency and the unfilled GD frequency, we assume that any residual GD that is too low in concentration to be detectable by ethidium bromide staining of the agarose gel (see Fig. 2) does not contribute significantly to the HSV-*tk* mutant frequency measured for fully hybridized GD.

## Polymerase accuracy on undamaged DNA templates

We have analyzed the frequency and specificity of errors produced in the HSV-*tk* assay by three 3′→5′ exonuclease (proofreading)-deficient DNA polymerases: DNA polymerase α-primase, polymerase β and Exo$^-$ Klenow polymerase (Table 2). DNA synthesis by Exo$^-$ Klenow polymerase increased the HSV-*tk* mutant frequency to $1.4 \pm 0.45 \times 10^{-3}$, ~5-fold over the background mutant frequency. Similarly, the mutant frequency was increased 8-fold after DNA synthesis by polymerase α-primase ($2.2 \pm 1.3 \times 10^{-3}$) and polymerase β ($2.4 \pm 0.79 \times 10^{-3}$). Plasmid DNA was isolated from individual FUdR$^R$ colonies obtained from at least two independent synthesis reactions for each polymerase and the DNA sequence changes within the target region were determined. These DNA sequence analyses of HSV-*tk* mutants demonstrate the characteristic mutational properties of each polymerase (Fig. 4).

**Table 2.** HSV-*tk* mutant frequencies on untreated DNA templates

| Polymerase | Viable cells (per ml) | | HSV-tk mutant |
|---|---|---|---|
| | Cm$^R$ | FUdR$^R$ Cm$^R$ | frequency ($\times 10^{-4}$) |
| None[a] | | | 2.9 (2.1) |
| Exo$^-$ Klenow | 42 000 | 42 | 10 |
| | 34 000 | 71 | 21 |
| | 48 000 | 61 | 13 |
| | 110 000 | 110 | 10 |
| Mean (SD) | | | 14 (4.5) |
| Pol α-primase | 43 000 | 170 | 40 |
| | 74 000 | 140 | 19 |
| | 290 000 | 220 | 7.6 |
| Mean (SD) | | | 22 (13) |
| Pol β | 920 000 | 3200 | 35 |
| | 54 000 | 110 | 20 |
| | 48 000 | 83 | 17 |
| Mean (SD) | | | 24 (7.9) |

[a]Mutant frequency obtained by hybridization of wild-type SF obtained from restriction enzyme digestion of pGTK2 plasmid DNA to Cm$^R$/ Cm$^S$ GD.

**Table 3.** Summary of polymerase-induced HSV-*tk* mutations on untreated templates

| Type of mutation | Number of mutants observed (proportion) | | |
|---|---|---|---|
| | Exo$^-$ Klenow | Pol α-primase | Pol β |
| Base substitutions | 37 (0.66) | 20 (0.41) | 25 (0.27) |
| Transitions | 22 | 15 | 10 |
| Transversions | 15 | 5 | 15 |
| One base frameshifts | 11 (0.20) | 27 (0.55) | 53 (0.56) |
| Deletion | 10 | 22 | 45 |
| Addition | 1 | 5 | 8 |
| 2 frameshifts | 6 (0.11) | 2 (0.04) | 12 (0.13) |
| TATATA | 0 | 1 | 11 |
| Other site | 6 | 1 | 1 |
| Other[a] | 2 (0.04) | 0 | 4 (0.04) |
| Total sequenced | 56 | 49 | 94 |
| Overall frequency | $11 \times 10^{-4}$ | $11 \times 10^{-4}$ | $26 \times 10^{-4}$ |

[a]Other changes in the Exo$^-$ Klenow spectrum are complex changes resulting in CCT at positions 226–229 and GGGAA at positions 237–244. Other changes in the polymerase β spectrum are a complex change resulting in CCT at positions 226–229 and three closely spaced multiple changes: ΔAT at 215–219 and ▲ G at 209–211; ΔACC at 244–252 and ΔG at 237–240; C→T at 198 and C→T at 208.

The observed base substitution mutant frequency is similar for all three polymerases, ranging from 4.5 to $7 \times 10^{-4}$ (Table 3). The eukaryotic polymerases display a preference for the misinsertion of purine dNMP over pyrimidine dNMP. This insertional bias is

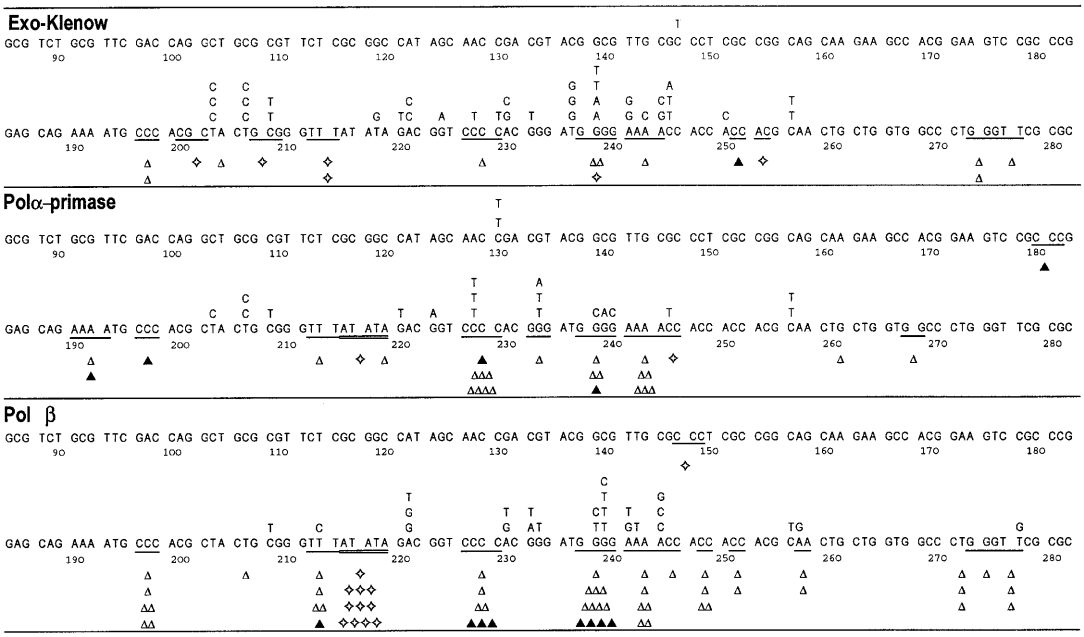


**Figure 4.** Polymerase error specificity on untreated DNA templates in the HSV-*tk* forward mutation assay. The DNA template sequence shown for each polymerase is the *Mlu*I–*Eco*RV mutational target that includes the ATP binding site (nt 208–246). Letters above the line indicate base substitutions, symbols below the line represent frameshift mutations. Repetitive motifs are underlined for clarity. Δ, One base deletion; ▲, one base insertion; ✧, two base deletion.

6-fold for polymerase α-primase but only 2-fold for polymerase β. In addition, the order of preference of polymerase α-primase for all potential misinsertions (A > G > T > C) differs from that observed for polymerase β (A > C > G > T), resulting in a significantly different transition to transversion ratio for the two polymerases (polymerase α, 3:1; polymerase β, 0.67:1; $P = 0.03$).

The proportion of one base frameshift mutations observed for the eukaryotic polymerases (~50%) differs significantly from that observed for the prokaryotic polymerase (20%) ($P \leq 0.0002$) (Table 3). Comparing the eukaryotic polymerases, the frequency of one base frameshifts produced by polymerase β ($15 \times 10^{-4}$) is 2-fold higher than that observed for polymerase α-primase ($6.1 \times 10^{-4}$). Both polymerases are biased toward the loss or gain of bases within repeated sequences, as compared with unique sequences, by at least 10-fold. In addition, the majority of one base frameshift events are the deletion of a base rather than addition of a base (Table 3).

The polymerase β spectrum is marked by the loss of two bases in the dinucleotide repeat TATATA at positions 214–219 (Fig. 4). These events constituted 12% of all detected polymerase β mutations (11 independent occurrences) but only 2% of polymerase α-primase mutations (one occurrence), resulting in a 14-fold higher frequency for polymerase β ($3.0 \times 10^{-4}$) compared with polymerase α-primase ($0.22 \times 10^{-4}$). This type of mutation was not observed for the prokaryotic enzyme, although two base deletions were observed in the Exo⁻ Klenow spectrum at a similar frequency ($1.2 \times 10^{-4}$) elsewhere in the target (Table 3).

Approximately 6% of the mutants isolated as FUdR-resistant displayed a leaky phenotype during the trimethoprim selection, defined as an elevated survival relative to fully *tk*⁻ cells at 2 μg/ml trimethoprim. Leaky mutants are fully sensitive to trimethoprim at 4 μg/ml, a concentration which does not adversely affect survival of *tk*⁺ plasmid-bearing cells (data not shown). We assume that the leaky mutations inactivate the thymidine kinase enzyme sufficiently to allow growth of the bacteria in the presence of FUdR but have some residual thymidine kinase activity allowing growth of the cells at 2 μg/ml trimethoprim. DNA sequence analysis of the leaky mutants revealed that the mutations were restricted to a few amino acid residues within or near the ATP binding site: codons 55 (D→G), 57 (P→H,L), 58 (H→R, L or Y), 59 (G→E,R) and 65 (T→P).

## Polymerase response to ENU modification

To demonstrate the applicability of the HSV-*tk* assay to the analysis of DNA damage, we have examined the responses of DNA polymerases to lesions produced by ENU. As depicted in Figure 5A, random ENU treatment of the DNA template resulted in a dose-dependent inhibition of DNA synthesis by Exo⁻ Klenow polymerase (Fig. 5A).

Even at the highest degree of inhibition analyzed, DNA synthesis proceeded past the 5′-end of the target for mutagenesis (position 82) and no fragments smaller than ~200 bases were observed (Fig. 5 and data not shown). Therefore, the products of the DNA synthesis reactions using ENU-modified templates were further analyzed for HSV-*tk* mutations. As described above, our procedure ensures that the observed mutant frequency is a direct measure of the polymerase errors directed by the DNA lesions and is not due to mutational processing of the lesions in *E.coli*. A dose-dependent increase in mutation frequency was observed in three independent template modifications and polymerase reactions (Fig. 5B). As the DNA sequence we analyzed constitutes only 2.5% of the total DNA modified and ENU has a short half-life in PEN buffer (5–10 min; 29), the observed mutational variability is most likely due to the stochastic nature of the modification reaction. Treatment of the DNA template with 20 mM ENU prior to DNA synthesis by Exo⁻ Klenow polymerase resulted in a mean mutant frequency of $100 \times 10^{-4}$, a significant increase over the 1 mM induced mutant

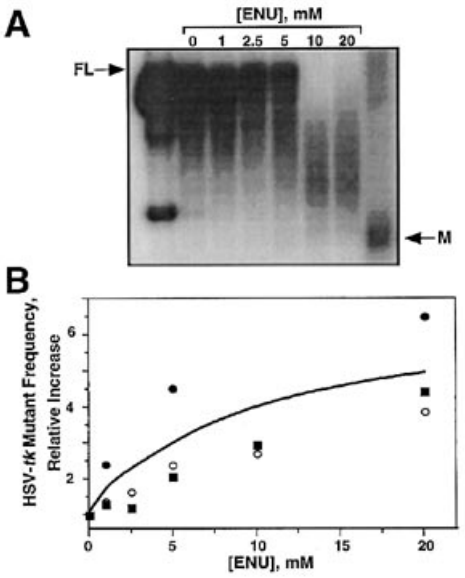


**Figure 5.** Exo⁻ Klenow polymerase response to ENU damage. Oligonucleotide-primed ssDNA was randomly modified by incubation with various concentrations of ENU (DMSO solvent) in phosphate buffer, pH 7.4, and used as a substrate for *in vitro* DNA synthesis reactions. (**A**) The extent of DNA synthesis was determined by supplementing the reactions with [α-$^{32}$P]dCTP, separating the DNA products by agarose gel electrophoresis and visualization by autoradiography. FL, full-length marker shows complete DNA synthesis around the ssDNA circle; M, minimal synthesis marker shows position of a 203 bp *Mlu*I–*Eco*RV fragment hybridized to the template. (**B**) The products of the synthesis reactions were analyzed for HSV-*tk* mutations as depicted in Figure 2 and described in Materials and Methods. The resulting HSV-*tk* mutation frequencies were normalized to the solvent control mutant frequency for each experiment. Symbols represent data from three independent ENU modification reactions.

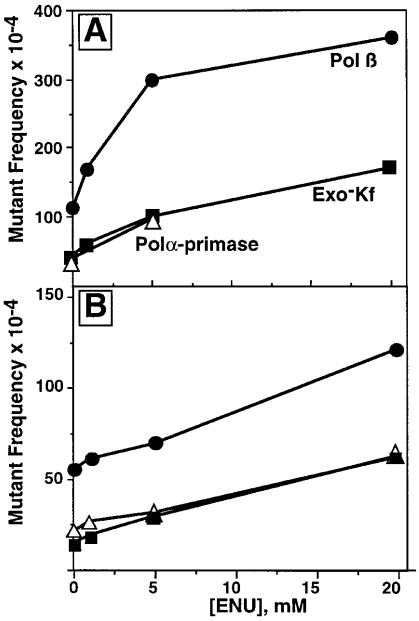


**Figure 6.** Comparison of DNA polymerase responses to ENU damage. Oligonucleotide-primed ssDNA was reacted with various concentrations of ENU (DMSO solvent). After purification, this modified template was divided into three portions and used as a substrate for *in vitro* DNA synthesis reactions with polymerase α-primase, polymerase β and Exo⁻ Klenow polymerase. The products of the synthesis reactions were analyzed for HSV-*tk* mutations as described in Materials and Methods. The results of two independent ENU modifications are shown.

frequency of $35 \times 10^{-4}$ ($P < 0.001$) (solvent-treated control frequency $23 \times 10^{-4}$).

We next used the *in vitro* HSV-*tk* forward mutation assay to test how differences among polymerases in the biochemical mechanisms of DNA synthesis affect the mutagenic potency of DNA alkyl adducts. In two independent experiments, ENU-modified template DNA preparations were divided into three portions and used as a substrate for *in vitro* DNA synthesis reactions with polymerase α-primase, polymerase β and Exo⁻ Klenow and the products were analyzed for HSV-*tk* mutations. As shown in Figure 6, similar dose–response curves were observed for the three polymerases. Treatment of the DNA template with 20 mM ENU increased the mutant frequency observed for polymerase β to $240 \times 10^{-4}$ (mean of two experiments) and for polymerase α-primase to $60 \times 10^{-4}$, 3-fold over solvent-treated controls ($82 \times 10^{-4}$ and $20 \times 10^{-4}$ respectively). The polymerase β and Exo⁻ Klenow dose–response curves do not differ significantly from one another ($P = 0.2$), suggesting that the nature of the polymerase does not quantitatively affect the overall mutagenicity of the DNA lesions. However, we have observed significant qualitative differences, suggesting that the types of mutations produced on each template are partially dependent on the DNA polymerase (30; Eckert,K.A., Vargo,P.L. and Hile,S.E., manuscript in preparation).

## DISCUSSION

We have developed an *in vitro* forward mutation assay to analyze polymerase-mediated events during DNA synthesis using chemically modified DNA templates. The experimental system is unique in that mutagenesis can be analyzed in human cells (22–24), in bacterial cells (20–21) or in polymerase *in vitro* synthesis reactions at a single DNA target sequence: the HSV-*tk* gene. This allows us to make direct quantitative and qualitative comparisons among the different levels of biological complexity. The HSV-*tk* system is general for any form of DNA damage, affording the opportunity to study the mutagenic potential of previously uncharacterized carcinogens in a human system.

Using the *in vitro* assay (Fig. 2), damage-induced DNA polymerase errors can be detected within a variety of sequence contexts along the 5′-region of the HSV-*tk* gene. Oligonucleotide-primed ssDNA is used as a template for DNA synthesis reactions. The experimental design ensures exclusive analysis of mutations derived from the DNA strand produced during *in vitro* synthesis. We have demonstrated that the resulting HSV-*tk* mutant frequency is a direct measure of the proportion of DNA fragments containing mutations (Fig. 3) and thus is a quantitative determination of the damage-induced polymerase error frequency. No selective loss of mutations in the linear fragments due to mismatch repair of the heteroduplex was observed (Table 1). Thus, our method quantitatively evaluates errors in the newly synthesized DNA strand and the modified template strand is not introduced into *E.coli* for mutational analyses. The method can be adapted to adduct site-specific studies wherein single adducts are placed in an oligonucleotide

which is hybridized to the GD (28), thus allowing direct comparisons of site-specific lesions with random mutagenesis in the same system.

The background HSV-*tk* mutant frequency for the assay is estimated to be $2.9 \pm 2.1 \times 10^{-4}$, comparable with other *in vitro* polymerase forward mutation assays (16,19). We have not yet saturated the HSV-*tk* mutational target with regard to all possible mutations that will be detectable by our selection scheme. To date, we have detected within the *Mlu*I–*Eco*RV region all 12 possible base substitution mutations at 58 sites and 18 repetitive sites at which we have detected one base frameshift mutations. Two base frameshift mutations, complex mutations and large deletions have also been observed in this region. The target sequence for this assay is operationally defined by the restriction enzymes used to construct the GD molecule. Therefore, the mutagenic target can be easily expanded to encompass a greater number of sequence contexts within the 1150 bp HSV-*tk* coding sequence by using a different pair of restriction enzymes.

We have determined the accuracy of three 3′→5′ exonuclease-deficient polymerases on undamaged DNA templates in the HSV-*tk* assay: calf thymus DNA polymerase α-primase, polymerase β and Exo$^-$ Klenow. DNA synthesis by all three polymerases increased the HSV-*tk* mutant frequency 5- to 8-fold over the background mutant frequency (Table 2). DNA sequence analyses of HSV-*tk* mutants illustrate the characteristic mutational properties of each polymerase (Fig. 4). The observed HSV-*tk* base substitution mutation frequency was similar for polymerase α-primase and polymerase β ($\sim 5 \times 10^{-4}$), however, the enzymes differ in their preferences for misinserted bases and exhibit significantly different transition to transversion ratios. While both eukaryotic polymerase spectra displayed a high proportion (50%) of one base frameshift mutations, the polymerase β spectrum is characterized by a high frequency of two base deletion mutations in a TATATA dinucleotide repeat motif (Table 3 and Fig. 4). These general tendencies of the polymerases to produce base substitution versus frameshift errors at the HSV-*tk* locus are the same as those previously described for the *lac*Z gap-filling forward mutation assay (31,32).

Our analysis of the HSV-*tk* data suggests that the relatively low accuracy of polymerase β is not general for all DNA sequences, but is restricted to the loss of bases within mononucleotide and dinucleotide repeated sequences. The observed base substitution accuracy of polymerase β is merely 1.5-fold lower than that of polymerase α-primase, whereas the observed one base frameshift accuracy of polymerase β is only 2.5-fold lower than that of polymerase α-primase (Table 3). However, the loss of two bases in a dinucleotide repeat is a high frequency event for polymerase β in the HSV-*tk* sequence (Fig. 4), constituting 12% of the total mutants observed. Moreover, the frequency of this type of mutation for polymerase β is at least 10-fold greater than that observed for either polymerase α-primase or Exo$^-$ Klenow. These observations suggest that during base excision repair of non-repetitive DNA sequences, polymerase β may be capable of accurate DNA synthesis in the proper sequence context.

The HSV-*tk in vitro* assay was developed to elucidate how the biochemical differences among polymerases affect mutagenic processing of DNA lesions. A dose-dependent inhibition of DNA synthesis and concomitant increase in mutation frequency was observed when DNA primer-templates were treated with ENU (Fig. 5). Moreover, for the three polymerases examined, the ENU dose–response mutation frequency curves do not differ significantly from one another (Fig. 6). We conclude that the identity of the polymerase does not quantitatively affect the overall mutagenic potency of the alkyl lesions. However, we have observed significant qualitative differences in ENU-induced mutational spectra among the polymerases (30); thus, the types of mutations produced on each template are partially dependent on the DNA polymerase that processes the lesions (Eckert,K.A., Vargo,P.L. and Hile,S.E., manuscript in preparation).

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