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SHORT COMMUNICATIONS

Cyclobutane pyrimidine dimers form preferentially at the major *p53* mutational hotspot in UVB-induced mouse skin tumors

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The most prevalent DNA lesion induced by UV irradiation is the cyclobutane pyrimidine dimer (CPD) which forms at positions of neighboring pyrimidines. In mouse skin tumors induced by irradiation with UVB (280–320 nm) lamps or solar UV simulators, a major mutational hotspot occurs at codon 270 (Arg→Cys) involving a sequence change from 5′-TCGT to 5′-TTGT. We have shown previously that CPD formation by UVB or sunlight is enhanced up to 10-fold at 5′-CCG and 5′-TCG sequences due to the presence of 5-methylcytosine bases. Sequence analysis showed that the CpG at codon 270 is methylated in mouse epidermis at a level of ~85%. Irradiation of mouse skin or mouse cells in culture produced the strongest CPD signal within exon 8 at the 5′-TCG sequence which is part of codon 270. Time course experiments showed that CPDs at this particular sequence persist longer than at several neighboring positions. The data suggest that formation of CPDs is responsible for induction of the major *p53* mutational hotspot in UV-induced mouse skin tumors.

Skin cancer is the most common type of tumor in the USA (1). Exposure to solar radiation is a principal factor in the development of skin cancer (1,2). Human skin malignancies and their precursor lesions commonly harbor mutations in the *p53* tumor suppressor gene (3–11). The predominant base changes are C→T and CC→TT mutations at dipyrimidine sequences, two types of base alterations specifically induced by UV light in many experimental systems (12–14). Of the various types of lesions formed in DNA after UV irradiation, the cyclobutane pyrimidine dimer (CPD) is considered the most mutagenic lesion based on its abundance, slow repair and distinct mutagenicity (15,16).

In the *p53* gene of human skin cancers ~32% of all mutations are C→T transition mutations within two unique trinucleotide sequences, 5′-TCG and 5′-CCG. 5′-CG (CpG) sequences in the human *p53* gene are methylated to form 5′-mCG (17). Thus, many skin cancer mutations may involve formation of pyrimidine dimers containing 5-methylcytosine bases. It was shown previously that CPD formation by sunlight or UVB is enhanced up to 10-fold at 5′-CCG and 5′-TCG sequences relative to UVC (254 nm) irradiation due to the presence of 5-methylcytosine (18,19). In the CpG-methylated *lacI* transgene, sunlight, but not UVC irradiation, produces mutational hotspots at dipyrimidine sequences that contain 5-methylcytosine (20).

In mouse skin tumors induced by UVB irradiation, *p53* mutations are also frequent (21–30). More than 80% of these mutations are C→T transitions at dipyrimidine sequences. The distribution of mutations along the *p53* gene of mouse skin cancers is shown in Figure 1. One dominant hotspot is seen at codon 270, which is mutated in 133 of 429 (31%) of sequenced mouse skin tumors irradiated with UVB or simulated solar light. This codon contains the sequence CpG wherein the cytosine is part of a dipyrimidine sequence.

In order to assess the role played by the selectivity of pyrimidine dimer formation in creating this distinct mutational hotspot, we have analyzed CPDs in mouse DNA along exon 8 of the *p53* gene. Hairless female mice, 6–8 weeks old, were obtained from Charles River Laboratories (strain SKH-1). Mice were irradiated in a cage in which they were able to move freely. The UVB source was a set of three Philips TL 20W/12RS lamps (peak emission 320 nm) filtered through cellulose acetate. Lamps were positioned above the cage. The UVB dose (~5000 J/m^2) was determined with a UVX radiometer (Ultraviolet Products, Upland, CA) and corresponds to 16 min irradiation. After irradiation, mice were killed immediately. The epidermis was isolated from dorsal skin by a trypsin flotation procedure (31). The epidermis was minced with scissors and lysis buffer (10 mM Tris–HCl, pH 8.0, 150 mM NaCl, 1 mM EDTA, 0.5% SDS, 100 µg/ml proteinase K) was added. After incubation for 16 h at 37°C, DNA was isolated by phenol/chloroform extraction and ethanol precipitation. The irradiation conditions introduced approximately one CPD every 2.5 kb of DNA as determined by alkaline agarose gel analysis (not shown). CPDs were mapped in the *p53* gene by ligation-mediated PCR (LMPCR). DNA isolation, enzymatic cleavage at CPDs and LMPCR were done as previously described (32). The following primers, specific for exon 8 of the mouse *p53* gene, were used in LMPCR: primer 1, 5′-AGCTCAACAGGCTCCTCC; primer 2, 5′-GCCTCCTTGGTCCCGCCTGC; primer 3, 5′-CCGCCTGCGTACCTCTCTTTGC. The LMPCR data were quantitated by phosphorimaging and by correcting for LMPCR amplification efficiency at individual sites using the Maxam–Gilbert T+C lanes as a reference. The numbers obtained are the relative CPD frequencies along a particular sequence.

Figure 2 shows an analysis of the upper strand of exon 8 where the common C→T transition at codon 270 occurs. The strongest CPD signal along the entire exon is seen at codon 270. Relative CPD frequencies are shown in Table I. Mouse codon 270 corresponds to human *p53* codon 273. Mutations at codon 273 are rare in human skin cancers, presumably because it is not part of a dipyrimidine sequence (5′-G-CGT-G). However, codon 273 is a prominent hotspot of mutation in many other tumors. This could be a consequence

Abbreviations: CPD, cyclobutane pyrimidine dimer; LMPCR, ligation-mediated polymerase chain reaction.

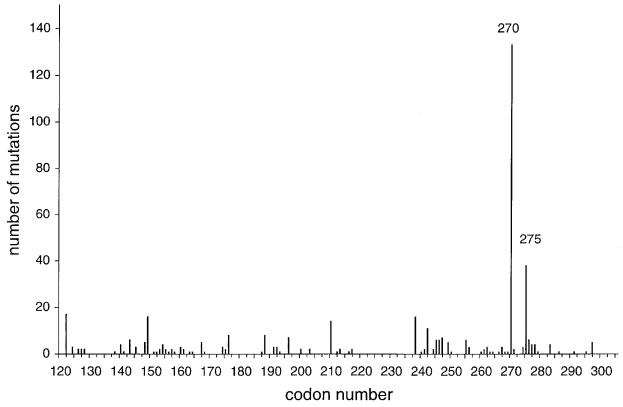

Fig. 1. Mutational spectrum of the *p53* gene in mouse skin cancer. The data include all *p53* mutations induced by UVB light sources and solar UV simulators in DNA repair-proficient and repair-deficient mice. Numbers were obtained from the literature (21–30).

of preferred targeting by mutational events at methylated CpG sequences (33–35) and/or tumorigenic selection. It is unlikely that mutations at codon 270 are produced by (6–4) photoproducts, since these form at very low levels and are suppressed at positions of 5-methylcytosines (16).

CPDs also form at codon 275, which is the second most common mutation site in mouse skin cancers (Figure 1), but they are much less pronounced than at codon 270. Mouse codon 275, which lacks a CpG sequence, corresponds to human codon 278. Interestingly, codon 278 is also a mutational hotspot in human skin cancer, similarly involving dipyrimidine transitions at a non-CpG sequence. Only moderate levels of CPDs are observed in human (36) and mouse *p53* (Figure 2 and Table I) at these sequences. One possibility is that mutation at codon 275/278 is particularly tumorigenic in skin. Although considered less likely, the major mutagenic lesion at codon 275/278 may be the (6–4) photoproduct (37) or even a minor DNA photoproduct.

Significant levels of CPDs are seen at codons 261/262. Mutations in UVB-induced mouse skin tumors are relatively rare at these codons (Figure 1). A C→T change at codon 262 would be silent (Leu→Leu), but a C→T mutation at 261 would produce a Leu→Phe change. This amino acid substitution may not be highly tumorigenic. Changes at the analogous human codon 264 are rare in the *p53* database of >11 000 entries (38). In fact, almost all mutations at this position in the human *p53* gene are frameshifts. Furthermore, although TT dimers are formed at high levels at several sites (Table I), they are thought to be only weakly mutagenic in eukaryotic cells (39–41).

The preferential formation of CPDs at codon 270 may be related to the presence of 5-methylcytosine. In order to determine if codon 270 is methylated *in vivo*, we extracted DNA from mouse epidermis and from embryonic mouse fibroblasts. CpG methylation was analyzed by chemical DNA sequencing and LMPCR (Figure 3). As controls for unmethylated and methylated DNA, respectively, a PCR product encompassing exon 8 of the mouse *p53* gene was either mock-methylated or was methylated *in vitro* with the CpG-specific DNA methyltransferase M*Sss*I. Methylated and unmethylated PCR products were then chemically sequenced. The presence of 5-methylcytosine is indicated by lack of a band due to lack of reactivity with hydrazine in the sequencing gels (Figure 3, lanes 5 and 6). Using the two control DNAs

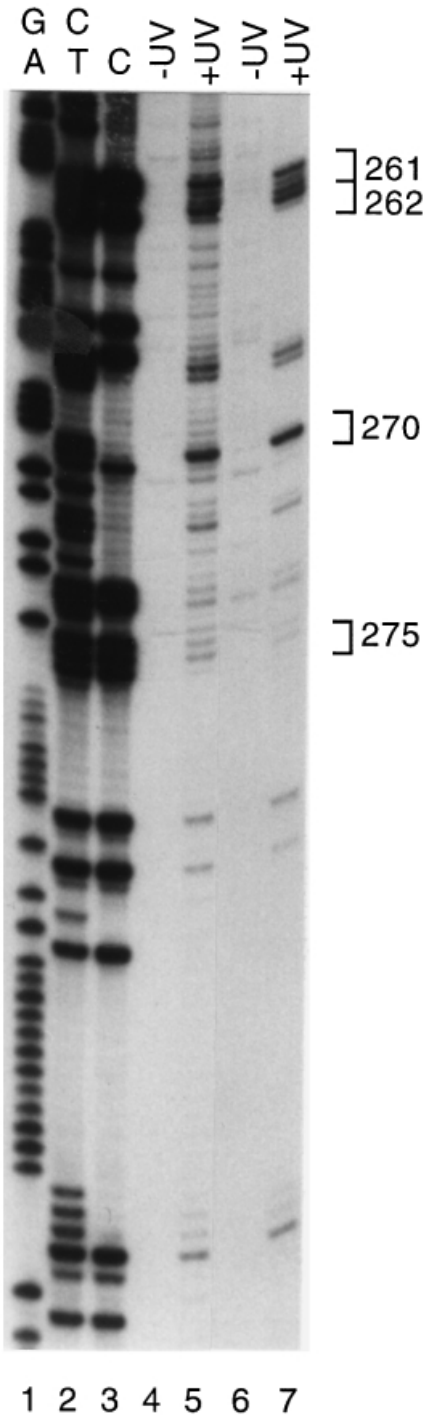

Fig. 2. Mapping of CPDs along exon 8 of the mouse *p53* gene. Maxam–Gilbert sequencing controls are in lanes 1–3. Unirradiated DNA from mouse fibroblasts and epidermis are in lanes 4 and 6, respectively. Hairless mice were irradiated with UVB (5000 J/m^2; lane 7). For comparison, mouse embryonic fibroblasts were irradiated with UVB (lane 5). DNA was isolated immediately after irradiation and cleaved at CPD sites with T4 endonuclease V and photolyase to create strand breaks detectable by LMPCR. CPDs were mapped by LMPCR using *p53*-specific primers. Selected codons are indicated by brackets.

as standards and comparing the CpG cytosine signal to the intensities of neighboring cytosines at non-CpG sequences, we calculate that the extent of methylation at codon 270 was 85% in mouse epidermis and 98% in embryonic fibroblasts (Figure 3). CpG sequences at codons 279, 280 and 287 were also

Table I. CPD frequency in exon 8 of the mouse *p53* gene

Sequence (5′-3′)	Codon	Relative CPD frequency[a]	PymCG ?	% CPDS remaining after 48 h repair[b]	Amino acid change (C→T)[c]
C∧CG	286/87	8.25	yes	n.d.	Arg-Cys
TT∧C	286	6.34	no	n.d	Phe-Phe
T∧TC	286	5.01	no	n.d.	TT dimer
T∧TT	285/86	1.79	no	n.d.	TT dimer
C∧CG	279/80	3.56	yes	32	Arg-Cys
C∧CG	278/79	8.04	yes	25	Arg-Cys
CC∧T	275	0.42	no	n.d	Pro-Leu
C∧CT	275	1.66	no	10	Pro-Leu
C∧CC	274/75	1.07	no	10	Pro-Ser
CC∧T	273/74	1.33	no	n.d	Val-Val
GC∧C	273	1.02	no	14	Ala-Val
TT∧T	271/72	4.3	no	15	TT dimer
GT∧T	271	1.96	no	n.d	TT dimer
T∧CGT	269/70	32.46	yes	28	Arg-Cys
GT∧T	269	0.03	no	n.d	TT dimer
TT∧T	267	5.64	no	5	TT dimer
T∧TT	267	8.96	no	2	TT dimer
C∧TT	266/67	1.06	no	3	Ser-Ser
C∧TG	262	1.12	no	n.d.	Leu-Leu
T∧CT	261/62	16.05	no	26	Leu-Leu
CT∧T	261	16.4	no	20	TT dimer
C∧TT	261	3.76	no	15	Leu-Phe
C∧CT	260/61	11.45	no	18	Leu-Phe

[a]Data are from Figure 2 and were quantitated as described in the text.
[b]Data are from Figure 4.
[c]The amino acid changes are for hypothetical C→T mutations at CPDs containing cytosine.

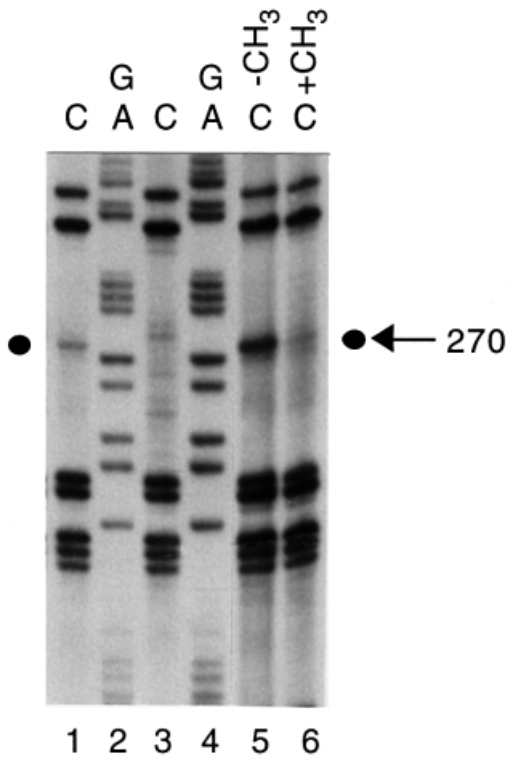

Fig. 3. Analysis of cytosine methylation in exon 8 of the mouse *p53* gene. The upper strand was analyzed by chemical sequencing and LMPCR. Lanes 1–4, C-specific and G+A-specific Maxam–Gilbert sequencing reactions of genomic DNA isolated from mouse epidermis (lanes 1 and 2) and genomic DNA isolated from mouse fibroblasts (lanes 3 and 4); lane 5, C-specific sequencing reaction of mock-methylated (-CH_3) mouse *p53* PCR products; lane 6, C-specific sequencing reaction of mouse *p53* PCR products methylated with the CpG-specific methylase M*Sss*I (+CH_3). The black dots indicate the positions of cytosines at the methylated CpG sequence of codon 270.

>80% methylated in skin (data not shown). The preferential targeting of a dipyrimidine within a methylated CpG sequence context is consistent with earlier data showing that methylation of cytosines dramatically enhances formation of CPDs in the UVB range (18,19). An additional possible pathway that may target UV mutagenesis to dipyrimidines containing 5-methylcytosine is that most UV-induced transition mutations at these sequences may result from correct DNA polymerase bypass of CPDs containing deaminated 5-methylcytosine (42).

We next measured repair rates for CPDs along exon 8 of the *p53* gene. Mouse embryonic fibroblasts (derived from C57BL/6 λLIZ transgenic mice; Stratagene) were irradiated with UVB as confluent monolayers (to prevent dilution of the LMPCR signal by cell division). The medium was removed and cells were washed in phosphate-buffered saline and irradiated for 80 s with the UVB light source (400 J/m^2). Then the medium was returned and the cells were incubated for periods of up to 48 h. Repair efficiency at individual CPD positions was calculated after densitometry of the LMPCR gels. The time course of CPD removal was monitored for up to 48 h. Most CPDs were at least partially removed after 48 h (Figure 4). At the 48 h time point there was a site-to-site difference in repair efficiency. The data were quantitated and are included in Table I. Codon 270 is a site at which repair of CPDs was less after 48 h compared with neighboring CPD positions. However, as can be seen for codons 278–280, it was not the only site at which significant levels of CPDs remained.

If a rather inefficient repair of CPDs is responsible, at least in part, for the occurrence of a mutational hotspot at codon 270, then one might expect that the hotspot will be diminished relative to other sites in DNA repair-deficient mice. The codon 270 hotspot was moderately reduced in XPC-deficient mice with wild-type *p53* (30). However, in another study of mice lacking XPC the hotspot was not reduced (28). The data on

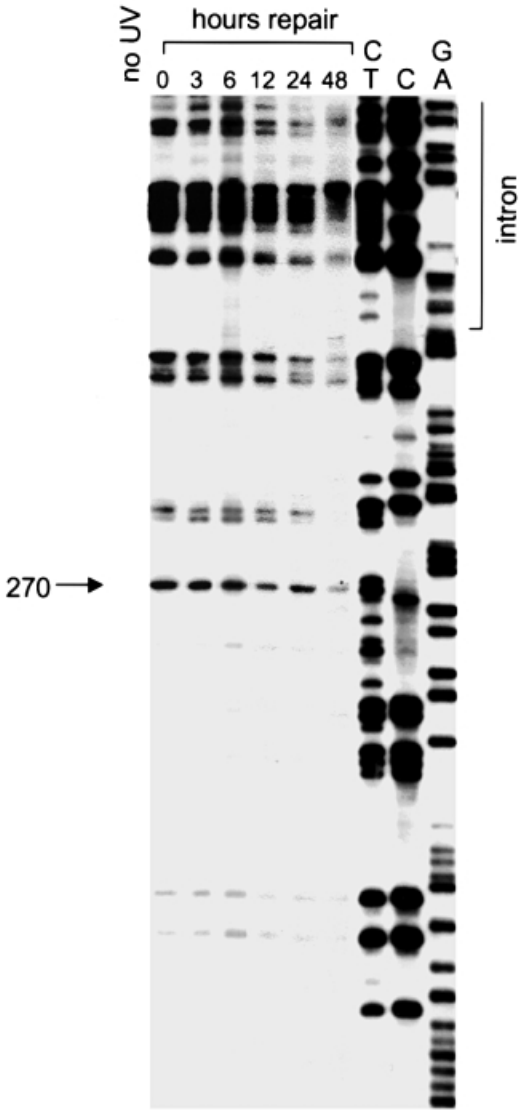

Fig. 4. Repair of UVB-induced CPDs in the mouse *p53* gene. Mouse embryonic fibroblasts were irradiated with UVB (400 J/m^2). DNA was isolated immediately after irradiation or at various time points following irradiation and cleaved at CPD sites with T4 endonuclease V and photolyase. CPDs were then detected by LMPCR using *p53*-specific primers. CPDs at different times following irradiation were measured along sequences of the upper strand of exon 8. Codon 270 is indicated.

repair-deficient animals would suggest that a high initial CPD frequency at a methylated trinucleotide is most important for the occurrence of a mutational hotspot. The relatively inefficient repair may play a role in repair-proficient animals.

In summary, the data show that the dominant *p53* mutational hotspot at codon 270 in UVB-induced mouse tumors correlates well with the site of strongest induction and relatively slow repair of CPDs. Other factors that are expected to contribute to this mutational hotspot are sequence-dependent deamination of 5-methylcytosine within the CPD, polymerase bypass efficiency and specificity and tumorigenic selection.

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