# The Spectrum of Mitochondrial DNA Deletions is a Ubiquitous Marker of Ultraviolet Radiation Exposure in Human Skin

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**We and colleagues have suggested that deletions of mitochondrial DNA may be useful as a biomarker of ultraviolet radiation exposure in skin. In this study using a southwestern approach involving monoclonal antibodies against thymine dimers we provide direct evidence for the presence of ultraviolet-induced damage in mitochondrial DNA purified from any nuclear DNA contamination. Previous studies have been limited, as they have focused on the frequency of a single mitochondrial DNA deletion. Therefore we have addressed the question of the spectrum of mitochondrial DNA deletions in skin and whether this can be used as an index of overall DNA damage. We have used a long polymerase chain reaction technique to determine the mitochondrial DNA deletion spectrum of almost the entire mitochondrial genome in 71 split skin samples in relation to sun exposure. There was a significant increase in the number of deletions with increasing ultraviolet exposure in the epidermis (Kruskal–Wallis test, $p = 0.0015$) but not the dermis ($p = 0.6376$). The findings in the epidermis are not confounded by any age-dependent increases in mitochondrial DNA deletions also detected by the long polymerase chain reaction technique. The large spectrum of deletions identified in our study highlights the ubiquitous nature and the high mutational load of mitochondrial DNA associated with ultraviolet exposure and chronologic aging. Compared with the detection of single deletions using competitive polymerase chain reaction, we show that long polymerase chain reaction is a sensitive technique and may therefore provide a more comprehensive, although not quantitative, index of *overall* mitochondrial DNA damage in skin.**
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The major determinant of skin cancer in individuals without protective pigmentation is ultraviolet radiation (UVR) (reviewed by Weinstock, 1998). An understanding of the genetics of the interaction between skin and UVR is therefore important for the understanding of skin cancer susceptibility. A major limitation of current studies relating genotype to phenotype of human skin cancer is the absence of reliable markers of exposure to UVR. We and colleagues have suggested that deletions of mitochondrial DNA (mtDNA), as opposed to nuclear DNA, may be useful as a biomarker of UVR exposure (Pang *et al*, 1994; Berneburg *et al*, 1997; Birch-Machin *et al*, 1998). The reasons for this are 3-fold. First, in contrast to nuclear DNA, there are no or limited mechanisms in mitochondria for the repair of DNA photoproducts [pyrimidine (6–4) pyrimidone photoproducts or cyclopyrimidine dimers] by the usual process of nucleotide excision (Clayton *et al*, 1974; Prakash, 1975; LeDoux *et al*, 1993; Croteau and Bohr, 1997; Pascucci *et al*, 1997; Sawyer and Van Houten, 1999). Second, mtDNA has a 10-fold higher mutation rate than nuclear DNA (Wallace, 1992). Third, mtDNA is ''very recessive'' as a consequence of there being (i) many mitochondrial genomes (two to ten copies) per mitochondria and (ii) many mitochondria per cell. Consequently, mitochondrial genomes can tolerate very high levels (up to 90%) of damaged DNA through complementation by the remaining wild type mtDNA (Chomyn *et al*, 1992; Sciacco *et al*, 1994). In addition mutated mtDNA can have a replicative advantage over wild type (Hayashi *et al*, 1991), and there is clonal expansion of mutated DNA (Moslemi *et al*, 1996; Brierley *et al*, 1998). Together these features suggest there may be little selection against cells harbouring mtDNA mutations, unlike most nuclear genes.

In this study using a southwestern approach, involving monoclonal antibodies raised against thymine dimers, we now provide direct evidence for the presence of UV-induced damage of mtDNA. Second, we have addressed the question of the range of mitochondrial deletions in skin. Previous studies have indicated that exposure of human skin to UVR leads to the accumulation of mtDNA harbouring the 4977 bp ''common deletion'' (Pang *et al*, 1994; Yang *et al*, 1995; Berneburg *et al*, 1997, 1999; Birch-Machin *et al*, 1998). The investigation of the frequency of a single deletion by these studies may not provide a reliable UV biomarker simply because they are identifying only the ''tip of the deletion iceberg''

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Abbreviation: mtDNA, mitochondrial DNA.

(Cortopassi *et al*, 1992; Melov *et al*, 1995). We have therefore extended these studies by using a long polymerase chain reaction (PCR) technique to amplify almost the entire 16,569 bp mitochondrial genome in order to determine the mtDNA deletion spectrum secondary to UV exposure. The study of the spectrum of DNA deletions as opposed to a single deletion may provide a more comprehensive picture of UV-induced mtDNA damage.

Recently mitochondria have been implicated in the carcinogenic process because of their role in apoptosis and other aspects of tumor biology (reviewed by Cavalli and Liang, 1998; Green and Reed, 1998). Furthermore homoplasmic mtDNA mutations (i.e., 100% mutated mtDNA) have recently been observed in human colorectal and gastric tumors (Habano *et al*, 1998; Polyak *et al*, 1998; Tamura *et al*, 1999). These findings together with the fact that UVR is important in the etiology of the common skin cancers (reviewed by Gilchrest and Yaar, 1992; Rees *et al*, 1997), and the access to clinical samples from different epidermal tumors, led us to study the spectrum of mtDNA deletions in both tumor and adjacent normal split skin. In an attempt to provide insight into the mechanism of mtDNA mutations we screened (i) a putative mutation hot-spot region in sun-exposed samples as well as (ii) the mtDNA spectrum in nucleotide excision repair mutants (i.e., patients with xeroderma pigmentosum).

## MATERIALS AND METHODS

**Cell culture** A spontaneously immortalized keratinocyte cell line (HaCaT) (Boukamp *et al*, 1988) was grown in Dulbecco's modified Eagle's medium containing 10% fetal bovine serum, 5 IU per ml penicillin, and 5 g per ml streptomycin.

**Immunocytochemistry** Cells were grown on baked glass coverslips that had been placed in 100 $mm^2$ tissue culture treated dishes. The cells attached to the coverslips following overnight incubation and were grown to approximately 50% confluency for analysis. Cells were washed twice in phosphate-buffered saline (PBS) and were then irradiated in PBS with 529 mJ per $cm^2$ of UVR [Helarium 40 W lamp (Wolff B1.01): 290–400 nm, peak emission at 325 nm]. This irradiation is the total unweighted UV dose measured by Dr. P. Farr and Professor B. Diffey (University of Newcastle) using a calibrated radiometer IL1700. The Wolff Helarium lamp consists of an array of four 60 cm UV fluorescent lamps. Seventy-five per cent of the output of this lamp lies within the UVA range and biologically significant amounts of UVC are not emitted (Farr *et al*, 1987). The cells were fixed to the coverslips using methanol:acetic acid (3:1) on ice for 3–5 min, washed in 100% ethanol, and dried. Cells were treated with 0.07 M NaOH/70% ethanol on ice for 2 min to denature DNA and were washed with PBS (five times). Cells were incubated with 1% bovine serum albumin (BSA) in PBS for 30 min and then with a 1:15 dilution of the TDM-2 monoclonal antibody raised against thymine dimers in PBS with 0.1% BSA (Mizuno *et al*, 1991) (a kind gift from Professor O. Nikaido, Kanazawa, Japan). After washing with PBS, cells were incubated in a 1:100 dilution of biotinylated goat antimouse IgG (Zymed) in PBS with 0.1% BSA. Antibody detection was then carried out using the StreptABC Complex/HRP system (Dako A/S, Denmark).

**UV irradiation of HaCaT cells and extraction of "pure" mtDNA** Cells were grown on coverslips to approximately 70%–90% confluency in 100 $mm^2$ tissue culture treated dishes, washed in PBS, and then irradiated in PBS with 529 mJ per $cm^2$ of UVR [Helarium 40 W lamp (Wolff B1.01) as described in the section *Immunohistochemistry*). Cells were then returned to a 37°C incubator (5% $CO_2$) for 30 min before being harvested by trypsinization. Pure mtDNA was isolated by differential centrifugation of the cellular components followed by DNase I treatment of intact mitochondria before lysis and purification of mtDNA (Collombet *et al*, 1997). This methodology gave very pure mtDNA, as confirmed by the absence of a $^{32}P$ signal (in the presence of a positive nuclear DNA control) in a southern blot analysis using a telomere probe (see next section for probe details) for nuclear contamination (data not shown).

**Southwestern blot analysis** DNA extracted from fifty 100 $mm^2$ Petri dishes was digested with BamHI and then separated on a 0.7% agarose gel. Transfer of the DNA to nylon membrane (GeneScreen Plus, NEN Life Sciences, Boston, MA) was carried out according to the manufacturer's instructions. The DNA was fixed to the membrane by drying in a vacuum at 80°C for 2 h. The membrane was then placed in a 10% milk solution in Tris-buffered saline (TBS) overnight at 4°C. The following day the membrane was washed in TBS (five times) and then incubated in a 1:1000 dilution of TDM-2 at room temperature for 1 h. A further three washes were carried out in 0.2% Tween 20 in TBS before incubation with a 1:1000 dilution of biotinylated goat antimouse IgG (Zymed) for 30 min. Washing was repeated and the membrane was incubated in a 1:10,000 dilution of streptavidin-conjugated horseradish peroxidase for 1 h. The membrane was washed in 0.2% Tween (five times) and then detection was carried out using SuperSignal chemiluminescent substrate (Pierce Chemical, Chester, U.K.). Following chemiluminescence detection, hybridization was performed using $^{32}P$-radiolabeled probes corresponding to the D loop region of the mitochondrial genome (nucleotides 15,977–16,420 bp) and a telomere probe to hybridize to nuclear DNA. The telomere probe (Wainwright *et al*, 1995) was made by PCR in 100 µl reactions consisting of 0.1 µM $(TTAGGG)_5$ and $(CCCTAA)_5$ oligonucleotides, 50 mM KCl, 10 mM Tris-HCl (pH 8.3), 1 mM $MgCl_2$, 200 µM of each dNTP and 2 units of *Taq* polymerase. The PCR profile consisted of 10 cycles of 1 min at 94°C, 30 s at 60°C, 90 s at 72°C, and one final step of 5 min at 72°C. Analysis of the hybridizations was carried out using the PhosphorImager and ImageQuant software (Molecular Dynamics, Chesham, U.K.)

**Clinical samples** Sun-protected skin (buttock and heel) was taken from postmortem samples (epidermis, n = 9, age range 69–85 y, mean age 77 y; dermis, n = 6, age range 69–84 y, mean age 75 y). Sun-exposed skin (face, hands, back, trunk, and legs) was taken from clinically normal perilesional skin of patients attending for skin cancer excision. These sun-exposed samples were prospectively classed as coming from sites of high exposure (face and hands) (epidermis, n = 28, age range 26–91 y, mean age 68 y; dermis, n = 13, age range 41–91 y, mean age 72 y) and from low or intermittent sun-exposed sites (back, trunk, and legs) (epidermis, n = 8, age range 24–83 y, mean age 54 y; dermis, n = 6, age range 24–83 y, mean age 62 y). None of the patients used for this study had a mitochondrial DNA defect. Epidermis and dermis were separated using 0.25% dispase at 4°C overnight and DNA was extracted as described by Jackson *et al* (1992). For the study of epidermal tumors and normal adjacent skin, adjacent skin samples (n = 8) were collected from patients attending for cancer excision and were classified as above. The cell lines from 30 xeroderma pigmentosum patients were classified into the following complementation groups: XPA (n = 6); XPB (n = 4); XPC (n = 4); XPF (n = 3); XPG (n = 5); XPV (n = 8).

**Long PCR of the mitochondrial genome** Mitochondrial DNA was amplified using the Expand Long Template PCR System (Boehringer Mannheim, Switzerland). Amplifications were performed in 50 µl reactions containing 16 pmol of each primer and 50–200 ng of total DNA. Two PCR reactions were carried out for each DNA sample to cover 11,095 bp and 5409 bp of the mitochondrial genome. The PCR primers used were those described by Kleinle *et al* (1997) covering the following regions of the Cambridge sequence (Anderson *et al*, 1981): DIA (nucleotides (nt) 336–363), DIB (nt 282–255), OLA (nt 5756–5781), OLB (nt 5745–5721). The PCR protocol consisted of a denaturing stage at 94°C for 2 min and then 10 cycles of 94°C for 10 s, 65°C for 30 s, and 60°C for 8–12 min, followed by a further 20 cycles of the same profile with an additional 20 s added to the elongation time every cycle and a final cycle with 7 min added to the elongation time. To ensure reproducibility, a known amount of DNA was separated on each gel and only samples that had at least the same amount of DNA were included in the analysis.

## RESULTS

**Direct evidence showing UV-induced thymine dimers in purified mtDNA** As mtDNA may be a significant target of UVR, the direct effect of UVR on mtDNA was investigated by looking at thymine dimer production using a specific monoclonal antibody, TDM-2 (Mori *et al*, 1991; Komatsu *et al*, 1997). First the TDM-2 antibody was used to show UV induction of thymine dimers in whole cells. This was seen as increased nuclear staining in HaCaT cells that had been exposed to 529 mJ per $cm^2$ of UVR (290–400 nm) compared with untreated cells (**Fig 1*a***, ***b***, respectively). Second, the same UVR dose was then used to treat HaCaT cells from which mtDNA was isolated and subsequently purified from any detectable nuclear contamination (see *Materials and Methods*). This purified mtDNA was linearized by digestion with BamHI prior to electrophoresis and probed with the TDM-2 antibody in a combined southwestern blotting analysis (**Fig 2*a***).

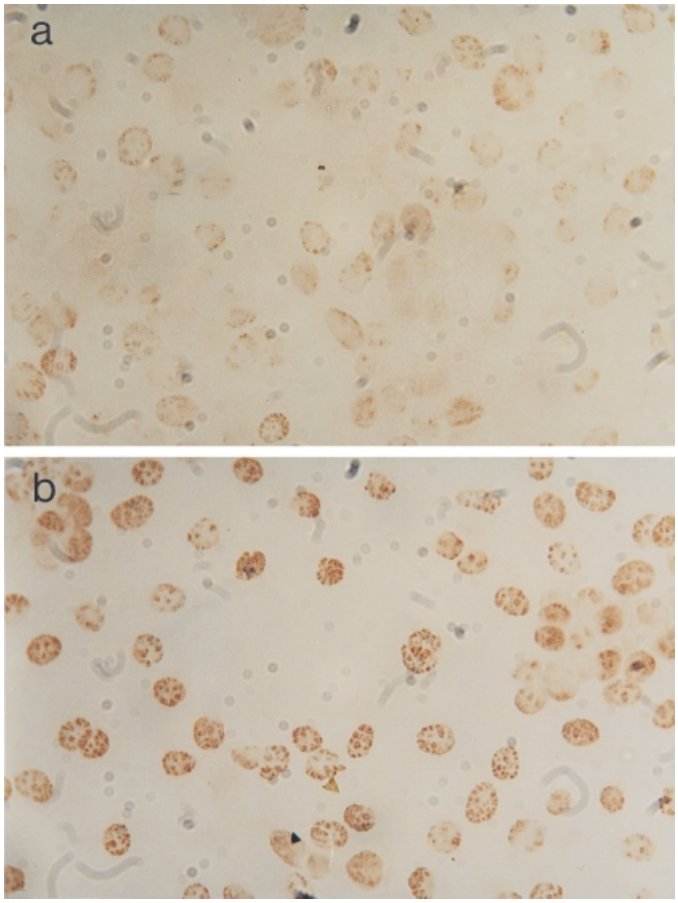


**Figure 1. Increased nuclear staining for thymine dimers in UV-irradiated HaCaT cells.** Immunocytochemistry of HaCaT cells with the TDM-2 monoclonal antibody for thymine dimers: (*a*) nonirradiated; (*b*) irradiated with 529 mJ per $cm^2$ UVR (290–400 nm).

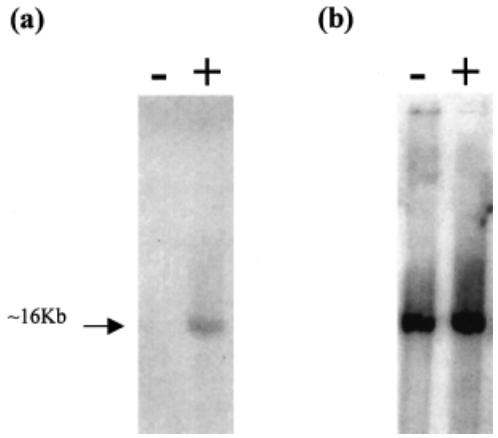


**Figure 2. Increased level of thymine dimers in mtDNA purified from UV-irradiated HaCaT cells.** Southwestern (*a*) and southern (*b*) analyses of mitochondrial DNA purified from HaCaT cells exposed to either 0 or 529 mJ per $cm^2$ of UVR (290–400 nm) (– and + *lanes*, respectively) and probed (*a*) with the TDM-2 monoclonal antibody raised thymine dimers and visualized with an enhanced chemiluminescence detection system or (*b*) a $^{32}$P-radiolabeled 470 bp PCR product from the D loop region of the mitochondrial genome (see *Materials and Methods*).

The results clearly show an increase (approximately 5–10-fold) in the level of thymine dimers in purified mtDNA from UV-irradiated HaCaT cells compared with nonirradiated cells. The same blot was probed with an mtDNA probe (D loop region) to show equal loading but to confirm also that the 16 kb band corresponded to full-length mtDNA (**Fig 2*b***).

Time course experiments were also performed to investigate the claims that repair of UV-induced thymine dimers is absent in mitochondria (Clayton *et al*, 1974; Prakash, 1975; LeDoux *et al*, 1993; Croteau *et al*, 1997; Pascucci *et al*, 1997; Sawyer and Van Houten, 1999). Thymine dimer formation was studied in HaCaT cells, which were harvested 30 min, 24 h, and 48 h following UV irradiation [529 mJ per $cm^2$ of UVR (290–400 nm)]. The absence of repair could not be determined unequivocally because of difficulties encountered through the death of UV-damaged adherent cells that resulted in the loss of thymine dimers into the culture medium (data not shown).

**An increase in the number of mtDNA deletions in epidermis is significantly associated with increasing UVR exposure** The long PCR technique is widely used to investigate the spectrum of mtDNA deletions in mitochondrial diseases and aging studies (Kleinle *et al*, 1997; Kovalenko *et al*, 1997; Nagley and Wei, 1998; Khrapko *et al*, 1999). Therefore mtDNA deletions were identified in human skin mitochondria through amplification of almost the entire mitochondrial genome in two sections (i.e., 5.4 kb and 11.1 kb) by long PCR. The spectrum of deletions in the mitochondrial genome is visualized in the assay as a DNA ladder of PCR products on an agarose gel (**Fig 3*a***). The procedure described by Melov *et al* (1995) was used to confirm that all the long PCR products observed were of mtDNA origin. Therefore the DNA fragments from **Fig 3(*a*)** were denatured and transferred to nylon filters by southern blotting and hybridized with a $^{32}$P-labeled full-length mtDNA probe (**Fig 3*b***). In agreement with the results of Melov *et al* all of the PCR products seen by ethidium bromide staining were also found to hybridize to the mtDNA probe, confirming that they are derived from the mitochondrial genome.

In an attempt to identify a UV-specific pattern of deletions long PCR was used to examine mtDNA in 71 split skin samples taken from body sites with high and intermittent sun exposure and from sun-protected body sites. For the 11.1 kb PCR the deletion sizes ranged from 4.0 to 10.5 kb, and for the 5.4 kb PCR the deletion sizes ranged from 2.3 to 5.0 kb. Each individual sample had a distinctive array of mtDNA deletions. Previous studies of mtDNA rearrangements in aging have analyzed the array of mtDNA deletions between samples in a qualitative manner by describing the increase in mtDNA damage as an increase in the number of deletions (Tengan *et al*, 1997; Wei, 1998). This methodology was therefore used to assess the differences in the array of mtDNA deletions isolated from the dermis and epidermis of sun-exposed *versus* sun-protected samples. As expected the 11.1 kb PCR assay produced a much greater array of mtDNA deletions compared with the 5.4 kb PCR because it encompasses the region of mtDNA in which the majority of deletions are found (Kleinle *et al*, 1997). We therefore restricted our analysis of results to those provided by the 11.1 kb PCR assay.

There was a significant increase in the number of deletions with increasing UVR exposure in the epidermis (Kruskal–Wallis test, $p = 0.0015$, **Fig 4*a***) but not the dermis ($p = 0.6376$, **Fig 4*b***). It is known from previous studies (reviewed by Nagley and Wei, 1998) that the spectrum of mtDNA deletions increases with age and it is therefore possible that this may confound our observations in **Fig 4**. To test this we performed further statistical analyses of our data. Not surprisingly we identified an age-associated increase in the spectrum of deletions in both the dermis (Spearman's rank correlation test, $p = 0.04$) and epidermis (although this failed to reach formal significance, $p = 0.058$) and also within UV exposure groups in the epidermis (Spearman's rank correlation test: $p = 0.003$ constant exposure; $p = 0.043$ sun-protected). These data do not confound our observations in **Fig 4**, however, because the analysis showed that in the epidermis there was a significant decrease of the median age as the degree of UVR exposure of the sample groups increased (Kruskal-Wallace test, $p = 0.0491$, **Fig 5*a***), which is a reversal of the trend in **Fig 4(*a*)**. In fact, the opposing nature of the trends observed in **Figs 4(*a*)** and **5(*a*)** suggests that the age-

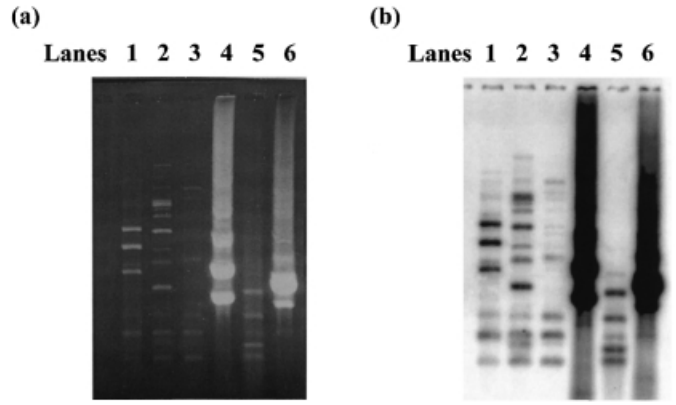


**Figure 3. The mitochondrial origin of the long PCR products were confirmed by southern blot analysis.** The spectrum of mtDNA deletions from six samples (*lanes 1–3*: whole skin samples; *lanes 4* and *6*: dermal samples; *lane 5*: epidermal sample) was visualized in the long PCR assay (using primers spanning the 11.1 kb mtDNA region) as a ladder of DNA products on an agarose gel (*a*). A southern blot of these products was probed with a $^{32}$P-radiolabeled 470 bp PCR product from the D loop region of the mitochondrial genome (*b*). All of the PCR products seen by ethidium bromide staining in (*a*) were also found to hybridize to the mtDNA probe in (*b*), confirming that they are derived from the mitochondrial genome. The long PCR assay was validated by the inclusion of several controls that included wild type mtDNA only (thereby amplifying either the 11.1 kb or 5.4 kb product alone) and mtDNA from a Kearns Sayre syndrome patient that harbours 75% of the common deletion (a gift from Professor D. Turnbull, University of Newcastle, U.K.) (results not shown).

associated increase in mtDNA deletions will contribute to an underestimation of the true increase of deletions with increasing UVR exposure. To test this we replotted our data from **Fig 4** using only the age-matched paired samples. As expected these paired data increased the degree of significance observed in the increase of the deletion number with increasing UVR exposure in the epidermis (i.e., $p = 0.0003$), thereby confirming the statistical associations observed in **Fig 4**(***a***).

**No evidence of increased mtDNA deletions in a range of epidermal neoplasms compared with surrounding perilesional epidermis** The findings of mtDNA deletions in human tumors (Habano *et al*, 1998; Polyak *et al*, 1998; Tamura *et al*, 1999) together with the fact that UVR is important in the etiology of the common skin cancers prompted us to study the spectrum of mtDNA deletions in epidermal tumors and the adjacent normal epidermis. Long PCR analysis was performed on a range of epidermal neoplasms (squamous cell carcinomas, $n = 4$; Bowen's disease, $n = 1$; and basal cell carcinomas, $n = 3$) taken from constant and intermittent sun-exposed body sites and sun-protected sites. As expected from our earlier results in **Fig 4** the epidermal samples showed an increase in the median number of deletions with increasing sun exposure. No deletions were detected in sun-protected and intermittent sun-exposed samples but median values of 2.5 and 4.0 were observed in constant sun-exposed samples from normal epidermis and tumor, respectively. The difference in the median values between the tumor and the perilesional epidermis was not statistically significant (two-tailed Mann-Whitney test, $p = 0.79840$).

**The mechanism of the mtDNA mutations** In an attempt to provide insight into the mechanism of mtDNA mutations we screened (i) a putative mutation hot-spot region in sun-exposed samples as well as (ii) the mtDNA spectrum in nucleotide excision repair mutants (i.e., patients with xeroderma pigmentosum).

*No evidence of UV-induced mtDNA mutations in a putative hot-spot region* Recent work has shown a UV-induced mutational spectrum in a coding region of mtDNA located within the

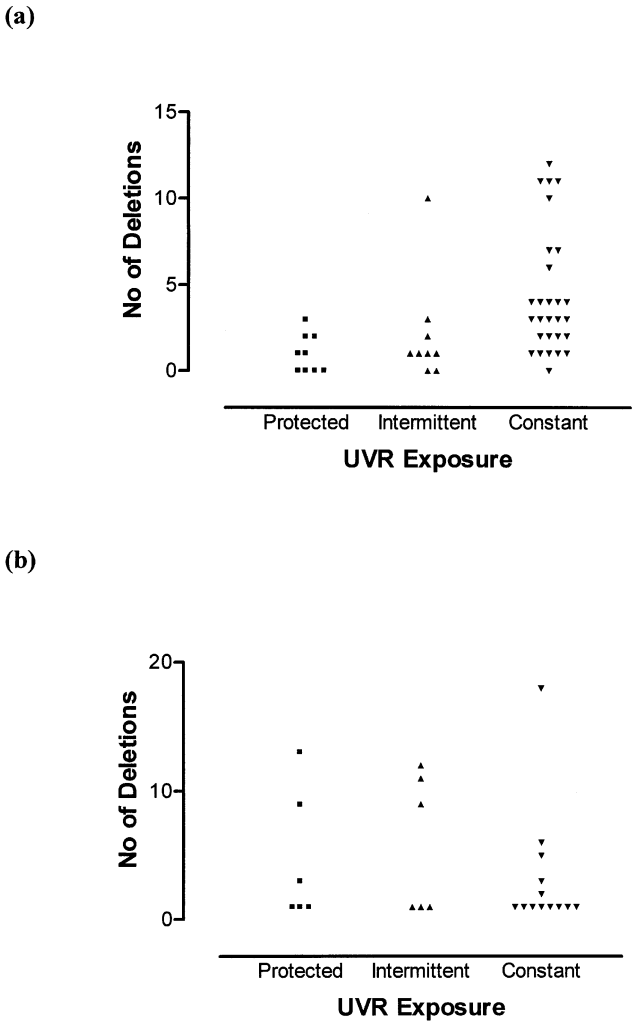


**Figure 4. A significant increase in the number of deletions with increasing UVR exposure is observed in the epidermis but not the dermis.** The number of deletions in (*a*) epidermal and (*b*) dermal samples with various levels of UVR exposure. Using the Kruskal–Wallis test, the medians were shown to vary significantly between each exposure group in the epidermis ($p = 0.0015$) but not in the dermis ($p = 0.6376$).

tRNA$^{leu}$ gene (Pascucci *et al*, 1997). Mutational analysis was therefore used to investigate a 287 bp PCR product from part of the same gene (i.e., specifically the tRNA$^{leu}$/16 s rRNA gene boundary) in a sun-exposed dermal sample known to harbour a high level (27%) of common deletion. Complete DNA sequencing of nine clones showed only wild type sequence (Anderson *et al*, 1981) apart from one clone in which a known polymorphism (T to C transition at position 3197) was identified (Hess *et al*, 1995).

*mtDNA deletions in xeroderma pigmentosum are not associated with complementation group* The mtDNA deletion spectrum was investigated in patients with xeroderma pigmentosum for several reasons. Apart from showing a deficiency in nucleotide excision repair, certain complementation groups, notably group A and also to a lesser extent group D (reviewed by Kraemer *et al*, 1994), exhibit the same neurodegeneration as is found in mitochondrial diseases, which are associated with elevated levels of the mtDNA common deletion (Wallace, 1992). It should be noted that although nucleotide excision repair should not be involved within mitochondria the pattern of mtDNA deletions between different complementation groups may give some insight into the deletion mechanism and/or nucleo–mitochondrial interactions. Long PCR analysis of DNA from 30 fibroblast cell lines from xeroderma pigmentosum patients (a gift from Professor Colin Arlet, MRC,

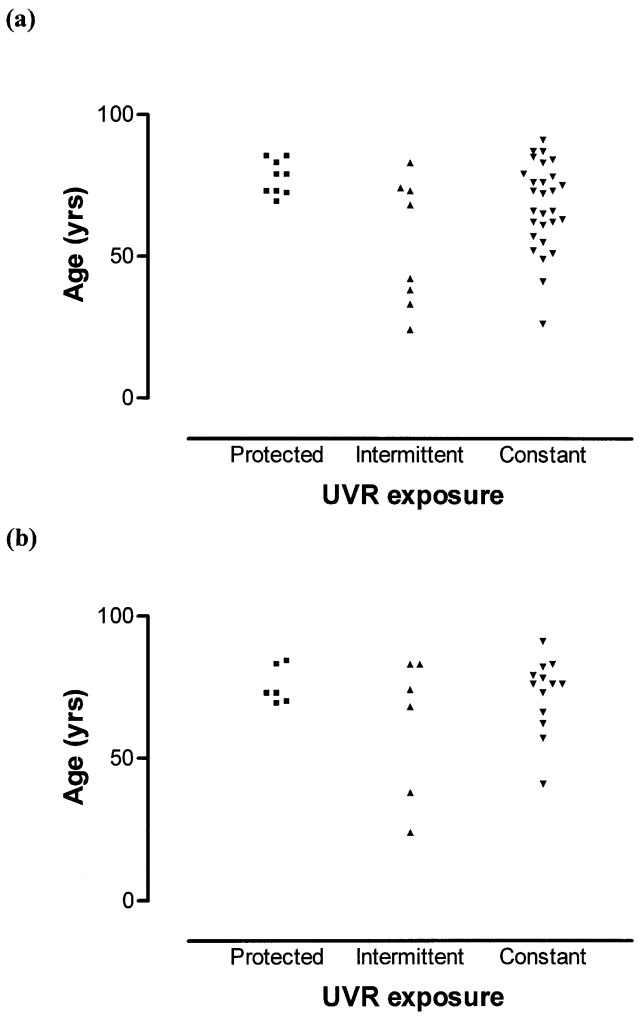


**Figure 5. The median age decreases significantly with increasing UVR exposure of the sample groups in the epidermis but not the dermis.** The ages of each epidermal (*a*) and dermal (*b*) sample are plotted from each UVR exposure group. Using the Kruskal–Wallis test the medians were shown to vary significantly between exposure groups in the epidermis ($p = 0.0491$) but not in the dermis ($p = 0.7333$).

Brighton, U.K.; see *Materials and Methods* for details of the complementation groups) showed only full-length wild type mtDNA irrespective of the complementation group.

## DISCUSSION

mtDNA is a significant target of UVR and the spectrum of mtDNA deletions is useful as a marker of overall mtDNA damage in the cell.

Using monoclonal antibodies raised against thymine dimers, our results provide direct evidence of photoproduct formation specifically in mtDNA. This suggests that mtDNA is a significant target of UVR but may also be indicative of the wider *overall* mtDNA damage in the cell. Previous studies (Yang *et al*, 1995; Berneburg *et al*, 1997, 1999; Birch-Machin *et al*, 1998) have focused on the incidence of a single deletion, which is likely to result in a distorted perspective of the overall somatic mtDNA deletion accumulation (Cortopassi *et al*, 1992; Melov *et al*, 1995). These studies have therefore investigated only a small portion of a much larger pool of deleted mtDNAs. In this study we have extended these previous investigations by using a long PCR technique to amplify almost the entire mitochondrial genome in order to determine the spectrum of mtDNA deletions secondary to UV exposure. There was a significant increase in the number of deletions with increasing UVR exposure in the epidermis. This finding is not accounted for by any age-dependent increases in mtDNA deletions also detected by the long PCR technique (reviewed by Nagley and Wei, 1998).

Summarizing this type of data is problematic as shown by the considerable debate in the current scientific literature surrounding the choice of methodology for determining the levels of mtDNA deletions (opposing viewpoints are reviewed in *Trends in Genetics* by Nagley and Wei, 1998, and Lightowlers *et al*, 1999). The major advantage of the long PCR technique is its increased sensitivity compared to the other methodologies that have been used to examine the incidence of a single deletion alone [e.g. our previous study (Birch-Machin *et al*, 1998) and the studies of colleagues (Yang *et al*, 1995; Berneburg *et al*, 1997)]. For example, our previous study identified the common mtDNA deletion only in the dermis from sun-exposed sites whereas the long PCR assay identified the entire spectrum of mtDNA deletions not only in the epidermis and the dermis but also in skin from sun-protected as well as sun-exposed sites. Furthermore there was no evidence in our previous study for the common deletion in epidermal tumors whereas the present work clearly shows mtDNA deletions in a similar range of epidermal neoplasms. Finally, a comparison of the observed deletion frequencies in sun-exposed sites shows that the long PCR assay (frequency of 96%) is more sensitive than the quantification of a single deletion alone (frequency of 27%) in our previous study.

The major disadvantage of the long PCR assay is that it can differentially amplify deleted genomes (results not shown; also work by Reynier *et al*, 1998; Melov *et al*, 1995; and reviewed by Lightowlers *et al*, 1999), thereby giving a qualitative rather than a quantitative profile of mtDNA molecules. In this respect the long PCR assay is similar to other semiquantitative PCR techniques where the absolute level of deleted mtDNA cannot be verified by southern analysis (Pang *et al*, 1994; Berneburg *et al*, 1999). This does not invalidate the use of long PCR as a biomarker of overall mtDNA damage in skin, however, particularly in epidermis, as shown by this study.

What is the functional significance of these mtDNA deletions? This is difficult to answer as the evidence for a functional deficit of mitochondrial respiration related to mtDNA deletions is unclear (as reviewed by Lightowlers *et al*, 1999). This is further complicated by the recessive nature of mitochondrial DNA (Chomyn *et al*, 1992; Sciacco *et al*, 1994) and the threshold of mtDNA damage that is required to give a biochemical consequence (Porteous *et al*, 1998). Finally, given the high mutagenic load of mtDNA one also needs to investigate its functional contribution to mitochondrial but also tissue dysfunction. It is of note, however, that even using the $\rho^{\circ}$*trans*mitochondrial system there has been no clear demonstration of a functional deficit of mitochondrial respiration in aging studies (Lightowlers *et al*, 1999). Whatever the functional consequences of the spectrum of mtDNA deletions detected by long PCR, again it does not detract from its primary use as a biomarker of overall mtDNA damage.

**Putative mechanism of mtDNA deletions** To provide insight into the mechanism of mtDNA mutations we screened a putative mutation hot-spot region (Pascucci *et al*, 1997) in sun-exposed samples as well as the mtDNA spectrum in patients with xeroderma pigmentosum. Both approaches failed to provide any evidence of mutation, however. Recent studies have shown homoplasmic mtDNA mutations in other coding and noncoding regions of mtDNA in human colorectal and gastric tumors and cell lines (Habano *et al*, 1998; Polyak *et al*, 1998; Tamura *et al*, 1999) and these may be worthy of future investigation in skin. The mechanism explaining our observations in skin is unclear, but prolonged UVR exposure could either directly or indirectly (by induction of free radicals, see Berneburg *et al*, 1999) affect structurally labile sites in the mtDNA (Hou and Wei, 1996), which would enhance intragenomic recombination, thereby eliciting an increase in deletions. Important in this respect is a

study that has identified a major mtDNA hot spot at the cytochrome b/tRNA$^{thr}$ junction with strong potential for secondary structure, which has been implicated in approximately 50% of the deletion spectrum in several tissues from aging rats (Van Tuyle *et al*, 1996).

In conclusion we provide the first direct evidence for the induction of thymine dimers in mtDNA by UVR. In addition, we have used long PCR to show that an increase in the spectrum of mtDNA deletions in the epidermis is significantly associated with increased UVR exposure in human skin. The large spectrum of deletions identified in our study highlights the ubiquitous nature and the high mutational load of mtDNA associated with UVR exposure. Compared with the detection of single deletions using competitive PCR, we show that long PCR is a sensitive technique and may therefore provide a more comprehensive, although not quantitative, index of *overall* mtDNA damage in skin. Within mitochondria the relationship between the mtDNA mutations described and any putative functional importance remains unproven (Lightowlers *et al*, 1999).

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