Spontaneous and photosensitiser-induced DNA single-strand breaks and formamidopyrimidine-DNA glycosylase sensitive sites at nucleotide resolution in the nuclear and mitochondrial DNA of Saccharomyces cerevisiae

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ABSTRACT

A system is described for mapping oxidative DNA damage (sites sensitive to formamidopyrimidine-DNA glycosylase and single-strand breaks) at nucleotide resolution in the nuclear and mitochondrial DNA of Saccharomyces cerevisiae. Our 3′ end labelling method is sensitive and was first developed using the well-studied inducer of oxidative DNA damage, methylene blue (MB) plus light. We treated yeast DNA in vitro with this so as to maximise levels of damage for assay development. Unfortunately, MB does not remain in yeast cells and yeast DNA repair mutants sensitive to active oxygen species are not sensitive to this agent, thus for in vivo experiments we turned to a polycyclic aromatic, RO 19-8022 (RO). This resulted in oxidative DNA damage when light was applied to yeast cells in its presence. The spectra of enzyme-sensitive sites and single-strand breaks induced by MB in vitro or by RO plus light in vivo or in vitro were examined in two yeast reporter genes: the nuclear MFA2 and the mitochondrial OLI1. The experiments revealed that most of the enzyme-sensitive sites and single-strand breaks induced by MB or RO plus light are at the same positions in these sequences, and that these are guanines.

INTRODUCTION

Oxygen is essential for cells but it also leads to the formation of extremely reactive oxygen species (ROS), either during metabolism or after exposure to exogenous factors such as ionising radiation or chemicals. ROS can react with DNA to produce a spectrum of damages including oxidised bases, abasic sites or single-strand breaks (SSB) (1,2). In the cell, mitochondrial DNA is particularly vulnerable to attack due to the presence of oxidative phosphorylation and the absence of protection from histones. Therefore, this genome may experience a higher extent of oxidative damage compared with nuclear DNA. A lack of repair or misrepair of these damages can lead to mutation or cell death (3). Additionally, there is evidence for the role of oxidative damages in carcinogenesis, ageing and various diseases (4,5). Thus, the study of oxidative DNA damage and its repair is central to elucidating the mechanisms by which cells preserve their genome integrity.

Here, we report the induction of a class of oxidised base damages by two photosensitisers and describe their detection at the level of the nucleotide in Saccharomyces cerevisiae. Exposure of cells to oxidants results in a spectrum of DNA damage which renders their measurement complex; our study is limited to the measurement of SSB and sites sensitive to the Escherichia coli formamidopyrimidine-DNA glycosylase (Fpg). This enzyme recognises formamidopyrimidines, apurinic sites and 8-oxoguanine (3,6). The latter lesion is one of the most abundant and mutagenic oxidised bases generated spontaneously in cells.

Oxidative DNA damage can be observed at the level of the nucleotide in DNA from untreated cells. To augment amounts of oxidative DNA damage for assay development, first we exposed DNA to methylene blue (MB) plus light (7). This is often used for inducing oxidative DNA damage and the types of damage are well documented. MB is a thiazine dye, and a photosensitiser that produces 8-oxoguanine and fapyguanine in the presence of light; the amount of 8-oxoguanine is ~20 times greater than that of fapyguanine (7). Unfortunately, MB does not remain in yeast cells, thus its use was restricted to in vitro experiments for assay development.

Subsequently, to examine induced oxidative DNA damages in cells we turned to a chemical produced by Hoffman la Roche: [R]-1-[(10-chloro-4-oxo-3-phenyl-4H-benzo[a]quinolizin-1-yl)-carbonyl]-2-pyrrolidinemethanol, or RO 19-8022 (RO) (8). This is a polycyclic aromatic that in the presence of light (primarily 425 nm) induces oxidative DNA damage. Here, a few sites are due to base loss but the majority are base modifications which are sensitive to Fpg. HPLC analysis has showed that most of these base modifications are 8-oxoguanine [74 ± 10% of Fpg-sensitive sites (FpgSS)] (8). This agent exhibited the potential to induce DNA oxidative damage in yeast cells.

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The technique used to detect FpgSS at the level of the nucleotide is a 3’ end labelling method previously developed by our group to detect cyclobutane pyrimidine dimers (CPDs) (9,10). Restricted DNA is cut at the damage sites with a DNA repair enzyme. The strand of interest is retrieved using a biotinylated probe and streptavidin beads. The labelling is performed by adding radioactive nucleotides at the 3’ end of the strand. The first aim of the work presented here was to modify the technique so as to detect oxidative DNA damage (i.e. FpgSS and SSB) at nucleotide resolution. Then, we used the method to define the spectrum of induced damage seen with MB plus light compared with that seen with RO plus light in vivo or in vitro in a nuclear and a mitochondrial gene.

The nuclear gene selected was MFA2 which codes for a2 factor, a protein involved in the pheromone response pathway (11). The transcriptional regulation of MFA2 is well understood (12,13) and it is easily controlled by investigating events in a or α mating type cells. In a cells, the gene is active and the chromatin structure is opened while in α cells the gene is silent and the chromatin structure is folded (14,15). The gene provides a good model for studying the effects of transcription and chromatin structure on DNA repair in yeast (10,16). In light of the data accrued on the induction and repair of CPDs in MFA2 at nucleotide resolution (10,16), it was logical to extend studies to include an examination of oxidative damages to DNA.

The mitochondrial gene selected was OLI1; it codes for subunit 9 of the mitochondrial ATPase complex. This subunit plays a key role in the proton translocating activity of the ATPase. OLI1 is the first gene of a transcription unit also coding for tRNAser and the Varl gene (17); its promoter is one of the strongest in the yeast mitochondrial genome (18). The gene confers sensitivity to oligomycin and it was initially identified through the analysis of oligomycin resistant mutants (oliR) (19). The selection of this gene will enable studies on the induction of oliR mutations at nucleotide resolution in relation to the types and frequencies of oxidative DNA damage observed.

Here we describe the development of the assay and the spectrum of FpgSS or SSB in both of the ‘reporter’ sequences described above.

MATERIALS AND METHODS

Yeast strains

The haploid W303 rad14 (a ade, his, leu, trp, CAN1) was constructed and donated by A. Scott (University of Wales Swansea, Swansea, UK). The yeast strains used for survival experiments were kindly given by S. Boiteux (CEA, Fontenay aux Roses, France) and A. Scott (University of Wales Swansea, Swansea, UK) and their genotypes are: FF 18733 (MATa, his 7-2, leu2-3, trp111-289, ura3-52, lys1-1), CD138 (ogg1::TRP1), CD186 (ogg1::URA3, ntg1::LEU2, ntg2::TRP1), rad14 mutant (rad14::URA3). Cells were incubated in complete media overnight at 28°C and grown to 3–4 × 10^7 cells/ml. They were then resuspended in cold phosphate buffer saline (PBS; 136 mM NaCl, 2.6 mM KCl, 8 mM Na2HPO4, 1.7 mM KH2PO4) at 2 × 10^7 cells/ml.

FpgSS and SSB induction

Chemicals. RO was a gift from Hoffman la Roche AG (Basel, Switzerland). MB was obtained from Sigma.

Treatment of cells. Exponential phase cells were resuspended in PBS at 2 × 10^7 cells/ml and were exposed to RO (6 µg/ml) plus visible light from a 1000 W halogen lamp (Osram) at a 10 cm distance on ice with vigorous stirring. The cells were then washed and resuspended in cold PBS, 50 mM EDTA. Total DNA was extracted from untreated or treated cells as follows (20). Cells were converted to spheroplasts at 4°C overnight using 20T zymolase (ICN) and lysed with 5 ml of a solution containing 1 vol of lysis buffer (4 M urea; 0.2 M NaCl; 100 mM Tris–HCl pH 7.5; 10 mM CTDA, 0.5% SDS) and 1 vol of PBS. The samples were then submitted to RNaseA (Sigma) and Proteinase K (Biometra-Amersco) digestions. One phenol/chloroform/isooamyl alcohol (25:24:1) and one chloroform/isooamyl alcohol (24:1) extraction were performed. Exposure to air of the samples was avoided in order to limit the induction of oxidative damages during the phenol/chloroform extraction. The DNA was then precipitated with isopropanol and finally resuspended in 1 vol of TE buffer.

In vitro DNA treatment. RO (6 µg/ml) or MB (2.5 µg/ml) was added to total yeast DNA (100 µg/ml) extracted as previously described (20). The RO plus light DNA treatment was performed as mentioned above. The MB treated DNA was exposed for 10 min to a 150 W Bulb (Claudfar) at a distance of 20 cm on ice. The DNA was washed by precipitation with ethanol at −20°C.

Identification of FpgSS and SSB in the MFA2 and OLI1 genes

Total DNA from untreated cells, DNA treated with MB plus light or RO plus light, or DNA from cells treated with RO plus light was restricted (Gibco BRL restriction enzyme) with 120 U of Rsal for analysis of the MFA2 gene (60 µg of DNA) and with 60 U of SstI for the analysis of the OLI1 gene (30 µg of DNA). Treatments were at 37°C for 1 h in a reaction volume of 300 µl. Complete digestion created an 869 bp MFA2 fragment or a 359 bp OLI1 fragment. Purification and precipitation of the DNA was performed as previously described (10). The digested DNA was resuspended in 200 µl TE buffer (10 mM Tris–HCl pH 7.5, 1 mM EDTA, pH 8) and then split in two. Fpg protein (provided by S. Boiteux, CEA, France) was added to one aliquot of a sample (0.5 µg Fpg/10 µg DNA) (+sample), the second aliquot was mock treated with a corresponding volume of TE buffer (−sample); all samples were incubated for 1 h at 37°C. A selected biotinylated probe (1 pmol) was added. FpgSS and SSB were detected in the transcribed strand (TS) using probe 1a (MFA2) or 1b (OLI1) and in the non-transcribed strand (NTS) using probe 2a (MFA2) or 2b (OLI1). The annealing at the corresponding annealing temperature (ta), binding with Dynabeads, washing and labelling steps were performed as previously described (10) except that [32P]dCTP (6000 Ci/mmol, Amersham) was used instead of [32P]dATP for the labelling with probes 1b and 2b. The electrophoresis conditions were performed as described by Teng et al. (10).

Probes/primers used for PCR reactions and 3’ end labelling

Sequences in lower case are overhang modification whereas sequences in upper case are MFA2 or OLI1 sequence.

For MFA2 fragments obtained with RsaI digestion. Probe/ primer 1a: 5′-Biotin-gatagattttACACCATCTACTACATAATTAGTATTTTTC-3′, ta = 55°C. Probe/primer 2a: 5′-Biotin-gatagattttACGGACTTTGATGACGAAAAAC- CATTATTTAAA-3′, ta = 57°C. Primer 3a: 5′-gatagACGGAC-
TTGATGCGATGAAAAACATATTATTTAA-3', \( ta = 57^\circ C \).

Primer 4a: 5'-gatgcACACCATCTACATAATTAGTAGTAGTTTCC+3', \( ta = 55^\circ C \).

For OLI1 fragments obtained with SSpI digestion. Probe/primer 1b: 5'-Biotin-atcggagggg ATTATGAAATTAGTACACCTC-3', \( ta = 55^\circ C \).

Primer 2b: 5'-Biotin-tatcaggggg ATTATGAAATTATATATATATATATAGAGTT-3', \( ta = 50^\circ C \).

Primer 3b: 5'-tagtcgaATTATATATATTATATATATATATATATAAGAATT-3', \( ta = 50^\circ C \).

Primer 4b: 5'-atcgagattATGCAATTATATTACAACTGCTTAT-3', \( ta = 55^\circ C \).

Sequencing ladders for MFA2 and OLI1 fragments

In order to determine the nucleotide position of the FpgSS, each damages sequence ladder was run with a sequencing ladder alongside. Each sequencing ladder [MFA2 (a) and OLI1 (b)] was obtained via PCR products. PCR reactions were carried out with primers 1 and 2 (the same sequences as probes 1 and 2) coupled with primers 3 and 4, respectively. Primers 3 and 4 were modified versions of primers 2 and 1, respectively. In order to run the sequencing ladder and the damage ladder alongside at the same nucleotide position, the primers 3 and 4 were shortened by 6 nt from the overhang. The sequencing reaction was performed according to the Sanger protocol with a sequencing kit from Amersham (T7 Sequenase version 2.0 DNA sequencing kit) using a single-stranded DNA template. The template was the biotinylated strand obtained from the PCR products and was isolated with Dynabeads as previously described (10).

Detection and quantification of FpgSS and SSB

For the detection of FpgSS and SSB, the sequencing gels were placed under a PhosphorImage screen which was then scanned with a PhosphorImager (Molecular Dynamics, Sunnyvale, CA) to detect 3’ end labelled bands.

The intensity of each band from each lane was quantified using ImageQuant Software (Molecular Dynamics, Sunnyvale, CA). The intensity of each band (top and lower bands) was individually quantified and totalled. The band frequencies were calculated as the ratio between each band intensity with the sum of the intensities of all bands in an individual lane. Using this method, effects due to variations in small differences of DNA quantity between one lane and another are eliminated. The frequencies of SSB and FpgSS were calculated as follows:

a) Spontaneous SSB frequencies = \((-U_{SSB}) = S_{SSB}\)
b) SSB induced by RO plus light frequencies = \((-RO_{SSB}) - S_{SSB}\)
c) SSB induced by MB plus light frequencies = \((-MB_{SSB}) - S_{SSB}\)
d) Spontaneous FpgSS frequencies = \((+U_{SSB}) - (U_{SSB}) = S_{Fpg}\)
e) FpgSS induced by RO plus light frequencies = \([+RO_{SSB}) - (-RO_{SSB}) - S_{Fpg}\]
f) FpgSS induced by MB plus light frequencies = \([+MB_{SSB}) - (-MB_{SSB}) - S_{Fpg}\]

\((U_{SSB})\) is the band frequency in the lane containing the DNA from untreated cells; \((RO_{SSB})\) is the band frequency in the lane containing DNA from yeast cells or DNA treated with RO plus light and \((MB_{SSB})\) is the band frequency in the lane containing DNA treated in vitro with MB plus light; minus lanes (−) represent DNA samples not incubated with Fpg protein; plus lanes (+) represent DNA samples incubated with Fpg protein.

The data are presented by plotting the band frequencies at the nucleotide position as determined from the sequence ladder.

The number of FpgSS or SSB per fragment (\( S \)) was estimated using Poisson distribution: \( S = -\ln P_0 \) (where \( P_0 \) is the fraction of fragments free of breaks).

RESULTS

As the induction of oxidative damage to DNA can be sequence dependent and it can vary with the location of the DNA in the cell, we examined at the level of the nucleotide the occurrence of SSB and FpgSS in the nuclear and mitochondrial DNA of S. cerevisiae.

First, in vitro treatments with MB plus light were carried out with DNA from untreated cells extracted as described in Materials and Methods. This provided nuclear and mitochondrial DNA in the same extract. Thus we could compare the induction of SSB and FpgSS in DNA from these two different organelles within the same treatment. Following exposure to light and the subsequent analysis of SSB and FpgSS in the nuclear MFA2 or the mitochondrial OLI1 sequences, autoradiographs typed by those in Figures 1 and 2 were obtained. Here, we see the results for the TS and the NTS of these genes. In each lane, the top band represents the full-length single-strand fragment containing no damage. Strands incised by Fpg protein at damaged sites or containing strand breaks migrate at lower positions in the lane. The presence of FpgSS is noticeable by the increasing intensity of the bands in the lanes containing DNA incubated with Fpg protein. The nature and position of the bands on the gel was determined by the nucleotide position in the sequence being compared with the reference sequence ladder alongside. As seen on the autoradiographs, most of the bands in the lanes with Fpg protein (FpgSS) or without Fpg protein (SSB), occurred at guanines in the sequence ladder.

The frequencies of FpgSS and SSB in the DNA from untreated cells or induced by MB plus light are given in Figures 3 and 4. In MFA2, hot spots occur for SSB, at nucleotide positions +169 to +183 in the NTS (Figs 1 and 3; Table 1). After treatment with MB plus light, most of the SSB occur at the same positions as FpgSS in both OLI1 and MFA2; however there are exceptions. For example, in MFA2 fragment on the TS at −196, SSB occur while no FpgSS are detected (Fig. 1).

Unfortunately, MB does not remain in yeast cells. Therefore, to examine events in vivo we turned to the less studied RO. This might be taken up by yeast cells and is also light activated. To better characterise the genotoxic effect of this chemical, survival experiments were undertaken on yeast mutants defective in genes having roles in base or nucleotide excision repair. As seen in Figure 5, the wild type (FF 18733), ogg1 (CD138) strains exhibit the same sensitivity ranging from 45 to 60% survival after treatment with RO plus 7.5 min light, while the rad14 mutant survival is only 11%. The triple mutant ogg1∫intg∫ntg2 shows an even higher sensitivity, since after 7.5 min only 1% survival is observed. These data show RO plus light is able to induce DNA damage in yeast cells. To analyse the spectrum of SSB and FpgSS at the level of the nucleotide induced by RO plus light, cells were treated as in Materials and Methods, and the DNA extracted from yeast cells treated or not with RO plus light. Because the spectrum of oxidative DNA damage may vary depending on the chromatin structure of the DNA in the yeast cells, experiments in vitro with RO plus light were also undertaken, using the same conditions as in vivo experiments and as described in Materials and Methods. To compare the spectrum of damages induced by both photo-sensitisers we undertook the processing and analysis of DNA....
Figure 1. Induction of SSB and FpgSS in the MFA2 RsaI fragment for the TS and the NTS. The lanes contain: DNA from untreated cells (U); DNA treated in vivo or in vitro with RO plus light (RO); DNA after in vitro treatment by MB plus light (MB). DNA was incubated (+) or not incubated (−) with Fpg protein. The map of the RsaI fragment consists of the promoter region (white box), from nucleotides −225 to −43 (with part of the Mcm1 region at −225 and the TATA box at −125); the transcribed region of MFA2 (dark grey box), from nucleotides −43 to +285; and the end of the RsaI fragment (light grey box), from nucleotides +285 to +435. +1 represents the start of the coding sequence. Arrows indicate particular nucleotide positions highlighted in the Results.

Table 1. Sequence context of hot spot damages described in Results

<table>
<thead>
<tr>
<th>Sequence</th>
<th>GENE Strand</th>
<th>HOT SPOT DAMAGE/TREATMENT</th>
</tr>
</thead>
<tbody>
<tr>
<td>CGT</td>
<td>M62 NTS</td>
<td>SSB/MB + light</td>
</tr>
<tr>
<td>CTACG</td>
<td>M62 NTS</td>
<td>FpgSS/RO+ light in vivo</td>
</tr>
<tr>
<td>TACAG</td>
<td>M62 TS</td>
<td>SSB / RO + light in vivo</td>
</tr>
<tr>
<td>AAAGG</td>
<td>M62 TS</td>
<td>FpgSS/RO+ light in vivo</td>
</tr>
<tr>
<td>TTG</td>
<td>M52 TS</td>
<td>FpgSS/RO+ light in vivo</td>
</tr>
<tr>
<td>GCAG</td>
<td>OLI TS</td>
<td>SSB / RO + light in vivo</td>
</tr>
</tbody>
</table>

The sequence and the nucleotide position surrounding the hot spot is given. The column hot spot damage/treatment indicates the damage type and the treatment used to induce the damage.

either treated in vitro with MB plus light or with RO plus light, or DNA from cells treated with RO plus light, side by side using the same reaction conditions. Thus we could directly compare the damage positions induced with RO plus light in vivo and in vitro with those with MB plus light.

Following analysis of MFA2 and OLI1, autoradiographs as shown in Figures 1 and 2 were obtained. They show that yeast cells incubated with RO and treated with light had higher amounts of both SSB and FpgSS compared with untreated cells (or cells treated with light or RO alone; data not shown). This indicates that this photoactivated agent provides a means of augmenting the levels of oxidative damage found in the DNA from untreated cells. In general, guanines appear to be converted to SSB or FpgSS. However, a few exceptions occur. For example, in the NTS of MFA2, one SSB is detected at +419 which is at a thymine (Fig. 1). In the TS, SSB and FpgSS are also detected at thymine or adenine; these are essentially localised in the promoter region, for example as seen at −194 nucleotide position (Fig. 1). In the DNA treated with RO plus light, it clearly appears that as in vivo, guanines are damaged and transformed as an SSB or an Fpg sensitive site. However, the intensities of the bands are weaker compared with those after in vivo treatment; this is true for MFA2 and OLI1 and for both strands (Figs 1 and 2). Additionally, in vitro the relative levels between damage sites varies compared with in vivo. For example, in the NTS of MFA2, the levels of FpgSS at guanines position +169/171, +179 and +183 are, in vitro, +183 > +169/171 > +179. However in vivo, they follow a different order:
Figure 3. SSB (left) and FpgSS (right) in relation to the nucleotide position within the MFA2-containing RsaI fragment. Each histogram represents the band frequency at a precise nucleotide position and is the mean value of three independent experiments. Standards errors are not integrated to give the graphs more clarity. The profiles represent damaged sites in DNA from untreated cells (plot A); from cells treated with RO plus light (plot B); in DNA after in vitro treatment with MB plus light (plot C); and in DNA after in vitro treatment with RO plus light (plot D). The map of the RsaI fragment is shown and is as described in Figure 1.

The spectrum of SSB and FpgSS in MFA2 and OLI1 from cells untreated or treated with RO plus light, or from DNA treated with MB plus light or RO plus light is given in Figures 3 and 4. In MFA2, after RO plus light treatment in vivo, one can identify two bands of stronger intensities for SSB at nucleotide positions –211 and –181 on the TS, two hot spots for FpgSS at +311 and +131 on the TS and one hotter spot for FpgSS at +169 on the NTS (Table 1). In the OLI1 fragment, one hot spot for SSB is found at nucleotide position +70 of the TS (Table 1). As with MB plus light, although most of the SSB occur at the same positions as FpgSS in both OLI1 and MFA2, there are exceptions. For example, after in vivo RO plus light treatment in the MFA2 fragment, two hot spots for SSB occur at –211 and –181 on the TS but these nucleotide positions are cold spots for FpgSS (Fig. 3). On the contrary, two hot spots for FpgSS are present at +131 and +311 of the TS but these are cold spots for SSB. Similar exceptions occur at different sites for the OLI1 sequence.

In order to compare the induction of DNA damages between the two specific genes studied (i.e. MFA2 and OLI1) we have expressed the overall DNA damage induction levels in TS and NTS in both genes in Table 2. Here, we show the induction of SSB and FpgSS per OLI1 or MFA2 fragment after treatment with RO or MB plus light. In general, there are more damage sites/kb (SSB and FpgSS) in the TS of MFA2 compared with the NTS, while in OLI1, NTS contains more damage than the TS. The SSB frequency in the untreated sample is increased in OLI1 compared with the MFA2. In MFA2 and OLI1, both RO in vivo and in vitro and MB plus light induce SSB and FpgSS, but the induction is higher with MB. The induction of SSB by MB plus light in the TS of MFA2 is appreciably higher compared with that in the NTS or to that in OLI1 (TS or NTS). Some FpgSS are detected in the untreated DNA in OLI1 and MFA2, but are at a lower frequency compared with SSB. There are more SSB and FpgSS induced in vivo after RO plus light treatment in both strands of MFA2 compared with OLI1. After RO plus light in vivo treatment, less SSB and FpgSS are detected in both strands of MFA2 and OLI1 compared with the RO plus light in vitro treatment and the in vitro MB plus light treatment.

The data presented in Figures 1–4 show that in vivo RO plus light treatment induces SSB and FpgSS. This suggests that either the chemical or ROS produced outside the cells in the presence of the light and RO enter yeast cells. To check whether ROS like H₂O₂ are
Figure 4. SSB (left) and FpgSS (right) in relation to the nucleotide position within the OLI1-containing SspI fragment. Each histogram represents the band frequency at a precise nucleotide position and is the mean value of two independent experiments. Standard errors are not integrated to give the graphs more clarity. The profiles represent damaged sites in DNA from untreated cells (plot A); from cells treated with RO plus light (plot B); in DNA after *in vitro* treatment with MB plus light (plot C); and in DNA after *in vitro* treatment with RO plus light (plot D). The map of SspI fragment is shown and is as described in Figure 2.

Figure 5. Sensitivity of strains after RO plus light treatment. The strains studied were: wild type FF18733 strain (∗), *ogg1* mutant CD138 (■), the *rad14* mutant (▲) and the *ogg1ntg1ntg2* CD186 mutant (X). Cells were treated as described in Materials and Methods and exposed to light for different lengths of time. Cells untreated or treated with RO plus light were spread on YC plates and colonies were counted after 3 days incubation at 28°C. The results are the mean of three experiments.

produced outside the cells, the survival of various mutants defective in base or nucleotide excision repair was undertaken in the presence or absence of catalase according to protocols previously described (21,22). Similar survival for all yeast strains in the presence or absence of catalase indicates there is no detectable formation of H₂O₂ outside the cells in presence of RO plus light. Furthermore, an identical spectrum of SSB and FpgSS was found after RO plus light in the presence or absence of catalase (data not shown).

**DISCUSSION**

The first purpose of this study was to adapt the 3’ end labelling technique previously developed by us to detect CPDs at the level of the nucleotide (9,10) for the detection of oxidative damage. This would provide the basis for examining the fate of oxidative DNA damage in yeast nuclear or mitochondrial sequences at nucleotide resolution.

Here, we show that this method is a powerful tool to detect and define the spectrum of oxidative damage at the level of the nucleotide. At best, the system can detect 10⁶ copies of DNA...
other repair enzymes such as oxidative damages can be extended to detect other lesions using the quality of the sequencing gels it provides. The study of yeast homologue of Fpg protein (28), may increase the precision oxidised pyrimidines. Alternatively, the use of Ogg1 protein, the H$_2$O$_2$ outside the cell. Hence, either the chemical enters yeast plus light in the presence of catalase exclude the production of DNA oxidative damage. Data obtained after treatment with RO presence of light are capable of entering the cells to induce the chemical itself or some ROS produced outside the cells and in the oxidative damage, i.e. FpgSS and SSB. This suggests that the substrate (26). Thus LMPCR is technically very demanding and has advantages only when the amount of DNA (and hence the number of copies of a sequence) is limited, as with studies involving mammalian cells.

Our 3' end labelling technique is attractive for its simplicity and the quality of the sequencing gels it provides. The study of oxidative damages can be extended to detect other lesions using other repair enzymes such as endonuclease III (27) to detect oxidised pyrimidines. Alternatively, the use of Ogg1 protein, the yeast homologue of Fpg protein (28), may increase the precision in the definition of the damage induced by treatments like RO plus light; Fpg protein cuts with equal efficiency at fapy and at 8-oxoguanines sites while Ogg1 protein cuts preferentially at 8-oxoguanine formation in DNA with singlet oxygen as a mediator (30). In the presence of D$_2$O, the lifetime of singlet oxygen is 10 times or more longer than in H$_2$O. An experiment in the presence of D$_2$O and RO plus light would provide information on the possible involvement of singlet oxygen in the production of oxidative damage by RO plus light.

In the selected nuclear and mitochondrial reporter genes, a comparison of the spectrum of FpgSS induced in vivo or in vitro by RO plus light with that induced in vitro by MB plus light shows that virtually all FpgSS induced by the RO plus light are at the same position as FpgSS induced by MB plus light, which are at guanines. Our data and a previous analysis by HPLC (7) leads us to conclude that in both genomes, these damages are 8-oxoguanines. With respect to FpgSS, the frequency induced by MB plus light is higher than that induced by RO plus light (Figs 1–4). The RO plus light in vitro treatment gave less SSB and FpgSS compared with in vivo whilst, as for MB plus light treatment, the in vitro treatment was performed on naked DNA. This indicates that the high level of SSB and FpgSS induced by MB plus light treatment as well as the induction of different hot spots for MB compared with RO plus light is due to the chemical itself and likely due to differences in the mechanism of formation of oxidative lesions; it does not reflect a difference due to naked DNA versus chromosomally complexed DNA. This could be because MB is an intercalator, (31) whereas whether RO intercalates DNA is unknown. Furthermore, yeast metabolism could be important for the production of oxidative damage after RO plus light treatment as there is more damage induced in vivo compared with in vitro.

It should also be noted that all guanines are hit, but to different magnitudes. The fact that this observation occurs in vivo and in vitro on naked DNA leads us to conclude that the differences in induction are due to sequence context. The sequence context of hot spot damages (SSB or FpgSS) described in Table 1 indicates that most of the hot spots occur at the position of guanines when two or three guanines are present, regardless of the strand or the gene. At–181 nucleotide position, only one guanine is present but then an adenine is present in 5', Ito et al. (32) described that exposure of double-stranded DNA to 365 nm in the presence of riboflavin induced cleavages after treatment by photodimerisation specifically at the 5' site of 5'-GG'3' sequences and that the most preferred site of 5'-GGG-3' was the central guanine. Figure 6 shows the site specificity of DNA cleavage (SSB and FpgSS) after treatment by MB plus light or RO plus light in part of MFA2 and OLI1 genes. It appears clear that after MB plus light treatment, the guanine on 5'-GG-3' is more sensitive than the guanine in 3', and that with 5'-GGG-3' the guanine in the middle is more susceptible to damage. This is also true but is less specific after RO plus light treatment. The reason for this heterogeneity is unknown.

In MFA2, the TS contains more damage sites/kb than the NTS (Table 2). This primarily reflects the higher content of guanines in the TS (181 guanines) compared with the NTS (114 guanines) of the RsaI fragment. In OLI1, the NTS contains more damage sites/kb than the TS. This in part may reflect the higher number of SSB and FpgSS/kb in the TS or in the NTS of OLI1 or MFA2 fragments.

<table>
<thead>
<tr>
<th>Damaged sites/kb</th>
<th>OLI1</th>
<th>MFA2</th>
</tr>
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<tbody>
<tr>
<td></td>
<td>TS</td>
<td>NTS</td>
</tr>
<tr>
<td>SSB in U</td>
<td>0.176±0.028</td>
<td>0.223±0.028</td>
</tr>
<tr>
<td>SSB induced in vivo by RO 19-8022 + light</td>
<td>0.07±0.14</td>
<td>0.04±0.008</td>
</tr>
<tr>
<td>SSB induced in vitro by RO 19-8022 + light</td>
<td>0.019±0.003</td>
<td>0.036±0.006</td>
</tr>
<tr>
<td>SSB induced by MB + light</td>
<td>0.13±0.039</td>
<td>0.20±0.064</td>
</tr>
<tr>
<td>Fpg sites in U</td>
<td>0.04±0.008</td>
<td>0.01±0.006</td>
</tr>
<tr>
<td>Fpg sites induced in vivo by RO 19-8022 + light</td>
<td>0.05±0.014</td>
<td>0.01±0.028</td>
</tr>
<tr>
<td>Fpg sites induced in vitro by RO 19-8022 + light</td>
<td>0.02±0.004</td>
<td>0.01±0.001</td>
</tr>
<tr>
<td>Fpg sites induced by MB + light</td>
<td>1.28±0.084</td>
<td>1.67±0.139</td>
</tr>
</tbody>
</table>

DNA from untreated cells (U), DNA from cells treated by RO plus light or DNA after in vitro treatment by MB plus light or RO plus light. Calculations were as in Materials and Methods and are the mean values from two to three experiments.

(data not shown) but optimally at least 5 × 10^7 copies are preferred and the sensitivity of the assay allows the detection of damage in the range of at least 0.2 SSB/8.7 kb (Table 2), with the same efficiency in the nuclear and mitochondrial genomes.

Ligase-mediated PCR (LMPCR) is another method utilised to identify oxidative damage in nuclear and mitochondrial sequences (23,24). It involves the introduction of cuts at damage in the DNA via a repair enzyme or by a chemical treatment which generates ligatable ends; these are ligated to a linker (25). A gene-specific primer and a linker primer are then used to amplify by PCR the cut fragment. However, many types of DNA repair enzymes that cut at damage do not produce ligatable ends, like T4 endonuclease V; the cut sites require further processing to generate a ligatable substrate (26). Thus LMPCR is technically very demanding and has advantages only when the amount of DNA (and hence the number of copies of a sequence) is limited, as with studies involving mammalian cells.
of guanines in the NTS (44 guanines) compared with the TS (38 guanines) of the SspI fragment, but it also must reflect the sequence differences between this TS and NTS to account for the degree of difference observed. The frequency of damage sites/kb in OLII after treatment with RO plus light is lower than that in MFA2. Because the same is true for in vivo and in vitro treatments, we conclude that the lower induction of damages in OLII is likely to be due to sequence differences between MFA2 and OLII, rather than to a possible reduced mitochondrial uptake of RO compared with that by the nucleus. The yeast mitochondrial genome is a very AT-rich sequence and that would clearly affect the spectrum of damage. With respect to untreated DNA or DNA from untreated cells, there is a greater level of SSB in OLII compared with MFA2 (Table 2). This can be attributed to the higher production of ROS by oxidative phosphorylation in the mitochondria compared with the nucleus (33, 34).

Thus, RO plus light is a suitable regime to augment levels of oxidative DNA damage in cells for studying the removal of lesions at nucleotide resolution. After a 10 min irradiation, in repair competent cells, a 10% survival was observed with a clearly detectable spectrum of oxidative damage (data not shown). Thus, experiments can be undertaken at reasonable survival levels to monitor both the repair of these damages and, in the case of OLII, their biological consequences with respect to mutations. The survival experiments (Fig. 5) show that the wild type, ogg1 strains express the same sensitivity whilst rad14 and the triple mutant ntg1ntg2ogg1 exhibit a significantly higher sensitivity indicating roles for nucleotide and base excision repair in the removal of DNA damage induced by RO plus light. The same studies have been performed in presence of RO alone or light only. No decrease in sensitivity for any strains was observed (data not shown). Recent data suggest that Ntg1, the yeast homologue of Endonuclease III from E. coli, is coded by the same gene as Ogg2. Ogg2 is the yeast second 8-oxoguanine DNA glycosylase activity which is different from Ogg1 in its substrate specificity and which may be involved in the incorporation of 8-oxo dGTP during replication (35). The study of survival in mutants like ntg1, ntg2, ntg1ntg2, rad14ogg1, ntg1ntg2rad14 or ntg1ntg2ogg1rad14 will certainly provide important information on the contribution of these genes to the removal of DNA oxidative damage.

We are now examining the repair of these lesions at nucleotide resolution in repair competent strains and in mutants defective in nucleotide and/or base excision repair. To date, studies have shown that nucleotide excision repair and base excision repair (1–3, 20) are involved in the removal of oxidative damage from nucleotide sequences and that transcription coupled repair has a role (36). In mitochondria, there is no evidence for nucleotide excision repair (37), but base excision repair operates. The contributions of these processes to the repair of oxidative damage to DNA in yeast can now be dissected.

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REFERENCES