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Novel mitochondrial deletions in human epithelial cells irradiated with an FS20 ultraviolet light source in vitro

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Abstract

The formation of mitochondrial deletions brought about by FS20 irradiation was examined in a line of human epithelial cells. In this system, Mt DNA deletions were found to be induced within 24 h following a single irradiation as low as 1.4 mJ/cm^2 from an FS20 light source. We observed at least two distinct FS20-induced Mt deletions: the widely observed 4977 bp common deletion (CD) and a novel 5128 bp deletion flanked by TAGG repeats at nt 8247–8250 and 13,375–13,378 that has not, to our knowledge been previously described. While glutathione (GSH) at concentrations between 50 and 200 μM was found to block the formation of the CD in a dose-dependent manner, GSH at higher concentration induced the CD even in unirradiated cells. This is consistent with the idea that low dose GSH inhibits CD formation via reductive elimination of peroxides while higher concentrations may act as an electron donor to produce reactive oxygen species. Interestingly, in irradiated cells, GSH at intermediate concentrations (50 μM) induced a second, shorter, deletion similar to the novel deletion induced by FS20 alone but involving a different TAGG repeat spanning nt 13,175–13,178.

Keywords: Mitochondria; Tetrameric repeats; Deletions; UVB; Keratinocytes

1. Introduction

Mitochondrial DNA is highly susceptible to mutation. Many of the thousands of mitochondrial mutations that have been catalogued are deletions that often encompass large DNA segments. Mitochondrial deletions are known to accumulate with age [1–4] and are associated with a variety of pathological conditions. One widely observed deletion, encompassing a 4977 bp segment and known as the common deletion, has been associated with diseases of the nervous system [5–8], heart and eye muscle [9–12], thyroid [13–16], kidney [17–18] and skin [19–23]. While some studies suggest that the deletions may play a causal role in the development of the diseases with which they are associated, in most cases a direct causative link has not been established. On the other hand, there is substantial evidence that mitochondrial deletions result from DNA damage due to reactive oxygen species (ROS) including free radicals or superoxides [24,25] and that the presence of mitochondrial deletions is itself indicative of oxidative stress in the cells that harbor them. Given the widespread interest in the relationship between mitochondrial deletion and their associated disease states, it is surprising that there are so few reports which utilize cell culture systems to study the formation of the deletions in vitro. Here we have employed a model system of cultured human epidermal keratinocytes to study the induction of mitochondrial deletions after exposure to ultraviolet light. We employed this system to study the formation of mitochondrial deletions following irradiation with an FS20 ultraviolet light source. In the irradiated cells we not only observed induction of the common deletion but also of at least one novel deletion. A second novel deletion was observed in cells treated with the antioxidant, glutathione.

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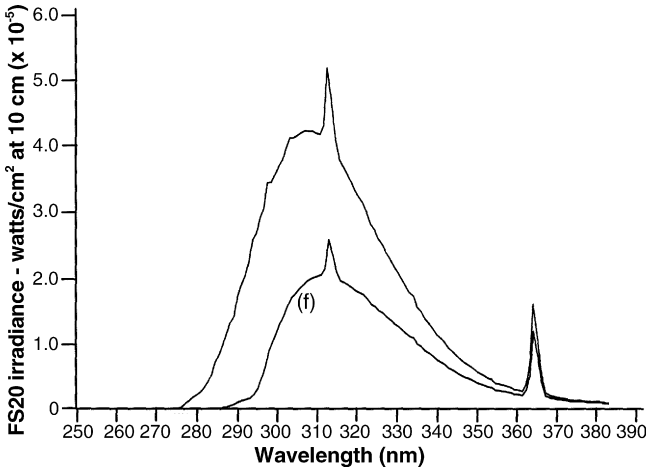

Fig. 1. The FS20 irradiation spectrum. FS20 irradiance in air, and through the wall of the plastic tissue culture flask (f) is shown.

2. Experimental/materials and methods

2.1. Cell culture and irradiation

Line 22 immortalized human epidermal keratinocytes at passages 31–39 were used for all experiments. The cells were grown in DMEM supplemented with 15% fetal bovine serum and hydrocortisone as previously described [26–28]. For irradiation, cells were grown to about 35% confluence in T25 flasks and irradiated from the bottom with an FS20 lamp (Light Source, Inc.) at a distance of 35 cm for various times as indicated. The FS20 covers the UVB range (290–320 nm) with an emission spectrum between 272 and 390 nm and a λ_{max} of 312.5 nm. Absorption of light by the plastic wall of the culture flask (Fig. 1) was measured using a Cary 500 spectrophotometer (Varian, Inc.); the flask absorbed more than 85% at wavelengths below 295 nm and about 51% of the light at λ_{max}.

2.2. Identification of mitochondrial DNA deletions

Deletion analysis was carried by the PCR-based technique of Soong and Arnheim [29] using the amplimer pair MT2 and MT1C for amplification of non-deleted sequences (nt 13,175–13,500; revised Cambridge reference sequence, GenBank no. NC_001807). The nested amplimers MT3 and MT2 paired with MT1A were used to detect mitochondrial fragments representing deletions within the segment spanning nt 8224 and 13,500. For sequencing, PCR reaction mixtures were run on low melting point agarose gels and the bands were visualized with a transilluminator and excised from the gel with a scalpel. The excised bands were used as templates for PCR reamplification with the appropriate primers. The reamplified PCR products were then precipitated 1 h with 1 volume of 4 M ammonium acetate and 2 volumes of isopropyl alcohol. The precipitate was pelleted and the pellet was washed with 70% ethanol, air dried and resuspended in TE buffer and sequenced in the RCMI sequencing facility at City College using the amplimers as a sequencing primers. The locations of the deletion(s) boundaries were determined by alignment of the PCR product sequence with the Cambridge mitochondrial DNA reference sequence.

2.3. Real-time PCR

The Roche LightCyler for Real-Time PCR was used to measure total mitochondria and the common deletion. Each capillary contained 1X LightCycler® FastStart DNA Master HybProbe, 300 nM each forward and reverse primer, 200 nM probe and 0.1 unit heat-labile uracil-DNA glycosylase (Roche Applied Science) in a total volume of 10 μl. The probe for the ensuing amplicon is located at nucleotides 13,461–13,480 of the Cambridge sequence and is tagged with the reporter FAM (6-carboxy fluorescein, λ_{max} 518 nm) at the 5′ end and the quencher BHQ1 at the 3′ end. Pogozelski et al. [30] designed two plasmids, one that contains the amplicon for the common deletion and the other that contains the amplicon for total mitochondria. In each run, as appropriate, the plasmids were run as external controls. For the common deletion, copy number per cell was determined from the plasmid control copy number and its threshold cycle.

3. Results

3.1. Mitochondrial deletions induced by FS20 in vitro

We used the technique described by Soong and Arnheim [29] to study the formation of mitochondrial deletions in vitro after FS20 irradiation of a line of human epithelial cells. This analysis makes use of the fact that large deletions result in the fusion of normally distal DNA segments so that PCR products from primers located within these distal segments generate aberrantly short products. In these experiments, we found that irradiation of line 22 keratinocytes with an FS20 light source lead to the induction of mitochondrial deletions within 24 h after a single exposure (Fig. 2). Irradiated cells were found to exhibit at least two distinct mitochondrial deletions that generated corresponding PCR products of about 300 and 150 bp with the MT1A and MT2 amplimer pair (Fig. 2, upper and middle panels). These experiments also showed that induction of the deletions increased in a manner related to irradiation dosage over exposure times ranging between 1 and 8 min (1.4–11.4 mJ/cm^2) although maximal band intensities were generally seen after 4 min of irradiation (5.7 mJ/cm^2) and all subsequent experiments were carried out using an irradiation time of 4 min. Fig. 3 also illustrates that, in the in vitro system, the intensity of the PCR bands corresponding to each deletion varied independently from one another and from one experiment to the next but were consistent within each experiment. We generally observed that one type of deletion predominated in a given experiment although in some experiments PCR products for both deletions were present in about equal quantity (one example is shown in Fig. 4, lower panel). PCR products corresponding to mitochondrial genomes carrying deletions could only be seen in the nested PCR reactions indicating that they represent only a small fraction of the total mitochondrial pool.

Real-time PCR showed that the common deletion was present even in non-irradiated cells but only at a level of

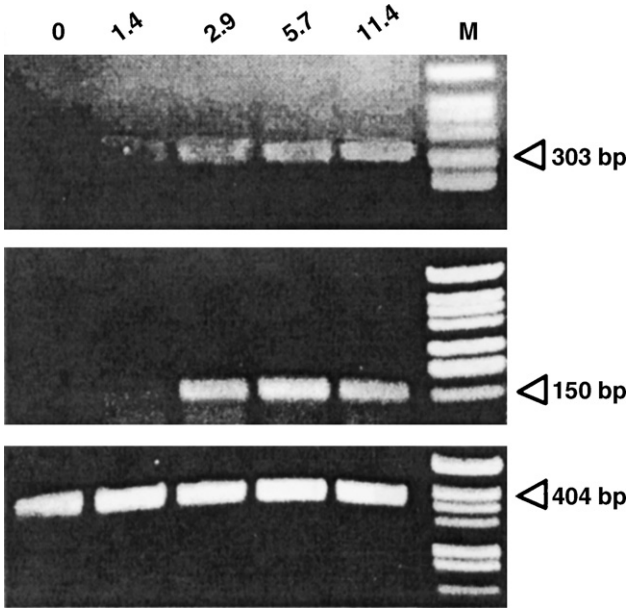

Fig. 2. Induction of mitochondrial deletions in cells exposed to an FS20 light source. T25 culture flasks of immortalized human keratinocyte line 22 cells were irradiated for various times ranging between 1 and 8 min with the FS20 lamp as described in Section 2. Twenty-four hours later, total DNA was prepared from the cells and used as a PCR template for deletion analysis using the amplimer pair, MT2/MT1A (upper and middle panels). Numbers represent radiation dosage in mJ/cm^2. Upper panel: dose-dependent appearance of a 303 bp PCR band derived from the 4977 bp common deletion; arrowhead points to the 298 bp marker band. Middle panel: dose-dependent appearance of a 150 bp PCR product corresponding to a novel 5128 bp deletion (Δ_{uv}); arrowhead points to the 154 bp marker band. Lower panel: presence of non-deleted mitochondrial sequences in irradiated cells. A 404 bp PCR product generated from the MT3/MT1C amplimer pair is seen in all lanes; arrowhead points to the 394 bp marker band.

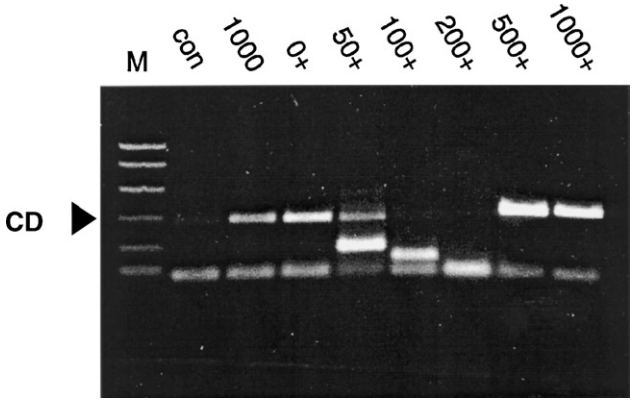

Fig. 3. Effect of glutathione pretreatment on formation of the FS20-induced mitochondrial common deletion. Line 22 cells at about 35% confluence were pretreated with glutathione. Twenty-four hours later cells were uv irradiated with an FS20 lamp for 4 min (5.7 mJ/cm^2). DNA was harvested 24 h after irradiation and analyzed for mitochondrial deletions. The numbers indicate the concentrations of glutathione in micromolar. Symbol '+' indicates that the cells were irradiated after pretreatment with glutathione; control cells (con) were neither irradiated nor pretreated. Arrowhead points to the 300 bp marker band; marker bands of 150 and 50 bp are seen below.

about 0.003 copies/cell or in about 0.04% of the total mitochondrial genomes. After 4 min irradiation, this increased to 0.013 copies/cell or about 0.12% of mitochondrial genomes. A dose-dependent increase in deletion formation was seen in cells irradiated for 1, 2 and 4 min (Fig. 5).

Mitochondrial deletions are widely believed to involve strand breaks caused by reactive oxygen species such as superoxides or oxygen free radicals. If the FS20-induced mitochondrial deletions involve ROS, then their formation ought to be suppressed by antioxidants, particularly those that act directly to eliminate ROS via reductive mechanisms. In order to determine whether ROS production plays a direct role in the formation of deletions in the FS20 irradiated cells, we examined the effects of the antioxidant, glutathione (GSH) on mitochondrial deletions in the in vitro system. When cells were pretreated 24 h prior to irradiation with GSH at concentrations ranging from 50 to 200 μM the antioxidant was found to block formation of the deletions in a dose-dependent manner (Fig. 3). However, irradiated cells treated with GSH at 100 and 200 μM also displayed two additional small MT1A/MT2 PCR bands of about 150 and 100 bp, respectively. Surprisingly, we also observed a PCR product of about 300 bp in cells treated with 1 mM GSH alone.

Sequence analyses carried out on the gel purified PCR bands in Fig. 2 showed that the PCR product of about 300 bp represented the well characterized 4977 bp deletion referred to as the common deletion (CD) that is generated from complementary cuts within two perfect 13 bp repeats that flank the deletion [29]. The smaller band was found to contain a fusion product derived from a larger deletion of 5128 bp (Δ_{uv}) with cut sites within the flanking tetramer; TAGG (mitochondrial nt 8247–8250) and its inverted complement ATCC (nt 13,375–13,378; Fig. 4) and appears to represent a novel deletion not previously described. In several cases, we were able to detect low levels of spontaneous deletion in control cultures. The spontaneous deletions all proved to be the 5128 bp deletion seen in FS20 irradiated cells (Δ_{uv}). In the Δ_{uv} deleted mitochondrial genomes, the fragments of the cut sites were present in the fusion sequence, TATCC, indicating a mechanism of deletion involving two possible cut schemes within the TAGG and ATCC motifs, either TA↓GG/A↓TCC or T↓AGG/↓ATCC. Sequence analysis of the PCR product of ∼150 bp in irradiated cells treated with 100 μM GSH showed it to be identical with the PCR product corresponding to the novel 5128 bp deletion induced by FS20 alone (Figs. 2, middle panel, and 4). The smaller product of ∼100 bp was not seen to exhibit homology to any mitochondrial sequences in an NCBI BLAST search. We also found that, in FS20 irradiated cells pretreated with GSH at the two higher concentrations (500 and 1000 μM), the MT1A/MT2 amplimers produced a more intense band with a slightly larger apparent size than the 303 bp product corresponding to the common deletion. The nucleotide sequence data showed that these bands represented a 4927 bp deletion distinct from either of the deletions produced by irradiation alone. Sequence alignments showed that this new deletion (Δ_{uv+GSH}) was generated by cuts within the Δ_{uv} upstream TAGG tetramer at nt 8247–8250 and a second TAGG direct repeat at nt 13,175–13,178. Sequence analyses of

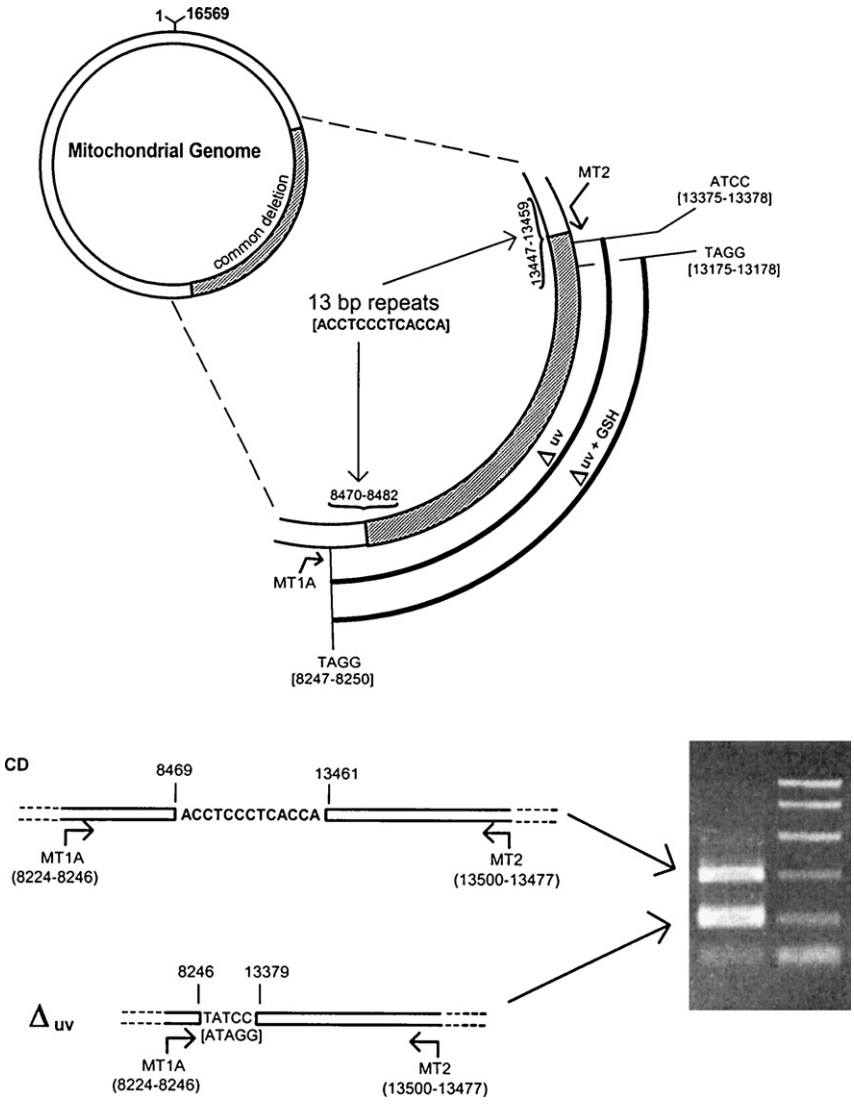

Fig. 4. Diagrammatic summary of the deletions induced by FS20 and FS20 + GSH. Upper panel: the location of the MT1A MT2 PCR primers, and the 13 bp direct repeats flanking the 4977 bp common deletion on the mitochondrial genome are shown. The locations of TAGG and ATCC tetramers that contain the cut sites for the Δ_{uv} and Δ_{uv+GSH} deletions are shown in the detail. Lower panel: the junctional sequences formed from splicing the cut ends of the common deletion (CD) and Δ_{uv} and the corresponding MT1A/MT2 PCR products in an experiment in which both deletions can be seen; the respective PCR products comigrate with the 300 and 150 bp marker (M) bands, respectively.

the PCR product of ∼300 bp seen in cells treated with 1 mM GSH alone (Fig. 3) showed that this product represented induction of the common deletion.

4. Discussion

In this report, we have described an in vitro model system of human epithelial cells in which mitochondrial genomic deletions were induced by FS20 irradiation. In addition to the 4977 bp deletion often observed in a wide variety of pathological conditions we also found at least two other deletions produced by FS20 or FS20 in combination with GSH that appear to be novel. The breakpoints of the vast majority of the mitochondrial deletions occur at sites that are within, or just adjacent to, segments that are repeated at or close to the breakpoints of the deletion (summarized on the MITOMAP website, http://www.mitomap.org). The breakpoints of the two novel deletions occurred either within repeats of the tetrameric sequence TAGG (Δ_{uv+GSH}) or within TAGG and its inverted complement, ATCC (Δ_{uv}). We found no other reports of deletions occurring at TAGG and/or ATCC sites although there is at least one report of CCTA used as a cut site in a case of mitochondrial myopathy [31]. We were unable to discern any significant features of the sequences flanking the TAGG cut sites nt 8247–8250 and 13,175–13,178 that might account for why deletions at these TAGG sites might be favored over any of the other 15 TAGG motifs that are present within the MT2/MT1A primer region. It has been suggested that the nucleotide environments surrounding the cut site motifs may play a role in determining site selection, possibly by inducing a favorable local tertiary structure [32]. The deletions removed segments of the mitochondrial covering complexes I, IV and V but the frequency of the deletions under the conditions tested was too small to cause significant damage to overall electron transport function.

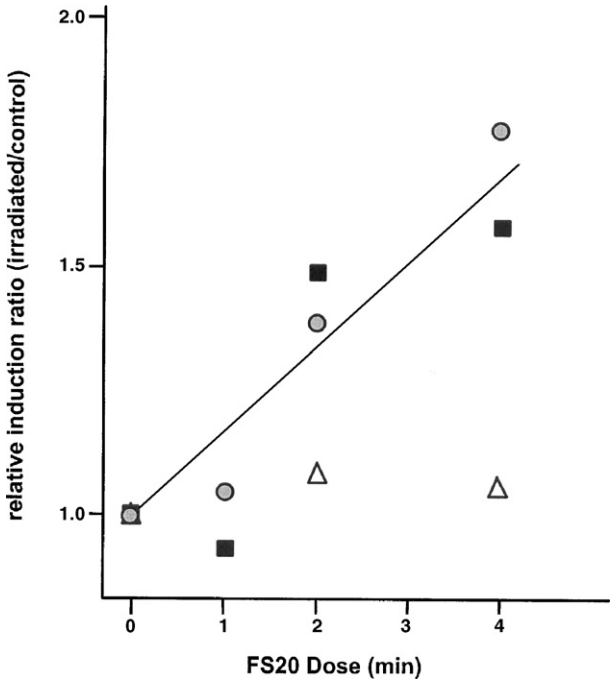

Fig. 5. Real-time PCR measurements of the induction of the common deletion in FS20 irradiated keratinocytes using real-time PCR. Mitochondrial DNA deletions in line 22 keratinocytes irradiated for 1, 2 and 4 min were examined by real-time PCR. Relative levels of deletion in irradiated vs. unirradiated cells are shown for the common deletion (○) and for the Δ_{uv} deletion (■). Relative levels of total mitochondria are indicated by symbol '△'. The Δ_{uv} deletion was amplified with MT2 and the deletion specific primer TTTGAAATATCCACAACCTT which anneals only to the junctional site created by the deletion.

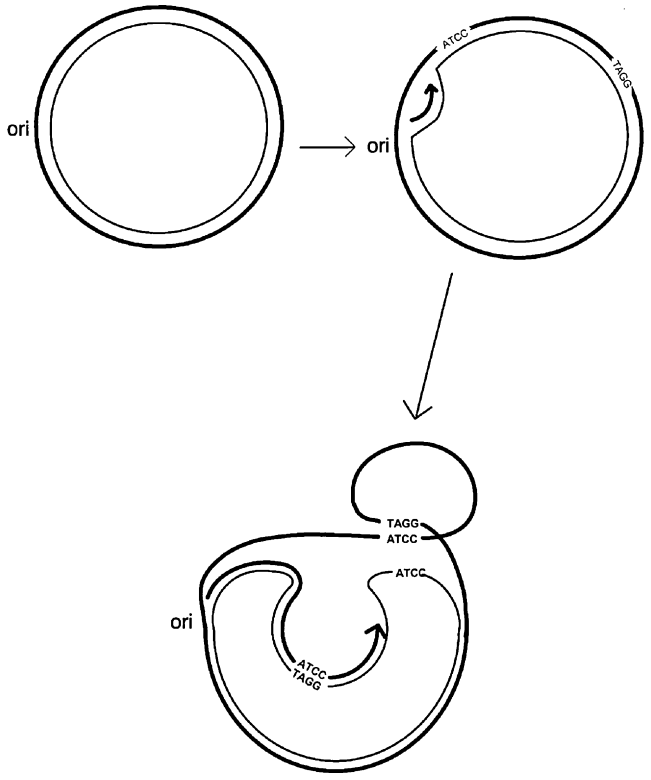

Fig. 6. Possible mechanism for the generation of the Δ_{uv} deletion. Replication of the mitochondrial genome proceeds asymmetrically along the L strand from the origin (ori) leaving an unpaired segment of the H strand (thick line) that forms a loop via parallel alignment of the tetrameric sequence, TAGG and its downstream reverse complement, ATCC. Hoogsteen base pairs stabilize the aligned tetramers in a duplex or possibly in a triple helix if the H and L strands are still base paired at the downstream tetramer. Nicking, followed by degradation of the single stranded loop and ligation of the ends (AT and AGG or A and TAGG) creates the deletion.

The mechanism by which deletion occurs in mitochondria is generally believed to involve a slipstrand intermediate in which the Heavy strand upstream repeat segment mispairs with the complementary sequences of its downstream counterpart; this leads to the formation of a single stranded loop which is subsequently removed to create the deletion [25]. However, the Δ_{uv} deletion cut sites are inverted complements of one another rather than direct repeats. Therefore, a similar slipstrand mechanism would require that the cut site sequences be aligned in parallel and the duplex stabilized by Hoogsteen base pairs. Hoogsteen base pairing is frequently seen in distorted DNA helices, particularly in triple helical DNA [33] but has also recently been demonstrated in duplexes of short oligonucleotides [34]. For the Δ_{uv} repeat sequences, it is likely that a parallel duplex could be generated via mispairing of the cut sites either via an intrastrand duplex or in a triple helical intermediate with the double stranded downstream cut site similar to the model proposed by Rocher et al. [35] (Fig. 6).

We were also able to use the in vitro system to test the effects of antioxidants on the formation of deletions. Depending upon concentration, glutathione pretreatment both suppressed and induced mitochondrial deletions. At low concentrations the FS20-induced deletions were suppressed by GSH pretreatment as well as other antioxidants including ascorbic acid (not shown) indicating that their formation was mediated by ROS. However, the induction of deletions by GSH alone or in concert with uv radiation was unexpected. The antioxidant effects of glutathione$_{red}$ stem from its ability to carry out the reduction of peroxides and free radicals. However, at the higher concentrations, GSH might, itself, also generate ROS by reduction of oxygen via electron carriers: $2GSH \rightarrow GSSG + H^+ + 2e^-$, $e^- + O_2 \rightarrow O_2{}^-$.

The majority (22 out of 27) of the FW20-induced deletions that we sequenced were not the 4977 bp common deletion but were rather the novel, 5128 bp deletion (Δ_{uv}). Although the reasons for this variability remain unknown, it seems likely that slight differences in ongoing metabolism and/or redox status of the cells in different experiments might produce differing rates of deletion at the different mitochondrial genomic loci. Berneburg et al. [20] studied UVA-induced mtDNA deletions in human fibroblasts. In that system, deletions were only seen after multiple irradiations using >1000-fold higher doses of ultraviolet radiation than used here but, in the keratinocyte system, using FS20, deletions were seen after a single irradiation.

5. Conclusions

UVB irradiation of keratinocytes produced novel mitochondrial DNA deletions involving repeats of sequence, TAGG. The finding that the antioxidant, glutathione, at high concentrations

induced formation of the common deletion or, in combination with UVB irradiation, enhanced formation of the TAGG-based deletions suggests the possibility that reductive antioxidants might, under certain conditions, actually act to enhance the formation of reactive oxygen species.

Acknowledgements

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