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Fidelity of Mammalian DNA Replication and Replicative DNA Polymerases†

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ABSTRACT: Current models suggest that two or more DNA polymerases may be required for high-fidelity semiconservative DNA replication in eukaryotic cells. In the present study, we directly compare the fidelity of SV40 origin-dependent DNA replication in human cell extracts to the fidelity of mammalian DNA polymerases α, δ, and ϵ using *lacZα* of M13mp2 as a reporter gene. Their fidelity, in decreasing order, is replication $\geq$ pol ϵ > pol δ > pol α. DNA sequence analysis of mutants derived from extract reactions suggests that replication is accurate when considering single-base substitutions, single-base frameshifts, and larger deletions. The exonuclease-containing calf thymus DNA polymerase ϵ is also highly accurate. When high concentrations of deoxynucleoside triphosphates and deoxyguanosine monophosphate are included in the pol ϵ reaction, both base substitution and frameshift error rates increase. This response suggests that exonucleolytic proofreading contributes to the high base substitution and frameshift fidelity. Exonuclease-containing calf thymus DNA polymerase δ, which requires proliferating cell nuclear antigen for efficient synthesis, is significantly less accurate than pol ϵ. In contrast to pol ϵ, pol δ generates errors during synthesis at a relatively modest concentration of deoxynucleoside triphosphates (100 μM), and the error rate did not increase upon addition of adenosine monophosphate. Thus, we are as yet unable to demonstrate that exonucleolytic proofreading contributes to accuracy during synthesis by DNA polymerase δ. The four-subunit DNA polymerase α–primase complex from both HeLa cells and calf thymus is the least accurate replicative polymerase. Fidelity is similar whether the enzyme is assayed immediately after purification or after being stored frozen. DNA sequence analysis of independent mutants generated by each enzyme shows that they all produce single-base substitution and frameshift errors, as well as larger deletions. However, the three polymerases have distinctly different error rates and error specificities, which has implications for their roles in the various stages of DNA replication.

In the past few years, major advances have been made in the field of eukaryotic DNA replication, largely due to the study of model systems such as simian virus 40 (SV40)[1] [for reviews, see Kelly (1988), Stillman (1989), Hurwitz et al. (1990), and Bambara and Jessee (1991)]. In fact, the nearly complete replication of SV40 origin containing DNAs has been reconstituted in vitro with purified proteins (Tsurimoto et al., 1990; Weinberg et al., 1990). Evidence from these and other studies suggests that at least two DNA polymerase systems are involved in synthesis of the leading and lagging strands at the eukaryotic replication fork (Prelich & Stillman, 1988; Focher et al., 1988; Downey et al., 1988; Weinberg & Kelly, 1989; Lee et al., 1989; Tsurimoto & Stillman, 1989; Matsumoto et al., 1990; Tsurimoto et al., 1990). One model that has emerged suggests that DNA polymerase δ (pol δ), in association with additional accessory proteins, may replicate the leading strand, while the DNA polymerase α–primase complex (pol α) generates Okazaki fragments on the lagging strand.

Studies with the reconstituted systems leave open the possibility that additional proteins, including DNA polymerases, may be involved in replication in crude extracts or in vivo. Recent studies with nuclei isolated from simian cells (Nethanel & Kaufmann, 1990) and with extracts of HeLa cells (Bullock et al., 1991) suggest that two polymerases may replicate the lagging strand. The final processing of Okazaki fragments in the SV40 system does not occur when replication is reconstituted with completely purified proteins (Tsurimoto et al., 1990; Weinberg et al., 1990), leaving open the question of which polymerase carries out this step. Since several studies have shown that replication can take place even when the only polymerase present is pol α (Murakami et al., 1986; Wold et al., 1989; Tsurimoto & Stillman, 1991), the definition of the roles of other polymerases in the reconstituted system may require a fuller understanding of the appropriate concentrations of all replication proteins.

More recently, yeast DNA polymerase II, the analogue to mammalian DNA polymerase ϵ (Bambara & Jessee, 1991; Burgers et al., 1990; Syväoja et al., 1990), was shown to be a third DNA polymerase essential for viability in *Saccharomyces cerevisiae* (Morrison et al., 1990). Moreover, the gene encoding a subunit of yeast pol II, *DBP2*, is essential for viability, and a temperature-sensitive mutant is partially defective in DNA synthesis at the restrictive temperature (Araki et al., 1991). The authors of those studies suggest that DNA replication in yeast may involve three DNA polymerases. They propose a model in which DNA polymerase ϵ is the leading strand polymerase, while DNA polymerases α and δ are responsible for lagging strand replication. This model is intri-

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[1] Abbreviations: pol α, DNA polymerase α–primase; pol δ, DNA polymerase δ; pol ϵ, DNA polymerase ϵ; yeast pol II, yeast DNA polymerase II; SV40, simian virus 40; T antigen, SV40 large tumor antigen; PCNA, proliferating cell nuclear antigen.

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guing given the many similarities between mammalian and yeast replication (Burgers, 1990).

Replication models involving more than one DNA polymerase are obviously highly relevant to how cells replicate DNA with exceptional fidelity. DNA polymerases synthesize DNA in vitro with distinctive fidelities, depending on differences in misinsertion and mispair extension rates [reviewed in Echols and Goodman (1991)] and on whether the polymerase has an associated proofreading exonuclease activity [reviewed in Kunkel (1988)]. Furthermore, the fidelity of a DNA polymerase varies depending on the type and position of the error [for reviews, see Kunkel (1990) and Echols and Goodman (1991)]. Therefore, it is likely that replication error rates are not uniform but reflect the DNA polymerase/accessory protein complexes responsible for synthesis during different stages of replication. These stages include synthesis at the origin, synthesis of the leading strand, synthesis of the lagging strand, and synthesis during replacement of RNA primers with DNA.

In initial studies of replication fidelity, we (Roberts & Kunkel, 1988) and others (Hauser et al., 1988) have established that SV40 origin-dependent DNA replication in mammalian cell extracts is highly accurate when considering a small number of single-base substitution errors. However, replication fidelity has not yet been established for each of the 12 possible mispairs or for base addition and deletion errors. Furthermore, extensive error specificity data exist only for one of the three mammalian DNA polymerases featured in the current eukaryotic replication models, DNA polymerase α (Kunkel, 1985; Mendelman et al., 1989, 1990; Kunkel et al., 1989, 1991). The fidelity of DNA polymerase δ has not been described previously, and the fidelity of DNA polymerase ϵ (then referred to as pol δII) has been determined only for a few base substitution errors that revert a nonsense codon (Kunkel et al., 1987). Thus, in the present study, we have used a forward mutation assay to define error rates for a variety of base substitutions, deletions, and additions, during replication in HeLa cell extracts and during gap-filling synthesis by DNA polymerases α, δ, and ϵ. Our eventual objective is to understand how high-fidelity DNA polymerization is achieved during each phase of replicative synthesis.

Experimental Procedures

Materials. *Escherichia coli* strains NR9099, MC1061, NR9162, and CSH50 and bacteriophages M13mp2 and M13mp2SV have been described (Roberts & Kunkel, 1988; Kunkel, 1985a; Kunkel & Soni, 1988a). Restriction enzymes and sequencing reagents were obtained from New England Biolabs or Boehringer Mannheim. [α-^{32}P] dCTP and [γ-^{32}P] ATP were purchased from Amersham Corp.

DNA Polymerases. HeLa cell DNA polymerase α–primase complex, provided by D. Weinberg and T. J. Kelly (Johns Hopkins University), was purified by immunoaffinity chromatography as described (Murakami et al., 1986; Wold et al., 1988). The preparation contains subunits of 180, 70, 55, and 45 kDa (Murakami et al., 1986; Wold et al., 1988). DNA polymerase α–primase was purified by immunoaffinity chromatography from calf thymus by the method of Wahl et al. (1984, 1986) and contained subunits of 180, 70, 65, and 60 kDa (Wahl et al., 1984, 1986). The 3'→5' exonuclease-containing, PCNA-dependent DNA polymerase δ was purified from calf thymus by conventional chromatographic methods (Lee et al., 1984; Ng et al., 1991). This polymerase contains two subunits of 125 and 48 kDa. It was unable to perform gap-filling DNA synthesis unless supplemented with PCNA (data not shown). The PCNA was purified from calf thymus as described (Tan et al., 1986). The 210-kDa, 3'→5' exonuclease containing DNA polymerase ϵ (Bambara & Jessee, 1991), formerly designated DNA polymerase δII, was purified from calf thymus as described (Crute et al., 1986). Polymerase unit definitions are as described in each reference for their purification. For replication studies, HeLa cell cytoplasmic extract was prepared by the method of Li and Kelly (1985). SV40 large T antigen was purchased from Molecular Biology Resources.

Forward Mutagenesis Assay. The assay scores errors in the wild-type *lacZ*α gene of M13mp2. Correct polymerization during gap-filling synthesis in a 390-base single-stranded gap (by purified polymerases) or during semiconservative replication produces DNA that yields dark blue M13 plaques upon transfection of an appropriate *E. coli* host strain. Errors are scored as lighter blue or colorless plaques. Since the assay measures loss of a gene function (α-complementation of β-galactosidase activity) that is not essential for phage production, a wide variety of mutations at many different sites can be recovered and scored. This includes 221 single base substitution errors at 114 different template positions, single-base frameshifts at 150 template positions, and a variety of other errors (Bebenek et al., 1990).

Studies of Replication Fidelity with a HeLa Cell Extract. Replication reactions (25 μL) were performed as described (Roberts & Kunkel, 1988), using a double-stranded, covalently closed circular M13mp2 DNA molecule containing the SV40 origin of replication (Roberts & Kunkel, 1988). Unreplicated molecules were inactivated by treatment with the restriction endonuclease *Dpn*I, and the replicated DNA was used for transfection of *E. coli* strain NR9162 (*mutS*) to score plaque colors on indicator plates, as described (Roberts et al., 1991).

Studies with Purified Polymerases. Assays with purified polymerases were performed with a gapped M13mp2 substrate (Kunkel, 1985a). HeLa pol α reaction mixtures (50 μL) contained 20 mM Tris-HCl (pH 7.5), 20% (v/v) glycerol, 10 mM $MgCl_2$, 2 mM dithiothreitol, 10 μg of bovine serum albumin, 300 ng of gapped M13mp2 DNA, either 100 or 500 μM each dNTP, and 3.8 units of DNA polymerase α–primase. Calf thymus DNA pol α and ϵ reaction mixtures (50 μL) contained 20 mM Tris-HCl (pH 7.5), 20% (v/v) glycerol, 10 mM $MgCl_2$, 10 μg of bovine serum albumin, 3 mM ATP, 300 ng of gapped M13mp2 DNA, each dNTP at the concentration indicated in Table I, and either 6 units of DNA polymerase α–primase or 10 units of DNA polymerase ϵ. DNA pol δ reaction mixtures (30 μL) contained 40 mM Bis-Tris, pH 6.5, 10% (v/v) glycerol, 5 mM $MgCl_2$, 1 mM dithiothreitol, 40 μg/mL bovine serum albumin, 200 ng of M13mp2 gapped DNA, 140 μg/mL PCNA, and 15 units of pol δ. For reaction mixtures containing nucleoside monophosphates, the concentrations are given in Table I. Following DNA synthesis, a portion of each reaction was analyzed by agarose gel electrophoresis to monitor the extent of synthesis. Except for reactions catalyzed by DNA polymerase δ (see below), all polymerase reactions reported here generated products that migrated coincident with fully double-stranded, replicative form II DNA. Aliquots of the remaining samples were used to transfect competent *E. coli* strain MC1061 cells by electroporation. After mutant frequencies were scored, independent mutants were isolated, and their DNA was sequenced. Details of the transfection procedure, plating conditions, scoring and sequencing of mutants, and calculation of error frequencies were as described (Kunkel, 1985a; Kunkel & Soni, 1988a).

Results

Using the forward mutation assay, we compared the fidelity

Table I: Mutation Frequency for DNA Replication and for Synthesis by DNA Polymerases

polymerization reaction	dNTP concn (μM)	plaques scored: total	plaques scored: mutant	mutation frequency ($\times 10^{-4}$)
control[a]		199655	128	6.4
replication				
minus T antigen	100	92046	61	6.6
plus T antigen, experiment 1	100	122410	85	6.9
plus T antigen, experiment 2	100	47635	39	8.2
calf thymus pol ϵ	20	16787	13	7.7
	50	16959	12	7.1
	1000[b]	24381	59	24
calf thymus pol δ + PCNA	20	21302	40	19
	100	19881	79	40
	100[c]	16622	60	36
calf thymus pol α	50	3951	37	94
	500	6495	26	190
HeLa pol α	100	10124	92	91
	500	17835	224	130

[a] Determined by the transfection of *E. coli* strain MC1061 with an artificially constructed RFII DNA substrate (Kunkel, 1985a). Such mutants are not necessarily independent. [b] Reaction mixture also contained 20 mM dGMP. [c] Reaction mixture also contained 1 mM AMP.

of semiconservative replication in HeLa cell extracts to that of gap-filling synthesis by DNA polymerases α, δ, and ϵ. The results, expressed as mutation frequencies, are presented in Table I. The products of bidirectional, semiconservative replication in HeLa cell extracts yielded mutation frequencies that were not significantly above background values obtained by transfection of *E. coli* with unreplicated DNAs, either untreated DNA (6.4×10^{-4}) or DNA incubated with the extract in the absence of T antigen (6.6×10^{-4}). This suggests that replication fidelity was high. Similarly, the products of gap-filling DNA synthesis performed by DNA pol ϵ with low dNTP concentrations yielded mutation frequencies that were not above background, suggesting that fidelity was high. To determine if the 3'→5' exonucleolytic activity associated with this enzyme was at least partly responsible for high-fidelity synthesis, reactions were performed using a high concentration (1000 μM) of the four dNTPs and 20 mM dGMP. These conditions reduce exonucleolytic proofreading by several exonuclease-containing DNA polymerases [for a review, see Kunkel (1988)]. Using this reaction condition, the mutation frequency increased to 24×10^{-4}, 3.8-fold above the background frequency of uncopied DNA.

Mutation frequencies above background were also observed for synthesis by DNA pol δ plus PCNA (Table I). This was so even at low (20 and 100 μM) dNTP concentrations and in the absence of added nucleoside monophosphate. The mutation frequencies for this enzyme may be underestimated, since even in the presence of PCNA, pol δ performed successful gap-filling synthesis on only ~60% of the molecules in the reaction (data not shown). Interestingly, when nucleoside monophosphates are included at concentrations known to inhibit the 3'→5' exonuclease activity of pol δ (Byrnes et al., 1977), no increase in the mutant frequency was observed (Table I, compare 40×10^{-4} to 36×10^{-4}).

DNA synthesis by both DNA polymerase α–primase preparations, at either low or high dNTP concentrations, generated higher mutant frequencies than for either pol δ or pol ϵ (Table I). The calf thymus enzyme yielded similar results whether assayed immediately after purification without freezing (Table I) or after storage at −70 °C for more than 1 year (100×10^{-4} with 50 μM dNTPs).

DNA Sequence Analysis. The DNAs isolated from independent mutants obtained in the forward assay were sequenced to define the error specificity. To establish the background mutant specificity, 128 mutants were analyzed. Seventy-eight mutants recovered from extract reactions were analyzed. Since synthesis by DNA polymerase ϵ produced a mutant frequency above background only when reactions contained 1 mM dNTPs plus 20 mM dGMP, all 59 mutants recovered from this reaction were sequenced. For synthesis by DNA polymerase δ plus PCNA, 79 mutants generated in reaction mixtures containing 100 μM dNTPs were analyzed, while 91 and 56 mutants generated by HeLa cell DNA polymerase α–DNA primase with 100 and 500 μM dNTPs, respectively, were sequenced.

Due to limitations in the amounts of available DNA polymerases δ and ϵ and because the sequence analysis of large mutant collections is labor-intensive, the error specificity analyses for the polymerases were not performed multiple times. However, past experience illustrates three facts relevant to the reproducibility of the approach: (i) for those experiments performed 3 or more times, standard deviations are typically 10–20% of mean values for overall mutation frequencies[2] (Kunkel et al., 1989; Kunkel & Soni, 1988b); (ii) similar error spectra were generated for the same polymerase when performed a second time, either several years apart [Kunkel (1985a) versus Kunkel and Soni (1988b)] or using a recombinant versus natural form of polymerase [Figure 1 in Bebenek et al. (1989)]; (iii) α-polymerases from different sources yield similar mutant frequencies and error spectra (Kunkel, 1985b; Kunkel et al., 1989; this study).

In all five mutant collections, single-base substitutions, single-base frameshifts, and other sequence changes were observed. Consistent with the lack of a replication-dependent increase in overall mutant frequency (Table I), the mutant specificity for DNA replicated in the extract was no different from the background mutant specificity of DNA not replicated in vitro (Figure 1). Thus, most or all of the mutants are presumed to be of background origin. On the basis of these data, we did not sequence mutants from reactions catalyzed by DNA polymerase ϵ with 20 or 50 μM dNTPs, because these mutant frequencies were also at the background value (Table I). However, when reaction mixtures contained 1 mM dNTPs plus 20 mM dGMP, DNA polymerase ϵ generated 3-fold and 6-fold increases, respectively, in mutant frequencies for single-base substitution and single-base frameshift errors (Figure 1). Similarly, DNA polymerases δ and α produced both types of errors at frequencies substantially above the background (Figure 1). The distribution of base substitution and frameshift errors by the DNA polymerases is given in Figures 2–4.

For errors by the purified polymerases copying a single-strand DNA template, the mispaired intermediate can be inferred, since the plus strand serves as the template for incoming dNTPs during gap-filling synthesis. For semiconservative replication of double-stranded DNA, either strand can serve as the template for producing an error. Thus, only the mutational outcome can be determined, not the intermediate. For example, a mutant sequence containing a T→C transition error could have resulted from replicative misincorporation of either dGTP opposite a template T or dCTP opposite a template A. The same logic can be applied to frameshift errors. For example, for a minus-T error in a template T run generated by a DNA polymerase copying single-stranded DNA, one can reason that the putative misaligned intermediate contained one unpaired template T. However, the same mutant recovered from a double-stranded

[2] K. Bebenek, J. Abbotts, S. Wilson, and T. A. Kunkel, unpublished results.

Table II: Base Substitution Error Rates per Detectable Nucleotide Polymerized by a HeLa Cell Extract and Replicative DNA Polymerases[a]

			replication		calf thymus pol ϵ		calf thymus pol δ		HeLa pol α	
mutation	mispair	sites	mutants	error rate	mutants	error rate	mutants	error rate	mutants	error rate
Transition Mispairs										
A→G	A·dCTP	15			1	1/210 000	1	1/180 000	4	1/29 000
T→C	T·dGTP	23			5	1/65 000	1	1/280 000	11	1/16 000
average		38	7	≤1/290 000	6	1/90 000	2	1/230 000	15	1/20 000
G→A	G·dTTP	21			1	1/300 000	4	1/63 000	14	1/12 000
C→T	C·dATP	23			11	1/30 000	7	1/39 000	8	1/22 000
average		44	15	≤1/160 000	12	1/52 000	11	1/48 000	22	1/16 000
Transversion Mispairs										
G→C	G·dGTP	19			1	1/270 000	1	1/230 000	6	1/25 000
C→G	C·dCTP	9			0	≤1/130 000	0	≤1/110 000	0	≤1/70 000
average		28	4	1/370 000	1	1/400 000	1	1/340 000	6	1/36 000
A→C	A·dGTP	13			1	1/180 000	0	≤1/160 000	0	≤1/100 000
T→G	T·dCTP	23			2	1/160 000	0	≤1/280 000	5	1/36 000
average		36	2	≤1/950 000	3	1/170 000	0	≤1/430 000	5	1/56 000
A→T	A·dATP	21			4	1/74 000	2	1/130 000	14	1/12 000
T→A	T·dTTP	16			0	≤1/230 000	4	1/48 000	8	1/16 000
average		37	0	≤1/2 000 000	4	1/130 000	6	1/74 000	22	1/13 000
G→T	G·dATP	23			7	1/46 000	8	1/34 000	6	1/30 000
C→A	C·dTTP	15			0	≤1/210 000	2	1/90 000	7	1/17 000
average		38	9	≤1/220 000	7	1/77 000	10	1/46 000	13	1/23 000

[a] For "≤" values, either no mutants were recovered or the frequency was less than 2-fold higher than the background frequency for uncopied DNA.

Table III: Frameshift Error Rates per Detectable Nucleotide Polymerized by a HeLa Cell Extract and Replicative DNA Polymerases[a]

frameshift mutation	detectable sites	replication		calf thymus pol ϵ		calf thymus pol δ		HeLa pol α	
		mutants	error rate	mutants	error rate	mutants	error rate	mutants	error rate
plus-one	150	0	≤1/8 100 000	8	1/270 000	0	≤1/1 800 000	5	1/240 000
minus-one, overall	150	8	≤1/1 000 000	11	1/200 000	31	1/56 000	37	1/32 000
at nonreiterated	118	1	≤1/6 300 000	1	1/1 700 000	19	1/72 000	12	1/78 000
at reiterated	38	7	≤1/290 000	10	1/56 000	12	1/37 000	25	1/12 000

[a] For "≤" values, either no mutants were recovered or the frequency was less than 2-fold higher than the background frequency for uncopied DNA. A nonreiterated minus-one frameshift error is defined as the loss of a base that is flanked by different bases or is part of a two-base run, whereas a reiterated minus-one frameshift is the loss of a base from a run of three or more of the same base.

replication reaction could have resulted from an unpaired nucleotide in either the T- or the A-containing template strand.

With this in mind, and given our extensive knowledge of the target size for each specific type of error, quantitative error rates per detectable nucleotide polymerized can be calculated and are presented in Tables II and III. Since replication did not increase the mutant frequency for any base substitution error above that of the background frequency, only "less than or equal to" error rates can be calculated for replication. Given the signal to noise ratios in Figure 1, 76, 77, and 95% of the base substitution mutants in the DNA polymerase ϵ, δ, and α collections, respectively, are calculated to have resulted from DNA synthesis in vitro. Similarly, 93, 97, and 98% of the frameshift mutants in the DNA polymerase ϵ, δ, and α collections, respectively, are calculated to have resulted from DNA synthesis in vitro.

In addition to single-nucleotide mistakes, DNA polymerase δ and DNA polymerase α–primase generated other errors at frequencies above the background (Figure 1). The mutant collection from the pol δ reaction contained 9 deletions ranging from 2 to 317 bases, and 1 complex deletion. The pol α spectrum contained four two-base deletions and one complex mutant. The pol ϵ spectrum included 2 large deletions and an 11-base duplication. For the replication products, the only mutation involving a change of more than one nucleotide was a three-base addition, which may have been a spontaneous change.

Discussion

Semiconservative replication of double-stranded DNA in eukaryotes requires several phases of polymerization that are potential sources of mutations. The first is the extension of RNA primers at the origin to initiate what becomes the leading strand at the replication fork. Since the primase activity associated with pol α is responsible for synthesis of RNA primers, the initial DNA synthesis from these is thought to be carried out by pol α. At some distance from the origin, a switch to a second DNA polymerase is thought to occur, and elongation continues to complete the leading strand of the replicon. DNA synthesis on the lagging strand initiates from RNA primers, again synthesized by the primase activity associated with pol α. These primers are extended into fragments ~150 bp in length, presumably by pol α and perhaps a second DNA polymerase as well (Nethanel & Kaufmann, 1990; Bullock et al., 1991; Morrison et al., 1990). Finally, RNA primer removal from the lagging strand and at the origin requires new DNA synthesis to generate complete double-stranded DNA products.

The involvement of more than one DNA polymerase in replication has implications for the enzymatic control of mutation rates in eukaryotes. Several previous studies have demonstrated that the fidelity of DNA polymerases with different biochemical properties can vary over a wide range [for reviews, see Kunkel and Bebenek (1988) and Echols and Goodman (1991)]. DNA polymerases α, δ, and ϵ indeed do have distinctly different subunit compositions, biochemical properties, and associated enzymatic activities [for reviews, see Fry and Loeb (1986), Burgers, (1990), and Bambara and Jessee (1991)]. Despite this, and despite their potential extensive roles in replication (see the introduction for models), only limited information has yet been published on the fidelity of DNA polymerases δ and ϵ, and on the fidelity of semi-

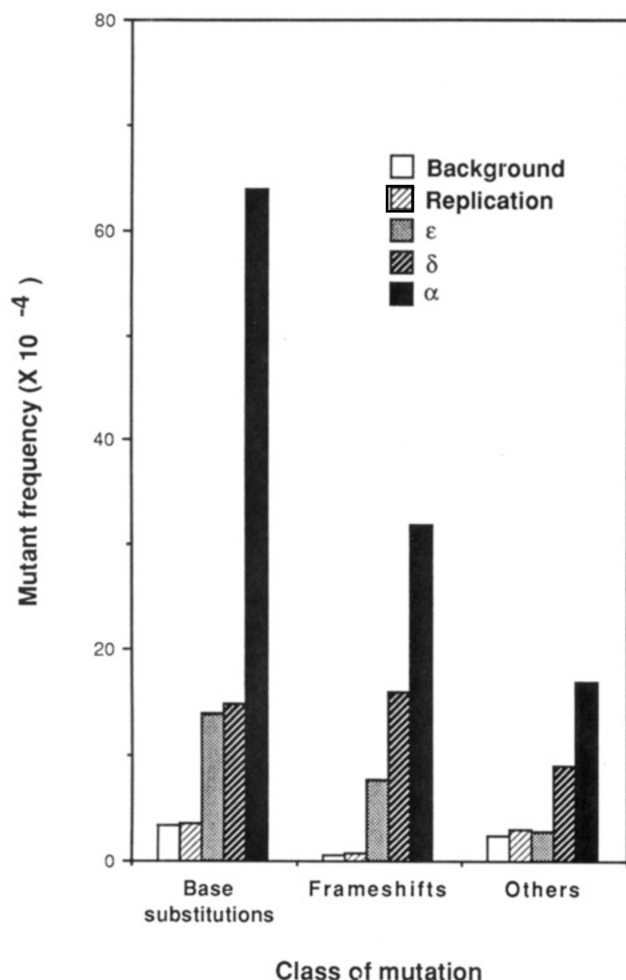

FIGURE 1: Mutation specificities by class for mammalian DNA replication by a HeLa cell extract and by purified replicative DNA polymerases. Mutants from various reactions presented in Table I were sequenced to determine mutant frequencies for base substitutions, frameshifts, and other mutations, which include deletions, duplications, multiple nontandem mutations, and mutants that exhibit a light blue phenotype with no change detected in the target. The specific reactions subjected to sequence analysis and the exact number of mutants sequenced are presented in the text. Since the spectra generated by HeLa cell DNA polymerase α–primase with 100 and 500 μM dNTPs were very similar, these data were combined for this analysis. Regarding the background, we have consistently obtained mutant frequency data between 3×10^{-4} and 7×10^{-4} from transfection of either single-stranded DNA, untreated double-stranded DNA, double-stranded DNA incubated in extracts without T antigen, or double-stranded DNA constructed [as described in Kunkel (1985a)] to mimic the products of a polymerase gap-filling reaction (the control value in the first row of Table I). Although the above background specificity data reflect the latter condition, similar data were obtained upon sequence analysis following transfection of either single-stranded DNA or double-stranded DNA first incubated in extracts without T antigen (data not shown).

conservative DNA replication in eukaryotes. This study presents a direct comparison of the fidelity of all three polymerases and of DNA replication in extracts of HeLa cells.

DNA Polymerase α–Primase. DNA polymerase α has been the most extensively studied eukaryotic replicative polymerase because it was the first to be discovered and could be obtained in sufficient quantity due to its relative abundance in cells. As the only replicative polymerase identified until relatively recently, primase-free pol α was typically purified from a number of sources as a partially proteolyzed, exonuclease-deficient catalytic subunit whose base substitution accuracy was ~ 1/10 000 [for a review, see Fry and Loeb (1986)]. The fact that this accuracy is low relative to that expected of a major replicative DNA polymerase has led to a continuing search for forms of pol α that have higher fidelity. This led to the observations that proteolysis (Brosius et al., 1983) and enzyme storage (Reyland & Loeb, 1987) diminish the fidelity of pol α for certain base substitution errors. It has also been demonstrated that the fidelity of the immunoaffinity-purified four-subunit DNA polymerase α–primase complex can be much higher than the fidelity of other forms of the enzyme for several base substitutions that revert an amber codon in φX174 DNA (Reyland & Loeb, 1987; Perrino & Loeb, 1989). Furthermore, several reports [reviewed in Kunkel (1988)] describe pol α preparations containing 3'→5' exonuclease activity, including preparations from *Drosophila melanogaster* (Cotterill et al., 1987; Reyland et al., 1988) and a human lymphoblast cell line (Bialek et al., 1989) that have high base substitution fidelity in φX174 amber codon reversion assays.

In contrast to the above reports of high-fidelity forms of pol α, the results of the present study clearly demonstrate that synthesis by pol α–primase is not highly accurate when a comprehensive spectrum of errors is considered. This interpretation is reinforced by earlier studies with the human KB cell pol α (Kunkel, 1985b), *Drosophila melanogaster* pol α (Mendelman et al., 1989, 1990), and yeast polymerase α–primase (Kunkel et al., 1989, 1991). It is unlikely that this reflects use of an enzyme preparation containing a proteolyzed polymerase catalytic subunit, since in this and two earlier studies (Kunkel et al., 1989, 1991) the polymerase catalytic subunit was 180 kDa. The error rates also do not reflect freeze–thawing or long-term storage of the polymerase–primase, since we obtained essentially the same results with the calf thymus (this study) or yeast (Kunkel et al., 1989) enzymes assayed immediately after purification or after storage for more than 1 year at −70 °C.

The fact that immunoaffinity-purified pol α–primase preparations from yeast, calf thymus, and human KB cells are not highly accurate is consistent with the fact that these enzymes have no detectable 3'→5' proofreading exonuclease activity (Reyland & Loeb, 1987; Perrino & Loeb, 1989; Kunkel et al., 1989; Copeland & Wang, 1991). When the polymerase catalytic subunit of either the yeast or human KB cell α-polymerases was overexpressed from the cloned genes, the purified protein lacked detectable 3'→5' proofreading exonuclease activity (Brooke et al., 1991; Copeland & Wang, 1991). This is consistent with recent deduced amino acid sequence alignments. These alignments suggest that α-polymerases lack five amino acid residues that are important for exonuclease activity in *E. coli* DNA polymerase I and that are invariant among aphidicolin-sensitive DNA polymerases with an associated proofreading activity (Morrison et al., 1991).

There are several possible explanations for the essential role of α-polymerase in high-fidelity replication, despite its apparent modest fidelity during DNA synthesis in vitro. For the model in which pol α performs the bulk of lagging strand replication, polymerase accessory proteins may enhance the fidelity of pol α (Carty et al., 1990) to that observed for lagging strand replication in HeLa cell extracts (Roberts et al., 1991). Given the recent data suggesting that exonucleolytic proofreading occurs during lagging strand synthesis (Roberts et al., 1991), a proofreading exonuclease is an obvious possibility. Candidates include those exonucleases already detected in several preparations of pol α (Chen et al., 1979; Skarnes et al., 1986; Ottiger & Hübscher, 1984; Grosse et al., 1983; Cotterill et al., 1987; Bialek et al., 1989), those associated with DNA polymerases δ and ε (Bambara & Jessee, 1991), or completely

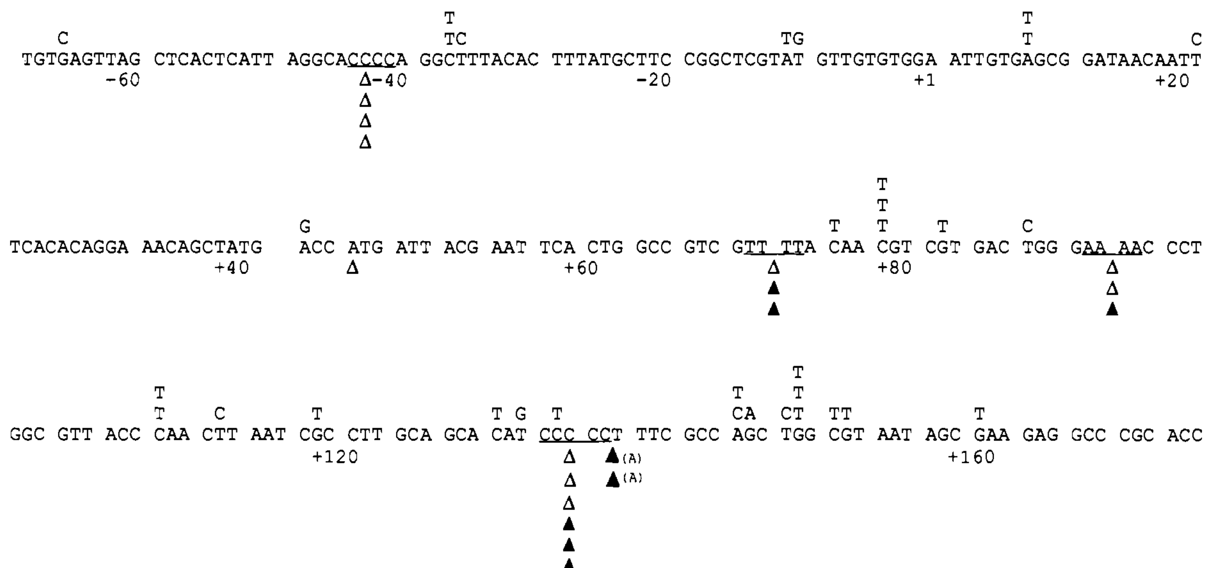

FIGURE 2: Spectrum of single-base mutations produced by calf thymus pol ϵ. Three lines of primary wild-type DNA sequence [of the viral (+)-template strand] are shown. Position +1 is the first transcribed base of the *lacZα* gene. The figure presents only single-base mutations, with base substitutions shown above each line of wild-type sequence and frameshifts shown below each line. The letters used for the base substitutions indicate the new base found in the viral template strand DNA sequence, in place of and directly above the wild-type base. For frameshift events, the loss of a base is indicated by an open triangle, whereas the addition of a base is indicated by a solid triangle. When a frameshift occurs at positions of iterated or reiterated bases (underlined), it is not possible to distinguish which base was lost or added; thus, the symbol is centered under the run. For two mutants, an A was added between two nucleotides as indicated. Other mutations found but not shown above include 2 large deletions, 1 11-base duplication, 1 nontandem double base substitution, and 3 mutants that exhibit a slight reduction in blue color intensity but for which no change was found from positions −84 through +174, the target sequence within the gap.

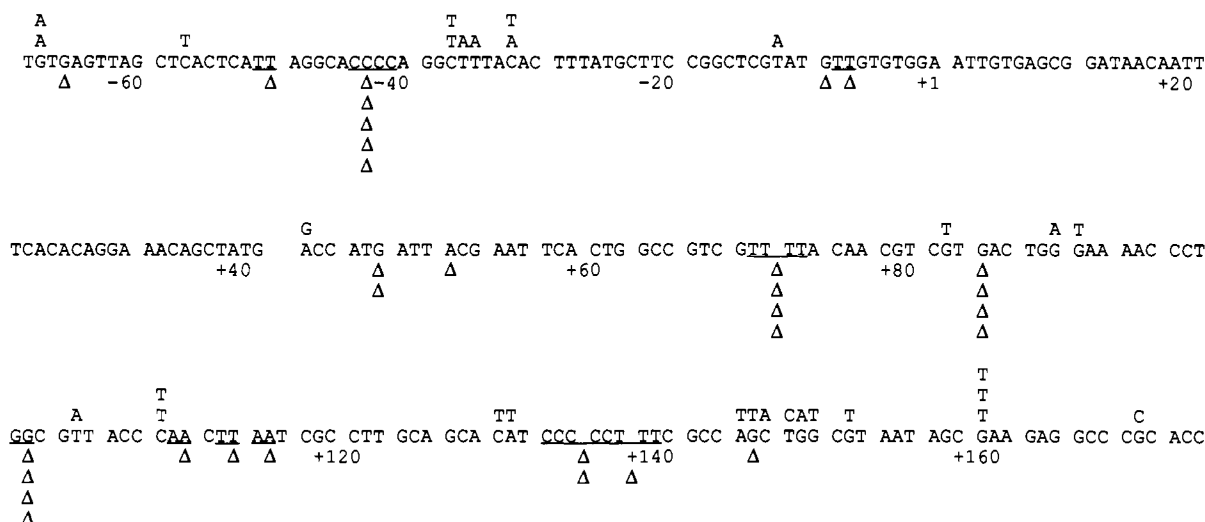

FIGURE 3: Spectrum of single-base errors by calf thymus pol δ. See the legend to Figure 2 for explanation. Other mutations not listed include 9 large deletions, 1 nontandem double mutant, 1 complex mutation, 1 deletion of the primer site for sequencing, and 6 mutants that exhibit a slight reduction in blue color intensity but for which no change was found from positions −84 through +174.

separate exonucleases (Mosbaugh & Meyer, 1980). Any such proofreading activity for pol α would be unique relative to all previously identified proofreading exonucleases. The latter either are an integral part of the polymerase polypeptide itself (e.g., *E. coli* DNA polymerase I) or are contained in a subunit that is very tightly associated with the DNA polymerase through numerous purification steps (e.g., *E. coli* DNA polymerase III), neither of which seems to be the case for yeast or mammalian pol α.

Alternatively, even if pol α fidelity is not affected by additional components of the replication apparatus, the fact that pol α is not as accurate as replication is not inconsistent with the model in which it provides a critical role in extending RNA primers but performs only limited polymerization before replication switches to another polymerase. Occasional errors at the origin or on the lagging strand could be corrected by heteroduplex repair or during replacement of RNA primers with DNA.

DNA Polymerase δ. Although this enzyme was discovered 15 years ago (Byrnes et al., 1976), prior to this work, examination of its fidelity has been limited to a single study using a synthetic polynucleotide in a manganese-activated reaction (Byrnes et al., 1977). In the present study, DNA polymerase δ did not perform gap-filling synthesis with the M13mp2 DNA substrate unless its accessory protein, PCNA, was added to the reaction. Even then, the enzyme did not complete synthesis on all DNA molecules, providing a minimum estimate of error rates. Such inefficient synthesis presumably reflects the fact that this polymerase requires additional accessory proteins to perform its role(s) in replication effectively.

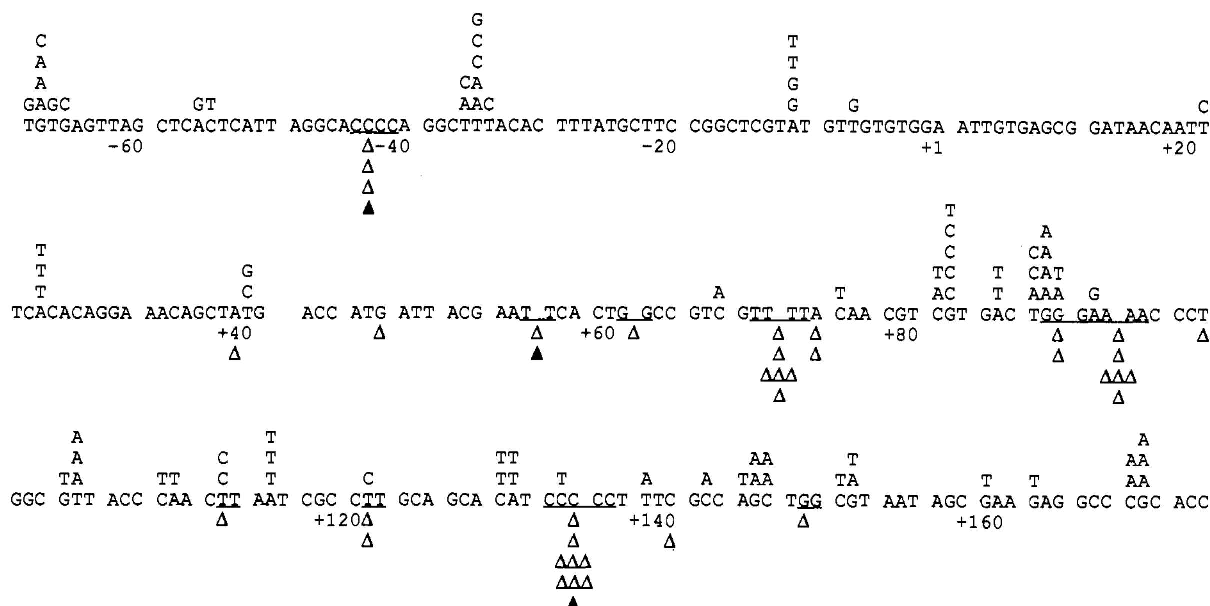

FIGURE 4: Spectrum of single-base errors by HeLa cell DNA polymerase α–primase. See the legend to Figure 2 for explanation. Two mutants not shown in the spectrum include the addition of an A between nucleotides at positions +135 and +136 and the addition of an A between positions +136 and +137. Other mutations not listed include 1 complex change, 12 nontandem double mutations, 1 nontandem triple mutation, 4 2-base deletions, and 4 mutants that exhibit a slight reduction in blue color intensity but for which no change was found from positions −84 through +174.

DNA polymerase δ has a tightly associated 3′→5′ exonuclease activity [reviewed in Bambara and Jessee (1991)] that may proofread errors. Consistent with this possibility, DNA polymerase δ is more accurate than pol α when considering base substitutions and frameshifts (Figure 1). The rates for 7 of the 12 base substitution errors, at less than 1/100 000 (Table II), are substantially lower than those typically observed with polymerases lacking exonuclease activity. For example, a number of studies (Grosse et al., 1983; Kunkel & Alexander, 1986; Tindall & Kunkel, 1988; Kunkel et al., 1989; Mendelman et al., 1989; Bebenek et al., 1990; Carroll et al., 1991) have shown that the misinsertion of dGTP opposite template T is one of the most frequent mistakes for exonuclease-deficient polymerases; the error rate is typically higher than 1/10 000. The resulting mispair is also the most easily extended of the 12 mispairs (Mendelman et al., 1990; Newton et al., 1989). Thus, an error rate of 1/280 000 for T·dGTP errors by pol δ (Table II) is consistent with a substantial contribution of proofreading to fidelity.

As an alternative to proofreading, the polymerase itself may be more selective against certain errors. We cannot exclude this yet because two attempts to provide evidence for exonucleolytic proofreading met with limited success. Increasing the dNTP concentration to favor extension from rather than excision of misinserted nucleotides gave only a 2-fold increase in mutant frequency (Table I, compare 19×10^{-4} to 40×10^{-4}). Addition of AMP[3] had no effect on the mutation frequency in the forward mutation assay (Table I, compare 40 $\times 10^{-4}$ to 36×10^{-4}). Furthermore, even when no monophosphate was added and the dNTP substrate concentration was 100 μM, pol δ was not highly accurate when considering several mispairs (Table II). It was also inaccurate when considering frameshift errors (Table III), and the error spectrum included nine errors wherein more than a single nucleotide was lost. One possible explanation for such infidelity is that the polymerase may require its replicative accessory proteins for optimal discrimination. It will be important to determine if these proteins improve fidelity via increased polymerase selectivity and/or proofreading efficiency.

Because the exonuclease activity of pol δ is inhibited by AMP (Byrnes et al., 1977), the absence of an AMP effect on fidelity was unexpected. We do not yet clearly understand this result. However, it is interesting to consider the implications for the role of pol δ in leading strand synthesis in light of the observation (Roberts et al., 1991) that the fidelity of leading strand synthesis for four transition errors is reduced by addition of monophosphate (in that instance, dGMP) to an SV40 origin-dependent replication reaction.

DNA Polymerase ε. This was the most accurate of the three polymerases examined. Although we did not sequence the mutants recovered from the high-fidelity pol ε reactions (Table I, 20 and 50 μM dNTPs), base substitution and frameshift error rates are presumably similar to the "less than or equal to" values shown in Tables II and III for extract reactions. This is because pol ε dependent synthesis, as for the replication in the extract, did not generate mutants above the background mutant frequency of uncopied DNA.

Pol ε dependent errors were only detected when dGMP was included in the synthesis reaction, suggesting that high fidelity without dGMP resulted partly from exonucleolytic proofreading. This is consistent with an earlier study (Kunkel et al., 1987) suggesting exonucleolytic proofreading of base substitution errors at the template T of an opal codon by pol ε (which at that time was designated pol δII). The present study also suggests proofreading of frameshift errors, which are readily detected (Figure 1) using a reaction condition that reduces proofreading. The 30-fold higher error rates for frameshifts at reiterated sites also suggest that the majority of pol ε dependent frameshift errors may result from a template-primer slippage (Streisinger et al., 1966). In contrast, the pol δ error rates for frameshifts at nonreiterated positions are within 2-fold of the error rate at reiterated sites. This suggests that another mechanism may be operative, e.g., errors initiated by nucleotide misinsertion (Bebenek & Kunkel, 1990).

[3] We used AMP because in earlier studies of pol δ, this had been shown to effectively inhibit the associated 3′→5′ exonuclease activity.

SV40 Origin-Dependent DNA Replication. Consistent with earlier observations (Roberts & Kunkel, 1988; Hauser et al,. 1988), the overall average fidelity of SV40 origin-dependent replication in HeLa cell extracts is high, as indicated by the observation that the mutant frequency of replicated DNA was not significantly different from that of unreplicated DNA (Table I). However, in six other studies with the forward assay[2,4] (Kunkel & Soni, 1988a; Kunkel et al., 1989; Roberts et al., 1990; Bebenek & Kunkel, 1990), we have detected significant differences in specificity even when little or no difference in mutant frequency was initially seen when two experimental conditions were compared. This prompted a sequence analysis of mutants recovered from the unreplicated as well as replicated DNA, to see if any differences could be found. As shown by the data in Figure 1 and Tables II and III, replication is highly accurate when considering all 12 base substitution errors as well as frameshift errors at both reiterated and nonreiterated sites. In only two instances was the mutant frequency for replication more than 2-fold higher than background (C·G→G·C transversions and minus one C·G base pair at the only five-nucleotide run in the target, positions 132–136). In both cases, the number of mutants recovered was small, suggesting that even these differences may not be significant. High replication fidelity for all error classes is interesting relative to the model derived from studies of the proteins required for SV40 origin-dependent replication in reconstituted systems, which suggests that pol α is the lagging strand polymerase and pol δ is the leading strand enzyme. Using identical dNTP concentrations (100 μM) for all synthesis conditions, neither polymerase is as accurate as replication when considering base substitutions, one-base frameshifts, or deletion errors.

A comparison of the template site preferences and error rates demonstrates that DNA polymerases α, δ, and ϵ have different error specificities. No template sites with more than two base substitution mutants each were common to any two enzymes (Figures 2–4). The enzymes had distinctly different error rates for certain base substitutions (Table II) as well as for plus-one and minus-one nucleotide errors (Table III). Such differences may eventually be useful for elucidating the roles of the polymerases in the several stages of DNA replication.

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