The Mutational Specificity of DNA Polymerase-β during in Vitro DNA Synthesis

PRODUCTION OF FRAMESHIFT, BASE SUBSTITUTION, AND DELETION MUTATIONS

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The frequency and specificity of mutations produced in vitro by eucaryotic DNA polymerase-β have been determined in a forward mutation assay using a 250-base target sequence in M13mp2 DNA. Homogeneous DNA polymerase-β, isolated from four different sources, produces mutations at a frequency of 4–6%/single round of gap-filling DNA synthesis. DNA sequence analyses of 460 independent mutants resulting from this error-prone DNA synthesis demonstrate a wide variety of mutational events. Frameshift and base substitutions are made at approximately equal frequency and together comprise about 90% of all mutations. Two mutational “hot spots” for frameshift and base substitution mutations were observed. The characteristics of the mutations at these sites suggest that certain base substitution errors result from dislocation of template bases rather than from direct mispair formation by DNA polymerase-β. When considering the entire target sequence, single-base frameshift mutations occur primarily in runs of identical bases, usually pyrimidines. The loss of a single base occurs 20–80 times more frequently than single-base additions and much more frequently than the loss of two or more bases. Base substitutions occur at many sites throughout the target, representing a wide spectrum of mispair formations. Averaged over a large number of non-pheno-typically detectable sites, the base substitution error frequency is greater than one mistake for every 5000 bases polymerized. Large deletion mutations are also observed, at a frequency more than 10-fold over background, indicating that purified DNA polymerases alone are capable of producing such deletions. These data are discussed in relation to the physical and kinetic properties of the purified enzymes and with respect to the proposed role for this DNA polymerase in vivo.

The low spontaneous mutation rates observed in eucaryotes (1) suggest that these organisms maintain the genetic information with high fidelity. Replication, repair, and recombination all contribute to this fidelity, and each of these processes depends on the synthesis of new DNA by DNA polymerases in association with other proteins. It is the primary effort of this laboratory to determine the mechanisms used by these proteins to achieve accurate DNA synthesis. The first step in this approach is to determine the frequency and specificity of errors produced by purified eucaryotic DNA polymerases during a single round of DNA synthesis in vitro.

The fidelity of in vitro DNA synthesis has previously been determined by misincorporation assays with defined polynucleotide templates or using φX174am3 DNA as a natural template (2). In the φX assay, accuracy is determined by measuring the reversion frequency of an amber mutation, observed upon transfection of the product of the in vitro DNA synthesis reaction into competent cells (3). This assay focuses on base substitution errors that restore an essential gene function and measures these events at one, two, or three template nucleotides. Since spontaneous and induced mutation rates reflect several different types of errors in addition to base substitutions, and since errors in DNA synthesis occur at many different sites within a gene, it is desirable to monitor a wide spectrum of errors. To this end, a new system is described here using M13mp2 DNA (4). The fidelity of in vitro DNA synthesis is determined for a 250-base target sequence in the lacZα gene, scoring for any error causing loss of a non-essential gene function (α-complementation). This forward mutational assay is thus capable of detecting frameshift, deletion, duplication, and complex errors in addition to a large number of different base substitution errors at many sites.

The system has first been applied to an analysis of the frequency and specificity of mutations produced in vitro by eucaryotic DNA polymerase-β. This class of DNA polymerase has been implicated in repair synthesis in higher organisms (5, 6) and may participate in other processes as well. DNA polymerase-β (Pol-β) has been purified, essentially to homo- geneity, from several sources and is well-characterized physically and kinetically (5–14). The purified enzyme consists of a single low molecular weight polypeptide (Mr = 30,000–45,000) containing no associated endo- or exonucleolytic activities. Pol-β prefers gapped DNA as a primer-template and fills gaps to completion. The enzyme has also been shown to be error-prone for base substitutions, producing such errors at position 587 in φX174am3 DNA at a frequency of one mistake for every 5000 correct incorporation events (15). These properties have prompted an analysis of Pol-β as the first to be examined with the forward mutational assay.

EXPERIMENTAL PROCEDURES

Materials—Escherichia coli strains NR9099 (Δ(pro-lac), recA-, ara-, thi'/F'(proAB, lacIqZ ΔM15)) and S90C (Δ(pro-lac), recA36, ara-, thi'/strA) were provided by Roeland Schaeper of this institute. E. coli CSH50 (Δ(pro-lac), ara-, thi'/F'(traD36, proAB, lacIqZ ΔM15)) and wild type bacteriophage M13mp2 were obtained from J. Eugene

¹The abbreviations used are: Pol-β, polymerase-β; Hepes, 4-(2-hydroxyethyl)-1-piperazineethanesulfonic acid.
LeClerc, University of Rochester, Rochester, NY. DNA polymerase-β from rat Novikoff hepatoma (hereafter called Rat Pol-β), homogenous fraction V (7), and from HeLa cells, Fraction VIII (9), were from Dale W. Mosbaugh, University of Texas, Austin, TX. DNA polymerase-β from chick embryos (chick Pol-β), homogenous fraction X, was prepared by Akira Shiroma, National Institute of Health Chikusa-Ku, Nagoya, Japan. Human liver Pol-β, homogenous fraction VII (10), was from T. S. F. Wang and D. Korn, Stanford University, Palo Alto, CA. Restriction endonucleases Aval, PvuII, and PstI were from Boehringer Mannheim.

Preparation of Gapped M13mp2 DNA—Bacteriophage M13mp2 was plated on minimal plates as described below, using E. coli NR9099 as a host strain. A single plaque was added to 1 liter of 2 × YT medium (containing, per liter, 16 g of Bacto-Tryptone, 10 g of Yeast Extract, 5 g of NaCl, pH 7.4) containing 10 μl of an overnight culture of E. coli NR9099. M13mp2-infected cells were grown overnight at 37°C with vigorous shaking. Cells were harvested by centrifugation at 5000 × g for 30 min, and replicative form DNA was prepared by the method of Birnboim and Doly (16). Phage were harvested from the clear culture supernatant by addition of polyethylene glycol 8000 to 7% and NaCl to 0.5 M. The phage pellet, obtained by centrifugation at 5000 × g for 30 min at 0°C, was resuspended in phenol extraction buffer (100 mM Tris-HCl, pH 8.0, 30 mM NaCl, 1 mM EDTA). The single-stranded phage DNA was extracted with phenol twice, followed by two chloroform/isoamyl alcohol (24:1) extractions. The M13mp2 DNA was precipitated with ethanol and resuspended in TE buffer (10 mM Tris-HCl, pH 8.0, 1 mM EDTA).

Replicative form M13mp2 DNA was digested at 37°C with restriction endonucleases PstI and PvuII in a reaction containing 10 mM Tris-HCl, pH 7.8, 10 mM MgCl₂, 50 mM NaCl, 1 mM dithiothreitol, replicative form DNA, and enough of each enzyme to achieve complete digestion as determined in small preliminary digestions before the large-scale preparative digestion). The digested DNA was subjected to electrophoresis in a 0.8% agarose gel in TAE buffer (containing 0.5 mg/ml ethidium bromide). The gel was then dried and used to expose Kodak X-ray film. Electrophoresis was at a constant 50 V for 16 hr and the 6.8-kilobase fragment was excised. The DNA electroeluted from this gel fragment was purified and concentrated on an Elutip-d filter paper (Schleicher and Schuell) according to the manufacturer's instructions. The fragment was then precipitated with ethanol and resuspended in TE buffer.

The gapped double-stranded circular molecule was then prepared by mixing 500 μg each of the restriction endonuclease fragment and single-stranded circular viral DNA (T796 bases) in 30 mM NaCl, 50 mM sodium citrate. The mixture was heated to 95°C for 10 min, cooled in an ice bath, and incubated at 65°C for 30 min. The gapped molecule was then purified by preparative agarose electrophoresis and affinity chromatography as for the restriction endonuclease fragment. The final preparation, when analyzed by agarose gel electrophoresis, contained approximately 80% gapped double-stranded circular DNA and 20% linear restriction endonuclease fragment, but no detectable single-stranded DNA.

DNA Synthesis Reactions and Product Analysis—DNA synthesis was performed in 50-μl reactions containing 20 mM Hepes, pH 7.8, 2 mM MgCl₂, 500 μg/ml calf thymus DNA, 500 μg/ml [α-32P]dATP, dCTP, dGTP, and [α-32P]dTTP (500–1000 cpm/pmol). 900 ng of gapped circular M13mp2 DNA, and either 0.4 unit of rat DNA polymerase-β, 0.8 unit of chick DNA polymerase-β, 0.10 unit of human liver DNA polymerase-β, or 0.5 unit of HeLa cell DNA polymerase-β. In each instance, the unit definition was that of the enzyme supplier, as determined in individual laboratories before shipment. The second primer was previously described (7, 9, 10, 12). Incubation was at 37°C for 60 min, and reactions were terminated by addition of EDTA to 15 mM. Twenty μl (120 ng of DNA) of each reaction were mixed with 5 μl of sodium dodecyl sulfate dye mix (20 mM Tris-HCl, pH 6.0, 5 mM EDTA, 5% sodium dodecyl sulfate, 0.5% bromphenol blue, 25% glycerol) and subjected to electrophoresis in a 0.8% agarose gel in TAE buffer containing 0.5 mg/ml ethidium bromide. Electrophoresis was at a constant 50 V for 16 hr. Bands were visualized by illumination with UV light and photographed. The gel was then dried and used to expose Kodak XAR film to produce autoradiographs.

Evaluation of DNA Product in Competent Cells—The remaining product of the polymerization reactions was used for transfection of E. coli S90C recA66 cells made competent as previously described using 75 mM CaCl₂ (17) or more recently using the procedure of Hanahan (18). With Ca⁺⁺-treated cells, the ratio of DNA molecules transfected and cells treated with cells treated by the procedure of Hanahan the ratio was either 1:1 or 2:1. The transfection and plating and the scoring and confirmation of mutant (light blue or colorless) phenotypes were performed as described previously (17). DNA sequence analysis of mutants was by the chain terminator method (19), using the oligonucleotides described previously (17). The 32P label needed to observe the sequence ladder was incorporated onto the 5′ ends of the oligonucleotides prior to hybridization using [γ-32P]ATP and T4 polynucleotide kinase.

RESULTS

The M13mp2 Mutagenesis Assay—The hybrid bacteriophage M13mp2, developed by Messing and co-workers (4), contains a small segment of the E. coli lac operon within the intergenic region of M13. This segment of DNA consists of the C-terminal coding sequence of the lacI gene, the lac promoter and operator regions and the DNA sequence coding for the first 145 amino acids of the N-terminal end of the lacZ gene (the α region). The E. coli host strain used for the assay (E. coli CSH50) contains a chromosomal deletion of the lac operon, but harbors an F episome to provide the remaining coding sequence of the lacZ gene. Functional lacZ gene product, β-galactosidase, is produced when the lacZ gene coding information missing on the F factor (for the host used here, nucleotides +71 through +163 where +1 is the first transcribed base) is provided by the information carried in the M13mp2 DNA. In this case, the two partial proteins produced within an M13mp2-infected host cell reconstitute enzyme activity by intracistronic α-complementation. This is detected by plating infected cells under conditions to monitor the production of blue color resulting from hydrolysis of the indicator dye 5-bromo-4-chloro-3-indolyl-β-D-galactoside by β-galactosidase. Decreased α-complementation resulting from a mutagenic event in the lacZα gene in M13mp2 will give rise to M13mp2 plaques having lighter blue or no color.

The general outline used to assay the accuracy of in vitro DNA synthesis is shown in Fig. 1. A gapped molecule is constructed (see "Experimental Procedures") in which the gap contains the target sequence. This is filled by a single cycle of in vitro DNA synthesis using the desired DNA polymerase and reaction conditions. A portion of the product is analyzed to assure complete synthesis, and the remainder is used to infect cells and assay for α-complementation. Certain errors during the in vitro DNA synthesis result in altered production of α-peptide, and upon scoring and confirming the mutant phenotype, the exact nature of the error can be determined by DNA sequence analysis of the single-stranded viral DNA.

This assay system has several major advantages for measurements of in vitro accuracy. Most importantly, the assay scores for loss of a non-essential gene function, allowing a wide variation of mutations to be tolerated. The use of a derivative of M13 permits the simple preparation of large amounts of pure viral DNA, from a small culture, in single-stranded form. This not only facilitates routine DNA sequence analysis of mutants, it also allows one to produce a specifically gapped molecule and to easily engineer sequence changes in the mutational target (see below). M13mp2 has been chosen over the other M13mp derivatives, as it produces a darker blue color on the plates, potentially allowing a wider spectrum of "down" mutants to be scored. This species derivative of the M13mp series of vectors also does not contain nonsense codons and can be grown on a suppressor minus host. This allows detection of nonsense mutations in the target sequence.

The gapped M13mp2 molecule depicted in Fig. 1 is a good primer-template for most purified DNA polymerases. The specific gap used for these studies, constructed using two
Gapped DNA position of the five sites for adenine methylation used to instruct mismatch correction cleavage with restriction endonucleases

The end product of the in vitro reaction is therefore fully methylated away from the end of the target (which is position -84, the first nucleotide after the lac gene termination codon), but still within the lac DNA. The 3'-OH primer terminus (on the right) is nucleotide +175, the middle nucleotide of the lacZa codon 45. The lacZa target is indicated by the darker line within the gap. The direction of DNA synthesis within the gap is right to left. The square (made with dashed lines) represents a competent E. coli cell.

Restriction endonucleases, has been chosen for several reasons. The complementary (minus) strand is almost full genome length and perhaps more likely to survive transfection in the host cell than partial minus strands (2, 3). The double-stranded portion of the molecule retains all adenine methylation sites used to instruct mismatch correction (20, 21). The end product of the in vitro reaction is therefore fully methylated, and polymerase errors in the newly synthesized DNA are less likely to be repaired in vivo by the mismatch correction repair system (22, 23). The 3'-OH primer terminus of the gap (at +174) is 11 bases beyond the last nucleotide missing (+163) on the F' (lacZ-AM15) of the host cell, thus including most of the coding sequence of the a-peptide needed for a-complementation. The 5'-phosphoryl terminus is at position -216, which is more than 100 bases beyond the regulatory sequences which control a-peptide production, but still within the non-essential DNA sequence in M13mp2. The gap thus contains the entire mutational target plus over 100 bases of non-essential sequence in which mutations are expected to be phenotypically silent. It is therefore not necessary to completely fill the entire gap in order to see mutations throughout the target. Only 250 of 390 bases need to be incorporated to observe a complete spectrum.

Mutation Frequency of Control and Pol-β-copied DNA—DNA polymerase-β prefers gapped DNA as a primer template and is capable of filling gaps to completion (9, 11). An agarose gel analysis of the product of a DNA synthesis reaction by homogeneous rat Pol-β on M13mp2 DNA containing a 390-base gap is shown in Fig. 2. The major band after a 60-min reaction, visualized either by fluorescence (lane B) or autoradiography (lane C), is in the position of fully double-stranded DNA, representing a clear shift from the position of the unfilled gapped molecule (lane A). Results similar to those

in Fig. 2 were obtained with each of the four Pol-β preparations used. The products of these reactions thus represent a relatively homogeneous population of copied molecules containing putative mutations resulting from one round of in vitro synthesis.

The results of transfection of rat Pol-β-copied DNA into competent cells are shown in Table I, compared to transfections of several uncopied control DNA molecules (see legend to Table I for description). The frequency of light blue or colorless mutant plaques is 2.8 x 10^-4 to 6.6 x 10^-4 for the control DNA molecules, compared to 640 x 10^-4 for DNA copied by Pol-β. This 100-fold increase is due to mutations in the newly synthesized strand, since denaturation to eliminate the biological activity of this (unligated) strand returns the mutation frequency to the background level.

The unexpectedly high mutation frequency resulting from synthesis by this enzyme led to an examination of three additional preparations of DNA polymerase-β, isolated from different sources. The mutation frequencies of DNA copied by Pol-β from chick embryo, HeLa cells, or human liver were all similar to results with the rat polymerase (Table II). Thus, not just a single enzyme but rather the β-polymerase class is highly inaccurate in vitro.

DNA Sequence Analysis of Mutants—Since loss of a-com-
phenotypes was observed, which when replated yielded both wild type
within the non-essential DNA), this construction was deemed most
appropriate for subsequent analyses of spontaneous mutants (Table
stranded circular with a single nick outside the mutational target but
Having been subjected to manipulations similar to the gapped mole-
buffer and used for transfection.

The nicked construct was made by cleaving replicative form DNA
with restriction endonuclease AvaII and then hybridizing the full
size of the sectors in the original infective center. The ratio of light
blue plaques and mutant plaques, in approximate proportion to the

Mutants include colorless plaques as well as those having lighter blue color than wild type mp2. Several light blue phenotypes were
observed varying in intensity from almost colorless to almost wild type. More than 95% of the mutants, when carefully removed from the
plate, diluted, and replated, were of only a single phenotype. Occasionally, a plaque having sectors of both colorless and blue phenotypes
was observed, which when replated yielded both wild type blue plaques and mutant plaques, in approximate proportion to the

The nicked construct was made by cleaving replicative form DNA
with restriction endonuclease AavII and then hybridizing the full
genome length complementary strand to the viral strand as for formation of the gapped molecule. The resulting completely double-
stranded circular molecule contains a nick at position +264, only 48
bases from the position of the nick in the Pol-β gap-filled molecule.

Occasionally, a plaque having sectors of both colorless and blue
phenotypes was observed, which when replated yielded both wild type
blue plaques and mutant plaques, in approximate proportion to the
size of the sectors in the original infective center. The ratio of light blue to colorless mutants was 2:1 for the nicked construct transfections but 1:2 for the Pol-β-copied DNA. This is consistent with the sequence analysis (Table IV), since many of the Pol-β frameshift mutants, produced at high frequency, are colorless.

The complete spectra of single-base framemshifts (shown below each line of primary viral DNA sequence) and single-base substitutions (above each line) are shown in Fig. 3 (rat Pol-β) and Fig. 4 (chick Pol-β). Mutations are distributed over both the coding and regulatory sequences, consistent with DNA synthesis throughout the target. Only two of 440 Pol-β mutants analyzed did not contain an error in the target within the gap. While in many instances mutations were found consistent with previously observed phenotypes (17, 25), many new detectable sites for mutation were observed. These new mutations were the only changes detected within the 254 bases shown in Figs. 3 and 4. As most of these have been observed several times as independent events and have the same phenotypes (i.e. intensity of blue color), it seems reasonable to conclude that the observed sequence changes are in fact responsible for the mutant phenotypes. In each case, the sequence change alters what is though to be an important base for regulation or alters the amino acid composition of the α-peptide.

A summary of the various classes of mutants is listed in Table III. In order to establish a background frequency for each class of events, 128 spontaneous mutants, obtained by transfection of nicked double-stranded DNA (see legend to Table I), were also analyzed.

<table>
<thead>
<tr>
<th>Source of Pol-β</th>
<th>Number of determinations</th>
<th>Plaques scored</th>
<th>Mutation frequency</th>
</tr>
</thead>
<tbody>
<tr>
<td>Rat hepatoma</td>
<td>5</td>
<td>10,644 669</td>
<td>640.0</td>
</tr>
<tr>
<td>Chick embryo</td>
<td>2</td>
<td>2,576 196</td>
<td>760.0</td>
</tr>
<tr>
<td>HeLa cell</td>
<td>2</td>
<td>4,435 161</td>
<td>360.0</td>
</tr>
<tr>
<td>Human liver</td>
<td>2</td>
<td>12,930 61</td>
<td>440.0</td>
</tr>
<tr>
<td></td>
<td></td>
<td>10,644 7</td>
<td>6.8</td>
</tr>
<tr>
<td></td>
<td></td>
<td>2,576 3</td>
<td>3.7</td>
</tr>
</tbody>
</table>

### Table I

**Mutation frequency of control and rat Pol-β-copied DNA**

The rat hepatoma DNA polymerase-β copying reactions, transfec-
tions, and plating were as described under "Experimental Procedures" and in Ref. 17. Mutation frequencies of viral and replicative form DNA were determined from the same preparations of DNA used to construct the gapped molecule.

<table>
<thead>
<tr>
<th>DNA</th>
<th>Number of determinations</th>
<th>Plaques scored</th>
<th>Mutation frequency</th>
</tr>
</thead>
<tbody>
<tr>
<td>Viral</td>
<td>2</td>
<td>10,597 7</td>
<td>6.6</td>
</tr>
<tr>
<td>Replicative form</td>
<td>1</td>
<td>28,655 8</td>
<td>2.8</td>
</tr>
<tr>
<td>Nicked construct&lt;sup&gt;a&lt;/sup&gt;</td>
<td>3</td>
<td>199,655 128</td>
<td>6.4</td>
</tr>
<tr>
<td>Pol-β-copied</td>
<td>2</td>
<td>10,474 669</td>
<td>640.0</td>
</tr>
<tr>
<td>Pol-β-copied, denatured&lt;sup&gt;b&lt;/sup&gt;</td>
<td>1</td>
<td>3,089 3</td>
<td>9.7</td>
</tr>
</tbody>
</table>

<sup>a</sup> Mutants include colorless plaques as well as those having lighter blue color than wild type mp2. Several light blue phenotypes were observed varying in intensity from almost colorless to almost wild type. More than 95% of the mutants, when carefully removed from the plate, diluted, and replated, were of only a single phenotype. Occasionally, a plaque having sectors of both colorless and blue phenotypes was observed, which when replated yielded both wild type blue plaques and mutant plaques, in approximate proportion to the size of the sectors in the original infective center. The ratio of light blue to colorless mutants was 2:1 for the nicked construct transfections but 1:2 for the Pol-β-copied DNA. This is consistent with the sequence analysis (Table IV), since many of the Pol-β frameshift mutants, produced at high frequency, are colorless.

<sup>b</sup> The nicked construct was made by cleaving replicative form DNA with restriction endonuclease AavII and then hybridizing the full genome length complementary strand to the viral strand as for formation of the gapped molecule. The resulting completely double-stranded circular molecule contains a nick at position +264, only 48 bases from the position of the nick in the Pol-β gap-filled molecule. Having been subjected to manipulations similar to the gapped molecule and having a similar configuration (i.e. completely double-stranded circular with a single nick outside the mutational target but within the non-essential DNA), this construction was deemed most appropriate for subsequent analyses of spontaneous mutants (Table III).

For the final transfection shown, the product of the Pol-β copying reaction was denatured at 95°C for 3 min and then diluted in TE buffer and used for transfection.

### Table II

**Mutation frequency of DNA copied by Pol-β from different sources**

Copying reactions, transfections, and plating were as described under "Experimental Procedures."

<table>
<thead>
<tr>
<th>Source of Pol-β</th>
<th>Number of determinations</th>
<th>Plaques scored</th>
<th>Mutation frequency</th>
</tr>
</thead>
<tbody>
<tr>
<td>Rat hepatoma</td>
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<td>2</td>
<td>2,576 196</td>
<td>760.0</td>
</tr>
</tbody>
</table>

*T. A. Kunkel, unpublished results.*
When considering only the $-1$-base frametshifts, these are observed to occur primarily in runs of 2 or more of the same base and (7-fold) more frequently at template pyrimidine sites than at template purine sites (Table IV B and Figs. 3 and 4). A more detailed consideration of the single-base loss frame-shifts (Table V) shows that these asymmetries are not due to a bias in the target size. For example, there are 12 2-base pyrimidine runs (4C, 8T) and 12 2-base purine runs (6G, 6A) which are potential frametshift mutational targets, yet $-1$-pyrimidine errors at 2-base runs occur 15 times more frequently than do $-1$-purine events (30 versus 2). Furthermore, the 12 2-base pyrimidine runs total 24 bases for which there are 20 mutations. In contrast, there are only two examples of $-1$-pyrimidine errors at 31 single-base pyrimidine sites (19 C residues and 12 T residues). None of the above-mentioned asymmetries in the frequencies of these frametshift errors can be explained by differential expression in the transfection assay of plus or minus frametshifts in artificially constructed heteroduplexes.  

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While $-1$-base frametshift errors occur more frequently in runs of a common base, other factors presumably influence the frequency of their occurrence. For example, there are three runs of 3 C residues in the lacZa coding sequence. The loss of a single C is frequent at two of these sites, but 5-fold less frequent at the third run (Table VI). Similarly, there is a substantial difference in the number of occurrences at 2-base C runs (compare 7 versus 2, versus 0, versus 0, Table VI).  

Base Substitution Errors—DNA polymerase-$\beta$, from either rat hepatoma or chick embryo, produces a wide variety of base substitution errors throughout the lacZa target (Figs. 3 and 4).  

Mutational Specificity of DNA Polymerase-β in Vitro

Fig. 4. Spectrum of single-base mutations produced by chick embryo Pol-β. See legend to Fig. 3 for explanation, and note the T → C silent base change at position +72. This change reduced the frequency of -T frameshifts events at positions +70 through +73 (see text). This reduced the forward mutation frequency somewhat (about 440 × 10⁻⁴, Table II, T at position +72 versus 350 × 10⁻⁴, Table III, C at position +72).

Table III

Frequency of various classes of mutations

<table>
<thead>
<tr>
<th>Mutational event</th>
<th>Spontaneous (6.4 × 10⁻⁴)</th>
<th>Rat Pol-β (640 × 10⁻⁴)</th>
<th>Chick Pol-β (350 × 10⁻⁴)</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>Number</td>
<td>Frequency</td>
<td>Number</td>
</tr>
<tr>
<td>Single-base frame shift</td>
<td>11</td>
<td>0.55</td>
<td>164</td>
</tr>
<tr>
<td>Single-base substitution</td>
<td>67</td>
<td>3.4</td>
<td>102</td>
</tr>
<tr>
<td>Deletions</td>
<td>32</td>
<td>1.6</td>
<td>11</td>
</tr>
<tr>
<td>Otherd</td>
<td>18</td>
<td>0.9</td>
<td>19</td>
</tr>
<tr>
<td>Total</td>
<td>128</td>
<td>6.4</td>
<td>296</td>
</tr>
</tbody>
</table>

* Spontaneous mutants are from transfection of the nicked construct DNA (see Table I) and are not necessarily independent.

The high frequency of their occurrence relative to spontaneous base substitutions (about 229 × 10⁻⁴ or 190 × 10⁻⁴ versus 3.5 × 10⁻⁴, Table III) clearly demonstrates that they represent misinsertions by DNA polymerase-β.

Table VII summarizes the base substitution errors shown in Figs. 3 and 4. Several common features are apparent for the two enzymes. The rat Pol-β spectrum includes 57 transitions (79%) and 18 transversions (21%), and the chick Pol-β
The data for single-base events are taken from Figs. 3 and 4. The numbers in parentheses are T-frame shift events at positions +70 to +73 and +103 to +104. The analysis in B excludes the hot spot frameshift mutants.

<table>
<thead>
<tr>
<th>Number of occurrences</th>
<th>Rat Pol-β</th>
<th>Chick Pol-β</th>
</tr>
</thead>
<tbody>
<tr>
<td>A. Comparison of all frameshift errors</td>
<td></td>
<td></td>
</tr>
<tr>
<td>Additions</td>
<td></td>
<td></td>
</tr>
<tr>
<td>1 base</td>
<td>2</td>
<td>3</td>
</tr>
<tr>
<td>&gt;1 base</td>
<td>0</td>
<td>0</td>
</tr>
<tr>
<td>Deletions</td>
<td>89 (73)</td>
<td>31 (16)</td>
</tr>
<tr>
<td>2 bases</td>
<td>2</td>
<td>0</td>
</tr>
<tr>
<td>3 bases</td>
<td>1</td>
<td>0</td>
</tr>
<tr>
<td>4 bases</td>
<td>1</td>
<td>0</td>
</tr>
<tr>
<td>B. Comparison of -1-base frameshift errors</td>
<td>84</td>
<td>29</td>
</tr>
<tr>
<td>At runs of the same base</td>
<td>5</td>
<td>2</td>
</tr>
<tr>
<td>At pyrimidine sites</td>
<td>78</td>
<td>27</td>
</tr>
<tr>
<td>At purine sites</td>
<td>11</td>
<td>4</td>
</tr>
</tbody>
</table>

**TABLE V**

Analysis of rat Pol-β single-base (deletion) frameshifts

The data are taken from Fig. 3 and utilize only the sequences within the target known to yield detectable phenotypes via -1 frameshift events (Refs. 17 and 25; Figs. 3 and 4; Footnote 2).

<table>
<thead>
<tr>
<th>Length of run</th>
<th>C</th>
<th>T</th>
<th>G</th>
<th>A</th>
</tr>
</thead>
<tbody>
<tr>
<td>bases</td>
<td>5</td>
<td>1</td>
<td>0</td>
<td>0</td>
</tr>
<tr>
<td>4</td>
<td>1</td>
<td>14</td>
<td>1</td>
<td>69</td>
</tr>
<tr>
<td>3</td>
<td>3</td>
<td>34</td>
<td>1</td>
<td>1</td>
</tr>
<tr>
<td>2</td>
<td>4</td>
<td>9</td>
<td>8</td>
<td>21</td>
</tr>
<tr>
<td>1</td>
<td>19</td>
<td>0</td>
<td>12</td>
<td>2</td>
</tr>
<tr>
<td>Total</td>
<td>58</td>
<td>93</td>
<td>8</td>
<td>3</td>
</tr>
</tbody>
</table>

**TABLE VI**

Analysis of rat Pol-β -1-base frameshifts at C runs

The data are taken from Fig. 3.

<table>
<thead>
<tr>
<th>Template DNA sequence</th>
<th>3' bases</th>
<th>Number of mutants</th>
</tr>
</thead>
<tbody>
<tr>
<td>AT</td>
<td>CCCCC</td>
<td>TT</td>
</tr>
<tr>
<td>CA</td>
<td>CCCC</td>
<td>AG</td>
</tr>
<tr>
<td>TA</td>
<td>CCC</td>
<td>AA</td>
</tr>
<tr>
<td>AA</td>
<td>CCC</td>
<td>TG</td>
</tr>
<tr>
<td>GG</td>
<td>CCC</td>
<td>GC</td>
</tr>
<tr>
<td>CG</td>
<td>CC</td>
<td>TT</td>
</tr>
<tr>
<td>GG</td>
<td>CC</td>
<td>GT</td>
</tr>
<tr>
<td>CG</td>
<td>CC</td>
<td>AG</td>
</tr>
<tr>
<td>GA</td>
<td>CC</td>
<td>AT</td>
</tr>
</tbody>
</table>

Within each spectrum, both site preferences and insertion preferences are observed. For example, in each spectrum, there are five T→C transitions at position +97, yet no T→C transitions at positions -36, -10, +103, +121, or +139 in either spectrum, although this change does produce a detectable phenotype at these sites. For both spectra, dAMP is the most frequently misinserted nucleotide, while dTMP is misinserted least frequently.

Deletions—Mutations resulting from the deletion of five or more bases were observed among the collection of both rat and chick Pol-β mutants. The frequency of deletion mutations is 15-fold (rat) or 20-fold (chick) above the background frequency (Table III). Also, the specificity of the Pol-β deletions is very different from that of the spontaneous deletions. Only 2 of 24 mutants from the Pol-β spectra were also observed in the spontaneous collection. The spontaneous deletions (30 of 32) represent only three different structures and may represent the progeny of a much smaller number of independently arising mutants. In contrast, the Pol-β deletions are of many different types and represent the loss of from 5 to over 300 bases. One particular 317-base deletion, between two CCCGC repeats within the 390-base gap, has been observed five times in the rat Pol-β spectrum and seven times in the chick Pol-β spectrum, while not occurring once in the spontaneous collection. In the rat Pol-β spectrum, three short deletions (of 5, 8, or 16 bases) as well as two -2-base frameshifts (Table IV) were clustered in one region of the lacZα coding sequence, while no spontaneous deletion events were observed in this region. All these observations strongly suggest that DNA polymerase-β is capable of producing deletion errors during *in vitro* DNA synthesis.

**DISCUSSION**

Analyses of the frequency and specificity of errors produced by purified DNA replication and repair proteins are intended to determine what properties of protein-nucleic acid interactions during DNA polymerization are important in avoiding (or producing) various types of mutations. This report establishes the frequency and specificity of mutations generated *in vitro* by purified DNA polymerase-β, a putative repair enzyme in mammalian cells (5, 6). The analysis is performed using a newly developed forward mutation assay capable of detecting a variety of mutational events, including frameshifts, base substitutions, and deletions. Each of these classes of muta-

**TABLE IV**

DNA polymerase-β frameshift error specificity

The numbers in parentheses are -T-frame shift events at positions +70 to +73 and +103 to +104. The analysis in B excludes the hot spot frameshift mutants.

<table>
<thead>
<tr>
<th>Number of occurrences</th>
<th>Rat Pol-β</th>
<th>Chick Pol-β</th>
</tr>
</thead>
<tbody>
<tr>
<td>A. Comparison of all frameshift errors</td>
<td></td>
<td></td>
</tr>
<tr>
<td>Additions</td>
<td></td>
<td></td>
</tr>
<tr>
<td>1 base</td>
<td>2</td>
<td>3</td>
</tr>
<tr>
<td>&gt;1 base</td>
<td>0</td>
<td>0</td>
</tr>
<tr>
<td>Deletions</td>
<td>89 (73)</td>
<td>31 (16)</td>
</tr>
<tr>
<td>2 bases</td>
<td>2</td>
<td>0</td>
</tr>
<tr>
<td>3 bases</td>
<td>1</td>
<td>0</td>
</tr>
<tr>
<td>4 bases</td>
<td>1</td>
<td>0</td>
</tr>
<tr>
<td>B. Comparison of -1-base frameshift errors</td>
<td>84</td>
<td>29</td>
</tr>
<tr>
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</tr>
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<td>27</td>
</tr>
<tr>
<td>At purine sites</td>
<td>11</td>
<td>4</td>
</tr>
</tbody>
</table>

**TABLE VII**

DNA polymerase-β base substitution specificity

The data are taken from Figs. 3 and 4. The numbers in parentheses reflect the T→G transition events at positions +70 and +103.

<table>
<thead>
<tr>
<th>Template mutation</th>
<th>Mismatch formed (template-dNMP)</th>
<th>Observed number of occurrences</th>
</tr>
</thead>
<tbody>
<tr>
<td>Rat Pol-β</td>
<td>Sites</td>
<td>Mutants</td>
</tr>
<tr>
<td>T→C</td>
<td>5</td>
<td>12</td>
</tr>
<tr>
<td>T→G</td>
<td>2 (2)</td>
<td>6 (2)</td>
</tr>
<tr>
<td>A→T</td>
<td>1</td>
<td>0</td>
</tr>
<tr>
<td>C→T</td>
<td>12</td>
<td>31</td>
</tr>
<tr>
<td>C→G</td>
<td>0</td>
<td>0</td>
</tr>
<tr>
<td>A→C</td>
<td>0</td>
<td>0</td>
</tr>
<tr>
<td>G→A</td>
<td>5</td>
<td>7</td>
</tr>
<tr>
<td>G→T</td>
<td>6</td>
<td>9</td>
</tr>
<tr>
<td>C→G</td>
<td>2</td>
<td>2</td>
</tr>
<tr>
<td>A→G</td>
<td>4</td>
<td>8</td>
</tr>
<tr>
<td>T→A</td>
<td>1</td>
<td>1</td>
</tr>
<tr>
<td>C→A</td>
<td>0</td>
<td>0</td>
</tr>
</tbody>
</table>

**FIGURE**

Comparison of all frameshift mutants.

- Deletions-Mutations resulting from the deletion of five or more bases were observed among the collection of both rat and chick Pol-β mutants. The frequency of deletion mutations is 15-fold (rat) or 20-fold (chick) above the background frequency (Table III). Also, the specificity of the Pol-β deletions is very different from that of the spontaneous deletions. Only 2 of 24 mutants from the Pol-β spectra were also observed in the spontaneous collection. The spontaneous deletions (30 of 32) represent only three different structures and may represent the progeny of a much smaller number of independently arising mutants. In contrast, the Pol-β deletions are of many different types and represent the loss of from 5 to over 300 bases. One particular 317-base deletion, between two CCCGC repeats within the 390-base gap, has been observed five times in the rat Pol-β spectrum and seven times in the chick Pol-β spectrum, while not occurring once in the spontaneous collection. In the rat Pol-β spectrum, three short deletions (of 5, 8, or 16 bases) as well as two -2-base frameshifts (Table IV) were clustered in one region of the lacZα coding sequence, while no spontaneous deletion events were observed in this region. All these observations strongly suggest that DNA polymerase-β is capable of producing deletion errors during *in vitro* DNA synthesis.

**DISCUSSION**

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tions was observed at frequencies substantially above the spontaneous background, clearly indicating that they are produced in vitro by Pol-β (Table III).

DNA polymerase-β was the first of the purified polymerase-β polymerases to be examined since it is the least complex of the eucaryotic DNA polymerases. When purified to >95% homogeneity (7, 9, 10, 12), Pol-β is a single-subunit enzyme containing no associated nuclease activities. It is the smallest DNA polymerase, with a catalytic polypeptide of between 32 and 45 kilodaltons, depending on the source (5–14). Pol-β, which has been implicated in short gap repair synthesis in vivo (5, 6), prefers gapped DNA templates and fills them to completion (9). Finally, this enzyme was a good first choice to establish the feasibility of the M13mp2 assay, since Pol-β is error-prone for base substitution mutations at a single nucleotide in φX174am3 DNA (15). This frequent production of base substitution errors has been confirmed here (Table III) and extended to a broad range of mispairs (Table VII).

The error-prone nature of Pol-β is a common property of this class of purified polymerases rather than a characteristic of a single enzyme (Table II). Furthermore, the mutational spectra of Pol-β from rat hepatoma and chick embryo exhibit similar frequencies (Table III) and specificities (Tables IV and VII, Figs. 3 and 4). This is the case for frameshifts and base substitutions, as well as for production of a 317-base deletion between a 5-base pair direct repeat. These data are encouraging for the purpose of correlating the frequency and specificity of the errors with the common biochemical properties of polymerase-β, regardless of the source (5, 6, 13).

Several properties of Pol-β seem relevant to its error-prone nature. The absence of an associated 3′→5′ exonuclease activity (2, 5, 6) excludes exonuclease-dependent proofreading from contributing to fidelity. DNA polymerase-β will bind tightly to either 3′-PO4 or 3′-OH termini at nicks where no single-stranded template DNA is available with which to interact (11). Pol-β can incorporate a base into a single-nucleotide gap (9), which defines the minimum interaction with single-stranded template at a primer-template terminus needed to achieve incorporation. The enzyme can incorporate onto artificially constructed primer-template termini containing 1–3 terminally mispaired bases (6, 11); base hydrogen bonding is therefore not absolutely required for incorporation. These relaxed primer-template requirements and the observation that Pol-β is the most distributive DNA polymerase known (5, 6) suggest minimal and perhaps weak interactions between the single polypeptide and the primer-template. Thus, Pol-β, perhaps in part due to its small size, may not itself be constrained by or put constraints on small distortions, such as mispaired or misaligned (i.e., looped out) bases, leading to the frequent production of base substitutions, frameshifts, and deletions.

Both the frequency and specificity of the Pol-β-dependent mutations are expected to reflect not only properties of the enzyme, but properties of the primer-template as well. For example, the two hot spots for -T and T → G mutations share a similar primary DNA sequence, CCGTAC. However, the ratio of frameshifts to base substitutions is very different. At the hot spot containing 4 consecutive T residues, frameshifts predominate, while base substitutions are most frequent at the hot spot having 2 T residue. The T → G transversion events are only observed at the 5′-most T residue in the run, adjacent to a template G. This Pol-β-dependent transversion occurs through the formation of a T(terminal)C(primer) intermediate. However, from a consideration of base hydrogen bonding and double-helix conformation (26, 27), this intermediate is predicted to be produced by misinsertion only rarely, and in fact is not observed in three other in vitro systems that utilize other protein and DNA molecules (28–30). These data suggest a model, first described by Fresco and Alberts (31) and later by Fowler et al. (32), in which the initiating event for production of both frameshift and base substitution mutations at the hot spots is dislocation (i.e., looping out) of a template T. Once Pol-β polymerizes A residues opposite all properly aligned T residues, the enzyme correctly incorporates dCMP opposite the next correct template base, a G. If the misaligned template T then realigns before yet another correct incorporation event by Pol-β, the T:C mispair will be produced despite the fact that Pol-β did not actually insert an incorrect base. In this case, a base substitution error is produced by a transient misalignment, not misinsertion. Alternatively, if Pol-β adds the second correct nucleotide before realignment of the looped-out T residue, the misalignment will be stabilized, leading to a -T frameshift. This model predicts that the ratio of frameshifts to base substitutions should increase as the length of the run increases, since more complementary base pairs (in this case, T:A pairs) would be available in the run to stabilize the misalignment. This is what is observed (Figs. 3 and 4). The model also predicts that the specificity of the base substitution errors at the 5′-most base in the template run will be determined by the next base in the template, a prediction which has not yet been examined. The validity of the model and the potential contribution of misalignment to base substitution mutagenesis both in vitro with Pol-β and other enzymes and in vivo are currently being examined and will be reported in detail in a later publication.

An examination of the frameshift mutations at sites other than the hot spots also suggests several properties of the interacting molecules which are important in determining both frequency and specificity. The distributive nature of the synthesis by Pol-β leaves the primer-template free of protein for some time between each incorporation step, potentially allowing misaligned structures to form more easily, thus accounting for the high frequency of frameshifts. It remains to be determined whether Pol-β takes an active role in formation of the necessary misaligned intermediates. The loss of a single base occurs at least 10 times more frequently than does the addition of 1 base or the loss of 2 or more bases (Table IV). Thus, Pol-β may interact with the DNA to permit incorporation onto a primer-template containing a single-template base misalignment (leading to a 1-base deletion) while excluding misalignments of 2 or more template bases or a misaligned base in the primer strand (which would lead to a 1-base addition).

Alternatively, Pol-β may utilize any of these misalignments equally well, and the relative frequency of various frameshifts may reflect the probability that any particular misaligned configuration exists as the polymerase incorporates the next base. This depends on the initial rate of formation and subsequent stability of the misaligned intermediates, which in turn depend on base hydrogen bonding and stacking interactions within the primer-template. Two of the frameshift mutation asymmetries might be explained on this basis, i.e., the 10:1 ratio of -1 to +1 frameshifts and the 7:1 ratio of -1 frameshifts in template pyrimidine compared to template purine runs. In order to produce a -1 frameshift, it is only necessary to unstack bases within the run. It is not necessary to disrupt any base hydrogen bonds to form the template misalignment (as long as the loop-out forms before the last complementary base within the run is incorporated). However a +1 addition within a run requires complementary bases to be incorporated within the run, followed by disruption of the
hydrogen bonds thus formed, unstacking of the bases to form the primer strand loop, and finally, reformation of hydrogen bonding for at least 1 base pair (although this last step may not be required by Pol-β). The added necessity of hydrogen bond disruption should result in a lower frequency of +1 additions. Similarly, the 7:1 preference for −1 frameshifts in pyrimidine runs may simply reflect the fact that pyrimidine:pyrimidine stacking interactions are known to be much weaker than purine:purine stacking (33, 34). Thus, template pyrimidine run misalignments may form much more frequently. This may be enhanced by an increased probability of incorporation of an incoming purine base when strongly stacked with its purine neighbor in the complementary strand run, which would stabilize the misalignment. 

The strong preference for frameshifts in runs of the same base is consistent with the strand slippage model (35) for generation of these mutations. Overall, one would expect such mutations to occur with a frequency approximately proportional to the length of the run. However, the paucity of frameshifts at the run of 5 C residues, the 5:1 difference in frequency at runs of 3 C residues, and the 7:1 difference at runs of 2 C residues (Table VI), demonstrates that a simple correlation of frequency with length does not exist. As for site-specific differences in base substitution frequencies, these frameshift site differences may reflect interactions with the DNA polymerase itself or neighboring base effects. For example, for the −C frameshift targets shown in Table VI, the differences observed in −1 frameshifts in runs of the same length may result from at least two parameters. If the preceding template base (3') is a pyrimidine, a template C misalignment may form more easily due to weak stacking (for example, compare CCT, 7 occurrences with CCA, 0 occurrences). Or, if the preceding base pair is weak (e.g. an A:T base pair), template misalignments might form more easily (for example, compare CCCA, 16 occurrences with CCCG, 3 occurrences). The quantitative contribution of each of these factors, as well as the distance over which they can exert influence on frameshift frequencies, is yet to be determined. 

As yet no model accounts for the Pol-β-dependent production of frameshifts at template bases not found in runs of a common base (Tables IV and V). It seems reasonable that the interactions necessary to produce these events differ from those occurring in runs, but exactly which properties of the enzyme or polymerase-β are relevant remains to be determined. 

One major goal of this work was to extend the understanding of base substitution fidelity by examining a broad spectrum of mismatches. Unlike previous in vitro studies of base substitution specificity using natural DNA (2), these results are obtained in a forward mutation assay containing many detectable sites, all observable without dNTP pool biases. Pol-β formed the mismatches described in Table VII under equivalent conditions in which all four substrates were available in equimolar concentration to compete for incorporation at any template site. If one assumes that approximately 100 bases within the target will eventually be found to be mutable when scoring for α-complementation, the 2% error frequency becomes 0.02%/nucleotide (i.e. 1/5000), which is the value obtained for reversion of a single nucleotide in αX174am3 DNA (15). Pol-β is actually even less accurate than this, however, since errors made in vitro are not expressed at 100% efficiency in the transfection assay. The conclusion that Pol-β has a high single-base misinsertion frequency is therefore justified even when averaged over many sites and for several different mismatches. The most frequent mismatches produced by Pol-β (excluding the T → G transversion at the two hot spots) were purine-pyrimidine and pyrimidine-purine mismatches (Table VII). The strong preference for frameshifts in runs of the same base is consistent with the strand slippage model for at least 1 base pair (although this last step may not be required by Pol-β). The added necessity of hydrogen bond disruption should result in a lower frequency of +1 additions. Similarly, the 7:1 preference for −1 frameshifts in pyrimidine runs may simply reflect the fact that pyrimidine:pyrimidine stacking interactions are known to be much weaker than purine:purine stacking (33, 34). Thus, template pyrimidine run misalignments may form much more frequently. This may be enhanced by an increased probability of incorporation of an incoming purine base when strongly stacked with its purine neighbor in the complementary strand run, which would stabilize the misalignment. 

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While a detailed consideration of each of the Pol-β-dependant deletions observed here is beyond the scope of this report, it is clear that purified Pol-β does produce deletion errors in vitro. The deletion of 317 bases between a direct repeat of only 5 bases, as observed several times in both Pol-β mutant collections, suggests several properties important to deletion mutagenesis. The simplest model is one in which Pol-β incorporates nine nucleotides, starting at position +174 (the provided 3′-OH terminus) through nucleotide +166. After each addition of a base, the enzyme dissociates. Without bound enzyme to constrain it, the primer strand “breathes” to allow the five nucleotide sequence GGCGG to search the available single-stranded DNA in the gap for its complement (found at positions −152 through −148). The resulting five G:C base pairs are sufficient to stabilize the loop and provide a primer-template for further in vitro elongation by Pol-β, leading to a 317-base deletion. Based on its error-prone nature, it is not too surprising that Pol-β could use such a relatively unstable primer-template configuration. This model further implies that the hydrogen bonding of at least five G:C base pairs was disrupted to initiate the event and that this sequence could search a considerable stretch of DNA for its complement. The limits placed on such phenomena are not currently known. Certain of the other deletions, observed less frequently, cannot be explained as easily and are probably produced by other mechanisms. The in vitro system described here provides one approach which should be useful for assessing the roles of both the proteins and nucleic acid sequences involved in producing this frequently observed class of mutations. 

A forward mutation frequency of 4-6% for in vitro synthesis by a eukaryotic DNA polymerase over a 250-base target is inconsistent with the low spontaneous mutation rates described in vivo (1). Obviously, other DNA polymerases and accessory proteins are involved in achieving high fidelity in vivo. It is possible that some of the in vitro mutants observed here arise as a consequence of cryptic damage to either the enzymes or DNA used, as both have been subjected to extensive purification. A more attractive possibility is that accessory proteins will be found which improve the fidelity of Pol-β. For example, mammalian DNase V is known to associate with Pol-β (8), contains both 5″→3″ and 3″→5″ exonuclease activity (36), and influences Pol-β synthesis of gapped mole-
cules (9). Less highly purified forms of Pol-β exist in association with other proteins (5, 6), one or more of which may alter fidelity. It is also possible that DNA polymerase-β is highly inaccurate in vivo. The consequences of this inaccuracy might not be too severe if this enzyme participated in repair of gaps only a few nucleotides long. In addition, a highly inaccurate DNA polymerase might be an advantage under some circumstances, as, for example, in bypassing an otherwise lethal lesion in DNA.

In vivo, highly inaccurate DNA polymerase might not be too severe if this enzyme participated in repair or for the generation of immunoglobulin diversity (38).

Independent of their immediate relevance to mutation rates in vivo, these data and the in vitro approach used here with defined proteins and nucleic acids should provide information on the properties of protein nucleic acid interactions relevant to various mutational end points. For example, the properties of several other purified DNA polymerases are distinctly different from those of Pol-β (5, 6, 11, 14). An analysis of the errors they produce should prove instructive.

Acknowledgments—I would like to express my appreciation to J. Liu and J. Motto for expert technical assistance and to J. Drake, R. Schaaper, and A. Sugino for critical evaluation of the manuscript.

REFERENCES