Mutagenic Spectrum Resulting from DNA Damage by Oxygen Radicals†

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ABSTRACT: Oxygen free radicals are highly reactive species that damage DNA and cause mutations. We determined the mutagenic spectrum of oxygen free radicals produced by the aerobic incubation of single-stranded M13mp2 DNA with Fe^{2+}. The Fe^{2+}-treated DNA was transfected into competent *Escherichia coli*, and mutants within the nonessential *lac* Zα gene for β-galactosidase were identified by decreased α-complementation. The frequency of mutants obtained with 10 μM Fe^{2+} was 20- to 80-fold greater than that obtained with untreated DNA. Mutagenesis was greater after the host cells were exposed to UV irradiation to induce the SOS "error-prone" response. The ability of catalase, mannitol, and superoxide dismutase to diminish mutagenesis indicates the involvement of oxygen free radicals. The sequence data on 94 of the mutants establish that mutagenesis results primarily from an increase in single-base substitutions. Ninety-four percent of the mutants with detectable changes in nucleotide sequence were single-base substitutions, the most frequent being G → C transversions, followed by C → T transitions and G → T transversions. The clustering of mutations at distinct gene positions suggests that Fe^{2+}/oxygen damage to DNA is nonrandom. This mutational spectrum provides evidence that a multiplicity of DNA lesions produced by oxygen free radicals in vitro are promutagenic and could be a source of spontaneous mutations.

Oxygen free radicals are generated in cells by a variety of normal metabolic processes. Among these processes are respiration, cell injury, phagocytosis, and drug toxicity (Naqui et al., 1986; Klebanoff, 1988; Fridovich, 1986). By sequential one-electron reductions of oxygen, a series of reactive species are produced that include the following: hydroxyl radicals, hydrogen peroxide, superoxide ions, and singlet oxygen. Of these, the hydroxyl radical is generally considered the most deleterious, and it has been proposed that much of the toxicity of superoxide and hydrogen peroxide in cells is due to hydroxyl radicals or a similarly reactive species generated by a Haber–Weiss type reaction, using iron as a catalyst:

$$H_2O_2 + O_2^{\bullet -} \xrightarrow[Fe^{2+}]{} O_2 + OH^- + OH^\bullet$$

Damage to cellular macromolecules by oxygen free radicals has been hypothesized to be a causal factor in chronic diseases, including cancer, joint diseases, atherosclerosis, and aging (Cerutti, 1985; Klebanoff et al., 1983).

There is a considerable evidence that oxygen free radicals are causative agents in mutagenesis. Substances that produce oxygen free radicals have been shown to be mutagenic (Ames, 1983; Farr et al., 1986; Hsie et al., 1986); exposure of cells to hyperbolic oxygen enhances mutagenesis (Lesko et al., 1985); and mutagenesis by oxygen free radical generators can be prevented by scavengers of oxygen free radicals (Imlay & Linn, 1988). Bacterial mutants defective in the repair of oxygen-induced DNA damage (Kow & Wallace, 1985) or in the production of oxygen scavengers (Farr et al., 1986; Fridovich, 1983) exhibit an elevated mutation frequency after exposure to agents that produce oxygen free radicals. Despite this strong evidence for oxygen radical induced mutagenesis, very little is known about the frequency and types of mutations produced by oxygen free radicals. The data are needed to evaluate two important questions: what is the contribution of oxygen free radicals to spontaneous mutagenesis in normal cells? and which DNA lesions produced oxygen free radicals contribute to mutagenesis?

Progress in the study of DNA damage and mutagenesis by oxygen free radicals has been hampered by both the diversity in the types of oxygen free radicals produced in cells (Cadet & Berger, 1985) and the multiplicity of DNA modifications produced by oxygen free radicals (Hutchinson, 1981). On the basis of alterations resultant from the exposure of nucleosides to oxygen free radicals in vitro (Cadet & Berger, 1985; Hutchinson, 1981), it can be estimated that at least 35 different base modifications are produced in DNA by oxygen free radicals. As a result, it has been difficult to assign a particular type of oxygen free radical or a modification in DNA to a specific type of mutation.

We previously demonstrated that Fe^{2+}-induced oxidative damage to ϕX174 *am*3 DNA results in the production of mutants following replication of the damaged DNA in SOS-induced *Escherichia coli* (Loeb et al., 1988). The ratio of mutations to lethal events at the *am*3 locus is greater than that produced by any other agent so far examined. However, these assays with ϕX174 *am*3 DNA only detect mutations occurring at a single adenine residue at the middle position of the *am*3 locus. To establish the spectrum of mutagenesis by Fe^{2+}-induced oxidative DNA damage, we have now utilized the M13mp2 forward mutation assay developed by Kunkel (1984). This assay scores for all 12 types of single-base substitutions, deletions, additions, frameshifts, and complex rearrangements at multiple sites within a nonessential gene. In these initial experiments, we report that DNA damage by oxygen free radicals causes a wide spectrum of mutations. Our results suggest that many different types of lesions produced in DNA by exposure to oxygen free radicals are mutagenic.

Materials and Methods

E. coli Strains. The *E. coli* strains were provided by Thomas A. Kunkel (National Institute of Environmental

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Health Sciences, Research Triangle Park, NC). NR9099 [Δ(*pro-lac*), *rec*A⁻56, *ara*⁻ *thi*⁻/*F'*(*pro*AB, *Lac*I_q⁻Z⁻ ΔM15] was the host bacterium for the production of single-stranded M13mp2 DNA. MC1061 [*hsd*R, *mcr*B, *ara*D, 139Δ-(*ara*ABC-*leu*), 7679Δ*lac*X74, *galU*, *galK*, *rpsL*, *thi*] was the host for transfection of the treated M13mp2 DNA. CSH50 [Δ(*pro-lac*)/F'*traD*36, *thi*⁻, *ara*⁻, *proAB*, *lac*I^{q}Z⁻ ΔM15] was used as an indicator for the infected *E. coli*.

HeLa cell AP endonuclease (Kane & Linn, 1981) was a generous gift from Dale Mosbaugh (Oregon State University, Corvallis, OR). $FeSO_4$ (reagent grade) was obtained from Baker, and deferoxamine was from CIBA Pharmaceutical. Other reagents were obtained as previously indicated (Loeb et al., 1988).

Treatment of DNA. M13mp2 single-stranded DNA (0.5 mg/mL) was incubated with $FeSO_4$ in 0.025 mL of 10 mM sodium phosphate buffer (pH 7.0) in opened 1.5-mL microcentrifuge plastic tubes for 30 min at 37 °C. Thereafter, 0.01 mL of 50 μM deferoxamine was added and the entire reaction was transfected into 20 volumes of competent SOS-induced *E. coli*.

Preparation of Competent SOS-Induced E. coli. Procedures for the transfection of M13mp2 DNA were those described by Kunkel (1984). Cells grown at a density of (4–8) × 10^8 cells/mL were irradiated at 50 J/m^2 and then treated with $CaCl_2$ to produce competent cells with the following modifications: UV-irradiated or unirradiated *E. coli* MC 1061 were harvested by centrifugation at 4000*g*, 20 min, 0 °C, gently resuspended in half the original culture volume of ice-cold 50 mM $CaCl_2$, and incubated for 20 min on ice. Cells were again pelleted, resuspended in 10% of the original culture volume of cold 50 mM $CaCl_2$, and incubated an additional 20 min on ice. The cells were pelleted a third time, resuspended in cold 50 mM $CaCl_2$ in 10% of their original volume, and then used immediately for transfection by M13mp2 DNA.

Transfection. The treated M13mp2 DNA was incubated with 20 volumes of competent cells, at 0 °C for 30 min, and then the mixture was heat shocked for 2 min at 42 °C. The efficiency of transfection varied by 3- to 5-fold between experiments. An amount of this transfection mixture, yielding between 100 and 300 plaques per plate (typically 5–50 μL), was added to 3 mL of liquid (42 °C) 0.8% Bacto-agar in 0.9% NaCl containing 0.08% X-Gal in dimethylformamide and 200 μL of *E. coli* strain CSH50 (OD_{550} ~ 1.0). This mixture was 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 agar gelled, the plates were inverted and incubated 24 h at 37 °C, and for an additional 24 h at room temperature, before scoring and counting plaques. All light blue and colorless plaques were isolated and replated in the presence of equal titers of wild-type M13mp2 phage (dark blue) to confirm the phenotype color change.

Sequence Analysis. Mutants were plaque purified and sequenced as described previously, using the dideoxy chain termination method (Sanger, et al., 1978). The oligonucleotide used as a sequence primer was a 15-mer that was complementary to the M13mp2 plus strand sequence between nucleotide positions +179 and +194 in the *lac*Z gene.

Results

In the initial studies on mutagenesis in vitro by oxygen free radicals, we incubated aerobically φX174 *am*3 single-stranded DNA with Fe^{2+} and then transfected the treated DNA into *E. coli* spheroplasts (Loeb et al., 1988). Mutagenesis by Fe^{2+} was 10- to 100- fold greater than controls, required the induction of the *E. coli* SOS repair system, and was dependent on the generation of oxygen free radicals. The high frequency of mutations per lethal event in the φX reversion assay suggested that a forward mutation assay could be used to catalogue the types of mutations produced by oxygen free radicals. To do so, we utilized the M13mp2 assay developed by Kunkel (1984). The loss in viability of single-stranded M13mp2 DNA upon aerobic incubation with increasing concentration of Fe^{2+} (Figure 1A) is of similar magnitude to that previously observed using single-stranded φX174 *am*3 DNA. From the results in Figure 1 we calculate that one lethal hit is introduced per M13mp2 single-stranded DNA by incubation with 2.5 μM Fe^{2+} for 30 min at 37 °C. This compares with our previous report of one lethal hit per φX DNA by 2.7 μM Fe^{2+} (Loeb et al., 1988). Analysis of the size distribution of the Fe^{2+}-treated φX DNA by polyacrylamide gel electrophoresis indicates that approximately one break per molecule corresponds to one lethal event (results not shown). Thus a major consequence of Fe^{2+}-induced DNA damage is cleavage of the phosphodiester DNA backbone.

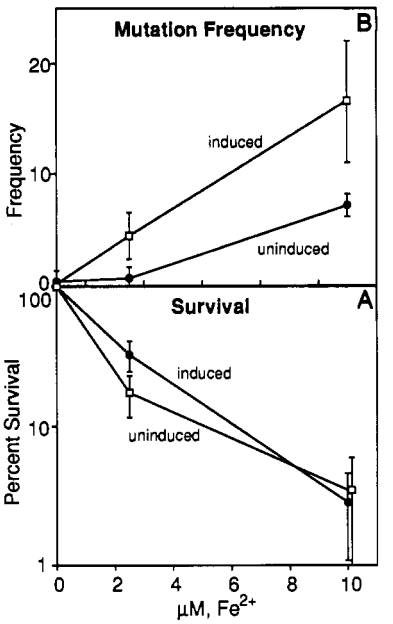

FIGURE 1: Survival of single-stranded M13mp2 DNA and mutagenesis after incubation with Fe^{2+}. M13mp2 was incubated in 0.025 mL of 10 mM sodium phosphate (pH 7.0) with indicated concentration of Fe^{2+} for 30 min at 37 °C in opened tubes as given under Materials and Methods. After addition of 0.01 mL of 50 μM deferoxamine the reaction mixture was immediately transfected into 20 volumes of competent SOS-induced *E. coli*. (A) Plot of the percentage of survivors obtained from triplicate assays after transfection of competent *E. coli*, both SOS and non-SOS. (B) Plot of the frequency of light blue and white plaques to total plaques after transfection in non-SOS- and SOS-induced *E. coli*. The vertical bar is one standard deviation from the average of the different experiments carried out in SOS-induced *E. coli*. In the three SOS-induced experiments, the total number of plaques counted at 0, 2.5, and 10 μM were 15414, 22268, and 4449. In the two SOS⁻ experiments, the total number of plaques counted at 0, 2.5, and 10 μM were 12673, 3303, and 827. The mutation frequency in the absence of Fe^{2+} was 0.4 × 10^{-3} and 0.2 × 10^{-3} in experiments with normal and SOS-induced *E. coli*, respectively.

Single-stranded m13mp2 DNA contains a 474-nucleotide sequence that codes for a portion of the *lac*Z gene and its regulatory sequences. Upon transfection into *E. coli* expressing the remaining portion of the *lac*Z gene, the M13mp2 DNA provides the coding information needed to produce a functional β-galactosidase via intracistronic α-complementation. *E. coli* expressing fully active β-galactosidase produce dark blue plaques on the indicator substrate, X-Gal (Kunkel, 1984).

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

additions	survival (% of control)	mutation frequency ($\times 10^3$)
none	100	0.6
Fe^{2+} (2.5 μM)	31	3.3
+mannitol (0.1 M)	59	1.9
+catalase (5.8 μg/mL)	131	1.2
+SOD (5.0 μg/mL)	40	1.6
+deferoxamine (50 μM)	49	1.8
Fe (10 μM)	1	21.3
+mannitol (0.1 M)	60	4.5
+catalase (5.8 μg/mL)	52	3.1
+SOD (5.0 μg/mL)	28	8.9
+deferoxamine (50 μM)	64	6.2

[a] Single-stranded M13mp2 DNA was incubated in 0.025 mL of 10 mM sodium phosphate (pH 7.0) and the added reactants for 30 min at 37 °C. Transfection assays were carried out in SOS-induced competent *E. coli*. Survival and mutation frequency were determined as given under Materials and Methods. In control samples without Fe^{2+}, the survival and mutation frequencies for mannitol, catalase, superoxide dismutase, and deferoxamine were respectively 89%, 93%, 123%, and 72% and 0.0016, 0.0014, 0.0011, and 0.0028.

Table II: Effect of Apurinic Endonuclease on Fe-Induced Mutagenesis[a]

additions	survival (% of control)	mutation frequency ($\times 10^3$)
none	100	1
+2.5 μM Fe^{2+}	13	17
+2.5 μM Fe^{2+} + AP Endo	12	10
+10 μM Fe^{2+}	1	31
+10 μM Fe^{2+} + AP Endo	1	50
+acid-treated	5	42
+acid-treated + AP Endo	8	2

[a] Single-stranded M13mp2 DNA was incubated with the indicated amounts of Fe^{2+} aerobically in 10 mM sodium phosphate (pH 7.0) for 30 min at 37 °C. Thereafter, deferoxamine was added to a final concentration of 50 μM. The DNA was precipitated with ethanol, dissolved in 50 μL of 25 mM Tris-HCl (pH 7.5), and incubated with 16 units of HeLa apurinic endonuclease (AP Endo) and 5 mM $MgCl_2$ for 2 h at 37°C. Reactions and transfections were carried out as indicated under Materials and Methods. Acid-treated M13mp2 DNA was made by incubation at pH 5.0 for 15 min at 70 °C, yielding approximately two apurinic sites per molecule (Loeb et al., 1988).

Mutations within the *lacZ*α segment of M13mp2 DNA reduce the production of or produce a less active β-galactosidase and yield light blue or colorless plaques. The data in Figure 1B indicate that the frequency of these mutant plaques is increased after incubation of M13mp2 DNA with Fe^{2+}. Since cleaved single-stranded DNA is not likely to yield viable phage after transfection into *E. coli*, these results suggest that an additional consequence of Fe^{2+}-induced damage is the production of other lesions that are responsible for mutagenesis. In normal competent *E. coli*, the mutation frequency increased from 0.4×10^{-3} after incubation in the absence of Fe^{2+} to 0.7×10^{-3} and 7.2×10^{-3} in the presence of 2.5 and 10 μM Fe^{2+}, respectively. A higher frequency of mutations was observed when transfection was into competent bacteria that were previously exposed to UV irradiation to induce the SOS response. The mutation frequency of Fe^{2+}-treated DNA (10 μM Fe^{2+}) in SOS-induced cells was a great as 80-fold greater than that of untreated DNA.

The ratio of mutagenic to lethal events can be gleaned by comparing the data in panels A and B of Figure 1. The mutation frequency per lethal event in SOS-induced cells is approximately 2×10^{-3}. The target size that can produce lethal damage is the entire M13mp2 genome since one phosphodiester cleavage of single-stranded circular DNA destroys its biological activity. The target size for mutagenesis is primarily the *lacZ*α fragment and its controlling regions; this target constitutes 6% of the M13mp2 DNA. If one assumes that breaks and mutagenesis are evenly distributed throughout the M13mp2 DNA, the most frequent lesions produced by Fe^{2+}-treatment are phosphodiester breaks. However, mutagenic events also occur at high frequency: approximately 0.03 mutagenic events occur per lethal event, at each nucleotide site damage by Fe^{2+}-produced oxygen free radicals.

The effects of oxygen free radical scavangers and metal chelators on Fe^{2+}-induced lethality and mutagenesis suggest that both processes proceed via the production of oxygen free radicals (Table I). The inhibition of both processes by mannitol and catalase indicates the involvement of hydroxyl radicals and hydrogen peroxide, respectively, and is similar to the results reported with ϕX174 *am*3 DNA. In our addition with M13mp2 DNA, inhibition of mutagenesis is also observed with superoxide dismutase. The inhibition with deferoxamine suggests this chelator forms an inactive complex with Fe^{2+} under the conditions used in this experiment (Klebanoff et al., 1989). The overall results of studies with inhibitors and oxygen free radical scavengers indicate that the major mutagenic effects of Fe^{2+} treatment of DNA can be ascribed to the generation of superoxide, hydrogen peroxide, and hydroxyl radicals via the Haber–Weiss reaction and support the tentative assignment of hydroxyl radicals as both the DNA-cleaving (Floyd et al., 1986) and the major mutagenic species. The overall similar requirements for mutagenesis by Fe^{2+} in both the ϕX reversion assay (Loeb et al., 1988) and the M13 forward mutation assay suggest that the same species of oxygen free radicals that produce mutations at the *am*3 locus also produce mutations at multiple sites in M13mp2 DNA. However, the differences in degree of inhibition by different agents also suggest the possible involvement of different types of oxygen free radicals in the M13 assay.

There is evidence suggesting that abasic sites or related lesions are produced in DNA by free radicals (Teebor et al., 1988). Abasic sites have been shown to be mutagenic in a variety of systems (Schaaper & Loeb, 1981) including the M13mp2 assay (Kunkel, 1984). Incubation of M13mp2 DNA with 2.5 and 10 μM Fe^{2+} reduces survival to 13 and 1%, respectively (Table II), and subsequent incubation with the HeLa apurinic endonuclease does not further reduce survival. While mutagenesis at 2.5 μM Fe^{2+} was partially reduced by the apurinic endonuclease, no reduction was observed at 10 μM Fe^{2+} in these experiments. In control reactions that form abasic sites, exposure of M13mp2 DNA to acid results in a 20-fold increase in mutagenesis, and most of this enhancement can be abolished by digestion of the acid-treated DNA with the apurinic endonuclease. Thus, the fact that mutagenesis of the Fe^{2+}-treated M13mp2 DNA is not abolished by an apurinic endonuclease that cleaves and inactivates single-stranded DNA containing abasic sites indicates that unmodified abasic sites are not the major species responsible for the mutagenic effects of Fe^{2+}.

We have collected a total of 240 Fe^{2+}-induced mutants and sequenced the region from the first nucleotide after the *lacI* termination codon through nucleotide +178 of the *lacZ* gene. The experiments that generated these mutants were carried out in SOS-induced *E. coli*, and the mutation frequencies observed were at least 20-fold greater than that obtained with the same DNA incubated in the absence of Fe^{2+}. Thus, 95% of all mutants were due to the presence of Fe^{2+} during incubation. The 3′-terminus of the sequencing primer was located

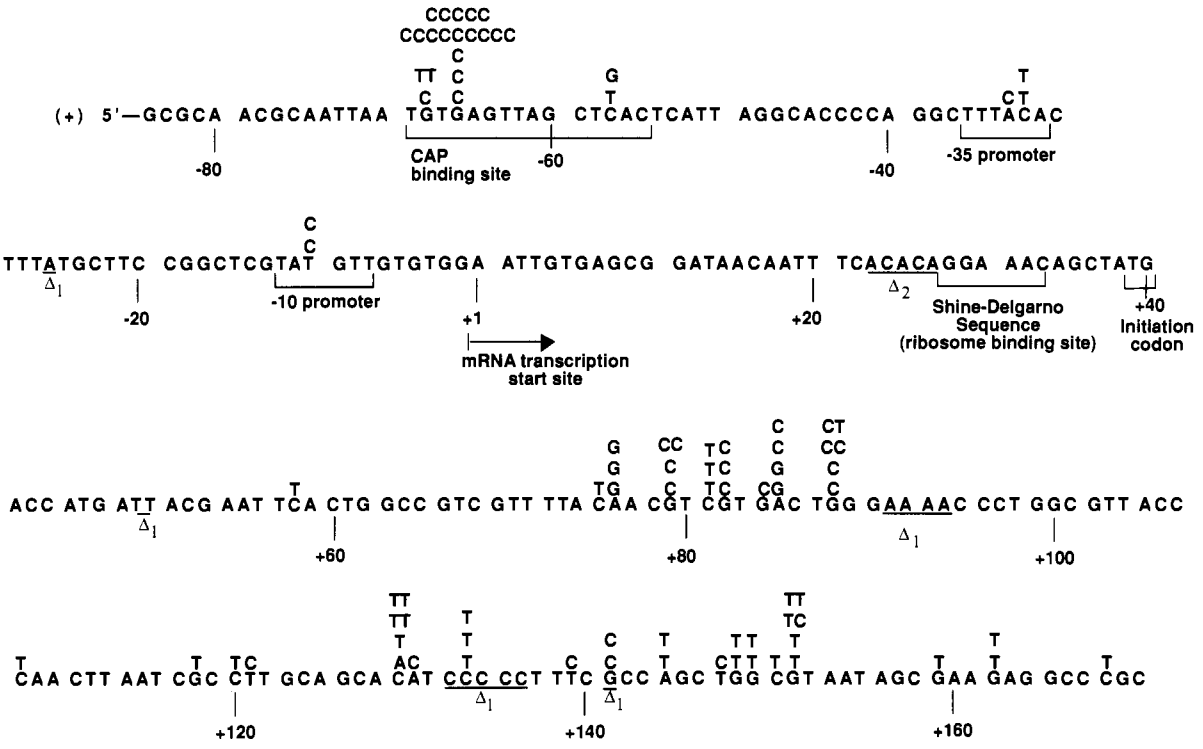

FIGURE 2: Spectrum of mutations induced by Fe^{2+}. The DNA sequence of the viral strand of M13mp2 starting at the first nucleotide after the *lac*I termination codon through the coding sequence of amino acid 47 of the *lac*Z gene. Single-base substitutions are displayed above the wild-type sequence and indicate the nucleotide found in the viral strand. Deletions are shown below the viral sequence and are indicated by the Δ_n, where n equals the number of deleted nucleotides; if present in a repetitive sequence, a line gives the possible origin.

opposite nucleotide position +179 of the *lac*Z insert, and the DNA stretch that was sequenced contains 250 nucleotides and corresponds to 32% of the β-galactosidase α-complementation segment present within M13mp2 DNA. A preliminary grouping of the types of mutations can be made on the basis of color (Kunkel, 1984). Most of the mutants are light blue and are thus likely to contain single-base substitutions. Only 10% are white, and these are most likely to be the result of deletions or other mutations that fail to complement the endogenous β-galactosidase fragment made in the *E. coli* host.

Mutations presumably occur throughout the β-galactosidase fragment within M13mp2 DNA. Thirty-nine percent of the mutations analyzed exhibited identifiable alterations in their nucleotide sequence, and this percentage is in accord with the fact that only 32% of the β-galactosidase insert was sequenced. The distribution of mutants within the 250 base sequence analyzed is presented in Figure 2, and the classes of mutants are summarized in Table III. Single-base substitutions are by far the most frequent nucleotide sequence alterations. Of the 94 sequence changes detected, 88 were single-base substitutions. Only six deletions were observed, and no additions or complex rearrangements were detected. This distribution is in agreement with the predominance of light blue colonies observed in the initial screening that included putative mutations in the region of the β-galactosidase α-fragment that was not sequenced, as well as mutations in other parts of the M13mp2 DNA that might alter the appearance of the phage plaques and thus mimic β-galactosidase mutants.

Even though single-base substitutions were observed throughout the segment sequenced, their distribution was clustered. The largest "hot spot" contains a C in place of a G at nucleotide −66. This substitution is presumably mediated by a lesion at G-66 in the single-stranded M13mp2 DNA and the modified G paired with an incoming dGTP during replication. Three smaller clusters, each consisting of six mutants, were observed at positions 88, 129, and 151, and two of these were also at a G, the other being at a template C.

Table III: Summary of Fe^{2+}-Induced Base Substitutions[a]

	single-base substitutions	number sequenced
A	→ C	4
	→ G	5
	→ T	2
		total 10
C	→ A	1
	→ G	1
	→ T	20
		total 22
G	→ A	0
	→ C	34
	→ T	16
		total 50
T	→ A	0
	→ C	5
	→ G	0
		total 5

[a] Compilation of the data in Figure 2.

A tabulation of types of single-base substitutions for each of the target nucleotides is in Table III. The most frequent substitutions were opposite template Gs (50/88) and Cs (21/88). The most frequent type of substitution was a cytosine in place of a guanosine, presumably via a G·G mispairing. If one eliminates from consideration the most prominent G → C cluster at position −66, the frequency of G → C transversions is similar to that of G → T transversions and C → T transitions. The C → T transition is presumably mediated by a modification of cytosine that favors a C·A pairing. In total, Fe^{2+}-induced mutagenesis in this system produces predominantly single-base substitutions; among these, transversions

(58/88) are more frequent than transitions (30/88).

Discussion

Mutagenesis by oxygen free radicals has been demonstrated in both procaryotic (Farr et al., 1986) and eucaryotic cells (Hsie et al., 1986). The mutations produced are usually considered to be the direct result of modifications of DNA by oxygen free radicals. The hypothesis directly linking oxygen damage to mutations is in accord with the plethora of oxygen free radical induced modifications of isolated nucleotides (Cadet & Berger, 1985) and DNA (Hutchinson, 1981). The most extensive documentation of the types of modifications produced by oxygen free radical damage to DNA has been provided by gas chromatography–mass spectrometry analysis of DNA after incubation with the hypoxanthine/xanthine oxidase system (Aruoma et al., 1989b) or with ferric ion chelates plus hydrogen peroxide (Aruoma et al., 1989a). Alterations produced include 8-hydroxyguanosine (8OH-G), thymine glycol, dihydroxycytidine and 2,6-diamino-4-hydroxy-5-formamidopyrimidine (FapyGua). On the other hand, a variety of mutational changes have been documented in cells after exposure to chemicals that produce oxygen free radicals. Direct DNA damage need not be the mechanism for mutagenesis by oxygen free radicals. Oxygen free radicals also damage other cellular macromolecules, and these presumably include enzymes involved in DNA repair and DNA replication. The resulting enzyme alterations could cause mutations by misincorporation during DNA synthesis (Fry & Loeb, 1986; Doshi & Preston, 1990). Our results support a direct mechanism in which oxygen free radical damage to DNA produces promutagenic lesions. Since we exposed purified single-stranded DNA to oxygen free radicals in vitro and measured subsequent alterations in biologic activity, we can directly relate modifications of specific nucleotides in DNA to mutagenesis. Most other studies on oxygen free radical mutagenesis have utilized double-stranded DNA (Decuyper-Debergh et al., 1987), and as a result one cannot unequivocally identify which of the nucleotides in each base pair is responsible for mutagenesis. Studies on single-stranded DNA could be the more relevant, since this is the structure of the DNA template within the active site of DNA polymerase (Derbyshire et al., 1988).

In our initial studies on oxygen mutagenesis in vitro, we incubated ϕX174 *am*3 DNA with Fe^{2+} in the presence of air and measured the frequency of revertant phage produced after transfection into SOS-induced *E. coli* spheroplasts (Loeb et al., 1988). Mutagenesis was SOS dependent, required oxygen, and could be abolished by the addition of catalase or mannitol. These combined results indicate that mutagenesis by Fe^{2+} proceeds via the generation of oxygen free radicals. Even though the ϕX174 *am*3 reversion assay is exceptionally sensitive, it only scores for single-base substitutions at one site on ϕX DNA, opposite the adenine at position 587. The spectrum of Fe^{2+}-induced mutations detected in the ϕX assay (predominantly A → T transversions) was restricted to a single nucleotide position and did not address the question of the different types of mutations that could be produced. In contrast, the M13mp2 forward mutation assay developed by Kunkel (1984) measures a wide variety of mutations. Moreover, on the same DNA, the mutations produced by oxygen free radicals can be compared with the literature on mutagenic spectra produced by DNA polymerase errors (Kunkel, 1985; Kunkel & Alexander, 1986) and damage by other agents (Kunkel, 1984; LeClerc et al., 1986).

The major mutagenic changes induced by exposure of M13mp2 DNA to Fe^{2+} are single-base substitutions. The most frequent single-base substitution is a C in place of a template G, presumably arising from a G·G mispairing during DNA replication (Table III). The data suggest that the template G is modified by oxygen free radicals so that it pairs at high frequency during DNA replication with dGTP instead of dCTP. The most extensively investigated modification of G by oxygen free radicals is of 8-hydroxyguanosine (Kasai & Nishimura, 1984; Floyd et al., 1986), and this could be the promutagenic lesion responsible for the G → C transversions. Sixteen of nineteen mutants induce in double-stranded M13 DNA were found by Hoebee et al. (1988) to contain C/G to G/C transversions, and it was proposed that these arose from mispairing between 8-hydroxyguanosine and guanosine. However, data from in vitro DNA polymerization studies do not support this mispairing scheme. The replication of an oligonucleotide containing a single residue of 8-hydroxyguanine by the large fragment of *E. coli* DNA polymerase I in vitro has been reported to result in the incorporation of noncomplementary A, T, and Gs with almost equal frequencies (Kuchino et al., 1987) or in the preferential incorporation of Ts (Shibutani et al., 1990), which would produce a G → A transition. Our studies carried out in *E. coli* under the SOS response and the types of mutations observed under these conditions may be different than those measured with purified DNA polymerases in vitro. Alternatively, the major mutagenic lesion may not be 8-hydroxyguanosine.

The second most frequent mutation in this spectrum is C → T transitions. In studies on γ-ray irradiation of double-stranded λ phage, the most frequent mutations observed were G·C → A·T transitions (Tindall et al., 1988); it was hypothesized that these arose during DNA replication by the deamination of cytosine to yield a template deoxyuridine that base-paired with A. In other studies, Ayaki et al. (1986) sequenced 15 mutants of single-stranded M13mp10 produced by exposure to ^{60}Co γ-rays; 10 were opposite template cytosine residues. Of these, 7 were C → T transitions. Mutagenesis by γ-rays is likely to be mediated by oxygen free radicals, and thus the spectrum might be similar to that obtained by exposure of DNA to Fe^{2+}. The chemical structure of the promutagenic cytidine modification remains to be established. The simple explanation that damage by irradiation or oxygen free radicals (Hutchinson, 1981) produces either uracil by deamination of cytosine or an abasic site by depyrimidation is unlikely, since the Fe^{2+}-treated DNA was transfected into *E. coli* that expressed both uracil glycosylase and apurinic endonucleases, unless the repair capacity of the *E. coli* was exceeded. Alternatively, Boorstein et al. (1989) demonstrated that UV irradiation of DNA yields a stable cytosine hydrate intermediate (6-hydroxy-5,6-dihydrocytosine), which could be the miscoding lesion responsible for the C → T transitions.

The third most frequent type of substitution, G → T transversions, could be mediated via depurination (Schaaper et al., 1983) or other noncoding lesions (Sagher & Strauss, 1983). Among the lesions produced in DNA by other sources of free radicals are apurinic sites (Teebor et al., 1988). Depurination occurs most frequently at G residues (Lindahl & Nyberg, 1972; Greer, 1962), and A substitutions are the most frequent mispairings opposite abasic sites in DNA (Schaaper et al., 1983). Since mutagenesis via abasic sites and perhaps other noncoding lesions is SOS dependent (Schaaper & Loeb, 1981), it will be interesting to determine if G → T transversions induced by Fe^{2+} are selectively decreased in the absence of the SOS response.

Mutations by Fe^{2+}-generated oxygen free radicals are not randomly distributed. The most prominent cluster, consisting

of G → C transversions, was found at position −66. This "hot spot" appears to be specific for Fe^{2+}-induced mutagenesis: it is not a "hot spot" in mutant spectra generated by copying errors of DNA polymerases or among those produced by acid treatment (Kunkel, 1984) or UV irradiation (LeClerc et al., 1986) of M13mp2 DNA. Furthermore, it is not present among the spontaneous mutants of M13mp2 sequenced by Kunkel (personal communication) or among a limited number sequenced by us using the same sample of M13mp2 DNA (results not given). The clustering of G → C transversions could be due to the preferential binding of Fe^{2+} to guanine with the localized generation of hydroxyl radicals (Imlay & Linn, 1986). This would be analogous to the enhancement of hydroxyl radical generation by the binding of Fe^{3+} to certain chelators and nucleotides (Inoue & Kawanishi, 1987). None of the "hot spots" for mutagenesis that we observed with Fe^{2+} correlate with the positions of cleavage by piperidine (unpublished results), suggesting that alkaline-sensitive lesions are not the major species for mutagenesis at these sites. We cannot rigorously eliminate the possibility that the G·G mispairings are due to the residency of Fe^{2+} on G in DNA with a change in base pairing properties of the Fe^{2+} guanosine complex, but this seems unlikely since the DNA was treated with deferoxamine, a potent Fe^{2+} chelator, prior to the transfection procedure and mutagenesis was reduced by oxygen free radical scavangers. An analysis of mutagenic spectrum by other agents that generate oxygen free radicals should allow one to determine if the G·G mispairings are unique to Fe^{2+} or are characteristic of DNA damage by a specific species of oxygen free radicals. So far, there is only limited data on ionizing radiation in mammalian cells (Grosovsky et al., 1988; Miles & Meuth, 1989).

The mutagenic potential of Fe^{2+}-generated oxygen free radicals is substantial. In studies with ϕX *am*3 174 DNA, 0.002 mutations were observed per lethal event after incubation with Fe^{2+} (Loeb et al., 1988). With M13mp2 DNA, this ratio is greater, 0.03, since multiple positions within the 250-base *lacZ* target can yield mutations. With double-stranded DNA in cells, the majority of phosphodiester breaks are ligated and might not result in lethal events. Thus, mutagenesis via damage to DNA by oxygen free radicals is likely to be more frequent in vivo.

The mechanism of oxygen free radicals in spontaneous mutagenesis remains to be established. The finding that incubation of Fe^{2+} with DNA produces a variety of mutations brings into focus the possibility that intracellular iron is mutagenic and is a contributor to pathological processes. Even though iron is an essential nutrient, used as a constituent of many enzymes, electron transport complexes, and oxygen carriers, excess iron can be toxic (Stevens et al., 1986). In eucaryotes, the great majority of iron is present in heme proteins, and these complexes have been shown to generate oxygen free radicals and to cleave DNA (Sadrzadeh et al., 1984). Iron is also found in strong association with cellular DNA (Andronikashvili et al., 1974) and could serve to generate hydroxyl radicals, particularly in the presence of hydrogen peroxide (Mello-Filho et al., 1984). Furthermore, on the basis of epidemiological studies it has been proposed that there is a causal relationship between iron stores and the risk of developing certain human cancers in men (Stevens et al., 1988).

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Ionic Strength Dependence of the Kinetics of Electron Transfer from Bovine Mitochondrial Cytochrome *c* to Bovine Cytochrome *c* Oxidase†

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ABSTRACT: The effect of ionic strength on the one-electron reduction of oxidized bovine cytochrome *c* oxidase by reduced bovine cytochrome *c* has been studied by using flavin semiquinone reductants generated in situ by laser flash photolysis. In the absence of cytochrome *c*, direct reduction of the heme *a* prosthetic group of the oxidase by the one-electron reductant 5-deazariboflavin semiquinone occurred slowly, despite a driving force of approximately +1 V. This is consistent with a sterically inaccessible heme *a* center. This reduction process was independent of ionic strength from 10 to 100 mM. Addition of cytochrome *c* resulted in a marked increase in the amount of reduced oxidase generated per laser flash. Reduction of the oxidase at the heme *a* site was monophasic, whereas oxidation of cytochrome *c* was multiphasic, the fastest phase corresponding in rate constant to the reduction of the heme *a*. During the fast kinetic phase, 2 equiv of cytochrome *c* was oxidized per heme *a* reduced. We presume that the second equivalent was used to reduce the Cu_a center, although this was not directly measured. The first-order rate-limiting process which controls electron transfer to the heme *a* showed a marked ionic strength effect, with a maximum rate constant occurring at μ = 110 mM (1470 s^{-1}), whereas the rate constant obtained at μ = 10 mM was 630 s^{-1} and at μ = 510 mM was 45 s^{-1}. There was no effect of "pulsing" the enzyme on this rate-limiting one-electron transfer process. These results suggest that there are structural differences in the complex(es) formed between mitochondrial cytochrome *c* and cytochrome *c* oxidase at very low and more physiologically relevant ionic strengths, which lead to differences in electron-transfer rate constants.

As a continuation of ongoing studies of the kinetics of electron-transfer processes involving biological redox proteins, we have investigated the effect of ionic strength on the reaction between bovine ferrous cytochrome *c* (cyt c^{2+})[1] and fully oxidized bovine cytochrome *c* oxidase (CcO). A large body of information has accumulated on the kinetics of CcO reduction by both cyt *c* and nonphysiological reductants. Included in the latter category are ferrocyanide and ferrous sulfate (Krab

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[1] Abbreviations: cyt c^{2+} and cyt c^{3+}, ferrous and ferric cytochrome *c*, respectively; CcO, cytochrome *c* oxidase; 5-DRF and 5-DRFH•, oxidized and semiquinone forms of 5-deazariboflavin, respectively; EDTA, ethylenediaminetetraacetic acid; Tris, tris(hydroxymethyl)aminomethane.

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