Mechanisms of Mutation by Oxidative DNA Damage: Reduced Fidelity of Mammalian DNA Polymerase β[†]

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ABSTRACT: Reactive oxygen species, produced in cells by a variety of mechanisms, damage DNA and cause mutations. To characterize the types of mutations produced in mammalian cells, we copied DNA damaged by reactive oxygen species with mammalian DNA polymerase β. Double-stranded circular M13mp2 DNA containing a 361-nucleotide single-stranded gap within the *lac*Z gene was damaged by aerobic incubation with Fe^{2+} and H_2O_2. The gap then was filled by purified recombinant rat DNA polymerase β, and the DNA was transfected into *Escherichia coli*. Mutations within the nonessential *lac*Z gene for β-galactosidase were identified by reduced α-complementation. In this system, oxidative damage increased the mutation frequency within the target region by an average of 4.3-fold. At certain sites, the base substitution rate is nearly 300 times greater than would be expected to result from a random distribution of damage. The oxidatively induced mutations fall into two categories: those apparently caused by direct miscoding of modified DNA and those associated with enhanced misincorporation at prexisting polymerase-specific hot spots. The latter group may be due to a conformational change in the DNA caused by oxidative modification and could be indicative of a novel mutagenic mechanism.

In procaryotic and eucaryotic cells, oxygen is metabolized by a series of one-electron reductions, generating a variety of oxygen free radicals and associated species. These highly reactive intermediates alter cellular macromolecules. The extent of DNA damage by reactive oxygen species in human cells has been estimated by Ames and co-workers (Shigenaga et al., 1989; Cathacart et al., 1984; Richter et al., 1988). On the basis of the recovery of 8-oxoguanine (Shigenaga et al., 1989) and thymine glycol (Cathacart et al., 1984) in human urine, it appears that the average human cell genome undergoes approximately 20 000 oxygen-induced alterations per day (Richter et al., 1988). Presumably, most of this damage is repaired by error-free mechanisms, yet it is likely that some escapes DNA repair. The unrepaired, modified bases are copied during DNA replication or repair synthesis and could be an important source of mutations in somatic cells.

Damage to DNA by reactive oxygen species (ROS)[1] results in a variety of chemical alterations. Exposure of DNA (Aruoma et al., 1989) or free nucleotides (Cadet & Berger, 1985) to systems that generate ROS in vitro produces more than 35 different base modifications (Fraga et al., 1990), many of which can be detected in DNA isolated from cells that have undergone oxidative stress (Hsie et al., 1986). Although the multiplicity of reactive oxygen species and the diversity of DNA modifications they cause have hindered progress in understanding oxidative mutagenesis, several important advances have been made recently. 8-OH-dGuo, one of the most common oxidative adducts in mammalian DNA (Floyd et al., 1986; Kasai et al., 1986), has been synthesized, incorporated into DNA templates, and found to code for G to T transversions (Wood et al., 1990; Shibutani et al., 1991; Cheng et al., 1992). Studies of other oxidative DNA lesions have shown that 2,6-diamino-4-hydroxy-5-*N*-methylformamidopyrimidine (FapyG) is a strong block to DNA synthesis (O'Conner et al., 1988) and thymine glycol can be a block to DNA synthesis (Ide et al., 1985) or code for T to C transitions (0.3%), but codes for its complementary nucleotide with greater than 99% accuracy (Basu et al., 1989).

In previous studies, we have determined the mutational spectra resulting from misincorporations during replication of oxidatively damaged DNA in *Escherichia coli* (Loeb et al., 1988; McBride et al., 1991, 1992; Tkeshelashvili et al., 1991; Reid & Loeb, 1992). We have used a forward mutation assay to examine the type and frequency of mutations caused by reactive oxygen species produced by metal-catalyzed reduction of H_2O_2 and O_2, methylene blue activated by white light, and the oxidative burst of neutrophils in culture (Loeb et al., 1988; McBride et al., 1991, 1992; Tkeshelashvili et al., 1991; Reid & Loeb, 1992). In each case, the mutations induced were not randomly dispersed but rather occur in clusters, "hot spots", specific to the source of the damage. All of these studies, however, have been limited to SOS-induced bacteria. The spectra observed presumably were generated by *E. coli* DNA polymerase III under error-prone conditions and may have limited applicability to higher organisms.

Our long-term goal is to understand the contribution of ROS to mutagenesis in mammalian cells. Our initial approach is to analyze the effect of oxygen free radical damage to DNA on the fidelity of DNA polymerase β (pol-β). Pol-β is a small, mammalian DNA polymerase that is present in resting cells and widely thought to be involved in DNA repair but may also have a role in DNA replication (Rein et al., 1990; Sweasy & Loeb, 1992). We have modified the *lac*Z forward mutation

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[1] Abbreviations: pol-β, mammalian DNA polymerase β; 8-OH-dGuo, 8-hydroxyguanosine; IPTG, isopropyl β-D-thiogalactopyranoside; ROS, reactive oxygen species; EDTA, ethylenedinitrilotetraacetic acid; HEPES, *N*-(2-hydroxyethyl)piperazine-*N*'-2-ethanesulfonic acid; fmol, femtomole; pmol, picomole.

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assay to study the effects of oxidative damage to the template on the fidelity of DNA synthesis. We treated an M13mp2 replicative form molecule, containing a 361-nucleotide single-stranded gap spanning the 5′ regulatory region and the first 145-nucleotides of the coding region of the β-galactosidase gene, with $FeSO_4$ and H_2O_2. We then determined the mutation frequency from gap-filling synthesis by purified pol-β. Mutations induced by oxidative damage fall into two categories: (1) hot spots observed only when the DNA had been damaged by the oxygen free radicals; (2) large increases in the frequency of mutations at sites that are preexisting mutational hot spots for pol-β. We present evidence that DNA damage by oxygen free radicals causes mutations by modifying nucleotide bases, which results in miscoding when the DNA is copied by DNA polymerase, and by altering the conformation of the DNA template.

MATERIALS AND METHODS

E. coli Strains. NR9099 [Δ(pro-lac), recA⁻56, ara⁻, thi⁻/F′proAB, lacI^{q}Z⁻ ΔM15] was the host strain for the preparation of replicating form M13mp2Δpvu. CJ236 [ung⁻1, dut⁻1, relA1, spoT1, thi⁻1, pCJ105] was the host strain for the preparation of single-stranded, uracil-containing M13mp2. MC1061 [hsdR, mcrR, araD, (139Δ⁻araABC⁻leu) 7679ΔlacX74, galU, galK, rspL, thi⁻] was the strain used for transfections. CSH50 [Δ(proBlac)/F′ traD36, thi⁻, ara⁻, proAB, lacI^{q}Z⁻ΔM15] was used as an indicator strain for infected *E. coli*. NR9099, MC1061, and CSH50 were provided by Thomas Kunkel (NIEHS, Research Triangle Park, NC), and CJ236 was provided by Barbara Bachmann (*E. coli* Genetic Stocks Center, Yale University, New Haven, CT).

DNA Reagents. M13mp2Δpvu contains a 361-base deletion relative to wild type and was constructed by removing the sequence between the *Pvu*II sites at positions 5959 and 6320 on the M13 map. This deletion spans the 145 base pairs at the 5′ end of the *lacZα* coding region as well as all of the upstream regulatory sequences. This plasmid has a unique *Pvu*II site derived from ligation of the sites at 5959 and 6320 of the wild-type sequence.

Gapped DNA was prepared by the method of Kunkel (1984) with the following modifications. M13mp2Δpvu was linearized by *Pvu*II. Endonuclease digestion was continued until no circular plasmid was observable on an ethidium bromide stained agarose gel and there was no detectable biological activity upon transfection into competent MC1061. Following phenol and chloroform–isoamyl alcohol (24:1) extractions, the DNA was passed through a Sephadex G-50 column, equilibrated with distilled H_2O. The linear DNA was denatured at 70 °C, in distilled H_2O, at a concentration of 167 μg/mL for 15 min. Single-stranded, uracil-containing M13mp2 was added such that the molar ratio of double-stranded linear to single-stranded circular was 2:1. After 2 min at 70 °C, 0.1 volume of 20 × SSC was added, and the mixture was transferred to 60 °C for 15 min, followed by 15-min incubations at 42 °C, 37 °C, and room temperature. The gapped DNA was purified by agarose gel electrophoresis and stored in small aliquots in TE buffer, at −70 °C.

Oligodeoxynucleotides used in this study were synthesized and HPLC-purified by Operon Technologies, Inc. (Alameda, CA). The template DNA for the pausing assay, 5′CGTCGT-GACTGGGAAAACCCTGGCGTTACCCAACT-TAATCGC3′, corresponds to nucleotides +78 to +119 of the *lacZ* coding region. The primer, 5′GCGATTAAGTTG3′, is complementary to the 3′-12 nucleotides of the template.

All buffers used for DNA were passed through Chelex-100 (Bio-Rad) columns to remove trace metal contamination.

Treatment of Gapped DNA. (See Figure 1). Gapped DNA was incubated in 10 mM sodium phosphate buffer (pH 6.9) with or without 10 μM $FeSO_4$ and 10 μM H_2O_2 for 1 h at 37 °C in uncapped 1.7-mL microcentrifuge tubes. Where indicated, 100 μM mannitol, 1 unit of catalase, 5 units of superoxide dismutase, 25 μM deferoxamine, or 100 μM bathocuproinedisulfonic acid were included. The reaction was quenched by a 1:1 dilution with 100 μM deferoxamine followed by ethanol precipitation in the presence of 10 μg of glycogen. The treated DNA was resuspended in H_2O. The gap was filled by recombinant rat DNA polymerase β, a generous gift from S. Wilson (University of Texas, Galveston, TX). Twenty-microliter reactions contained 40 fmol of control or treated gapped DNA, 25 mM HEPES, pH 8.5, 125 mM NaCl, 5 mM $MgCl_2$, 100 μM each of dATP, dCTP, dGTP, and dTTP, and 335 fmol of DNA polymerase β (the molar ratio of enzyme to DNA is 8.3:1). The reactions were incubated for 1 h at 37 °C and stopped by the addition of 80 μL of 25 mM EDTA followed by phenol and chloroform–isoamyl alcohol extractions and ethanol precipitation in the presence of 10 μg of glycogen.

Product Analysis. Products of gap-filling synthesis were analyzed by radionucleotide incorporation and restriction fragment length analysis. Control and damaged DNAs were subjected to gap-filling synthesis in 20-μL reactions identical to those used for the mutation experiments except for the substitution of 100 μM [α-^{32}P]dTTP for 100 μM dTTP. The reactions were stopped by the addition of 80 μL of 25 mM EDTA, and 2 μL ($^1/_{50}$th of the total) was added to 200 μg of salmon sperm DNA. The DNA was precipitated 3 times in 1 N perchloric acid and 50 mM sodium pyrophosphate. After the third precipitation, the DNA was filtered on 24-mm, no. 25 glass filters. Radionucleotide incorporation was determined by liquid scintillation counting. The remaining 98 μL of the gap-filling reactions was extracted once each with phenol and 24:1 chloroform–isoamyl alcohol and precipitated in ethanol in the presence of 10 μg of glycogen. Samples were resuspended in 10 μL of 1x NEBuffer-2 containing 0.5 unit of *Pvu*II restriction enzyme (New England Biolabs) and incubated at 37 °C for 45 min. Samples were passed through Sephadex G-50 columns and were analyzed by electrophoresis on 6% polyacrylamide gels containing 7 M urea.

Transfections and Isolation of Mutants and DNA Sequencing. MC1061 cells were made competent by $CaCl_2$ treatment as described by Maniatis (Sambrook et al., 1989). The treated DNA (100 ng) was incubated with 0.5 mL of competent cells for 45 min followed by a 2-min heat shock at 42 °C. Aliquots (10 μL) of the transfection mixture were added to 3 mL of top agar, consisting of 0.9% NaCl, 0.8% Bacto-Agar, 0.08% X-gal dissolved in dimethyl formamide, and 0.2 mL of log-phase (OD_{600} approximately 0.8) CHS50 cells, and layered onto plates (100 × 15 mm) containing 30 mL of solidified M9 medium containing 1.5% agar and 15 μM isopropyl β-D-thiogalactopyranoside (IPTG). After the top agar solidified, the plates were inverted and incubated overnight at 37 °C and then for 24 h at room temperature. Light blue and colorless plaques were isolated, mixed with an equal quantity of wild-type M13mp2 phage, and replated in order to confirm mutant phenotypes. DNA was isolated from confirmed mutants as described previously (McBride et al., 1991) and sequenced by the method of Sanger (Sanger et al., 1978) using a 15mer oligodeoxynucleotide primer comple-

mentary to the +179 to +194 region of the *lacZα* coding sequence.

β-Polymerase Pausing Assay. Two hundred picomoles of template oligonucleotide, with a sequence corresponding to +78 to +119 of the *lacZ* coding region (see DNA Reagents), was incubated in 100 μL of 10 mM sodium phosphate buffer, pH 6.9, containing varying amounts of H_2O_2 and $FeSO_4$, for 1 h at 37 °C in open 1.7-mL microcentrifuge tubes. Reactions were quenched by the addition of 100 μL of 100 μM deferoxamine and then precipitated with ethanol in the presence of 10 μg of glycogen. Treated oligonucleotides were resuspended in 20 μL of H_2O and stored at −70 °C.

5′ end-labeled primers were annealed to treated templates by incubating 200 fmol of primer and 40 fmol of template in 6-μL reactions containing 83 mM HEPES (pH 8.5) and 16.5 mM $MgCl_2$ at 70 °C for 10 min and then at room temperature for 30 min. Nucleotides and β-polymerase were added to a final volume of 20 μL containing 25 mM HEPES, 125 mM NaCl, 5 mM $MgCl_2$, 5 μM each of dATP, dCTP, dGTP, and dTTP, and a polymerase to DNA ratio of 8.3, the same as in the mutagenesis assay. Reactions were incubated at 37 °C for 30 min and stopped by the addition of 30 μL of 25 mM EDTA followed by a 10-min incubation at 70 °C. Samples were then passed through Sephadex G-50 columns and analyzed on 20% polyacrylamide gels containing 7 M urea. After autoradiography, the bands were excised from the gel and quantitated by scintillation counting.

RESULTS

DNA Treatment and Mutation Frequency. For our initial studies on the effects of oxidative DNA damage on the fidelity of DNA synthesis in vitro, we elected to use mammalian DNA pol-β and to damage the DNA template by preincubation with $FeSO_4$ and H_2O_2. $FeSO_4$ and H_2O_2 react via the Haber–Weiss reaction, producing hydroxyl radical, which is thought to be the most potent of the DNA-damaging ROS (Imlay & Linn, 1988). This system for generating ROS has been shown to cause a diverse variety of DNA lesions (Aruoma et al., 1989) and to be highly mutagenic when damaged DNA is transfected into *E. coli* (McBride et al., 1991). Furthermore, it may contribute to oxidative DNA damage in vivo as its components are present in significant quantities in mammalian cells.

To measure and characterize errors in DNA synthesis caused by unrepaired DNA damage, we modified the *lacZ* forward mutation assay developed by Kunkel (1984). The target sequence is a 361-nucleotide single-stranded gap spanning the *lacZ* regulatory region and the 145 5′ bases of coding sequence. This allows the scoring of mutations by reduced α-complementation. After incubation of the gapped DNA with $FeSO_4$ and H_2O_2, the gap was filled by complementary strand synthesis with purified pol-β. The filled molecules were transfected into competent MC1061 and plated with a CSH50 indicator strain (Figure 1). Upon transfection, the DNA template strand containing uracil residues is hydrolyzed by a combination of uracil glycosylase and apurinic endonucleaes present in the host bacterium. Control experiments indicated that the expression of the template strand with uracil residues was less than 0.1% of that obtained with the wild-type single-stranded template containing thymidine residues (results not shown). Thus, greater than 99% of the mutants isolated in this system represent sequence changes derived from the strand synthesized by pol-β.

E. coli strain MC1061 is wild type with respect to DNA repair, and it is possible that some of the misincorporations

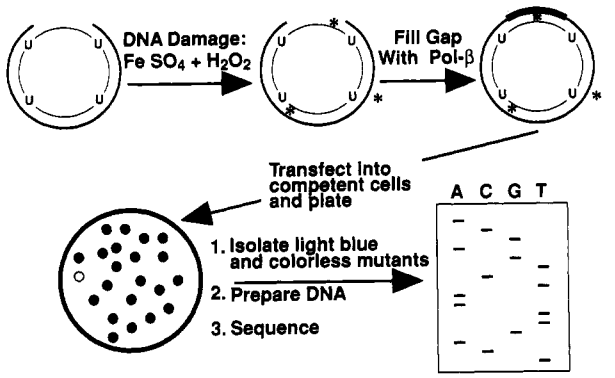

FIGURE 1: Schematic diagram of the assay used to measure the fidelity of pol-β on an oxidatively modified template. See text for experimental details.

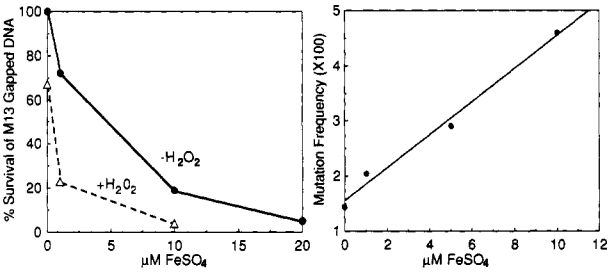

FIGURE 2: Dose responsiveness of killing and mutation frequency to oxidative DNA damage. Gapped DNA was incubated in $FeSO_4$, in the presence or absence of 10 μM H_2O_2, and then filled by DNA pol-β as described in the text. (Left) After incubation for 1 h at 37 °C with varying amounts of $FeSO_4$, the unfilled gapped DNA was transfected into MC1061. Fraction survival was calculated by dividing the phage titer resulting from damaged DNA by that of the undamaged DNA. (Right) DNA samples that had been treated with $FeSO_4$ and H_2O_2 were subjected to gap-filling synthesis, transfected into MC1061, and screened for mutations in *lacZα*. The mutation frequency of gapped DNA not filled by pol-β is 3.2×10^{-4}.

by pol-β are corrected after transfection. Most known DNA repair pathways, however, would either enhance or have no effect on the detection of the errors made by pol-β on oxidatively damaged DNA. Excision or glycosylase-mediated repair of lesions would use the pol-β-derived strand to direct resynthesis. Methyl-directed mismatch repair would have little effect because both DNA strands of the gapped molecule were generated in dam^+ strains (Modrich, 1989). MutY does catalyze the removal of an A incorporated by pol-β opposite 8-OH-dGuo (Michaels et al., 1992). Although mutY could cause an underestimation of 8-OH-dGuo-mediated G to T transversions, to do so it would have to compete with the uracil glycosylase mediated destruction of the template strand as well as several DNA repair pathways and replication.

The loss in survival and the enhancement in mutagenesis as a function of $FeSO_4$ concentration are shown in the left and right panels, respectively, of Figure 2. The extensive loss of survival could be the result of four mechanisms: (1) cleavage of the single-stranded region by hydroxyl radicals; (2) double-strand breaks (Mello-Filho & Meneghini, 1984); (3) damage-induced replication blocks; and (4) disruption of genes required for phage replication. At 1 and 10 μM $FeSO_4$, the addition of 10 μM H_2O_2 resulted in a 4–5-fold further loss in biological activity. On the basis of the loss of survival and enhancement of mutagenesis, we chose 10 μM $FeSO_4$ with 10 μM H_2O_2 as standard conditions in subsequent experiments to analyze mutagenesis by ROS.

The reaction conditions for gap-filling DNA synthesis were determined empirically, and the reaction products were

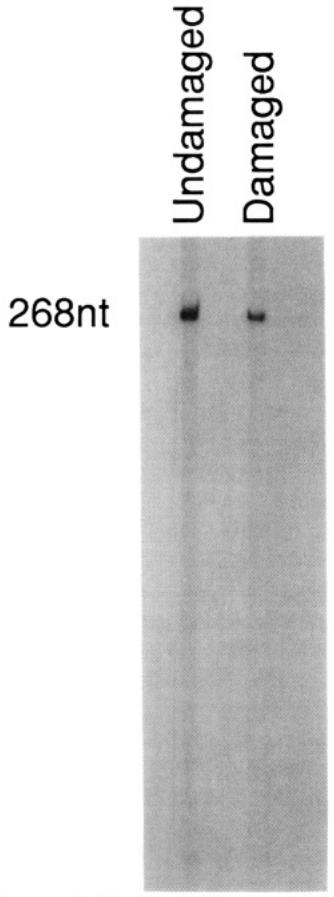

FIGURE 3: Product analysis. Gap-filling synthesis with pol-β and 100 μM dATP, dCTP, dGTP, and [α-^{32}P]dTTP was performed on undamaged template and template damage by incubation with $FeSO_4$ and H_2O_2. Products were treated with *Pvu*II restriction enzyme which cleaves the DNA at +145 and −123 of the *lac*Z sequence. The two restriction sites border the entire mutation target region used in these studies.

analyzed by radionucleotide incorporation and restriction fragment length analysis. In four independent experiments, reactions containing 100 μM dATP, dCTP, dGTP, and [α-^{32}P]-dTTP and an 8.3:1 molar ratio of pol-β to DNA, the average acid-precipitable counts incorporated into oxidatively damaged (10 μM $FeSO_4$ with 10 μM H_2O_2) gapped DNA was 91% of that incorporated into undamaged gap DNA. The acid-precipitable counts incorporated into the undamaged substrate were 84% of the theoretical value calculated for complete filling of all of the molecules in the reaction (data not shown). This indicates that the extent of gap-filling for damage and undamaged molecules is similar as well as nearly complete. This conclusion is further supported by restriction fragment analysis. The product DNA with incorporated [α-^{32}P]dTTP was also treated with *Pvu*II. This restriction enzyme cleaves M13mp2 at map positions 6320 (the start site for gap-filling synthesis) and 6052 (sites −123 and +145 of *lac*Z). A "full-length" product of 268 base pairs indicates DNA synthesis through the entire target region for the mutation assay while shorter products are indicative of abortive synthesis or strand breaks. Such an analysis is shown in Figure 3. Single-strand breaks within the gap will cause premature termination of pol-β. Because of the random distribution of the breaks, no single species is formed in sufficient quantity to generate a discrete band. The abundance of full-length products, relative to shorter products, is the same when synthesis is on undamaged or damaged templates. This confirms that, under the conditions used for the mutagenesis experiments, the extent of gap-filling synthesis for undamaged and damaged DNA is the same. Thus, any difference in mutation frequency secondary to oxidative damage is due to altered misincorporation by pol-β and not to reduced gap-filling.

Table I: Summary of Mutation Assay Data

	total plaques	total mutants	frequency ($\times 10^{-2}$)
	Undamaged Template		
−pol-β	9342	3	0.03
+pol-β			
expt 1	4761	51	1.1
expt 2	10489	80	0.76
expt 3	8045	74	0.92
total	23295	205	0.88
	Damaged Template: Fe^{2+} (10 μM) + H_2O_2 (10 μM)		
−pol-β	8644	0	>0.02
+pol-β			
expt 1	6894	251	3.6
expt 2	5589	225	4.0
total	12483	476	3.8

Table II: Inhibition of Fe^{2+}-Induced Mutagenesis by Antioxidants[a]

added	mutation frequency ($\times 10^{-2}$)	
	undamaged	Fe^{2+} + H_2O_2
nothing	0.86 (1.0)	3.9 (4.6)
mannitol (100 μM)	1.3 (1.5)	1.5 (1.8)
catalase (1 unit)	0.95 (1.1)	1.1 (1.3)
SOD (5 units)	0.85 (1.0)	1.5 (1.8)
deferoxamine (25 μM)	0.77 (0.9)	1.1 (1.3)
bathocuproindisulfonic acid (100 μM)	0.52 (0.6)	4.5 (5.2)

[a] Mutation rates are the average of two independent experiments. The phenotypes were confirmed by replating, but the mutants were not sequenced. Numbers in parentheses are relative mutation rates.

The mutagenesis by ROS is summarized in Table I. The mutation frequency of the undamaged, unfilled gap DNA is 3×10^{-4} and is increased by gap-filling synthesis by pol-β by an average of 30-fold. Thus, in the three experiments using the undamaged template, approximately 97% of the mutants were caused by misincorporations by pol-β. Damage to the template in the absence of in vitro DNA synthesis did not cause an increase in mutation frequency. This was expected since detectable ROS mutagenesis, on a single-stranded target gene, in *E. coli*, requires induction of the SOS response (McBride et al., 1991). The mutation frequency of gapped DNA that has been oxidatively damaged and then filled by pol-β is an average of 4.3-fold greater than that of undamaged, filled gap DNA ($p \leq 0.005$). Thus, 77% of the 476 mutants can be attributed to damage-induced errors by pol-β.

To demonstrate that the increase in mutation rate is due to DNA damage by ROS, DNA was treated with $FeSO_4$ and H_2O_2 in the presence of a hydroxyl radical scavenger, mannitol, catalase, superoxide dismutase, or metal ion chelators (Table II). These reagents inhibit the Haber–Weiss reaction in which Fe^{2+} reduces H_2O_2, forming hydroxyl radical and hydroxyl anion and Fe^{3+} which is reduced to Fe^{2+} by the trace amounts of superoxide generated in the reaction (Loeb et al., 1988). Pretreatment of DNA with mannitol, catalase, SOD, or deferoxamine (an Fe^{2+}-specific chelator) did not suppress mutations resulting from pol-β copying undamaged substrate. Inclusion of each of these in the damage reaction, however, inhibited the increase in mutation frequency 75–90%. These inhibitor studies provide evidence that hydroxyl radical, H_2O_2, superoxide, and Fe^{2+} are required for the increase in mutation frequency. Bathocuproinedisulfonic acid, a Cu^{2+} chelator, is included as a negative control to demonstrate that the inhibition is specific to the components of the ROS-generating system used to damage the DNA in this study.

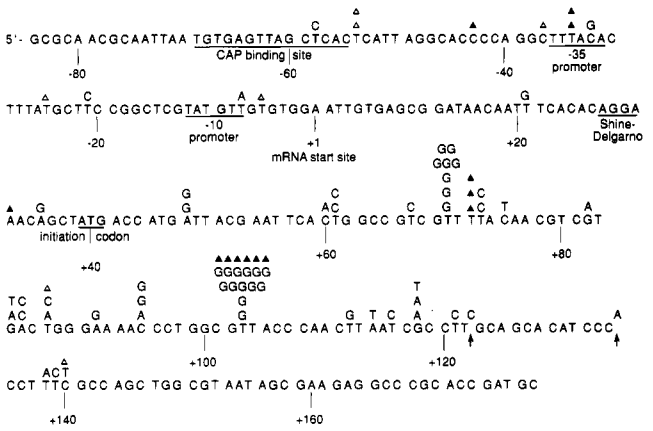

FIGURE 4: Mutation spectrum for DNA pol-β on an undamaged template. The five lines of numbered sequence represent the (+) strand of the wild-type *lac*Z gene of M13mp2. Gap-filling synthesis is initiated at +145, and extension is from right to left. Each symbol above the line represents a base change observed in a single mutant. For example, on undamaged template, we observed a T to G transversion at site +103 in 13 independently isolated mutants. Open triangles represent single base deletions. Closed triangles represent single base deletions with positions that could not be precisely defined because they occurred in runs of two or more identical nucleotides. An arrow indicates a single base addition.

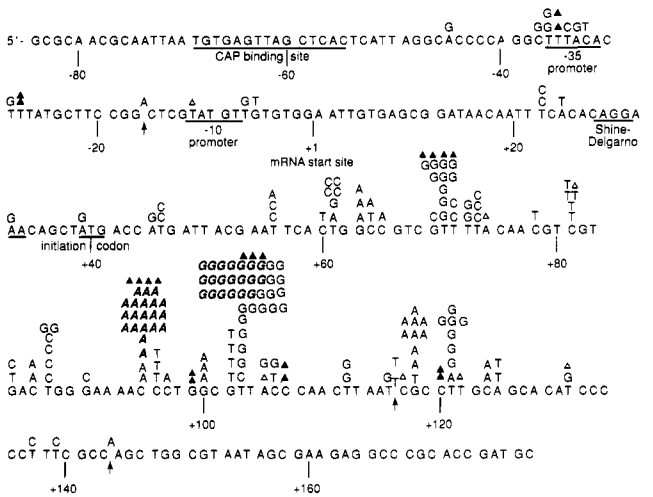

FIGURE 5: Mutation spectrum of pol-β with a template damaged by incubation with $FeSO_4$ and H_2O_2. The symbols and conventions are the same as in Figure 4. Twenty independently isolated mutants contained transversions at both site +95 and site +103. These are indicated by boldface.

Table III: Nucleotide Substitutions and Deletions by Pol-β[a]

nucleotide	substitution	undamaged template	Fe^{2+}-damaged template
A	→C	2	0
	→G	4	6
	→T	1	3
	Δ	1	2
C	→A	2	29
	→G	3	2
	→T	2	13
	Δ	3	10
G	→A	4	15
	→C	0	3
	→T	2	7
	Δ	0	2
T	→A	3	3
	→C	11	23
	→G	23	66
	Δ	16	14
deletions larger than 1 nucleotide			
2–10 nucleotides		0	5
>10 nucleotides		14	15
2 nontandem mutations		2	29

[a] Compilation of data from Figures 4 and 5.

Spectrum of ROS-Induced Mutations. We isolated and sequenced 205 mutants from undamaged templates and 476 mutants from templates damaged with $FeSO_4$ and H_2O_2. Of the 205 control mutants, 89 had detectable sequence changes, including 73 mutants with single base substitutions or deletions, 2 with multiple, nontandem, base substitutions, and 14 with deletions of 10 or more bases. Of the 476 Fe^{2+}-induced mutants, 189 had detectable sequence changes including 141 with single base subsitutions or deletions, 29 with multiple, nontandem, base substitutions, and 20 with deletions of 2 or more bases. The phenotype of the mutants for which no sequence change was detected was confirmed twice by replating. The fraction of mutants with detectable sequence changes for undamaged (43%) and damaged (40%) DNA is essentially the same, alleviating the concern that they might constitute a sampling error for the mutation spectra. The percentage of phenotypic mutants that yield sequence changes in the gap region is similar to previous studies using the *lacZ*α forward mutation assay (McBride et al., 1991; Reid & Loeb, 1992). Possible explanations for the cryptic mutations include sequence changes occurring further upstream of the coding region than we sequenced or that some mutations cause anomalous migrations of DNA fragments that mask the mutation on the sequencing gel.

The mutations observed after synthesis by pol-β with control and damaged templates are presented in Figures 4 and 5, and the nucleotide substitutions are catalogued in Table III. Pol-β most frequently makes single-base substitutions opposite template T's, especially T to G transversions. On damaged DNA, the profile of base changes is still dominated by T to G transversions, but C to A transversions and C to T transitions are more prominent.

The differences between the mutation spectrum of pol-β on undamaged and damaged templates can be grouped into two categories. First, there are three damage-dependent hot spots for mutation that were not prominent with the undamaged template: the C to T transition at +81, the G to A transition at +118, and the T to G transversion at +121. C to T transitions at +81 have also been observed with single-stranded M13mp2 DNA exposed to ROS-generating systems containing either methylene blue and white light or $FeSO_4$, and replicated in SOS-induced *E. coli* (McBride et al., 1991, 1992). The appearance of this mutation in multiple studies suggests that C81 is either particularly susceptible to oxidants or exceptionally mutagenic when modified. Second, we observed mutations on the damaged template that are also pol-β-specific hot spots. From a random distribution of oxidative lesions (Mello-Filho & Meneghini, 1984), one would predict no change in the mutation frequency at polymerase-specific hot spots to result from template damage. Suprisingly, the frequency of T to G transversions at +103 undergoes a 5.1-fold elevation from 5.6×10^{-4} (13 mutants per 23 295 plaques) to 2.9×10^{-3} (36 mutants per 12 483 plaques). The frequency of T to G transversions is also elevated at position +70. This indicates that the sequences or secondary structures that cause β-polymerase to have an elevated misincorporation rate are also hypersensitive to oxidative damage by $FeSO_4$ and H_2O_2 and/or that an oxidative lesion in the vicinity of such a sequence causes an exacerbation of an intrinsically mutagenic structure.

Template damage vastly increases the frequency of mutants sustaining more than one sequence change. The frequency of nontandem double mutations on undamaged DNA is 8.6 $\times 10^{-5}$ (2/23295) compared to 2.3 $\times 10^{-3}$ (29/12483) on damaged DNA, a 27-fold increase. Twenty of the damage-induced double mutants contained both a T to G transversion at +103 and a C to A transversion at +95 (indicated by boldface in Figure 5). These cannot be two independent events as the frequency of the mutants sustaining both changes, 1.6 $\times 10^{-3}$, is nearly 4 orders of magnitude greater than the product of the frequencies of mutants having one or the other, 2.1 $\times 10^{-7}$. Instead, these double mutants require another explanation.

Primer Extension Analysis. In order to elucidate the mechanism for the generation of the double mutants, we performed primer extension assays on an oligonucleotide template corresponding to nucleotides +79 to +120 of the *lac*Z-coding region. With the exception of reduced deoxynucleoside triphosphate concentrations (5 μM) and shorter incubation time (30 min), the DNA synthesis was performed at conditions identical to those used for the mutagenesis assay. Because of its highly distributive mechanism, β-polymerase yields a collection of products varying from 12 nucleotides, the unextended primer, to the fully extended product.

Figure 6 shows the primer extension analysis of undamaged and damaged templates. Extension on the undamaged template reveals a modest pause site at the GC residues corresponding to positions 102–101 in the *lac*Z sequence and a strong pause site at the CCA sequence corresponding to positions 96–94. In the absence of H_2O_2, treatment of the template with 1 or 10 μM $FeSO_4$ does not change the pausing pattern. Treatment of the template with 1 or 10 μM $FeSO_4$ in the presence of 10 μM H_2O_2, however, causes a marked decrease in pausing at the CCA site and an accumulation of more fully extended products. When the template is treated with 100 μM $FeSO_4$ in the presence or absence of H_2O_2, there is an increase in the fraction of the molecules that are fully extended although the relief of the pause site at C95 is not as great as seen with lower $FeSO_4$ concentrations. This could be due to the combination of relieved pausing and increased strand breaks in the template due to incubation with 100 μM $FeSO_4$.

In order to quantitate extension, the bands were excised from the gel, and the radioactivity in each was quantitated by scintillation counting. The results of the undamaged template and that treated with 10 μM H_2O_2 and 10 μM $FeSO_4$ are shown in Figure 6B. In the absence of damage, only 6.4% of the molecules are extended beyond C95 compared to 43.5% for the damaged template. This indicates that the structure that inhibits β-polymerase extension at C95, at low nucleotide concentrations, is altered by the ROS damage. This increased bypass, at conditions closely approximately those of the mutagenesis experiments, occurs at the same position as the damage-induced transversion hot spot (position 95). Furthermore, the other pause site that is relieved by the damage (position 102) also corresponds to a site of elevated mutation frequency.

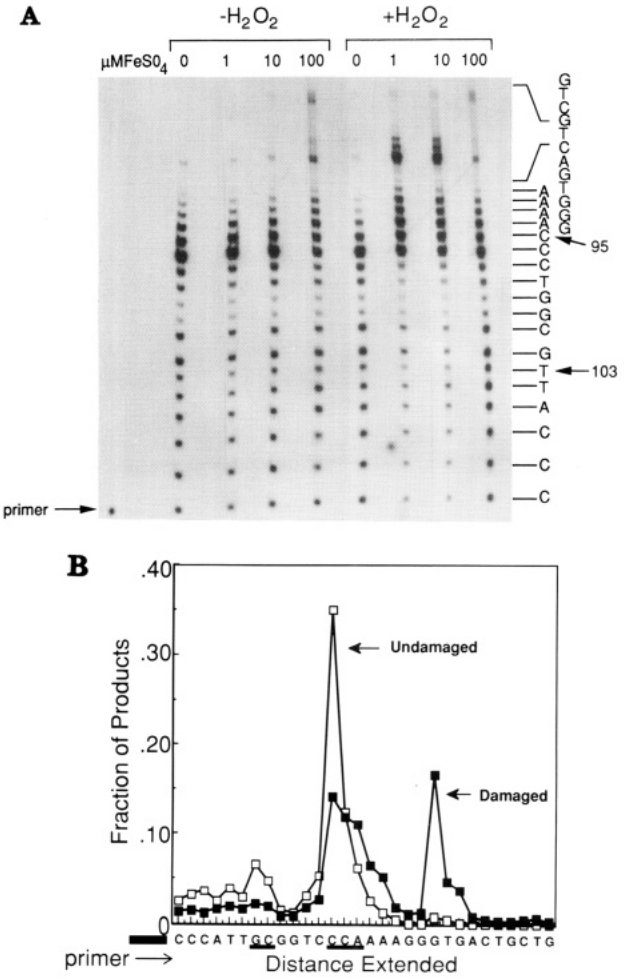

FIGURE 6: Primer extension analysis of undamaged and damaged oligonucleotides spanning the double mutation hot spot. The template strand sequence corresponds to +78 to +119 of the *lac*Z sequence. The 5′ end-labeled primer is complementary to +119 to +108. Damage, annealing, and extension were performed as described in the text. (A) Extended products were electrophoresed on a 20% strand-separating polyacrylamide gel. The leftmost lane is unextended primer. The sequence of the template is indicated at the right. (B) Bands were excised from the gel and quantitated by scintillation counting. The fraction of products extended to each site in the sequence is plotted against the template sequence. Data for extension coded by the undamaged template and those treated with 10 μM $FeSO_4$ and 10 μM H_2O_2 are shown. The underscored nucleotides on the abscissa designate the locations of the pause sites observed in (A).

DISCUSSION

There is considerable evidence for a role of reactive oxygen species in mutagenesis (Imlay & Linn, 1988). ROS are produced by several normal metabolic processes as well as many known mutagens, including some transition-metal ions (Ames, 1983; Sunderman, 1989; Kawanishi et al., 1989; Hsie et al., 1990; Kasprzak, 1991). Furthermore, oxygen radical scavengers specifically suppress the increase in mutation rate caused by many of these compounds (Ewing & Walton, 1991), and bacterial strains deficient in one or more antioxidant systems have a hypermutator phenotype (Fridovitch, 1983; Kow & Wallace, 1985; Farr et al., 1986). We now require more knowledge about the potential for ROS to cause errors during DNA synthesis in eucaryotic cells. We have begun to address this need to studying the effect of ROS damage to DNA on the fidelity of DNA pol-β.

Pol-β is the smallest and, at least in terms of subunit composition, the simplest of the mammalian polymerases. Although the physiological function of pol-β has not been unequivocally established, most of the data suggest a role in DNA repair: moderately specific inhibitors of pol-β inhibit DNA repair (Chen & Becker, 1981; Fry & Loeb, 1985; Wiebauer & Jiricny, 1990), pol-β is expressed at a constant level throughout the cell cycle (Zmudzka et al., 1988) and exposure of cells to certain DNA-damaging agents causes an induction of pol-β expression (Fornace et al., 1989). In

contrast, recent work in our laboratory suggests that pol-β has the potential to participate in DNA replication. It can complement a polymerase I defect in *E. coli*, at least in part, by mediating the rapid joining of Okazaki fragments (Sweasy & Loeb, 1992). Because of its relative simplicity and its potential to function in repair and replication, we elected to use it for our initial studies on the potential of ROS to cause errors in DNA synthesis.

The mechanism and the fidelity of pol-β have been studied on undamaged templates. Polymerization is highly distributive and proceeds through an ordered bi-bi reaction mechanism (Tanabe et al., 1979; Wang & Korn, 1982): the polymerase binds to the template-primer followed sequentially by the binding of Mg^{2+}-dNTP, nucleotide addition, release of pyrophosphate, and dissociation of the polymerase from the template. Studies by Kunkel have established the mutational spectrum of pol-β on the *lacZ*α gene (Kunkel, 1985a).

The spectrum of mutations, on undamaged DNA, that we demonstrate in our study can be directly compared with the results reported previously (Kunkel, 1985a). The set of mutational hot spots that we observe are nearly identical; however, the mutation frequency for pol-β on undamaged template is approximately 3-fold lower. Several factors probably contribute to this. To produce gapped DNA, we engineered M13mp2ΔpvuII (see Materials and Methods), eliminating the need to gel-purify the double-stranded linear molecule prior to hybridization to the single-stranded circular molecule. In our hands, this reduces the mutation frequency, in assays using the gapped product, approximately 3-fold (data not shown). Also, during gap-filling synthesis, we used a 5-fold lower deoxyribonucleoside triphosphate concentration, and our pol-β was isolated from a different source. The misincorporation frequency of pol-β isolated from different sources has been reported to vary over a 3-fold range (Kunkel, 1985a). In accord with the spectrum reported by Kunkel (1985a), our control spectrum is dominated by two major hot spots: T to G transversions in the pyrimidine runs, at sites +70 and +103. The model for this suggests that the nascent strand can partially dissociate from the template strand in pyrimidine runs and become misaligned such that the terminal nucleotide is correctly paired but the nascent strand is displaced one nucleotide in the 3′ direction. Continued DNA synthesis will result in a single base deletion. Alternatively, a single nucleotide incorporation event, followed by the return of the nascent strand to the original register, will result in a base substitution. This model is supported by the specificity of substitutions by β-polymerase in *lacZ* at the +70 and +103 hot spots (Bebenek & Kunkel, 1990; Kunkel, 1990) and is reconfirmed by the mutations in our studies.

We can analyze the primer extension data in light of what is known about the mechanism and fidelity of pol-β. Pause sites are sites at which a polymerase, more frequently than at other sites, does not add the next nucleotide to the nascent strand. For a highly distributive enzyme such as pol-β, a pause site may reflect a lower than usual binding affinity for the template-primer or a reduced rate of nucleotide addition. On the undamaged template, there are two pause sites (Figure 6): a weak pause at +103 to +102 and a stronger one at +95 to +94. Previous studies have shown that certain polymerase pause sites are positions of frequent misincorporation by DNA polymerases (Fry & Loeb, 1992). The locations of the two pause sites observed in this study correspond to two hot spots for mutagenesis: a pol-β-specific T to G transversion and a damage-specific C to A transversion. The fraction of molecules paused at these sites is reduced by oxidative damage to the template. Analysis of this sequence by a nucleic acid folding program (GCG Fold; Devereux et al., 1984) suggests that this region of the single-stranded template may assume a base-paired structure. Assuming the existence of this or another stem–loop structure, the pause sites would be at regions with fewer than 5 non-base-paired template residues downstream of the 3′ nascent strand terminus. Wang and Korn have shown that pol-β has reduced affinity for such structures (Wang & Korn, 1982) which would be manifested as the observed pause sites. An oxidative lesion on a base, sugar, or phosphate within this putative stem–loop could destabilize it, yielding a form for which pol-β has higher affinity and on which pol-β is less likely to pause.

Oxidative damage to DNA could cause a structural change that enhances bypass synthesis at sites of increased mutation frequency. Because both the T to G transversions at +103 and the C to A transversions at +95 occur at the end of pyrimidine runs, both are potential sites for dislocation mutagenesis (Kunkel, 1985a, 1990; Bebenek & Kunkel, 1990). A modification that destabilizes the secondary structure may also decrease the stability of the template–nascent strand duplex, increasing the probabilities of misalignment and realignment between nucleotide additions. An increased frequency of slippage between the template and the nascent strand secondary to oxidative damage is also supported by the damage-induced G to T transversions at +102. These mutations can be explained by misalignment and realignment in the opposite direction than that leading to the T to G transversions at +103.

The types and positions of the mutations derived from the damaged template are fairly similar to those derived from undamaged template. Both are dominated by changes at T's, 69% and 54% of the single base changes for the undamaged and damaged, respectively, particularly T to G transversions. On undamaged template, the T to G transversions are primarily at the +70 and +103 hot spots. While both of these sites are present at elevated frequency after damage, several new sites, T to C at +60, T to C/G at +87, and T to G at +121, become prominent, and the frequency of T deletions becomes less so. As our studies of oxidatively damaged DNA in *E. coli* have not revealed an elevation of substitutions at T's, these mutations reflect a property of pol-β, not simply the types of lesions produced by ROS.

The damage-induced spectrum also includes a marked increase of C to T and C to A substitutions. These types of mutations, particularly C to T transitions, were observed at high frequency in our studies with single-stranded M13mp2 with damage induced by $FeSO_4$, CuCl, or HL60 cells (McBride et al., 1991; Tkeshelashivili et al., 1992; Reid & Loeb, 1992). Thus, after treatment of DNA with any one of several ROS-generating systems, C residues are highly mutagenic. This suggests that one or more of the oxidized products of C is particularly mutagenic but the chemical lesion(s) responsible for the C to T or C to A changes induced by ROS damage remain(s) to be determined.

In summary, the mutations caused by oxidative damage to DNA occur primarily in hot spots and are not broadly distributed as one might have predicted on the basis of the random cleavage of phosphodiester bonds induced by incubation of single-stranded DNA with Fe^{2+} and H_2O_2 (Mello-Filho & Meneghini, 1984). This suggests either that certain nucleotide sequences are hypersensitive to damage or that in certain sequences oxidative lesions are particularly mutagenic. Many of the mutations observed in this study, however, are associated with pol-β-specific hot spots. DNA damage causes

an elevation in the frequency of base substitution at the sites where the polymerase is already prone to error. The primer extension data reveal that pause sites, which may be due to DNA secondary structures, are associated with mutational hot spots. ROS damage to the template causes an alteration in its conformation which correlates with an increase in mutations at or near polymerase-specific hot spots. Thus, it appears that the observed spectrum results from the combined influences of primary and secondary structures of the DNA, the properties of pol-β, and the ROS damage itself. As other DNA polymerases have markedly different mutational spectra on *lacZ*α (Kunkel, 1985a; Kunkel & Bebenek, 1988), one might predict that base changes secondary to ROS damage would also be dependent upon the properties of those polymerases. This suggests that studies on the spectrum of mutations in mammalian cells, at least those concerned with ROS damage to DNA, must consider the specific DNA polymerase(s) that will encounter the damage in vivo. Conversely, an analysis of the types of mutations found in mammalian cells exposed to ROS should provide clues to the identity of the polymerases involved in mutagenesis by oxygen free radicals.

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