Singlet oxygen induced mutation spectrum in mammalian cells

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ABSTRACT
In order to characterize the molecular nature of singlet oxygen (\(^{1}\text{O}_2\)) induced mutations in mammalian cells, a SV40-based shuttle vector (\(\tau\)SVPC13) was treated with singlet oxygen arising from the thermal decomposition of the water-soluble endoperoxide of 3,3'-(1,4-naphthylidene) dipropionate (NDPOJ). After the passage of damaged plasmid through monkey COS7 cells, the vector was shuttled into \(E.\text{coli}\) cells, allowing the screening of \(\text{sup}^F\) mutants. The mutation spectrum analysis shows that single and multiple base substitutions arose in 82.5% of the mutants, the others being rearrangements. The distribution of mutations within the \(\text{sup}^F\) gene is not random and some hotspots are evident. Most of the point mutations (98.4%) involve G:C base pairs and G:C to T:A transversion was the most frequent mutation (50.8%), followed by G:C to C:G transversion (32.8%). These results indicate that mutagenesis in mammalian cells, mediated by \(^{1}\text{O}_2\)-induced DNA damage, is targeted selectively at guanine residues.

INTRODUCTION
Intracellular DNA damage caused by reactive oxygen species is known to be mutagenic and is possibly involved in both the ageing process and the initiation and progression of many human diseases, including cancer (1). Among the reactive oxygen species that are likely to be generated inside the cells, singlet oxygen (\(^{1}\text{O}_2\)) can be produced under conditions of oxidative stress simultaneously with/or as a result of radical production (2). Singlet oxygen is of great importance in biological systems due to its high reactivity and involvement in physiological and pathological processes. It has been shown to be generated in biological systems and implicated in (i) defense mechanisms of living organisms such as phagocytosis, (ii) hormonal activity of prostaglandins, (iii) photochemotherapy utilising the photodynamic action of synthetic dyes, (iv) clinical manifestations of toxic agents like psoralens and (v) inborn errors of metabolism exemplified by erythropoietic porphyria (3,4).

The reactivity of \(^{1}\text{O}_2\) with molecular regions of high electron density, such as unsaturated compounds, sulfide and amino groups arises from its electrophilicity. Thus, biological targets for \(^{1}\text{O}_2\) bearing the above functional groups include unsaturated fatty acids, proteins and DNA. Its reactivity towards nucleic acid components and DNA has been reported (for review, see 5 and 6). Strand breaks and alkali-labile sites are detected on DNA molecules exposed to different sources of \(^{1}\text{O}_2\) (7,8). Earlier evidence of the specific reaction of \(^{1}\text{O}_2\) with guanosine upon treatment of free nucleotides (9) is also being confirmed for DNA. Phosphodiester breaks were found to be at guanine sites (10). Using \(\textit{in vitro}\) models, it has been shown that DNA polymerases may be blocked when replicating single-stranded DNA templates containing \(^{1}\text{O}_2\) induced lesions (11,12,13). Most of these DNA polymerization blocks are related to guanine residues, demonstrating the specificity of damages at this base. Quantification of these blocks indicates that they are twenty fold more frequent than single strand breaks (13). Actually, DNA damage profiles characterized by enzymatic probes, in a cell-free system, have demonstrated that single-strand breaks account for less than 5% of the total \(^{1}\text{O}_2\) induced DNA lesions (14). Recently, 8-hydroxy-2'-deoxyguanosine (8-OH-Gua) has been implicated as the more frequent base alteration which arises from the \(^{1}\text{O}_2\) mediated oxidation of guanine in DNA, when compared to breaks (15) or 2,6-diamino-4-hydroxy-5-formamidopyrimidine (Fapy-Gua)(16).

These base lesions in DNA are obvious premutagenic candidates \(\textit{in vivo}\) and several reports indicate \(^{1}\text{O}_2\) mutagenicity either in bacteria (17,18) or in mammalian cells (18,19). Decuyper-Debergh and coworkers (17) have focused on the characterization of the molecular nature of \(^{1}\text{O}_2\) induced mutations in M13 bacteriophage RF DNA. The mutation spectrum obtained for the \(\text{lac}Z\) gene shows that, in bacteria, \(^{1}\text{O}_2\) damaged phage DNA is mutated preferentially due to base substitutions. Among these base substitutions, G:C to T:A transversions are the most frequent mutations.

In the present study, a \(^{1}\text{O}_2\) damaged SV40-based shuttle vector was used for investigation of mutagenesis specificity within mammalian cells. The vector was exposed to a chemical clean

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source of $^{13}O_2$ (20) and transfected into mammalian COS7 cells, where it could be repaired and replicated. Then, the progeny molecules were shuttled back into bacteria for mutation analysis. The tRNA suppressor supF gene, carried by the vector, was used as mutation target. This locus is particularly interesting for this purpose, since extensive studies have demonstrated that base substitutions at almost any site, in the 85 structural base pairs of the tRNA, inactivate the supF function, with few silent mutations (21). This procedure allowed us to determine the pattern of DNA sequence changes that arose as a result of damaged DNA processing in eukaryotic cells.

**MATERIAL AND METHODS**

**Plasmid, cell line and bacterial strain**

The plasmid used in this work, $\pi$SVPC13, has been described in detail previously (18); it carries the chloramphenicol acetyl transferase and the supF genes, as well as the SV40 and bacterial origins of replication. Monkey COS7 cells (22), which produce the T antigen necessary for plasmid replication, were grown in Dulbecco's modified Eagle's medium supplemented with 7% fetal calf serum and antibiotics in a humid atmosphere with 5% CO$_2$. The E.coli MBM7070 strain was used for the screening of supF mutants among plasmid progeny (23). The amber mutation in this host chromosome's lacZ structural gene can be suppressed by a functional supF plasmid gene.

**DNA treatment**

Plasmid DNA $\pi$SVPC13 (2 $\mu$g/200 $\mu$l) was incubated with various amounts of the water soluble endoperoxide of disodium 3,3'-([1,4-naphthylidene) dipropionate (NDPO$_2$), yielding 3,3'-([1,4-naphthylidene) dipropionate (NDP), $^{13}O_2$ and triplet molecular oxygen (20). DNA damage was generated by treating the plasmid in 50 mM sodium phosphate buffer in D$_2$O, pH 7.4, at 37°C, for 1.5 h. After treatment, DNA samples were sterilized and purified with chloroform prior to transfection into mammalian cells.

**DNA transfection and mutagenesis analysis**

The passage of damaged plasmids through mammalian cells, their shuttling back to bacteria and mutant screening were previously described (18). Basically, subconfluent COS7 cultures were transfected with plasmid DNA (1 $\mu$g per 90 mm Petri dish) by the DEAE-dextran method (24). Seven days later, cells were harvested and the replicated episomal DNA was extracted, using the method of Birnboin and Doly (25), adapted for mammalian cells. The plasmid DNA recovered from monkey cells was shuttled to competent E.coli MBM7070 cells (26). Transformed colonies were plated on LB-agar medium, in the presence of chloramphenicol (34 $\mu$g/ml), X-Gal (5-bromo-4-chloro-3-indolyl-\beta-D-galactoside, 0.08 mg/ml) and IPTG (isopropyl-\beta-D-thiogalactoside, 0.1 mM). Mutants were identified as white or light blue colonies against a background of normal bright blue colonies of MBM7070 transformed cells. Selected colonies were then isolated and restreaked three times on the same medium, to confirm the phenotype. The mutation frequency was calculated as the ratio of white and light blue colonies to the total amount of bacterial clones. Mutants were analysed from six independent transfections in monkey COS7 cells. Plasmid DNA was prepared by the alkaline lysis method (25) and analysed by agarose gel electrophoresis. The supF locus of these plasmids was then sequenced by the Sanger chain termination method (27) using the Sequense Version 2.0 procedure (United States Biochemical Corporation). Two different 17-mer oligonucleotides, which hybridize with either one of the complementary strands, adjacent to the supF gene, were used as primers for the sequencing reactions. Only independent mutants were integrated in the mutation spectrum: those carrying the same modification(s) were considered as independent only if they were isolated from distinct transfection experiments in COS7 cells.

**RESULTS**

$^{13}O_2$-induced mutagenesis

NDPO$_2$-treated $\pi$SVPC13 vector was transfected into monkey COS7 cells, where plasmids replicated for seven days. They were then recovered, purified and shuttled back into E.coli cells for mutant screening, at the supF locus. The data presented in table 1 confirm the enhancement of the number of supF mutants in NDPO$_2$-treated $\pi$SVPC13 vector (18). As shown in figure 1, the mutation frequency is increased linearly with NDPO$_2$ doses, up to a factor 12, in comparison to plasmids submitted to the same experimental conditions, without NDPO$_2$.

![Figure 1](image_url)

Figure 1. Enhancement of mutation frequency at the supF locus of $^{13}O_2$ treated $\pi$SVPC13 plasmid, following replication in monkey COS7 cells. The enhancement factor is relative to the mutation frequency observed in vector treated without NDPO$_2$.

<table>
<thead>
<tr>
<th>Plasmid treatment</th>
<th>Mutants/total colonies analysed</th>
<th>Mutation frequency*</th>
</tr>
</thead>
<tbody>
<tr>
<td>NDPO$_2$ 0 mM</td>
<td>17/19,528</td>
<td>$8.7 \times 10^{-4}$</td>
</tr>
<tr>
<td>10 mM</td>
<td>27/13,111</td>
<td>$2.1 \times 10^{-3}$</td>
</tr>
<tr>
<td>20 mM</td>
<td>41/14,880</td>
<td>$2.5 \times 10^{-3}$</td>
</tr>
<tr>
<td>50 mM</td>
<td>65/12,764</td>
<td>$5.1 \times 10^{-3}$</td>
</tr>
<tr>
<td>100 mM</td>
<td>54/4,937</td>
<td>$1.1 \times 10^{-2}$</td>
</tr>
</tbody>
</table>

The data include those presented in reference 18.

*Mutation frequency is the ratio between the number of white or light blue colonies and the total number of transformed bacterial colonies.

The data are statistically different from DNA treated with 0 mM NDPO$_2$, p < 0.01, according to Bross' test.
The damaging action of \(^1\)O\(_2\) in DNA is reduced by replacing D\(_2\)O by H\(_2\)O, as expected from the shorter lifetime of \(^1\)O\(_2\) in H\(_2\)O, or in the presence of 1 mM sodium azide, a \(^1\)O\(_2\) quencher (18,19). The increase in mutation frequency is not observed if DNA is treated by 3,3',4-(1,4-naphthylidene) dipropionate (NDP), a product of NDPO\(_2\) thermolysis (18). These data, together with the evidence that NDPO\(_2\) is a highly specific source of \(^1\)O\(_2\) (20), indicate that this is the ultimate DNA modifying species, and thus the responsible for the mutagenic effect described here.

It should be noted that the high levels of \(^1\)O\(_2\)-induced mutagenesis are achieved at NDPO\(_2\) doses which still result in low biological inactivation rate, e.g., 45% of transforming ability is maintained after 100 mM NDPO\(_2\) (18). These levels of inactivation are much lower than those found when treated DNA is directly transfected into bacterial cells (17,18). Thus, one can infer that the \(^1\)O\(_2\)-induced damage is incorrectly processed in mammalian cells by DNA repair and/or replication mechanisms, which are efficient in recovering the vector's biological activity, but yield mutations.

The possibility that the mutations studied here arise in bacteria, due to low levels of remaining lesions after plasmid passage through mammalian cells, can be discarded. The mutation frequency of \(^1\)O\(_2\)-damaged vector, directly transformed in bacteria, is below the levels detected after COS7 cells transfection (18). Therefore, it is highly probable that the mutations described here actually arose in the mammalian host.

### Types of \(^1\)O\(_2\)-induced mutations

The supF locus of the mutated plasmids was sequenced. Table 2 summarizes the results obtained with 57 independent mutants. Most of the mutations induced by \(^1\)O\(_2\) involve single base substitutions. Some of the detected mutations have more than one base change: among the multiple ones, five contain tandem double base changes. Three of these multiple mutations include alterations close to the base substitutions: two of them present insertion of one base pair (A:T) and the other a three nucleotides deletion. The ten rearrangements detected include three deletions of less than 30 base pairs and seven gross alterations (for details, see legend of Table 2).

The types of mutation detected in these experiments are considerably different from the data obtained for spontaneous mutations in the supF gene of SV40-based shuttle vectors after passage through mammalian cells (28,29,30). Most of the independent mutants analyzed (49 of 57) were obtained from experiments where the level of induced mutagenesis is, at least, 3 fold over the background. Therefore, the majority of the observed mutations actually originates from the processing of \(^1\)O\(_2\)-induced DNA lesions in mammalian cells.

### \(^1\)O\(_2\)-induced mutation spectrum in the supF gene

A compilation of the types of base substitutions induced by \(^1\)O\(_2\) is presented in Table 3. Transversions G:C to T:A are clearly favored with respect to the total number of mutation sites. This modification represents 50.8% of the observed substitutions. More significant is the fact that 98.4% of the point mutations are on the G:C base pair. Changes involving a T:A base pair are observed in only one mutant.

Figure 2 shows the spectrum of base substitutions in the supF gene carried by the shuttle vector, after passage into mammalian cells. All the detected base substitutions are located in the tRNA coding region of the supF gene. The distribution of mutations within the supF gene is not random and some hotspots are evident. One major (position 65) and three additional (positions 102, 110 and 118) hotspots are observed in the \(^1\)O\(_2\) induced mutation spectrum of double stranded supF gene. The majority of base substitutions that give rise to hotspots are due to single rather than multiple changes in supF gene, particularly the one at position 65. Among these three positions in supF gene, it is interesting to note that all of them are located in regions containing two or more G:C adjacent base pairs.

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**Table 2. Types of mutants in \(^1\)O\(_2\)-treated \(\times\)SVPC13 plasmid replicated in COS7 cells.**

<table>
<thead>
<tr>
<th>Type</th>
<th>Number of Mutants</th>
<th>%</th>
</tr>
</thead>
<tbody>
<tr>
<td>Rearrangements</td>
<td>10</td>
<td>17.5</td>
</tr>
<tr>
<td>Single base substitutions</td>
<td>35</td>
<td>61.4</td>
</tr>
<tr>
<td>Multiple base substitutions</td>
<td>12</td>
<td>21.1</td>
</tr>
<tr>
<td>Total</td>
<td>57</td>
<td>100.0</td>
</tr>
</tbody>
</table>

* only independent mutants were considered.

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**Table 3. Types of base substitutions* detected in \(^1\)O\(_2\)-treated \(\times\)SVPC13 plasmid replicated in COS7 cells.**

<table>
<thead>
<tr>
<th>Type</th>
<th>Number of Mutations</th>
<th>%</th>
</tr>
</thead>
<tbody>
<tr>
<td>Transition</td>
<td></td>
<td></td>
</tr>
<tr>
<td>G:C - A:T</td>
<td>9</td>
<td>14.8</td>
</tr>
<tr>
<td>A:T - G:C</td>
<td>1</td>
<td>1.6</td>
</tr>
<tr>
<td>Transversion</td>
<td></td>
<td></td>
</tr>
<tr>
<td>G:C - C:G</td>
<td>20</td>
<td>32.8</td>
</tr>
<tr>
<td>G:C - T:A</td>
<td>31</td>
<td>50.8</td>
</tr>
<tr>
<td>A:T - T:A</td>
<td>-</td>
<td>-</td>
</tr>
<tr>
<td>A:T - C:G</td>
<td>-</td>
<td>-</td>
</tr>
<tr>
<td>Total</td>
<td>61</td>
<td>100.0</td>
</tr>
</tbody>
</table>

* Substitutions include single and multiple mutants.

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**Figure 2. Spectrum of DNA bases changes detected at the supF locus after transformation of COS7 cells with \(^1\)O\(_2\)-damaged DNA. Bases involved in multiple point mutations are underlined, arrows indicate the insertions. Asterisks denote the beginning and the end of the final transcript.**
DISCUSSION
This work confirms previous observations (18) on the mutagenicity of $\text{O}_2^-$-induced DNA damage, after replication of the shuttle vector $\pi$SVPC13 in mammalian cells. We have investigated the molecular nature of mutations in the $\text{supF}$ gene by DNA sequencing. The data show that base substitutions constitute the majority of these mutations. These point mutations occurred predominantly at sites which can be specifically oxidized by singlet oxygen, i.e., G:C base pairs. Although we cannot state precisely which of these two bases is actually responsible for the mutation, our data clearly suggest that the extracellular modification of the shuttle vector $\pi$SVPC13 by $\text{O}_2^-$ treatment results in the formation of miscoding guanine damage upon replication in mammalian cells.

The G:C to T:A transversion is the most frequent base substitution observed here. This is in good agreement with the results obtained after bacterial transfection of M13lacZ bacteriophage DNA treated with $\text{O}_2^-$ (17). The sequence of the mutated lacZ gene revealed, in general, types of mutations similar to those described here. This suggests that the mechanisms responsible for the mutagenesis due to $\text{O}_2^-$-induced DNA damages are alike in eucaryotic and procaryotic cells. Both data indicate that the modified guanine residue mispairs at high frequency with dATP during DNA replication. Recent work provided evidence that 8-OH-Gua is a frequent lesion detected in DNA exposed to $\text{O}_2^-$ (15,16) and this could be the premutagenic lesion responsible for the detected G:C to T:A transversions. In fact, Wood and coworkers (31) have found that E.coli transformation with a single-stranded M13 DNA, bearing a unique 8-OH-Gua, yields a high frequency of progeny phage showing G to T transversions at the position of the original lesion. Moriya et al (32) obtained similar results using a gapped duplex vector containing a single 8-OH-Gua. More recently, Cheng et al (33) used two different M13 bacteriophage assays to determine the miscoding properties of 8-OH-Gua. Their results indicate that this guanine analogue has the monospecific mutagenic ability to pair with A, generating transversions in E.coli. Considered together, these data provide support for the assumption that 8-OH-Gua residues are the major premutagenic lesion present on $\text{O}_2^-$-damaged DNA, resulting in G:C to T:A transversions. Alternatively, the replication apparatus favors the incorporation of dA opposite a non-instructive template, as the proposed 'A-rule' (34). In this case, the G:C to T:A transversion would be the consequence, regardless the modification at the guanine residue.

As in bacteria (17), G:C to C:G transversions and G:C to A:T transitions were frequent events in vectors mutated in mammalian cells (32.8% and 14.8%, respectively). Hoebee et al (35), working with the M13lacZ system in E.coli, found that gamma-rays also induce high levels of G:C to C:G mutations. The authors proposed that in vivo the 8-OH-Gua may also mispairs with a guanine, yielding such transversions. On the other hand, lesions such as Fapy-Gua (16), alkali- and piperidine-labile sites (7) and phosphodiester chain breaks (7,8,10) have also been found on $\text{O}_2^-$-damaged DNA. Although these lesions are less frequent than 8-OH-Gua, their role on $\text{O}_2^-$-induced mutagenesis is not known yet, and must be considered. They may be responsible, at least partially, for some of the mutations described here, including base substitutions and rearrangements.

In this work, multiple mutations account for an important fraction of the detected base substitutions. The possibility that these mutations arise from vectors containing two or more proximal lesions cannot be discarded, although there is a lack of correlation between the appearance of such mutations and the increase in the NDPO2 dose used in DNA treatment (data not shown). The explanation proposed by Seidman and coworkers (36) also fits the data presented here. They suggested that multiple mutations occur as a result of error-prone DNA polymerase activity during the repair of lesions. This presupposes that $\text{O}_2^-$-induced lesions are repaired during shuttle-vector passage through mammalian cells, which remains to be established. In prokaryotes, it has been demonstrated that base modifications induced by $\text{O}_2^-$ are recognized and repaired by Fpg protein and UvrABC endonuclease (37,38). In eukaryotes, the evidence is still indirect. Kasai and coworkers (39) have suggested that 8-OH-Gua can be repaired in vivo, since a time-dependent decrease in the amount of 8-OH-Gua in the liver DNA of gamma irradiated mice was noted following exposure. The presence of repair mechanisms strengthens the importance of these lesions. It should also be pointed out that multiple mutations are frequently observed in SV40-based shuttle vector mutagenesis reports (28,29,36). Thus, the presence of this kind of mutation may be a specific feature and a limitation of such vectors.

An important concern on the use of shuttle vectors to study mutagenesis in mammalian cells is to which degree the results reflect what would actually happen in a chromosomal gene. In fact, SV40-based shuttle vector systems, such as the one used here, are unable to detect gross DNA rearrangements as, for instance, large deletions. However, there seems to have a striking similarity on the specificity of induced point mutations at endogenous chromosomal loci and the data obtained with shuttle vectors (40). Thus, the observations described here probably correspond to the mutagenic events that occur at the chromosomal level, if $\text{O}_2^-$ reaches the cellular genome.

The pattern and the distribution of mutations induced by $\text{O}_2^-$ in $\text{supF}$ gene are quite different from what has been reported for similar systems, on which mutations are due to DNA treatment by agents that generate other oxygen radicals (29,30). These differences are certainly the consequence of a specific and distinct spectrum of premutagenic lesions induced by $\text{O}_2^-$. For this excited molecule, DNA lesions resulting from guanine oxidation seem to be responsible for the observed biological consequence, such as mutagenesis.

One interesting feature of the mutational pattern described here is that it appears to be involved in important biological events such as those responsible for carcinogenesis process. For example, analysing hepatocarcinomas, transversions in guanine sites were recently detected in p53, a tumour suppressor gene (41, 42).

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