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Signature *p53* mutation at DNA cross-linking sites in 8-methoxypsoralen and ultraviolet A (PUVA)-induced murine skin cancers

(psoriasis/PUVA therapy/DNA damage)

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ABSTRACT **A combination of psoralen and ultraviolet A radiation (PUVA) is widely used in the treatment of psoriasis. However, PUVA treatment increases the risk of developing skin cancer in psoriasis patients and induces skin cancer in mice. Since the DNA damage induced by PUVA is quite different from that induced by UV, we investigated whether PUVA-induced mouse skin cancers display carcinogen-specific mutations in the *p53* tumor suppressor gene. The results indicated that 10 of 13 (77%) PUVA-induced skin tumors contained missense mutations predominantly at exons 6 and 7. In contrast, tumor-adjacent, PUVA-exposed skin from tumor-bearing animals did not exhibit *p53* mutation in exons 4–8. Interestingly, about 40% of all mutations in PUVA-induced skin tumors occurred at 5′-TA sites, and an equal number of mutations occurred at one base flanking 5′-TA or 5′-TAT sites. Since PUVA induces DNA cross-links exclusively at these sites and since UV "signature" mutations were rarely detected in PUVA-induced skin cancers, we can conclude that PUVA acts as a carcinogen by inducing unique PUVA signature mutations in *p53*. This finding may have implications for identifying the etiology of skin cancer in psoriasis patients who have undergone PUVA therapy.**

A combination of psoralen and ultraviolet A radiation, commonly referred to as "PUVA," is being widely used in the treatment of psoriasis (1, 2). This therapy consists of oral or topical administration of 8-methoxypsoralen (8-MOP) followed by exposure to long-wave UVA radiation (320–400 nm). However, the widespread use of PUVA therapy has made its potential long-term side effects an issue of concern and debate. PUVA follow-up studies revealed an increased risk for the development of squamous cell carcinoma in patients receiving PUVA therapy (3, 4), although the European studies appeared to be at odds with these reports (5, 6). However, a closer examination of the European studies revealed substantial differences with regards to the duration of follow-up, percent of patients enrolled who received follow-up examinations, and above all, total PUVA exposure (3). When differences in exposure and population demographics were factored into the European study (3), these results seem to agree with the findings of Stern *et al.* (3). These results suggest that the increased risk of developing skin cancer in PUVA-treated patients is quite complex and is dependent on patient's genetic background, the number and intensity of PUVA treatments, and prior exposure to other carcinogens. Nonetheless, although the mutagenic and carcinogenic effects of PUVA treatment have been well established (7–13), the molecular mechanisms by which PUVA induces skin cancer are obscure.

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The etiology of skin cancer in psoriasis patients who have undergone PUVA therapy is unknown. However, several theories have been put forward to explain the increased incidence of skin cancer in PUVA-treated patients. First, since PUVA is mutagenic and carcinogenic, skin cancers arising in PUVA-treated patients could in fact be initiated by PUVA. Second, PUVA may not be the primary carcinogen, but it may act as a promoter or cocarcinogen for some other carcinogen such as UV, arsenic, methotrexate, or ionizing radiation (3–6). Third, PUVA treatment causes an immunologic alteration that permits the growth of skin cancers induced by other carcinogenic agents (14, 15). There is very little evidence to support any of these possibilities.

Mutations in the *p53* tumor suppressor gene occur at a high frequency in human and UV-induced murine skin cancers, and predominantly they are of the UV "signature" type, i.e., C→T or CC→TT transitions (16–22). Since the DNA damage induced by PUVA is quite different from UV-induced DNA damage, we hypothesize that mutations induced by these two carcinogenic agents may also be different. While UV radiation induces primarily cyclobutane-type pyrimidine dimers and pyrimidine (6–4) pyrimidone photoproducts (23), PUVA induces monoadducts and interstrand diadducts (cross-links) in cellular DNA (7, 24, 25). Since photoactivated psoralen cross-links occur exclusively at 5′-TA and 5′-TAT sites (7, 8, 25, 26), it is quite possible that PUVA induces signature mutations at these sites in the *p53* tumor suppressor gene leading to the development of skin cancer. If so, it may be possible to use *p53* gene mutations as a molecular marker and to determine whether skin cancers arising in PUVA-treated patients have a UV etiology or a PUVA etiology. We therefore examined PUVA-induced murine skin cancers for carcinogen or site-specific mutations in the *p53* tumor suppressor gene. In addition to PUVA-induced skin tumors, PUVA-exposed skin from tumor-bearing animals were also analyzed for *p53* mutation.

MATERIALS AND METHODS

Induction of Skin Cancers by PUVA in Skh-Hr1 Hairless Mice. The dorsal skins of 3-month-old animals were treated topically with 10 μg of 8-MOP (Sigma) in 100 μl of ethanol. At 20 min after painting, the animals were exposed to 10 kJ/m^2 of filtered (through 6-mm-thick window glass) UVA (320–400 nm) from a bank of two Sylvania F40/350BL lamps (National Biologics, Twinburg, OH) positioned 14 cm above the dorsa of the animals. This window glass filtered out all wavelengths

Abbreviations: PUVA, psoralen + UVA; 8-MOP, 8-methoxypsoralen; SSCP, single-strand conformation polymorphism.

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below 315 nm, and <0.0001% of the total energy was between 315 and 320 nm. The fluence rate was 6.94 W/m^2, as measured by a calibrated Eppley circular thermopile. This PUVA treatment regimen was administered 3 times a week for 42 weeks. The cumulative UVA dose was 1,260 kJ/m^2. Groups of mice were treated with 8-MOP alone or UVA alone for the duration of the experiment. All mice treated with PUVA developed tumors by week 42, whereas none of control mice treated with 8-MOP or UVA alone developed tumors. A total of 13 PUVA-induced tumors and matching, noncancerous PUVA-exposed skin from 10 mice were excised and frozen for molecular analysis. Dorsal skin from unirradiated mice were used as controls. Histologic evaluation revealed that all 13 tumors were squamous cell carcinomas.

PCR Single-Strand Conformation Polymorphism Analysis (SSCP). DNA was extracted from frozen tissues. Then, 60 ng of DNA from each sample was amplified by PCR in a 25-μl solution containing 10 mM Tris·HCl (pH 8.3); 50 mM KCl; 1.5 mM $MgCl_2$; 0.001% gelatin; 75 μM each of dATP, dGTP, dCTP, and dTTP; 2.5 μCi of [α-^{32}P]dCTP/upstream and downstream primers (200 nM each); and 1.5 units of AmpliTaq (PE Xpress) for 30 cycles as described previously (20). The amplification primers spanning the intron-exon boundaries, buffers, cycling conditions, and negative controls used were also as described previously (20). SSCP analysis was performed using a mutation detection enhancement ultra-high-resolution gel (AT Biochem, Malvern PA). Extreme precaution was taken to prevent contamination of PCR reactions, which included isolation of tissue samples and PCR reactions, use of blank PCR controls without DNA templates, and inclusion of normal mouse skin DNA in every PCR reaction. PCR-SSCP analysis was performed twice to confirm the presence or absence of aberrant *p53* bands.

Nucleotide Sequencing. Wild-type and mutant bands were excised from dried SSCP gels, dissolved in 100 μl of water, and heated to 55°C for 2 hr. The supernatants were purified by spinning through Microcon-30 tubes (Amicon) and were used as templates for PCR amplification with exon-specific primers. PCR products were subcloned into the pCR II vector (Invitrogen) and transformed according to the manufacturer's instructions in the TA cloning kit (Invitrogen). Six to nine colonies from each sample were sequenced bidirectionally using M13 (rev) and M13 (−40) primers with Sequenase version 2.0 (USB). In some cases, genomic DNA from SSCP-positive tumors were amplified by PCR and sequenced to confirm the mutation. Similarly, genomic DNAs from SSCP-negative PUVA-exposed skin were amplified by PCR and sequenced. In addition, to rule out PCR-generated mutations, normal mouse skin DNA was analyzed simultaneously in every PCR and sequencing reaction. In no case did we uncover *p53* mutation in normal mouse skin DNA.

RESULTS

PCR-SSCP Analysis of *p53* in PUVA-Induced Skin Tumors. Tumor cell DNA from a total of 13 PUVA-induced skin tumors from 10 Skh-Hr1 mice were analyzed by PCR-SSCP for *p53* mutations in exons 4–8, the region where over 95% of mutations have been reported (27). Six of 13 (46%) tumors exhibited altered SSCP bands in exon 6, five of 13 tumors (38%) in exon 7, and one of 13 (8%) in exon 5. PCR-SSCP analysis of genomic DNAs from PUVA-exposed skin from tumor-bearing mice and from normal Skh-Hr1 mice did not reveal aberrant bands in *p53* exons 4–8. Fig. 1 is a representative SSCP profile showing the presence of aberrant bands in exon 7 of *p53* in some of the PUVA-induced tumors but not in PUVA-exposed or normal skin. Two of 13 PUVA-induced skin tumors revealed mutant bands in more than one *p53* exon (data not shown). Whereas tumor 2T had mutations in exons 5 and 6, tumor 6TI had mutations in exons 6 and 7. It is interesting to note that most of the mutations occurred at exons 6 and 7, which are also rich in 5′-TA sites, suggesting a correlation between the number of mutations detected and the number of cross-linkable 5′-TA sites in the *p53* exons 4–8 (Table 1).

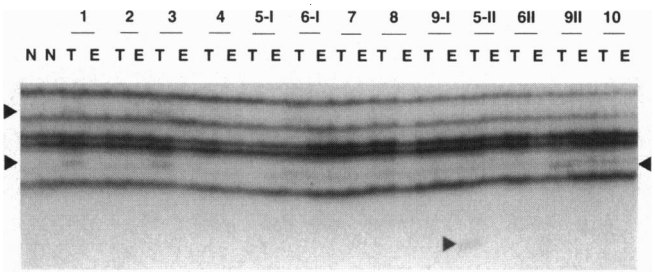

FIG. 1. SSCP profile of *p53* exon 7 in PUVA-induced murine skin cancers. Lanes marked N represent normal mouse skin. Lanes marked E and T represent PUVA-exposed skin and tumor, respectively. Numbers 1–10 represent different PUVA-induced skin tumors. Tumors 5I and 5II, 6I and 6II, and 9I and 9II are two different skin tumors from the same mouse, respectively. Shifted bands are indicated by arrowheads.

Missense *p53* Mutations in PUVA-Induced Skin Cancers. The aberrant bands from SSCP-positive gels were cut out, reamplified by PCR, and sequenced to identify the nature of the *p53* mutations. Ten of 13 SSCP-positive tumor DNAs contained missense point mutations (Table 2). In addition, 3 of 13 tumors contained multiple *p53* mutations. For instance, tumor 6TI contained two mutations in exon 6 at codons 196 and 200 and two mutations in exon 7 at codons 226 and 233 (Table 2). On the other hand, tumors 10T and 9TII displayed two *p53* mutations each; tumor 10T had a codon 192 mutation in one allele and a codon 200 mutation in the other, whereas tumor 9TII had a mutation at codon 225 in one allele and a mutation at 235 in the other allele. In addition to mutant *p53* alleles, all of the tumors contained a wild-type *p53* allele. This could be due to the presence of contaminating normal cells such as fibroblasts, endothelial cells, and inflammatory cells in primary tumors. Analogous to PUVA-induced skin tumors, apparently normal-looking SSCP bands from several PUVA-exposed and normal skin were also subcloned and sequenced. However, in all cases, only the wild-type sequences were detected. In addition, PCR amplification of genomic DNA followed by subcloning and sequencing of 8–10 subclones from PUVA-exposed and normal mouse skin also did not reveal any mutations in *p53* exons 4–8. More importantly, repeat analysis of genomic DNA from several tumors by PCR followed by subcloning and sequencing revealed *p53* mutations that were identical to those found in the first analysis.

PUVA Signature Mutations. Five of seven mutations in exon 6 occurred at codon 200, and four of them were identical C→G transversions, predicting a Pro → Ala substitution. Similarly, three of seven base changes in exon 7 of *p53* were identical

Table 1. Correlation between frequency of *p53* mutation and number of DNA cross-linkable sites in PUVA-induced mouse skin cancers.

Exon	Incidence of *p53* mutation*	No. of 5′-TA sites†
4	0/13 (0%)	2
5	1/13 (8%)	4
6	6/13 (46%)	5
7	5/13 (38%)	7
8	0/13 (0%)	1

*One tumor (2T) displayed alterations in both exon 5 and 6, while another tumor (6TI) had alterations in both exon 6 and 7.
†Number of 5′-TA sites in each exon of the murine wild-type *p53* gene was calculated from published sequences (38).

Table 2. *p53* Missense mutations in PUVA-induced murine skin cancers.

Tumor	Exon	Codon	Base change	Sequence change (5′→3′)*	Amino acid change
2T	5	161	A→C	TAC AAG → TAC CAG	Lys→Gln
6T1	6	196†	G→A	GGA AAT → GAA AAT	Gly→Glu
	6	200†	C→G	TAT CCC → TAT GCC	Pro→Ala
7T	6	200	C→G	TAT CCC → TAT GCC	Pro→Ala
8T	6	200	C→G	TAT CCC → TAT GCC	Pro→Ala
9T1	6	200	C→G	TAT CCC → TAT GCC	Pro→Ala
10T	6	192†	T→C	CTT ATC → CTT ACC	Ile→Thr
	6	200†	C→A	TAT CCC → TAT ACC	Pro→Thr
1T	7	233	T→A	AAG TAC → AAG AAC	Tyr→Asn
3T	7	233	T→A	AAG TAC → AAG AAC	Tyr→Asn
5TII	7	235	T→G	TGT AAT → TGG AAT	Cys→Trp
6T1	7	226†	T→A	TAT ACC → TAA ACC	Tyr→Stop
	7	233†	T→A	AAG TAC → AAG AAC	Tyr→Asn
9TII	7	225†	A→T	GAG TAT → GTG TAT	Glu→Val
	7	235†	G→T	TGT AAT → TTT AAT	Cys→Phe

*Wild-type bases representing sites for PUVA-induced DNA cross-links are underlined. Wild-type and corresponding mutant bases are shown in bold.
†Mutations in two different alleles.

T→A transversions at codon 233 (Table 2). The mutational profile of three representative PUVA-induced skin tumors with base substitutions at 5′-TA sites in exon 6 or 7 along with the corresponding wild-type sequences from normal skin are shown in Fig. 2. Interestingly, 14 of 15 (93%) missense mutations detected occurred at or within 2 bases of 5′-TA sequences (Table 2). Of these, six mutations were at 5′-TA or 5′-TAT sites, six mutations were at 5′ or 3′ bases immediately adjacent to 5′-TA or 5′-TAT sites, and two mutations were two bases away from 5′-TA or 5′-TAT sites. Missense mutations, such as C→T and CC→TT transitions, which are hallmarks of UVC and UVB-induced mutations, were conspicuously absent in PUVA-induced mouse skin tumors, with the exception of a single C→T mutation at a dipyrimidine site in tumor 6TI. Even though 6 of 15 *p53* mutations occurred at cytosine-cytosine residues, only one them was a C→T transition (Table 2).

Silent *p53* Mutations in PUVA-Induced Skin Cancers. In addition to missense mutations, some of the tumors displayed silent mutations in that they encoded the same amino acid. Of the 25 silent base changes detected, a sizable number (17 of 25; 68%) were at or near (within 2 bases of) 5′-TA sites (data not shown). These silent mutations were mainly C→A, G→A, C→T, or T→C substitutions occurring at codons 193, 201, 212, and 217, respectively (data not shown). Multiple silent mutations were seen in exon 6 in all six tumors, further suggesting that mutations in *p53* might have arisen independently within the same tumor environment. In contrast to tumors, none of the PUVA-exposed skin contained silent mutations.

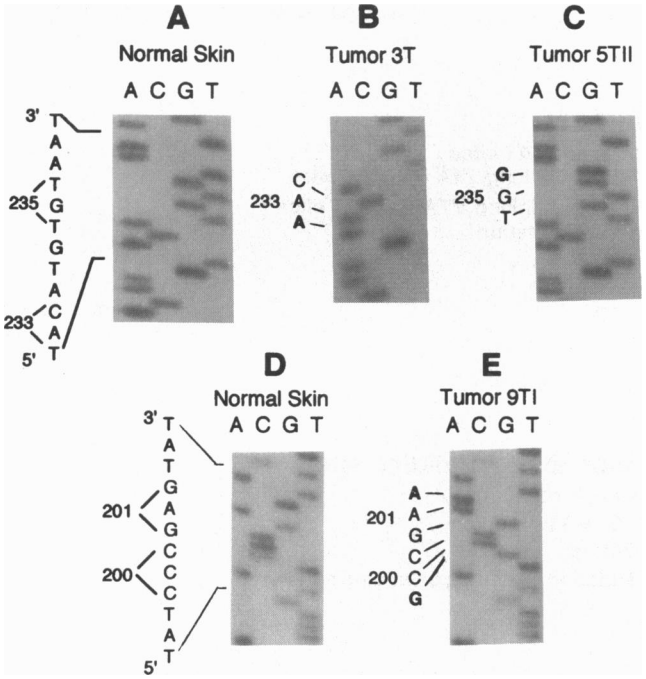

FIG. 2. Missense *p53* mutations in representative PUVA-induced skin cancers. (*A*) Wild-type sequence surrounding codons 233 and 235 from normal mouse skin. (*B*) Codon 233 mutation in tumor 3T. (*C*) Codon 235 mutation in tumor 5TII. (*D*) Wild-type sequence surrounding codons 200 and 201. (*E*) Mutations at codons 200 and 201 in tumor 9TI. The G→A mutation at codon 201 is silent; both GAG and GAA encode glutamic acid.

DISCUSSION

With the wide-spread use of PUVA therapy for the treatment of psoriasis and other skin disorders, considerable attention has been focused on understanding the mutagenic and carcinogenic effects of PUVA. Several studies have shown that PUVA is a potent mutagen and a carcinogen (7–13). *In vitro* studies have revealed that PUVA induces mutations in mammalian cells predominantly at 5′-TA sequences, which are sites for formation of both monoadducts and interstrand DNA cross-links (7, 8, 13). Although it is known that PUVA-induced DNA cross-links and monoadducts play a direct role in mutagenesis, their role in tumorigenesis is unclear. We therefore examined PUVA-induced murine skin cancers for site-specific genetic alterations and found that 77% of the tumors harbored *p53* mutations. In contrast, *p53* mutations were not detected in PUVA-exposed skin from tumor-bearing mice or in normal mouse skin. The absence of *p53* mutation in PUVA-exposed skin suggests that the mutations detected in PUVA-induced skin tumors did not arise during PCR due to the possible persistence of psoralen–DNA adducts, although there is precedence for DNA polymerases having difficulty at or near an adduct (28, 29). In addition, the possibility that the mutations found in PUVA-induced skin cancers were due to PCR artifacts can be ruled out because identical mutations were detected by two separate PCR and sequencing analysis of genomic DNA from some tumors. Nonetheless, 93% of all missense *p53* mutations occurred at or near (within one or two bases of) 5′-TA or 5′-TAT, potential sites for psoralen-DNA cross-link and monoadduct formation (Table 2). Five of 15 missense *p53* mutations occurred at codon 200, and three of 15 mutations occurred at codon 233, suggesting that these two

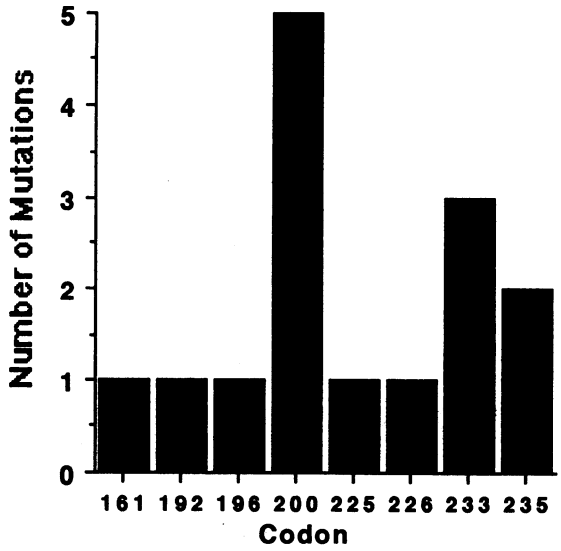

FIG. 3. p53 mutation hot spots in PUVA-induced murine skin cancers. Number of mutations at each codon was calculated from Table 2. A total of 15 missense mutations were detected in 10 tumors.

codons may be hot spots for PUVA-induced mutation (Fig. 3). Analogous to PUVA-induced skin tumors, *p53* mutation hot spots have also been reported for UV-induced skin cancers (17), which are quite distinct from those found in PUVA-induced mouse skin cancers. Interestingly, *p53* mutation hot spots in UV-induced skin cancers are associated with slow repair of UV-induced DNA lesions (30).

The propensity of *p53* mutations at exons 6 and 7 could be explained by the fact that these two exons are much richer in 5′-TA sequences than other *p53* exons (Table 1). Analogous to the UV-repair system (30), differential repair rates of psoralen-DNA cross-links, perhaps due to chromatin folding, might also explain why mutations are targeted at only certain 5′-TA sites. The susceptibility of 5′-TA sites in the *p53* gene to PUVA-induced mutations appears to fit the model proposed for PUVA-directed mutagenesis at cross-linkable 5′-TA sequences (13, 25), in which psoralen adducts on the transcribed strand are preferentially excised, followed by an error-prone (mutagenic) translesion synthesis across the gap by DNA polymerases. In addition to missense mutations at 5′-TA sites, a number of silent mutations were observed at GC sequences (data not shown). Similar mutations at GC sequences have also been detected in the *supF* tRNA gene following PUVA treatment (25).

Three PUVA-induced mouse skin tumors (6TI, 9TII, and 10T) contained multiple *p53* mutations. While the 10T tumor harbored *p53* mutations at codons 192 and 200 on different alleles, the 9TII tumor had mutations at codons 225 and 235 on different alleles. The 6TI tumor, on the other hand, contained four different *p53* mutations: two in exon 6 and two in exon 7 (Table 2). It is difficult, however, to tell whether the mutations detected in exons 6 and 7 in tumor 6TI are located on the same allele or on different alleles because these two exons were analyzed in separate PCR reactions using primers encompassing either exon 6 or exon 7. Nonetheless, the presence of multiple *p53* mutations in PUVA-induced mouse skin cancers suggests that clones harboring an initial mutation on one allele were targets for a second mutational event on the other allele or that these mutations may have arisen independently, perhaps in different clonal subpopulations during tumor development. The presence of multiple *p53* mutations have also been found in human and UV-induced mouse skin cancers (17, 20, 21) as well as in human bladder cancer (31, 32).

A comparison of the *p53* mutational spectra in PUVA- and UV-induced murine skin cancers reveal important insights into the mechanisms by which these two carcinogens induce skin cancer. First, PUVA-induced skin cancers, with a single exception, do not display C→T or CC→TT transitions (Fig. 4*A*), whereas UV-induced skin cancers display these mutations at a high frequency (Fig. 4*B*) (16–22). Second, PUVA-induced skin tumors display transversions at 5′-TA or 5′-TAT sites at

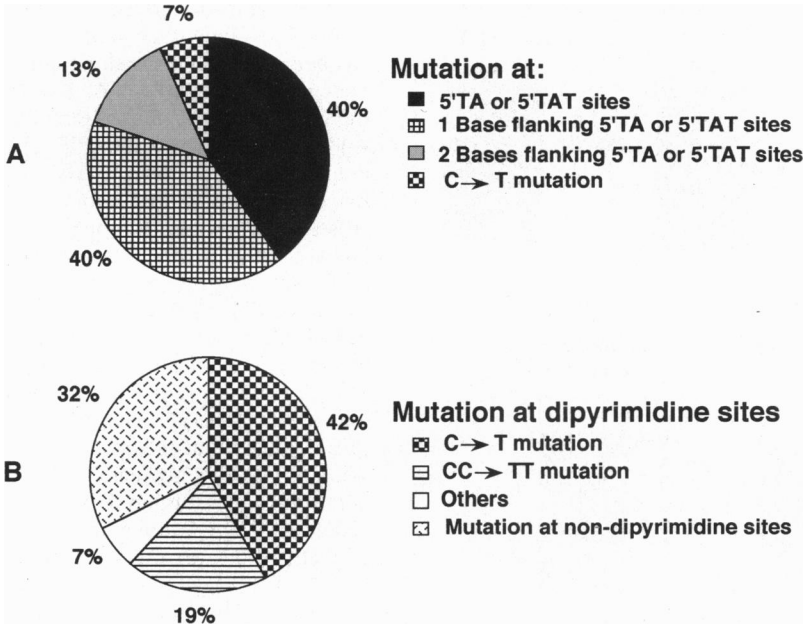

FIG. 4. p53 mutational spectra in PUVA- and UV-induced murine skin cancers. (*A*) Location and frequency of base substitutions in PUVA-induced murine skin cancers. Six of 15 (40%) mutations occurred at 5′-TA or 5′-TAT sites; six of 15 (40%) occurred at residues flanking 5′-TA or 5′-TAT sites; and two of 15 (13%) were seen 2 bases away from 5′-TA or 5′-TAT sites. One of 15 (7%) mutations was a C→T transition. (*B*) Type and frequency of *p53* mutations detected in UV-induced murine skin tumors. Mutation data were pooled from Kress *et al.* (19) and Kanjilal *et al.* (20). UV-specific mutations (C→T and CC→TT transitions) at dipyrimidine sites occurred at a frequency of 61%, while other types of mutations occurred at a frequency of 39%. None of the UV-induced mouse skin cancers contained mutation at 5′-TA sites. Only one mutation occurred at 2 bases away from a 5′-TA site (19).

a high frequency, whereas UV-induced skin tumors do not. This difference in *p53* mutation spectra between PUVA- and UV-induced mouse skin tumors could be due to the fact that PUVA induces interstrand cross-links and monoadducts in the DNA at 5′-TA sites (7, 24–26), whereas UV induces pyrimidine dimers and (6–4) photoproducts at dipyrimidine sequences (23). Errors in repair of these lesions leads to the generation of site-specific mutations. The fact that C→T and CC→TT transitions were not detected in PUVA-induced mouse skin cancers rules out the involvement of UVB-induced DNA damage in the induction of *p53* mutation. In addition, the possibility that *p53* mutations in PUVA-induced mouse skin cancers could have risen from UVA-induced DNA damage can also be ruled out because T→G transversions, which are characteristic of UVA-induced mutations (33), were rarely detected. Interestingly, however, a majority of the mutations in PUVA-induced mouse skin cancers originated from the nontranscribed strand of the *p53* gene, suggesting a strand bias for mutation induction. Sage *et al.* (13) also found that over 90% of PUVA-induced mutations in Chinese hamster APRT locus resulted from a damaged T residue located on the nontranscribed strand. These results are in accordance with the principle of preferential repair (34–37).

In summary, our studies demonstrate that PUVA induces signature mutations in the *p53* tumor suppressor gene that are quite different from UV signature mutations. An important implication of this funding is that if PUVA therapy is responsible for the increased incidence of skin cancer in psoriasis patients who have undergone this therapy, it may be possible to use the *p53* gene as a molecular marker and to determine whether or not PUVA is directly associated with the induction of skin cancer, based on the presence or absence of PUVA signature mutations. Such studies are currently in progress.

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