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# Detection of tandem CC → TT mutations induced by oxygen radicals using mutation-specific PCR

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**Abstract**

DNA lesions caused by reactive oxygen species (ROS) are considered to be one of the major contributors to DNA damage and mutagenesis. In this study, we developed a modification of allele-specific PCR to detect CC → TT mutations caused by oxidative damage. These tandem mutations have been previously demonstrated to be indicative of oxygen damage in the absence of UV-irradiation. Using a CC target site in the rat DNA polymerase β (pol β) gene and a thermostable restriction enzyme that cuts the wild type sequence but not the TT mutation, we demonstrate that the TT mutation can be preferentially amplified from plasmid DNA damaged by oxygen radicals but not other DNA-damaging agents. We evaluated the potential utility of this assay in screening for mutations in cells and in analyzing those that arise during clonal proliferation in carcinogenesis. 

*Keywords:* Oxygen free radical; DNA damage; Oxygen mutagenesis

## 1. Introduction

Oxygen, an element required for life, is mutagenic in a wide range of organisms. Active or free radical oxygen intermediates, collectively referred to as reactive oxygen species (ROS), act directly or indirectly to damage cellular DNA. Partially reduced oxygen molecules, including the hydroxyl ($\cdot$OH) and superoxide radicals and hydrogen peroxide ($H_2O_2$), are generated in vivo by the stepwise reduction of dioxygen to water. These ROS and singlet oxygen ($^1O_2$) are products of metabolic processes including mitochondrial respiration, cytochrome *P*450 reactions, and the oxidative burst from activated inflammatory cells [1]. They are also generated by ionizing radiation, xenobiotic agents and some transition metals [2–4]. The oxygen intermediates produced by these processes are highly reactive and damage neighboring molecules, including DNA.

The array of chemical modifications, which occur in DNA, damaged by ROS leads to a broad spectrum of mutations [5]. These DNA modifications have been postulated to play a role in the initiation and progression of cancer [6,7] and the decline of cellular function associated with aging [8,9] as well as with many degenerative diseases [10]. However, a direct correlation between these physiological events and

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ROS-induced mutations has been difficult to establish, in part because the mutation spectrum produced by ROS is difficult to distinguish from errors in DNA synthesis as well as from other mutagenic insults.

Previous studies from this and other laboratories have indicated that the most common mutation resulting from oxidative damage to DNA is a C → T transition [11–16]. But because this mutation is not unique to oxygen damage, it is not useful as a biomarker for assessing the role of oxidative damage in disease processes. However, we have also accumulated evidence that a less frequent double tandem mutation might be diagnostic for oxygen damage. Using single-stranded DNA in either a forward mutation or reversion assay, CC → TT mutations have been shown to occur as a result of oxygen-mediated DNA damage generated with metal ions [14,17,18] or by ROS produced by activated human leukemia cells [17]. Since ultraviolet light is the only other mutagen demonstrated to cause CC → TT mutations [18,20,21], these mutations are an ideal candidate to serve as a marker for oxidative damage in cells and/or tissue not exposed to UV.

Double tandem CC → TT mutations induced by UV-irradiation have been detected in human genomic DNA using a technique referred to as mutant allele-specific PCR or PCR amplification of specific alleles [21]. The methodology for this technique has been reviewed in detail [22]. The basic concept of the assay relies on the fact that PCR primers which contain a 3′-terminal or penultimate mismatched base are not an efficient substrate for DNA polymerases. Thus, it is possible to design PCR primers which will preferentially amplify a mutant allele in the presence of a large excess of the wild type allele; the alleles need only vary by 1 or 2 bp.

In this study, we have used mutant allele-specific PCR to establish the occurrence of CC → TT mutations resulting from oxidative damage to double-stranded DNA. This assay preferentially amplifies a mutant allele containing this double tandem transition. Our approach includes a modification of the earlier technique that has been applied to demonstrate mutations in the *p53* gene [21] and to determine polymorphisms or haplotypes in human populations [22]. In these applications, the mutations detected were present at a relatively high frequency relative to the wild type sequence. The modification we present relies on a thermostable restriction enzyme that specifically cleaves the wild type sequence and enriches the population of mutant molecules to increase the sensitivity of detection. Using this approach, we demonstrate that CC → TT mutations occur in double-stranded plasmid DNA in response to damage with metal ions plus $H_2O_2$ but not with an alkylating agent. We also demonstrate the advantages and limitations of the modified mutant allele-specific PCR in assessing mutation frequency in human genomic DNA.

## 2. Materials and methods

### 2.1. Bacterial strain and plasmid

*E. coli* strain MC1061 (*hsdR, mcrB, araD, 139* Δ(*araABC-leu*), 7679Δ *lacX74, galU, galK, rpsL, thi*) was the host strain for all experiments. The plasmid used for this study, pBL, was obtained from Dr. Joann Sweasy (Yale University). It is derived from pHSG576, a low copy number plasmid which contains the pSC101 replicon, the chloramphenicol resistance gene [23] and the rat DNA polymerase β gene (pol β).

### 2.2. DNA damage and transfection

For treatment with metal plus $H_2O_2$, 1 μg pBl in 50 μl of water with the indicated concentrations of metal ion and $H_2O_2$, was incubated for 30 min at 37°C in a loosely capped microfuge tube. When $Ni^{2+}$ was used to generate ROS, the tripeptide glycine–glycine–histidine was included to enhance the production of ROS [24,25]. After incubation, the $H_2O_2$ and metals were removed by placing the 50-μl reaction mixture in a microconcentrator with a 30,000-molecular weight cutoff (Amicon) and washing three times with 8 vol. of water. Plasmid DNA (100 ng) was then electroporated into 80 μl SOS-induced MC1061 cells [14] ($OD_{600}$ approximately 0.8) using a BioRad Gene Pulser (25 μF, 400 W, 2.0 kV; time constants of 8.0–9.0).

For treatment with the alkylating agent, methyl methanesulfonate, 100 ng plasmid DNA was suspended in 100 μl 10 mM Tris–EDTA (pH 7.4) containing the indicated concentration of MMS. The samples were incubated for 20 min at 37°C and

washed in a microconcentrator as described above. The plasmid (40 ng) was transfected into 40 μl SOS-induced MC1061 cells.

For UV-irradiation, 200 ng of plasmid DNA was suspended in 30 μl 10 mM Tris–HCL (pH 7.4) and irradiated with 254 nm light in an uncovered plastic Petri dish. Thereafter, 50 ng of UV-irradiated plasmid was electroporated into 40 μl SOS-induced MC1061 cells.

After transfection, the cells were incubated in 1 ml Luria broth (LB) for 1 h at 37°C then transferred to 20 ml LB with 30 μg/ml chloramphenicol and incubated at 37°C. After 24 h, the plasmid DNA was recovered using a Promega mini-prep kit.

### 2.3. Tissue culture and oxidative damage to HeLa cells

HeLa cells were grown in Dulbecco's modified Eagle's medium (DMEM) supplemented with 10% fetal bovine serum (FBS) at 37°C in a humidified incubator (6% $CO_2$). To induce oxygen-mediated DNA damage, approximately $2 \times 10^7$ cells in a 75-$cm^2$ flask cells were rinsed twice with phosphate-buffered saline, then treated with 20 μM $H_2O_2$ in serum-free DMEM for 30 min. The cells from four replicate cultures and four untreated control cultures were harvested by trypsinization and centrifuged at $1000 \times g$. The cell pellets were rinsed twice with ice-cold PBS and then genomic DNA was extracted using phenol:chloroform and ethanol precipitation.

### 2.4. Mutant allele-specific PCR

Mutant allele-specific PCR has been used to detect CC → TT mutations in the *p53* gene of cultured human keratinocytes exposed to UV-irradiation or in sun-exposed human skin [21]. We have modified this approach by using a thermostable restriction enzyme and DNA pol β as a target gene. The target site for detecting CC → TT mutations in double-stranded plasmid DNA is in exon 13 of the rat DNA pol β gene at the region corresponding to the cDNA at positions 987–988 [26]. The target cytosines at this locus constitute part of a restriction site for the thermostable restriction assay, *Bsa*J1 (United States Biochemical). The recognition site for this enzyme is C ^CNNGG. Thus, the enzyme will cut the wild type sequence but not the mutant. The target site for detecting CC → TT mutations in human genomic DNA is also in exon 13 of the human pol β gene at the region corresponding to the cDNA at 1012–1013 [27,28]. Since the human and rat genes are highly homologous; the target site in the wild type human pol β gene is also a substrate for the thermostable restriction enzyme.

Before the human pol β target site from genomic DNA was assayed in the mismatch PCR, all of exon 13 of pol β was first amplified using complementary primers [29]. Each 50 μl reaction mix contained buffer (50 mM Tris, pH 8.3; 12.5 μg bovine serum albumin; 0.5% Ficoll; 15 mM $MgCl_2$), 200 μM of each of the four dNTPs, 1 μM each primer, 1.5 μg bovine serum albumin, and 1 U Taq polymerase (Perkin-Elmer). The denaturing temperature used was 94°C and the annealing temperature was 45°C. Because the capillary PCR machine allows virtually instantaneous heat transfer, these temperatures were held for less than 1 s. The temperature during the 30-s elongation phase was 75°C. The resulting 141-bp fragment was purified on a NuSieve agarose gel (FMC), restricted with the *Bsa*J1 restriction enzyme, and used in the mismatch PCR amplification.

We assayed the CC target site from three different contexts in the mismatch assay. First, in order to validate the assay, we used a synthetic oligonucleotide template that was homologous to the human pol β CC target site. Secondly, we used whole plasmid DNA containing the rat pol β gene. Thirdly, we used the 141-bp fragment amplified from human genomic DNA as described above. All primers and oligonucleotide templates were prepared by Operon Technologies (Alameda, CA); the sequences are given in Table 1.

All of the allele-specific PCR reactions were carried out under the same conditions in 10-μl capillary tubes in an air-cooled thermocycler (Idaho Technology). Each 10 μl reaction mixture contained 50 mM Tris–HCl (pH 8.3); 12.5 μg bovine serum albumin; 0.5% Ficoll; 3 mM $MgCl_2$, 25 μM of each of the four dNTPs, 0.2 μM 5q-$^{32}$P-end-labeled complementary primer, 0.2 μM mutant allele-specific primer, and 0.1 U Taq polymerase. The reactions also contained 1 mM tartrazine dye to facilitate loading into capillary tubes [30]. The indicated amount of template DNA was included in each

Table 1
Oligonucleotide templates and primers

| | |
|---|---|
| *Templates* | |
| Human pol β | 5′-CTC ATG CCC TAG AAA AGG GTT TCA CAT ACA CCA TCC GTC CCT |
| homolog (wild type) | TGG GAG TCA CTG GT-3′ |
| Human pol β | 5′-CTC ATG CCC TAG AAA AGG GTT TCA CAT ACA CCA TCC GTT TCT |
| homolog (mutant) | TGG GAG TCA CTG GT-3′ |
| Rat pol β | 5′-CGC ATG CCC TGG AAA AGG GCT TCA CAA TCA CGA TCC GCC CCC |
| homolog (wild type) | TGG GGG TCA CTG GG-3′ |
| Rat pol β | 5′-CGC ATG CCC TGG AAA AGG GCT TCA CAA TCA CGA TCC GCT TCC |
| homolog (mutant) | TGG GGG TCA CTG GG-3′ |
| *Primers* | |
| Human exon 13 A | 5′-GAT CAG TAT TAC TGT GGT GT-3′ |
| Human exon 13 B | 5′-CAT GGA CAC TCA CCA GTG AC-3′ |
| Human upstream | 5′-CTC ATG CCC TAG AAA AGG GTT TCA-3′ |
| Human wild type-specific | 5′-ACC AGT GAC TC CAA GGG-3′ |
| Human mutant-specific | 5′-ACC AGT GAC TC CAA GAA-3′ |
| Rat upstream | 5′-CGC ATG CCC TGG AAA AGG GCT TCA-3′ |
| Rat wild type-specific | 5′-ACC AGT GAC CCC CAG GGG-3′ |
| Rat mutant-specific | 5′-ACC AGT GAC CCC CAG GAA-3′ |

reaction along with 1 unit of the thermostable restriction enzyme, *Bsa*J1, where stated. The denaturing and annealing temperatures were 94 and 55°C, respectively, with a 30-s interval at 75°C for primer elongation.

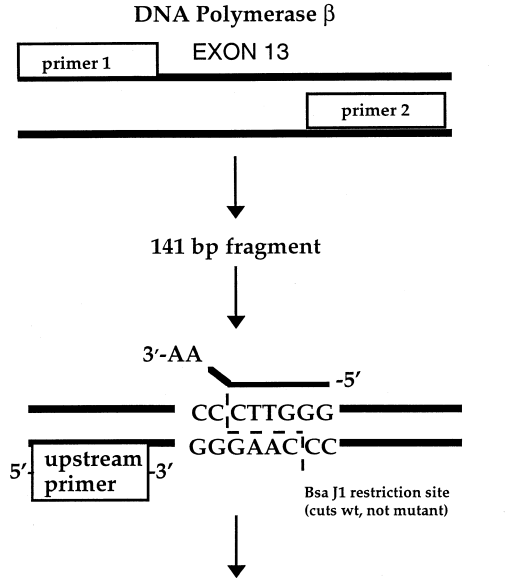


Fig. 1. Scheme of mismatch PCR assay for the detection of CC → TT mutations in the DNA pol β gene.

The PCR products were run on a 3.5% Metaphor agarose (FMC) gel or a 10% native acrylamide gel and analyzed with a PhosphorImager Model 400 S using ImageQuant software (Molecular Dynamics). The linear range of detection spans at least five orders of magnitude [31]. All data presented was verified in at least three independent experiments.

The scheme for the mismatch PCR is outlined in Fig. 1. The figure illustrates the entire nested PCR used for mutation detection from HeLa cell DNA. The second step of the PCR is the mismatch detection step. When using synthetic oligonucleotide or plasmid DNA template, the allele-specific PCR was employed directly, omitting the first round of amplification.

## 3. Results

### *3.1. Detection of CC → TT mutations in oligonucleotides*

In order to optimize the mutant allele-specific PCR assay with respect to the target site in pol β, we

first utilized synthetic oligonucleotides to serve as template DNA. These oligomers corresponded to the part of exon 13 of the human pol β sequence including nucleotides 964–1025 [27] plus the first two nucleotides of intron 13 [28]. We excluded the portion of the sequence between nucleotides 994 and 1002 so the PCR reaction product generated from the 56-mer would be nine bases shorter and thus distinguishable from that generated from human genomic DNA. This avoids the potential problem of cross-contamination by human DNA in the PCR reaction. Two templates were constructed that were identical except that one contained the wild type (CC) target sequence and the other contained the TT mutant sequence. The sequence of each of these is given in Table 1.

The mutant allele-specific PCR is dependent on the fact that the polymerase used does not efficiently extend two mispaired bases on the 3′ end of the primer. Although the frequency of mismatch extension is very low, it is not zero. Mismatch extension places an upper limit on the number of wild type molecules which can be added to the PCR reaction before amplification of the mismatched primer on the wild type target generates a high background. That is, since the frequency of extension of a 3′ mismatch primer is about $10^{-5}$, the addition of $10^{7}$ wild type molecules will theoretically generate 100 molecules of misextended product in the first round of PCR that will be exponentially amplified thereafter. Under our conditions, we include up to $10^{6}$ wild type oligomers without generating significant background amplification from the mutant primer (Fig. 2).

By mixing varying numbers of molecules of the mutant template oligomer with the wild type, we have determined that our assay is sufficiently sensitive to detect one mutant molecule in the presence of $10^{6}$ wild type molecules (Fig. 3).

### 3.2. Detection of ROS-induced CC → TT mutation

We used the allele-specific PCR assay to detect the double tandem mutation in plasmid DNA which had been treated with ROS. The pβL plasmid used, a derivative of pHSG576, is a low copy number plas-

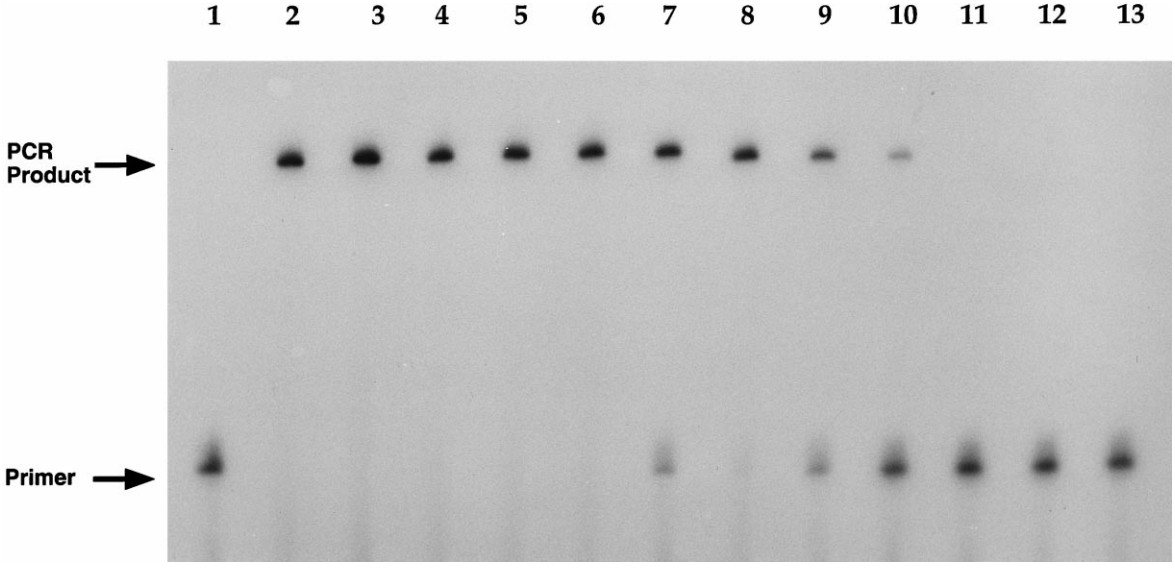


Fig. 2. Limits of detection of the mismatch PCR assay. Decreasing numbers of molecules of the 56-nt oligomer homologous to human wild type pol β containing a CC at the target site were amplified by either the matched wild type primer (lanes 2 through 7) or mismatched primer (lanes 8 through 13). Lane 1 is the PCR reaction with the wild type primer in the absence of template DNA. Lanes 2 and 8 contain $10^{9}$ template molecules and each subsequent lane represents a 10-fold dilution of template molecules. Amplification from the mismatched mutant primer is detected when $10^{7}$ wild type template molecules are included in the reaction (lane 10), but not when $10^{6}$ wild type molecules are present (lane 11).

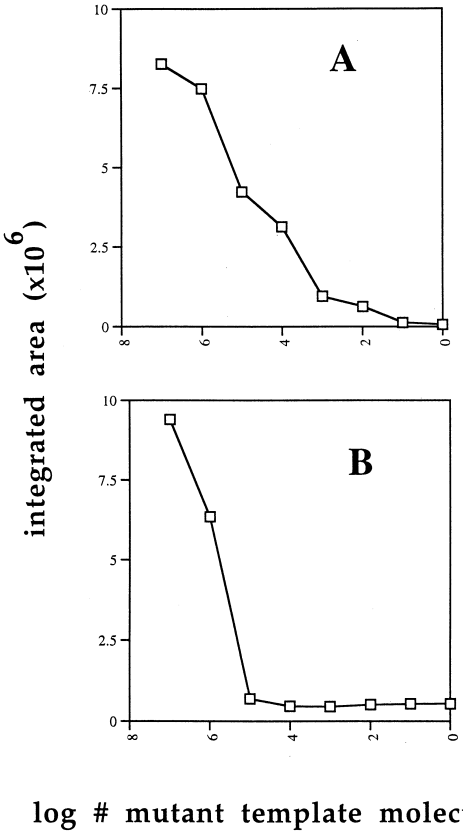


Fig. 3. Amplification of rare mutant molecules in the presence of excess wild type molecules. The number of wild type molecules in each PCR reaction were held constant at $10^6$, and decreasing concentrations of mutant molecules were added to determine the sensitivity of identifying a few mutant molecules present in a population of wild type molecules. Under these conditions, we can detect 1–10 mutant molecule in a population of $10^6$ wild type molecules (A). When the number of wild type template molecules present is increased to $10^7$, the ability to distinguish fewer than 10,000 mutant molecules is lost (B).

mid carrying a chloramphenicol resistance gene and the rat DNA pol β gene. The rat pol β gene contains the CC target site at positions 787–788 [26]. The plasmid was grown in *E. coli* strain DH5α and isolated therefrom. Purified plasmid DNA was then exposed to ROS generated by transition metal ions plus hydrogen peroxide. Plasmid DNA was also exposed to the alkylating agent, MMS, to assess the specificity of damage as well as the possible contribution of single base mutations that might facilitate extension of the primer and result in false positives in our assay. After the reactions were terminated, the DNA was transfected into SOS-induced *E. coli* strain M1061. The transfected bacteria were grown overnight in broth culture under chloramphenicol selection. The following day, the cultures were harvested and plasmid DNA isolated for the mutant allele-specific assay.

The PCR reactions were performed as described in Section 2. Equal amounts of plasmid DNA (50 ng) from each treatment group were pre-digested for 1 h at 50°C with 1 U *Bsa*J1 in the restriction enzyme buffer supplied by USB. The digest was diluted 1:1000 in PCR buffer and an aliquot containing $10^7$ restricted copies of plasmid DNA was used in each PCR reaction. The entire 10-μl reaction volumes were run out on a gel and visualized by PhosphorImager analysis (Fig. 4). The products from the PCR reaction containing the primer complementary to the mutant sequence, indicative of the amount of mutant (at the CC target site) pol β molecules in the sample, were quantitated. Compared to plasmid DNA not exposed to ROS, DNA which has been UV-irradiated (600 J/m$^2$) showed a marked increase in band intensity (data not shown), indicating the occurrence of the double tandem mutation as has been previ-

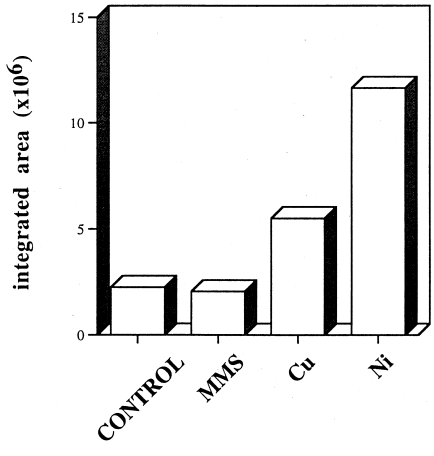


Fig. 4. CC → TT mutations in double-stranded plasmid DNA. Plasmid DNA containing the CC target site in the rat pol β gene was exposed to either the alkylating agent, MMS, or to $Cu^{2+}$ and $H_2O_2$, or $Ni^{2+}$ and $H_2O_2$ plus GlyGlyHis, as described in Section 2. The plasmid was then transfected into SOS-induced *E. coli* and allowed to replicate. The plasmid DNA was recovered and the relative amount of CC → TT mutations compared by mismatch PCR.

ously described for mutagenesis with UV light [20,21].

To generate ROS, we used $H_2O_2$ in the presence of different transition metals. These combinations generate the very reactive hydroxyl radical via a metal-catalyzed Fenton reaction [1]. When DNA was incubated with $Cu^{2+}$ plus $H_2O_2$, a 2.5-fold increase in band intensity was evident. As was previously observed in single-stranded DNA [19], $Ni^{2+}$ plus $H_2O_2$ and the tripeptide, glycine–glycine–histidine, produces an even greater number of CC → TT mutations as shown by the 5.2-fold increase in band intensity (Fig. 4).

This increase in CC → TT mutations was not observed in plasmid DNA exposed to MMS. This agent has been shown to cause single base mutations, predominately C → A substitutions, at a CCC reversion site in the hisG46 allele of *Salmonella typhimurium* [32]. Thus, a DNA-damaging agent which does not function via an oxidative radical mechanism does not generate CC → TT mutations or yield a positive result in our assay.

### 3.3. Detection of CC → TT mutations in human genomic DNA

Since we were able to demonstrate the presence of the CC → TT mutation in double-strand DNA, the next step was to determine whether we could detect the mutation in human genomic DNA from cells that had been exposed to oxidative stress and allowed to replicate. Using the same target site in the pol β gene and using the nested PCR approach described in Section 2, we compared the levels of PCR product obtained with the mutant-specific primer using 200 ng template DNA from control HeLa cells vs. HeLa cells damaged with 20 μM $H_2O_2$ and allowed to replicate for up to six passages in culture. This represents DNA pooled from approximately 32,000 cells or 64,000 initial copies of the DNA pol β gene.

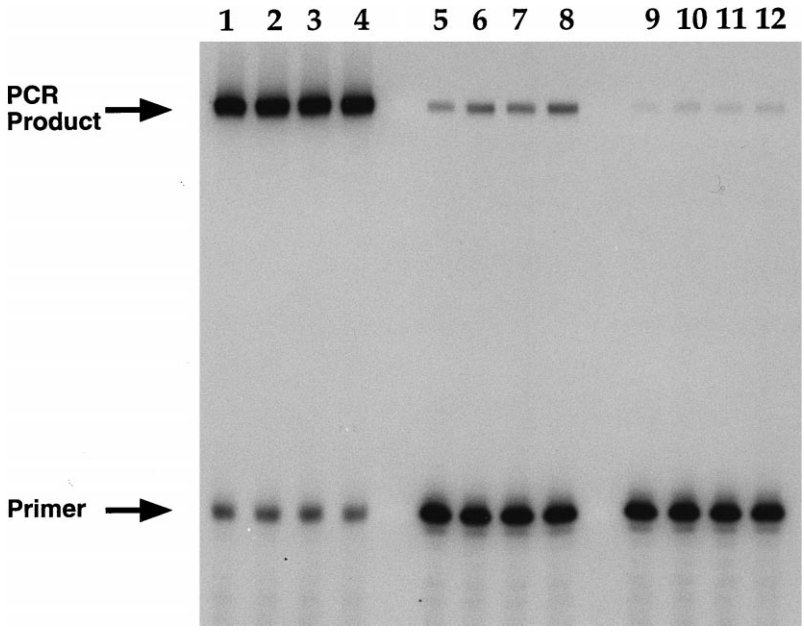


Fig. 5. Application of mismatch PCR to human genomic DNA. HeLa cells treated for 30 min with 20 μM $H_2O_2$ or irradiated with 20 J/m$^2$ UV were allowed to replicate for the indicated number of passages in culture before harvesting the DNA for PCR analysis. This is the 66-bp product from the second step of the nested PCR reaction. Lanes 1–4 are the products from the wild type primer using DNA from untreated control cells (lane 1), from cells damaged with $H_2O_2$ and allowed to replicate for three (lane 2) or four (lane 3) passages in culture, or from cells damaged with UV and allowed to replicate for six passages. This demonstrates equivalent amounts of DNA in each reaction. Lanes 5–8 are equivalent amounts of DNA from the same treatment groups amplified with the mutant primer. Lanes 9–12 represent a 10-fold dilution of the template concentration compared to lanes 5–8. The uniform decrease in band intensity indicates that the reactions are within the linear range of the PCR. There is no difference in band intensity between the treatment groups.

If the TT mutation were present in any cell, it should be amplified along with the rest of exon 13 in the first step of the nested PCR and thus be detectable in the subsequent step of mismatch PCR. Under these conditions, we were unable to detect an increase in band intensity over the background of the assay using the mutant-specific primer (Fig. 5). However, we were also unable to detect an increase over background in DNA from a similar number of cells irradiated with 20 $J/m^2$ UV that were also allowed to replicate for six passages after irradiation.

## 4. Discussion

Previous work from this laboratory has provided a strong argument that CC → TT mutations are diagnostic for oxygen damage. This work encompassed two different assays. The first, a forward mutation assay developed by Kunkel [33], uses the *lacZα* gene of *E. coli*, encoded in the DNA of bacteriophage M13mp2, as a target for mutation. Single-stranded M13mp2 DNA was treated with ROS in vitro, then transfected into SOS-induced *E. coli*, and tested for loss of α complementation. Mutants were then sequenced to determine the mutational spectrum. The second assay, a reversion assay referred to as M13G*1 [34] also used single-stranded M13mp2, but the target was a mutant *lacZα* gene containing an altered codon (GCC → CCC) at position 141–143. Using these two protocols, the mutational spectra of ROS produced by several different methods demonstrated that the CC → TT mutation occurs in runs of three or more cytosines in response to ROS generated by $Cu^{2+}$, $Fe^{2+}$, $Ni^{2+}$, and by the human leukemic cell line, HL-60 [16–19]. When M13G*1 DNA was treated with $H_2O_2$ plus $Ni^{2+}$ in the presence of GlyGlyHis, 20% of all mutants produced were CC → TT tandem mutations. This was the most efficient system for inducing CC → TT mutations [19].

The work described here presents additional evidence that double tandem CC → TT mutations are indicative of oxygen damage in the absence of UV light. We have shown that plasmid DNA carrying a eukaryotic DNA pol β gene damaged with reactive oxygen demonstrated an increase in this mutation when replicated in SOS-induced *E. coli*. This extends our previous work to show that the double tandem mutation also occurs in double-stranded DNA. This is significant in demonstrating the potential for using the mutation as a signature for oxidative damage in vivo and addresses the concern that the oxygen-induced CC → TT results from an adduct unique to single-stranded DNA [35].

The assay we have developed can in principle detect one tandem CC → TT mutation in less than $10^6$ nucleotides. Since the assay is directed to a single site it can only be utilized for the detection of mutations that occur with relatively high frequency. Such mutants are likely to be infrequent in the absence of clonal selection. In contrast to UV-induced CC → TT mutations in the *p53* gene amplified from patients with skin cancer [21], mutations at our target site in the pol β gene may not confer a selective growth advantage. Thus, the mutation would not be clonally expanded and would remain below our current limit of detection. No increase in CC → TT mutations were obtained using DNA obtained from cells exposed either to oxygen free radicals or to UV-irradiation. Since others have clearly demonstrated that UV-irradiation results in CC → TT mutations [21,21] we concluded that our assay lacked the sensitivity to detect this mutation at a single specific site in the genome.

At least two factors set a lower limit on the sensitivity of detection of mutations in genomic DNA using allele-specific PCR. The first is that errors generated during the first round of the nested PCR increase the background of the assay. This is suggested by the lower background observed using synthetic oligonucleotide templates or plasmid DNA compared to DNA amplified from a genomic sequence. The second, and probably the overriding factor, is that while mismatched primers are a poor substrate for DNA polymerase, they are still amplified at a low frequency. The frequency of extension of a single 3′-mismatch is about $10^{-5}$ [36].

Though the use of the thermostable restriction enzyme to eliminate the wild type DNA does not offer sufficient advantage for the detection of TT mutations in cells exposed to ROS or UV, it can be used to enhance the specificity of allele-specific PCR when used for detection of germline mutations or haplotypes. This modification could be especially useful in the application of allele-specific PCR to

screening populations of individuals for rare polymorphisms. In this assay, genomic DNA from individuals is pooled and amplified with primers specific for a rare variant DNA sequence [22]. The use of the thermostable enzyme should increase the number of individuals that can be included in the DNA pool and thus improve the throughput of that assay.

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