

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

PRODUCTION OF FRAMESHIFT, BASE SUBSTITUTION, AND DELETION MUTATIONS*

(Received for publication, November 9, 1984)

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The fidelity of *in vitro* DNA synthesis has previously been determined by misincorporation assays with defined polynucleotide templates or using ϕ X174am3 DNA as a natural template (2). In the ϕ X 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 *lacZ α* 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 ¹) has been purified, essentially to homogeneity, from several sources and is well-characterized physically and kinetically (5-14). The purified enzyme consists of a single low molecular weight polypeptide (M_r = 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 ϕ X174am3 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.

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

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EXPERIMENTAL PROCEDURES

Materials—*ara*⁻, *thi*⁻/F'(*ara*⁻, *thi*⁻, *strA*)*E. coli* CSH50 and wild type

¹The abbreviation hydroxyethyl)

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of Columbia University in the
City of New York
IPR2020-00988, -01065,
-01177, -01125, -01323

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specificity of errors produced by purified eucaryotic DNA polymerases during a single round of DNA synthesis *in vitro*.

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EXPERIMENTAL PROCEDURES

Materials—*Escherichia coli* strains NR9099 (Δ (*pro-lac*), *recA*[−], *ara*[−], *thi*[−]/F'(proAB, *lacIqZ* Δ M15)) and S90C (Δ (*pro-lac*), *recA*56, *ara*[−], *thi*[−], *strA*[−]) were provided by Roeland Schaaper of this institute. *E. coli* CSH50 (Δ (*pro-lac*), *ara*[−], *thi*[−]/F'(traD36, proAB, *lacIqZ* Δ M15)) and wild type bacteriophage M13mp2 were obtained from J. Eugene

¹ The 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), homogeneous fraction VI (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), homogeneous fraction VIII (12), was provided by Akio Matsukage, Aichi Cancer Center, Chikusa-Ku, Nagoya, Japan. Human liver Pol- β , homogeneous fraction VII (10), was from T. S.-F. Wang and D. Korn, Stanford University, Palo Alto, CA. Restriction endonucleases *Ava*II, *Pvu*I, and *Pvu*II 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 \times YT medium (containing, per liter, 16 g of Bacto-Tryptone, 10 g of Yeast Extract, 5 g of NaCl, pH 7.4) containing 10 ml 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 \times g for 30 min, and replicative form DNA was prepared by the method of Birnboim and Doly (16). Phage were precipitated from the clear culture supernatant by addition of polyethylene glycol 8000 to 3% and NaCl to 0.5 M. The phage pellet, obtained by centrifugation at 5000 \times g for 30 min at 0 °C, was resuspended in phenol extraction buffer (100 mM Tris-HCl, pH 8.0, 300 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 *Pvu*I and *Pvu*II 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 (40 mM Tris acetate, pH 8.3, 2 mM EDTA) containing 0.5 mg/ml ethidium bromide. Bands were visualized by a brief illumination with UV light, and the 6.8-kilobase fragment was excised. The DNA electroeluted from this gel fragment was purified and concentrated on an Elutip-d affinity column (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 μ g each of the restriction endonuclease fragment and single-stranded circular viral DNA (7196 bases) in 30 mM NaCl, 30 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 gel 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- μ l reactions containing 20 mM Hepes, pH 7.8, 2 mM dithiothreitol, 10 mM MgCl₂, 500 μ M each dATP, dGTP, dCTP, and [α -³²P]dTTP (500–1000 cpm/pmol), 300 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 each individual laboratory before shipment, as 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 μ l (120 ng of DNA) of each reaction were mixed with 5 μ l of sodium dodecyl sulfate dye mix (20 mM Tris-HCl, pH 8.0, 5 mM EDTA, 5% sodium dodecyl sulfate, 0.5% bromophenol 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 h. Bands were visualized by illumination with UV light and photographed. The gel was then dried and used to expose Kodak XAR film to produce autoradiograms.

Expression of DNA Product in Competent Cells—The remaining product of the polymerization reactions was used for transfection of *E. coli* S90C *recA56* cells made competent as previously described using 75 mM CaCl₂ (17) or more recently using the procedure of Hanahan (18). With Ca²⁺-treated cells, the ratio of DNA molecules to cells was held constant at 10:1, while with cells treated by the procedure of Hanahan the ratio was either 1:1 or 2:1. The transfec-

tions 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 *lacZ* α 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 then 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 variety 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 early 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

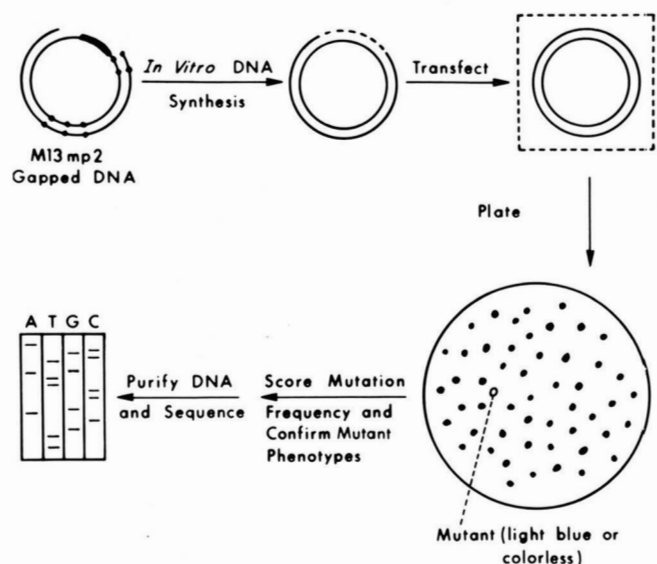


FIG. 1. Experimental outline of M13mp2 mutagenesis assay. The five pairs of dots on the gapped molecule indicate the position of the five sites for adenine methylation used to instruct mismatch correction (20–23). The gap extends from positions +174 to –216 (where +1 is the start of transcription) and is determined by cleavage with restriction endonucleases *PvuI* and *PvuII*. The 5' end (on the left) of the 390-base gap is therefore more than 100 bases away from the end of the target (which is position –84, the first nucleotide after the *lacI* 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 *lacZ α* codon 45. The *lacZ α* 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 Δ M15*) of the host cell, thus including most of the coding sequence of the α -peptide needed for α -complementation. The 5'-phosphoryl terminus is at position –216, which is more than 100 bases beyond the regulatory sequences which control α -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 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 these

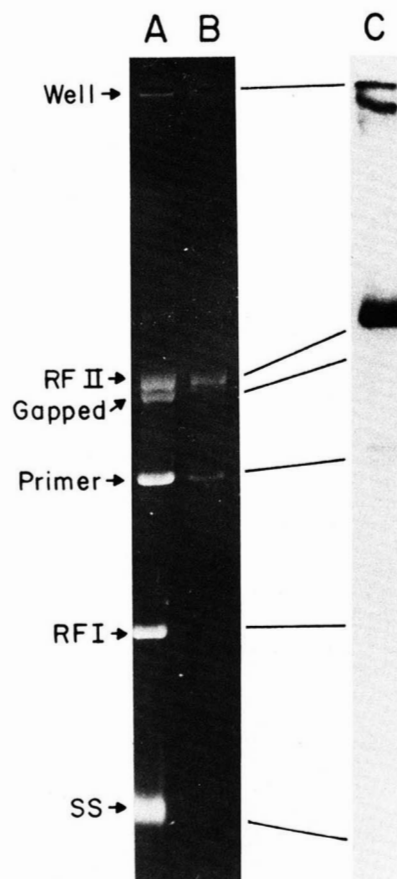


FIG. 2. Agarose gel analysis of the product of the rat hepatoma Pol- β copying reaction with gapped M13mp2 DNA. Lane A, standards, including an amount of uncopied gapped DNA equivalent to the copied DNA shown in lane B (Primer refers to the linear fragment used to make the gapped template); lane B, rat hepatoma Pol- β copied, 60 min, 37 °C; lane C, Pol- β copied, autoradiograph of ^{32}P incorporation. The analysis was performed as described under "Experimental Procedures." Lanes B and C were obtained from two separate agarose gel electrophoretic analyses of the same DNA preparation. RF, replicative form; SS, single strand.

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 2.8×10^{-4} to 6.6×10^{-4} for the control DNA molecules, compared to 640×10^{-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-

TABLE I

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

The rat hepatoma DNA polymerase- β copying reactions, transfections, 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.

DNA	Number of determinations	Plaques scored		Mutation frequency $\times 10^{-4}$
		Total	Mutant ^a	
Viral	2	10,597	7	6.6
Replicative form	2	28,655	8	2.8
Nicked construct ^b	3	199,655	128	6.4
Pol- β -copied	5	10,474	669	640.0
Pol- β -copied, denatured ^c	1	3,099	3	9.7

^a 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.

^b The nicked construct was made by cleaving replicative form DNA with restriction endonuclease *Ava*II 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).

^c 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."

Source of Pol- β	Number of determinations	Plaques scored		Mutation frequency $\times 10^{-4}$
		Total	Mutant	
Rat hepatoma	5	10,474	669	640.0
Chick embryo	4	13,930	611	440.0
HeLa cell	2	4,435	161	360.0
Human liver	2	2,576	196	760.0

plementation can result from several different types of events, mutants were analyzed by DNA sequencing to determine the exact nature of the errors made by Pol- β . For this purpose, a collection of randomly selected, independent mutants were isolated, and pure stocks were prepared and scored for the intensity of blue color in direct comparison to wild type M13mp2 blue plaques (17). Infrequent (<5%) false positives due to initial plating artifacts were discarded. This analysis of color phenotypes showed a common feature of all four Pol- β mutant collections; each contained 60–70% colorless mutants.

Mutational Hot Spots—The analysis of the first 159 rat mutants, obtained from three independent experiments, identified the occurrence of two mutational hot spots within the

coding sequence of the *lacZ α* gene. These were observed at a run of 4 consecutive T residues at positions +70 through +73 and at 2 T residues at positions +103 and +104. Both hot spots have similar dinucleotide neighbors, a CG on the 5' side and an AC on the 3' side. Two types of mutations were produced at these sites: the loss of a single T in the run (a colorless phenotype) or a T \rightarrow G transversion at +70 or +103, the 5'-most T residue (resulting in a valine \rightarrow glycine change, a light blue phenotype). The relative proportion of frameshift and transversion errors is different for the two sites. These hot spots are indeed a result of errors by Novikoff hepatoma Pol- β , since among 128 spontaneous mutants sequenced, not one error was observed at these two sites. An analysis of 20 independent colorless mutants generated in the chick embryo Pol- β copying reactions showed a similar high frequency of deletion frameshifts at +70 through +73. Thus, the hot spots seem to be a common feature of Pol- β mutational spectra with this target. The frequency of -T frameshifts at +70 to +73 (51 of 159 mutants analyzed) was sufficiently high to interfere with the generation of an extensive spectrum of other Pol- β errors. For this reason, site-specific mutagenesis techniques (24) were used to change the T residue at +72 to a C. This genetically silent change interrupts the run of 4 T residues and should effectively reduce the frequency of -T frameshifts at this hot spot. Using this target, differing by a single silent change from wild type, an additional 137 Novikoff hepatoma Pol- β mutants were analyzed. As expected, the total forward mutation frequency was reduced (from 640×10^{-4} to 490×10^{-4}), and the proportion of -T frameshifts at this site relative to the total mutants analyzed was decreased (18/137). This target was also used to generate the mutational spectrum of errors produced by chick Pol- β (144 mutants sequenced).

Spectrum of Errors by Pol- β —The complete spectra of single-base frameshifts (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 events² 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 thought 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.

Frameshift Errors—Both rat hepatoma Pol- β and chick embryo Pol- β produce frameshift errors at frequencies more than 200 times the background frequency and at frequencies similar to their base substitution error rates (Table III). The deletion of 1 or more bases is much more frequent than the addition of a single base. Furthermore, 1 base deletions predominate over 2-, 3-, or 4-base frameshift events (Table IV).

² T. A. Kunkel, unpublished results.

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