The spectrum of mutations generated by passage of a hydrogen peroxide damaged shuttle vector plasmid through a mammalian host

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ABSTRACT

Treatment of a plasmid shuttle vector (pZ189) with a combination of hydrogen peroxide and a ferric iron/EDTA complex prior to transfection and passage in simian (CV-1) cells increases the frequency of mutations at the *supF* locus by up to 60-fold over the spontaneous background. This increase in mutation frequency is abolished when the inhibitors desferrioxamine, superoxide dismutase, catalase or dimethyl sulfoxide are included in the initial reaction or when the iron/EDTA complex is omitted, a strong indication that the premutagenic damage arises as a result of direct attack by hydroxyl radical generated in a superoxide driven Fenton reaction. DNA sequence analysis of the mutated plasmids shows that 1) Deletions occurring in combination with base-substitutions arise in 22.5 percent of the induced mutants compared with only 3 percent of spontaneous mutants 2) Sixty percent of all induced deletion mutations involve the loss of a single base and 77 percent of these (20 out of 26) occur at two adenine-containing sites 3) The base-change spectrum of mutants arising in the treated plasmid population is marked by the predominance of mutants containing a single base-change and by an increase in changes at AT base pairs. These results provide direct information concerning the nature of mutations arising in mammalian cells as a result of hydroxyl radical mediated DNA damage.

INTRODUCTION

In addition to being a normal metabolite generated in low quantities by aerobic cells, hydrogen peroxide is generated intracellularly during the biotransformation of xenobiotics (1) and as a result of exposure to external physical agents including UVA (320-380 nm) radiation (2). The ultimate damaging effect of hydrogen peroxide in cells is probably the result of the generation of hydroxyl radicals in a superoxide and iron-dependent Haber-Weiss reaction (3; 4). In particular, damage to DNA may be caused by the localised generation of hydroxyl radicals (5). The ultimate mutagenic consequences of such a reaction are therefore of considerable interest.

Hydrogen peroxide has been shown to lead to phenotypic mutation in both bacterial (6; 7) and eucaryotic cells (8) and will mutate a shuttle vector plasmid present in treated cells (Moraes *et al.*, in preparation). A reaction similar to that believed to occur *in vivo* can be generated *in vitro* using hydrogen-peroxide in combination with a ferric-EDTA complex in order to specifically generate hydroxyl radicals (3). Such a system leads to extensive DNA breakage (9). Oxygen free radicals (presumably hydroxyl) generated by Fe²⁺ in aqueous solution in the absence of added H₂O₂ are mutagenic to single-stranded bacteriophage (10). In the present study, we have treated shuttle vector plasmid (pZ189) DNA with H₂O₂ in the presence of a ferric-EDTA complex and

examined the sequence changes that arise as a result of passage of the plasmid through a simian host cell.

MATERIALS AND METHODS

Cells and plasmids

The shuttle vector plasmid pZ189 contains the active bacterial *supF* suppressor tRNA gene (11) and was prepared using standard techniques. The indicator host bacterial strain used to distinguish between wild type and mutant plasmids was *E. coli* MBM 7070 which has the genotype [F⁻ *lacZ*(Am) CA7020 *lacY1 hsdR hsdM Δ(araABC-leu) 7679 galU galK rpsL thi*].

African green monkey kidney cells (CV-1) were used as the mammalian host and were grown as monolayers in Earle's MEM supplemented with 15 percent foetal calf serum in a 5 percent CO₂ atmosphere at 37°C.

Plasmid treatment

Isolated plasmid was treated with H₂O₂ in a freshly prepared solution containing 100 mM Tris pH 8.0, 10 mM EDTA, 10 mM MgCl₂ and 100 μM FeCl₃. H₂O₂ was added to a concentration of 44 mM at the start of the treatment and incubation continued for appropriate times at 37°C.

The reaction was quenched by the addition of 50 μg/ml bovine catalase and incubation continued for a further 10 min. The reaction products were then ethanol precipitated overnight and resuspended in TBS buffer (25 mM Tris HCl pH 7.5, 5 mM KCl, 0.9 mM Na₂HPO₄, 130 mM NaCl, 0.5 mM MgCl₂, 0.7 mM CaCl₂). Inhibitors were added directly to the reaction mix at the time of H₂O₂ addition to give final concentrations of 100 μM desferrioxamine (Ciba-Geigy, Basel), 50 μg/ml superoxide dismutase (SOD) and 4 percent dimethylsulfoxide (DMSO, Fluka, Buchs). For the experiments in which plasmid was treated intracellularly with hydrogen peroxide (*in vivo*, Fig. 4), cells were incubated for 6 h following transfection (see below) and then treated with hydrogen peroxide at a concentration of 7 or 10 mM in phosphate buffered saline. Incubation and extraction were then continued as described below. A complete description of the mutations derived by *in vivo* H₂O₂ treatment of the plasmid will be described elsewhere (Moraes *et al.*, in preparation).

Transfection and DNA extraction

CV-1 cells were seeded in 9 cm Petri dishes 3-4 days prior to transfection and allowed to reach 50 percent confluence. Treated or untreated plasmid DNA (50 ng) was introduced into the cells by the DEAE-dextran method (12) Cells were then incubated for an additional 48 h to allow processing and replication of the plasmid and then vector DNA was extracted from the cells using the method of Hirt (13) as modified by McMaster *et al.* (14).

Bacterial transformation and mutant selection

Competent cells were prepared by the simple procedure for frozen cells described by Hanahan (15) and transformed with appropriate quantities of treated or control plasmid. Bacteria

1) Frequency of mutants arising in the *supF* gene of the pZ189 plasmid as a result of treatment with hydrogen peroxide and passage through CV-1 cells. A ferric/EDTA complex is either included (x) or absent (o) from the reaction mix. The data is derived from a single experiment (minimum 6 mutants per data point) except for the 20 and 30 minute time points which are the average of 4 independent experiments shown with associated standard errors.

containing mutant plasmid were then selected on Luria Broth plates containing ampicillin (50 µg/ml), IPTG (12 mg, isopropyl-β-D-thiogalactoside) and X-gal (2mg, 5-bromo-4-chloro-3-indolyl-β-D-galactoside) and purified for storage in stab cultures as previously described (16). Using a similar procedure, Hauser *et al.* (17) established that all mutations arise in the mammalian cells and not in the bacterial host. In addition, plasmid treated with 44 mM H₂O₂ in the presence of the ferric iron/EDTA complex for up to 30 min and then transformed directly into the bacterial host was not detectably mutated.

Molecular analysis of mutant plasmid

Plasmid DNA was prepared for further analysis by a modification of the alkaline lysis procedure (18). The DNA was first analysed by agarose gel electrophoresis to distinguish those plasmids with altered mobility and which therefore carried large deletions or insertions. Only those DNA's which comigrated with control plasmid were sequenced. The entire tRNA region of the pZ189 vector was then sequenced using a modification of the Sanger dideoxyribonucleotide procedure which allows sequence determination directly from double-stranded plasmid DNA (19). Mutants obtained from a single transfection and which showed an identical mutation pattern were excluded from further analysis to ensure that all mutants were of independent origin. Mutants containing more than one mutation were included provided that at least one of the changes was unique to that transfection.

TABLE 1
Frequency of mutant plasmid after various conditions of H₂O₂ treatment
in the presence and absence of inhibitors

Treatment Conditions	Number of mutants	Number of colonies	Mutant frequency	Number of mutants	Number of colonies	Mutant frequency
	10 min treatment			15 min treatment		
H ₂ O ₂ /Fe ³⁺ -EDTA alone	13	11076	1.17 x 10 ⁻³	15	5681	2.64 x 10 ⁻³
H ₂ O ₂ /Fe ³⁺ -EDTA + desferrioxamine	8	99448	8.04 x 10 ⁻⁵	14	53468	2.62 x 10 ⁻⁴
H ₂ O ₂ /Fe ³⁺ -EDTA + DMSO	3	11490	2.61 x 10 ⁻⁴	15	47425	3.16 x 10 ⁻⁴
H ₂ O ₂ /Fe ³⁺ -EDTA + SOD	10	33244	3.01 x 10 ⁻⁴	3	9593	3.13 x 10 ⁻⁴
H ₂ O ₂ /Fe ³⁺ -EDTA + catalase	not done			7	40342	1.74 x 10 ⁻⁴

TABLE 2
General classification of mutations arising spontaneously
or after hydrogen peroxide treatment of the plasmid

	HYDROGEN PEROXIDE	SPONTANEOUS
ALTERED GEL MOBILITY	⁺ 13(15.5)	37(20)
ONLY DELETION	21(24.5)	49(26)
ONLY BASE CHANGE	31(36.5)	94(50)
ONLY INSERTION	-	02(1)
BASE CHANGE + DELETION	19(22.5)	05(3)
INSERTION + DELETION	1(1)	-
TOTAL	85	187
BASE CHANGE	50(59)	99(53)
DELETION (except altered gel mobility)	41(48)	54(29)

⁺ The values in parenthesis are the percentage of total mutants containing mutations in this class.

RESULTS

Induction of mutants by treatment of plasmid DNA with hydrogen peroxide.

Purified vector DNA was treated with H_2O_2 in the presence of a ferric iron/EDTA complex and then transfected into CV-1 cells which were incubated for 2 days to allow plasmid replication. The DNA extracted from these cells was then transformed into *E. coli* MBM 7070 and the bacteria were plated under selective conditions to allow identification of bacteria containing plasmid mutated at the *supF* locus. The frequency of mutated plasmid increases as a function of treatment time with H_2O_2 to reach a value of 60 times over spontaneous after 60 min (Fig. 1). To obtain additional mutants for analysis from independent transfections, plasmid was treated with the H_2O_2 /Ferric/EDTA mixture for either 20 or 30 min prior to transfection. All induced mutants were pooled for subsequent analysis. Also shown on Fig. 1 is the absolute requirement for the ferric complex for the generation of pre-mutagenic lesions during treatment of the plasmid with H_2O_2 . However incubation of the plasmid with the complex for 20 min in the absence of H_2O_2 does enhance the mutation frequency from a value of 2.00×10^{-4} (34 mutants scored from 181227 colonies) to 7.73×10^{-4} (15 mutants scored from 19417 colonies).

Effect of inhibitors on induction of mutants by the $\text{H}_2\text{O}_2/\text{Fe}^{3+}$ -EDTA complex

In order to confirm that the pre-mutagenic damage arises in the plasmid as a result of the action of hydroxyl radical generated by a superoxide driven Fenton reaction, we examined the effect of adding inhibitors to the $\text{H}_2\text{O}_2/\text{Fe}^{3+}$ -EDTA reaction mix. The results in Table 1 demonstrate that all four inhibitors tested (desferrioxamine, superoxide dismutase, catalase and dimethylsulfoxide) reduce the mutant frequency to spontaneous levels.

General classification of mutations induced by H_2O_2 at the *supF* locus.

DNA from all mutant plasmids was first screened by agarose gel electrophoresis to determine those with altered gel mobility i.e. containing large deletions or insertions. Only plasmids showing unaltered mobility were subjected to sequence analysis. Mutants were analysed from a total of 34 independent transfections which included 11 transfections using plasmid treated with H_2O_2 and 23 using untreated plasmid. To ensure analysis of independent mutants, plasmids isolated from the same transfection but which showed an identical pattern of sequence changes were excluded from the analysis. A general classification of the mutants arising either spontaneously or as a result of treatment of the plasmid with H_2O_2 is shown in Table 2. The most marked difference between the two populations is that a high percentage (22.5) of mutant plasmids in the induced population contain both deletions and base-changes, a pattern that occurs in only 3 percent of the spontaneous mutants.

Base-change mutations induced by hydrogen peroxide.

The percentage of mutants containing base changes is quite similar for the induced and spontaneous populations (Table 2). However, further analysis shows that 94 percent of the induced mutants contain only one or two base-changes whereas in the spontaneous population

+ Values in parenthesis are the percentage of total base-change mutants which occur in each category

1 2 3 4 5 6 7 8 9 10 11 12 13 14 15 16 17 18 19 20 21 22 23 24 25 26 27 28 29 30 31 32 33 34 35 36 37 38 39 40 41 42 43 44 45 46 47 48 49 50 51 52 53 54 55 56 57 58 59 60 61 62 63 64 65 66 67 68 69 70 71 72 73 74 75 76 77 78 79 80 81 82 83 84 85 86 87 88 89 90 91 92 93 94 95 96 97 98 99 100

5. *Conclusions*

SINGLE BASE CHANGES

HYDROGEN PEROXIDE (in vitro)

SPONTANEOUS

3) The spectra of base changes that are detected at the *supF* locus of hydrogen peroxide treated plasmid when only mutants containing a single base-change are considered.

only 58 percent fall in this category (Table 3). Base-change spectra have been derived either from all base-changes observed (Fig. 2) or only from mutants showing a single base change (Fig. 3). For the total base-change spectra (Fig. 2) only sites that contain 5 or more changes in the *supF* tRNA coding region of the induced spectrum or 7 or more changes in the spontaneous spectrum can be considered as significant hot spots (binomial probability distribution, $P < 0.05$). None of the three "hot-spot" locations in the induced spectrum (Fig. 2; bps 118, 159 and 169) contain significantly more changes than the corresponding sites in the spontaneous spectrum. The majority (94.5 percent) of the base-changes that arise in the spontaneous mutants appear at the right hand base pair of either a 5' T C 3' : 3' A G 5' or a 5' C C 3' : 3' G G 5' sequence (Fig. 4). This remarkable sequence specificity has been observed previously for spontaneous and UVC (254 nm) or UVB (313 nm) induced mutants (16; 17). Although this specificity also occurs in mutants generated by H₂O₂ treatment of transfected plasmid, it is clearly not the case for mutants arising from plasmid treated extracellularly with H₂O₂ since more than one third of the base changes occur at other sites (Fig. 4). This loss in specificity for changes at G-C base-pairs is also reflected in the overall analysis of base-changes in individual mutants (Table 4) which reveals that 15 percent of the induced base-changes occur at AT base pairs whereas less than 2 percent of the spontaneous base changes fall in this category.

Deletion mutations induced by hydrogen peroxide.

The most remarkable feature of the spectrum of mutations arising from the treated plasmid is that single base deletions account for 60 percent of all deletions of less than 100 bp whereas only 12.5

4) Histogram showing the relative frequency of base-changes arising at 1/ the right hand base of the 5' TC 3' : 3' AG 5' sequence 2/ the right hand base-pair of the 5' CC 3' : 3' GG 5' sequence or 3/ other sites. Data are shown for spontaneous mutants, for mutants arising as a result of hydrogen peroxide treatment of isolated plasmid (*in vitro*) and for mutants arising as a result of plasmid treated with hydrogen peroxide 6h after transfection of the plasmid into the host cell (*in vivo*)

percent of the spontaneous deletions (<100 bp) fall in this class (Fig. 5). A detailed description of the induced deletion mutants is shown in Fig. 6 from which it is evident that 20 out of the 26 single base-pair deletions occur at either bp 130 (4 mutations) or at the adenine doublet at bps 165/166 (16 mutations).

TABLE 4
Types of base-changes in spontaneous and hydrogen peroxide induced mutant.

NUMBER (%) OF MUTANTS OBSERVED		
BASE CHANGE	HYDROGEN PEROXIDE	SPONTANEOUS
GC to AT	35(43.5)	123(51)
GC to TA	19 (23.5)	71(29)
GC to CG	15(18.5)	44(18)
AT to GC	03(3.5)	01(0.5)
AT to CG	02(2.5)	01(0.5)
AT to TA	07(8.5)	02(1)

5) Histogram showing the relative frequency of deletions of less than 100 bps that occur in various size categories in untreated plasmid or in plasmid that has been treated with hydrogen peroxide.

Of the 17 induced deletions that we have observed between 2-100 bp, 7 contain direct 2-3 bp repeats lying immediately outside one deletion terminus and immediately inside the second terminus on the opposite side. Such mutants also arise frequently in populations of untreated plasmid (results not shown). Although direct molecular proof is still lacking, such sequence directed mutational events have been explained according to a "slipped mispairing" model (21) which postulates DNA strand slippage and mispairing of the two repeated elements during replication.

DISCUSSION

When SV-40 derived plasmid that has been treated with H_2O_2 in the presence of a ferric-EDTA complex is passaged through a permissive mammalian host, high levels of mutation arise which can be detected by suitable selection of bacterial populations that have been transformed with the processed DNA (Fig. 1). Direct transformation of the treated plasmid into the bacterial host leads to very low levels of mutation, a finding which further supports the notion that mutations arise in the mammalian host cell.

No mutations arise when the plasmid is treated with H_2O_2 alone (Fig. 1). This suggests that the reaction requires both components and proceeds via an iron-catalysed Haber-Weiss (superoxide-driven Fenton) reaction to generate the highly reactive hydroxyl radical as the principle active species (22). This mechanism is further supported by the strong inhibitory effect of desferrioxamine (Table 1) which prevents the initial reaction by chelating ferrous ion. In a previous study, damage to deoxyribose by hydrogen peroxide and a ferric-EDTA complex was

6) Deletions induced in the *supF* gene of the pZ189 plasmid treated with hydrogen peroxide. Single base deletions are arbitrarily indicated at the 5' base of a run of the same base. Where direct repeats occur immediately inside one deletion terminus and immediately outside the second terminus on the opposite end, the alternative that includes the direct repeat at the 5' end is indicated by the solid line. The second possibility is indicated by the second start site and the traced line at the 3' end. Deletions that run out of the gene are indicated by the dotted line at the 3' end. The size of the deletion is indicated where known.

completely prevented by superoxide dismutase, which in turn prevents the reduction of the Fe^{3+} complex by O_2 (3). In our system, a similar inhibition by superoxide dismutase is seen for the induction of pre-mutagenic damage. Finally DMSO, a powerful scavenger of hydroxyl radical, is also effective in preventing mutation induction (Table 1). Taken together these results strongly suggest that mutations that arise in the damaged plasmid have their origin in pre-mutagenic damage induced by the specific action of the hydroxyl radical.

The mutations that arise as a consequence of *in vitro* treatment of the plasmid appear to be largely lesion directed. Most of the induced mutants (both base-change and deletion) involve changes at a single or at most 2 base-pairs (Table 3; Figs 5 and 6). Base-change mutations that arise spontaneously in the shuttle vector as well as those induced by treatment with UVC and UVB radiations (16; 17), occur predominantly at GC base-pairs. Although this is also true for base-changes induced by H_2O_2 treatment, approximately 15 percent of the base-changes are now at AT base-pairs. Furthermore experiments in which plasmid was treated intracellularly with H_2O_2 (Fig. 4) or extracellularly with UVC or UVB radiations (16; 17) have shown that the majority of base changes occur at the right hand base pair of either the 5'-TC-3' : 3'-AG-5' or the 5'-CC-3' : 3'-GG-5' sequence. This sequence specificity is relaxed for the mutants arising from plasmid treated *in vitro* with H_2O_2 (Fig. 4). Since the specificity for changes at GC base-pairs or at sites with a specific adjacent 5' base is believed to arise as a result of the influence of

polymerase specificity or nucleotide pool levels (23; 24), the lack of such specificity in the spectrum of mutations induced by H₂O₂ *in vitro* suggests that the nature of the initial lesion has a more important influence on the mutagenic outcome.

The initial lesion may also influence the spectrum of deletion mutations induced by H₂O₂ treatment. Sixty percent of these deletions occur by elimination of a single base (Figs 5 and 6). Only 3 of the single base deletions observed occur at runs of 3 or more bases and can therefore be explained on the Streisinger model (25) which proposes that a break occurring at or near a region containing a run of identical bases would increase the probability of mispairing at the repeating bases which in turn would lead to small additions or deletions during new DNA synthesis. There is no other obvious structural feature which guides the specificity of the deletion mutations. Recent studies using copper and hydrogen peroxide have shown site-specific damage at polyguanosines in pZ189 DNA (26). Although hydroxyl radical appears to be involved, the reaction is not modified by superoxide dismutase and therefore appears to be quite different from that involved in the system we have employed. On the other hand, treatment of DNA with hydrogen peroxide in the presence of ferric chloride liberates all four bases from DNA (27) and Yamafuji and Uchida (28) demonstrated specific liberation of adenine from DNA by hydrogen peroxide. Thus it is noteworthy that almost half (21 out of 43) of all the deletions of less than 100 bp observed after H₂O₂ treatment are deletions of a single adenine base (Figs 5 and 6). This finding is indicative that a direct formation of an apurinic (apyrimidinic) site by hydrogen peroxide may lead to loss of an AT base pair during processing of the damaged DNA in the mammalian host. Alternatively the deletions may arise as a result of glycosylase action on a modified base. At present we have no explanation as to why bps 130 and 165/166 prove to be particularly susceptible to this type of alteration.

In summary a high level of mutation arises in plasmid DNA that has been damaged by H₂O₂ in the presence of a ferric-EDTA complex and transfected into a mammalian host. The majority of mutants analysed contain point mutations (deletion or base-change) involving a single base. The study has also identified hotspots for single base deletions in the coding region of the *supF* gene.

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