Proc. Natl. Acad. Sci. USA
Vol. 92, pp. 2350–2354, March 1995
Genetics

A role for ultraviolet A in solar mutagenesis

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Communicated by Richard B. Setlow, Brookhaven National Laboratory, Upton, NY, December 12, 1994 (received for review October 17, 1994)

ABSTRACT **It is well established that exposure to solar UVB (290–320 nm) gives rise to mutations in oncogenes and tumor suppressor genes that initiate the molecular cascade toward skin cancer. Although UVA (320–400 nm) has also been implicated in multistage photocarcinogenesis, its potential contribution to sunlight mutagenesis remains poorly characterized. We have determined the DNA sequence specificity of mutations induced by UVB ($\lambda > 290$ nm), and by UVA ($\lambda > 350$ nm), at the adenine phosphoribosyltransferase locus of Chinese hamster ovary cells. This has been compared to results previously obtained for simulated sunlight ($\lambda \geq 310$ nm) and 254-nm UVC in the same gene. We demonstrate that T → G transversions, a generally rare class of mutation, are induced at high frequency (up to 50%) in UVA-exposed cells. Furthermore, this event comprises a substantial proportion of the simulated sunlight-induced mutant collection (25%) but is significantly less frequent ($P < 0.05$) in cells irradiated with either UVB (9%) or UVC (5%). We conclude that the mutagenic specificity of broad-spectrum solar light in rodent cells is not determined entirely by the UVB component and that UVA also plays an important role.**

The alarming rise in the frequency of skin cancer in human populations is often blamed on depletion of stratospheric ozone and the concomitant increase in environmental levels of highly genotoxic UVB (290–320 nm) in sunlight (1). Much less attention has focused on the potential contribution of solar UVA (320–400 nm), which is not absorbed by ozone and is orders of magnitude less deleterious to DNA than UVB on the basis of incident energy (2). However, UVA is mutagenic in cultured cells (3) and a complete carcinogen in rodents (4). Relative to UVB, it comprises a substantially larger proportion of the total energy in sunlight (2) and is much more efficient in penetrating the actively dividing basal layer of the epidermis (5), where photocarcinogenesis is most likely initiated through mutations. In addition, notwithstanding the very recent availability of broad-band (UVB plus UVA) sunscreens, the previous widespread use of UVB-specific blocking agents allowed greatly prolonged periods of recreational suntanning, accompanied by dramatic increases in human exposure to UVA. These considerations collectively argue that the mutagenic effects of UVA, in addition to those of UVB, may have contributed substantially to the increased incidence of skin cancer over the past number of decades.

The precise identification of DNA sequence alterations induced by environmental carcinogens in cultured cells constitutes a powerful approach for probing the genotoxic basis of multistage carcinogenesis in humans (6) as well as fundamental mechanisms of mutagenesis (7). Such investigations have indicated that dipyrimidine photoproducts—i.e., cyclobutane pyrimidine dimers (CPDs) and/or pyrimidine-pyrimidone(6-4) photoproducts—are the major premutagenic lesions in cells irradiated with UVC or UVB and preferentially target C → T and some tandem CC → TT events at dipyrimidine sites (8–14). It was subsequently shown that mutated p53 tumor suppressor genes frequently recovered from human basal and squamous cell carcinomas bear this "molecular fingerprint," directly demonstrating the importance of UVB-induced dipyrimidine photoproducts in nonmelanoma skin cancer (15, 16). However, in the case of malignant melanoma, UVB exposure may not constitute the only critical predisposing factor. Indeed, accumulating epidemiological and molecular evidence (reviewed in ref. 17) actually supports the notion that UVA also plays a major role in the complex etiology of this particular disease. For example, the UV action spectrum for melanoma induction in fish indicates that the majority of tumors may be attributable to the mutagenic effects of wavelengths >320 nm (18). Unfortunately, any attempt to directly establish a genotoxic role for UVA in human photocarcinogenesis using DNA sequence analysis would be hampered by the fact that nothing has been reported on the precise molecular nature of UVA-induced mutations, in either prokaryotes or eukaryotes.

In addressing this problem, we have characterized the mutational specificity of UVB ($\lambda > 290$ nm), and of UVA ($\lambda > 350$ nm), at the adenine phosphoribosyltransferase (*aprt*) locus in Chinese hamster ovary (CHO) cells. Together with results previously obtained for broad-spectrum simulated sunlight (SSL; $\lambda \geq 310$ nm) and 254-nm UVC in the same gene (13, 14, 19), this comprises the most comprehensive collection of UV-induced DNA sequence alterations in any system to date. Analysis of this unique data base reveals direct evidence that wavelengths in the UVA region can significantly influence the DNA sequence specificity of sunlight-induced mutations in mammalian cells.

MATERIALS AND METHODS

Irradiation Conditions, Mutant Selection, and Sequence Analysis. The CHO strain used for collection of mutants (AT3; kindly provided by Gerald Adair, University of Texas) is a DNA nucleotide excision repair-proficient, *aprt* hemizygote (+\0). Cells were routinely propagated in α-minimal essential medium, containing 5% fetal calf serum (GIBCO). Twenty-four hours prior to mutagenic treatment, 1×10^6 cells were seeded on 35-mm dishes (Nunc). Exponentially growing monolayers (i.e., 3×10^6 cells) were then washed with Dulbecco's phosphate-buffered saline (PBS), and irradiated in PBS at 0°C using fluorescent 25-W lamps emitting either UVB (Spectroline model XX25B; Fig. 1*A*) or UVA (GE model F25T8-BL; Fig. 1*B*). The incident light was purified using 2-mm-thick glass filters (Schott, Mainz, Germany) to eliminate contaminating wavelengths below 290-nm in the case of UVB (filter WG 305) and below 350 nm for UVA (filter WG 360). The incident UVB ($\lambda > 290$ nm) and UVA ($\lambda > 350$ nm) dose rates were measured using a Spectroline DRC 100X digital radiometer equipped with DIX 300 and DIX 365 sensors, respectively. Following a 6-day phenotypic expression period, clonally independent *aprt*$^-$ mutants induced either by UVA (λ

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Abbreviations: *aprt*, adenine phosphoribosyltransferase; CHO, Chinese hamster ovary; CPD, cyclobutane pyrimidine dimer; ROS, reactive oxygen species; SSL, simulated sunlight.

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> 350 nm) or UVB (λ > 290 nm) were isolated by phenotypic selection using the base analogue 8-azaadenine according to established protocols (13). Mutant *aprt* alleles were amplified by the polymerase chain reaction, and the nontranscribed strand was sequenced by the Sanger method using *Taq* DNA polymerase as described (19). The protocols employed for 254-nm UVC and SSL (λ ≥ 310 nm) irradiations, and subsequent analysis of mutants, have been outlined in detail previously (13, 14, 19). Unless otherwise indicated, the terms UVA, UVB, and SSL will hereafter correspond to λ > 350 nm, λ > 290 nm, and λ ≥ 310 nm, respectively.

RESULTS

Mutation Induction by UVA and UVB at the CHO *aprt* Locus. *aprt*$^-$ mutants were selected following irradiation of CHO cells with UVB and UVA generated by fluorescent lamps (Fig. 1). The doses used were 500 kJ·m^{-2} (dose rate, 75 J·m^{-2}·s^{-1}) for UVA and 100 J·m^{-2} (dose rate, 1 J·m^{-2}·s^{-1}) for UVB. Each mutagenic treatment yielded a relative survival of 100% and a significant induction of *aprt*$^-$ mutants over spontaneous background—i.e., 20- to 30-fold for UVB and 3- to 5-fold for UVA. (It should be noted, therefore, that *aprt*$^-$ variants of spontaneous origin constituted ≈20–30% of the UVA mutant collection.) Although the UVB-induced mutant frequency increased in a dose-dependent manner within the range of 25–750 J·m^{-2} (data not shown), it was not possible to generate a dose–response curve in the case of UVA. We were constrained by the relatively weak output of the UVA lamp as well as by normal experimental variation in the yield of spontaneous *aprt*$^-$ mutants. To observe a UVA-induced mutant frequency that was clearly distinguishable above background, it was necessary to irradiate cells for ≈2 hr in PBS (i.e., corresponding to a dose of 500 kJ·m^{-2}). Significantly shorter irradiation times did not yield a clear induction of mutations, while longer ones compromised cell viability. However, we note that the 5000-fold difference in dose used for UVA and UVB treatments produced a 10-fold lower induction of UVA- vs. UVB-induced mutations. This indicates that UVB was 5 × 10^4-fold more mutagenic than UVA, in accord with other published data on solar UV mutagenesis (18). We also emphasize that the doses chosen for collection of *aprt*$^-$ mutants are within the range of those encountered during casual exposure to natural sunlight. For example, 500 kJ·m^{-2} of UVA could correspond to one minimal erythemal dose received within a period of 5 hr by an individual wearing a UVB sunprotection factor sunscreen (20).

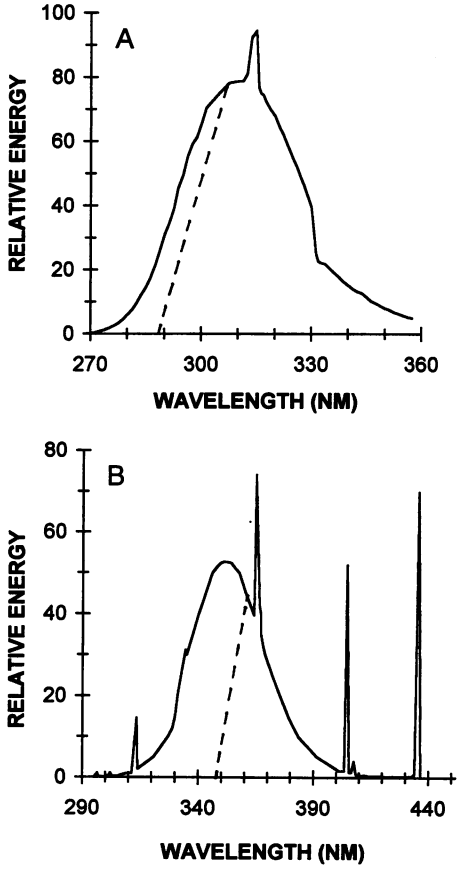

FIG. 1. Wavelength emission spectra of fluorescent UVA (*B*) and UVB (*A*) lamps. The dashed lines indicate the UV wavelength cutoff, using glass filters as described in the text.

To ensure that the UVA mutant collection did not reflect any effects due to leakage from the relatively small, but highly mutagenic, UVB component of the UVA lamp (Fig. 1*B*), we performed the following control. The source depicted in Fig. 1*A*, in conjunction with the glass filter (WG 360) described in Fig. 1*B*, was used to irradiate a UV-hypersensitive, DNA excision repair-deficient CHO strain (UVL-9; kindly provided by G. Adair, University of Texas) with 10,000 J·m^{-2} of UVB radiation. (This highly genotoxic UVB dose corresponds to that actually emitted by the UVA lamp under our experimental conditions.) No cytotoxic or mutagenic effects were observed. Likewise, mutagenesis and cytotoxicity were abolished following exposure of the same repair-deficient strain to 500 J·m^{-2} of 254-nm UVC, which was first purified using the WG 305 filter as in Fig. 1*A*. We also note that the UVB lamp emitted some energy >320 nm that could not be eliminated. However, the total UVA output in this case, not exceeding 20 J·m^{-2}, was unlikely to have any significant biological effect.

Mutational Specificity of UVA and UVB at the CHO *aprt* Locus. A total of 74 UVB- and 54 UVA-induced, clonally independent mutant *aprt* alleles was enzymatically amplified by PCR and directly sequenced. All DNA changes resulted in amino acid substitutions or alteration of a splice junction consensus sequence. The distribution by class of UVA- and UVB-induced mutations at the CHO *aprt* locus is presented in

Table 1. Classes of mutation induced by UVC, UVB, UVA, and SSL at the CHO *aprt* locus

	− UV*		UVC†		UVB		UVA		SSL‡	
Mutation	No.	%	No.	%	No.	%	No.	%	No.	%
Transitions										
G·C → A·T	22	26	33	57	49	66	12	22	13	36
A·T → G·C	9	10	4	7	0	0	5	9	1	3
Transversions										
G·C → T·A	11	13	0	0	1	1	2	4	0	0
G·C → C·G	9	10	5	9	1	1	5	9	0	0
A·T → T·A	2	2	3	5	2	3	1	2	2	6
A·T → C·G	2	2	3	5	7	9	20	37	9	25
Doubles										
Tandem	0	0	6	10	5	7	6	11	11	31
Nontandem	1	1	4	7	6	8	2	4	0	0
CC → TT§	0	0	3	5	4	5	3	5	9	25
Insertions	1	1	0	0	0	0	0	0	0	0
Deletions	18	21	0	0	0	0	0	0	0	0
Frameshifts	6	7	1	2	2	3	1	2	0	0
Duplications	5	6	0	0	1	1	0	0	0	0
Total	86		59		74		54		36	

*From ref. 21.
†Data pooled from investigations using the endogeneous *aprt* locus (13) as well as the *aprt* cDNA carried on a stably integrated, single-copy retroviral shuttle vector (14). The mutation spectra obtained using each of these systems were shown to be broadly similar.
‡From ref. 19.
§Also included as part of the "tandem" category.

Table 1, together with the results obtained previously for UVC- and SSL-exposed cells, as well as for spontaneously arising mutants. The types and proportions of events generated by UVB vs. UVC were largely comparable—i.e., a predominance of C → T transitions (66% vs. 57%), and some CC → TT events (5% in each case), at dipyrimidine sites. As previously discussed, the prevalence of these "UV signature" mutations in SSL-exposed cells confirms an important role for dipyrimidine photoproducts in solar mutagenesis. However, for SSL- vs. UVB-exposed cells, a significant decrease in C → T transitions (36% vs. 66%, $P < 0.05$, two-tailed Fisher's exact test) was accompanied by significant increases in the frequency of CC → TT tandem double events (25% vs. 5%, $P < 0.05$) and T → G transversions (25% vs. 9%, $P < 0.05$), in accord with the previously observed situation for SSL vs. UVC. Moreover, T → G was clearly the predominant event induced by UVA, constituting 37% the mutant spectrum. This is likely an underestimate, since up to 30% of the UVA collection may be comprised of spontaneous mutants. Therefore T → G events, which are only very rarely recovered spontaneously at the CHO *aprt* locus (ref. 21; Table 1), may constitute as much as 50% of actual UVA-induced mutations. We also note that tandem double CC → TT transitions were recovered following UVA irradiation in the same proportion as for UVC and UVB (i.e., 5%).

Site Specificity of Mutations Induced by UVA, UVB, UVC, and SSL at the *aprt* Locus. A composite representation of spontaneously occurring *aprt*$^-$ mutations in CHO cells, in addition to those induced by UVA, UVB, UVC, and SSL, is presented in Fig. 2. Despite the aforementioned heterogeneity in the frequency of mutational classes, important similarities in the site specificity of mutations induced by each wavelength region were observed. Indeed, 60% of the entire UV mutant collection was distributed among just 12 potential dipyrimidine target sites within the *aprt* coding sequence, and fully one-half of these mutations occurred within a single pyrimidine run located in exon 2—i.e., CCTCCTTCC 238–246. It is remarkable that the molecular fingerprint distinguishing mutations induced by each wavelength region at the *aprt* locus (i.e., C → T for UVC and UVB; CC → TT and T → G for SSL; T → G for UVA) are all manifested within this highly mutable pyrimidine run. In addition, we note that a CC → TT mutational hotspot was recovered at positions 241–242 in UVA- and SSL-exposed cells, whereas this site was dominated by single-base C → T events at position 241 following irradiation with either UVB or UVC.

DISCUSSION

We have determined the DNA sequence specificity of mutations induced by UVA and UVB at the *aprt* locus in CHO cells using fluorescent UV lamps. A comparison with results obtained earlier for SSL and 254-nm UVC in the same gene reveals further information about mechanisms of DNA damage processing in sunlight-exposed mammalian cells. Most notably, our data provide direct evidence that wavelengths in the UVA region play a significant role in solar mutagenesis. We observed previously that T → G transversions constituted a significant proportion of the SSL-induced mutant collection (25%; Table 1). In general, these events are only rarely induced in cultured cells, either spontaneously or following treatment with diverse mutagens. The frequent recovery of T → G

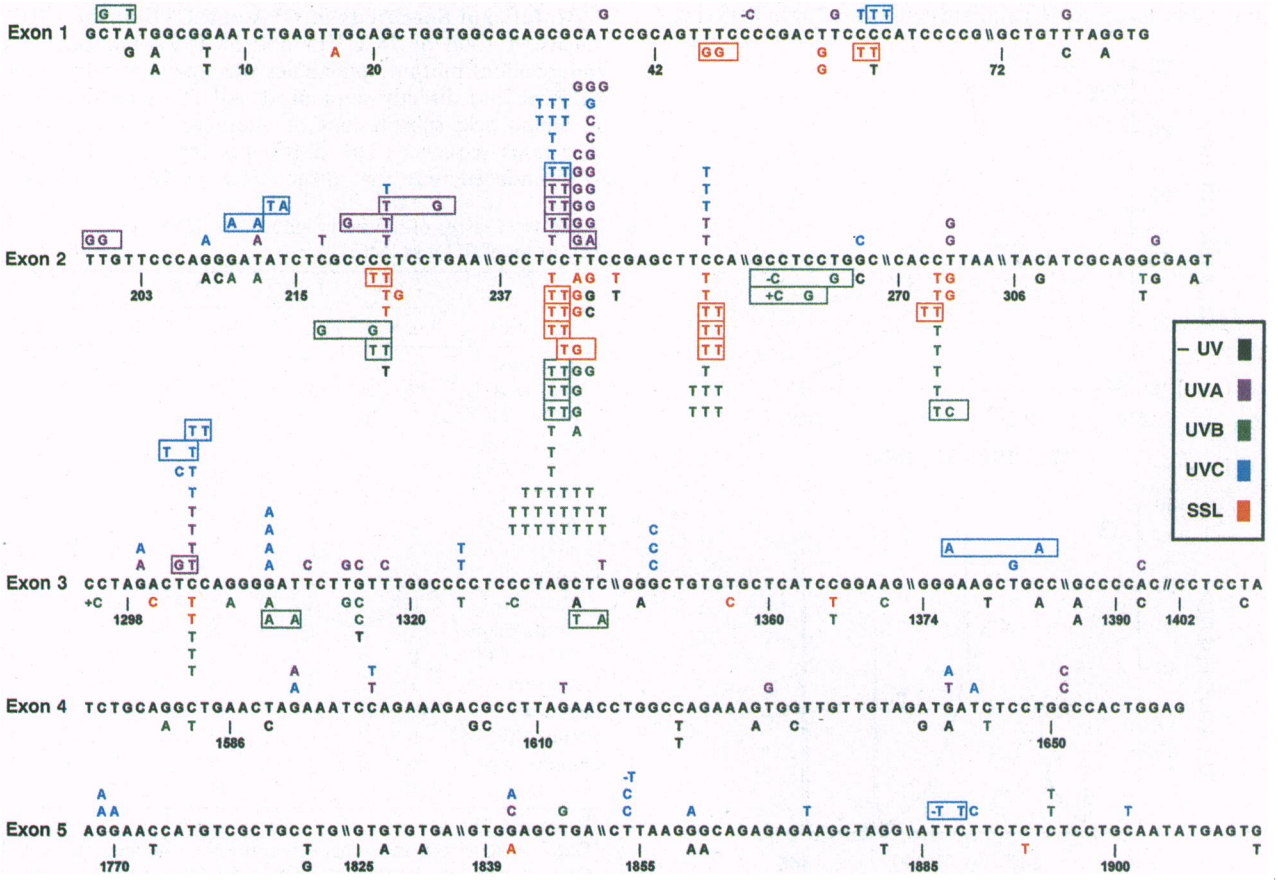

FIG. 2. DNA sequence specificity of spontaneous mutations ($n = 86$) in the CHO *aprt* gene, as well as those induced by UVC ($n = 59$), UVB ($n = 74$), UVA ($n = 54$), and SSL ($n = 36$). The five sequences from top to bottom represent portions of the nontranscribed strand for exons 1–5, respectively, and comprise 80% of the *aprt* coding region. A double backslash (\\) indicates a break in the sequence. Tandem and nontandem double events are boxed.

mutations in SSL-irradiated cells can now be attributed to the UVA component of SSL, with little or no direct contribution from UVB, since (*i*) this event is clearly the most prevalent mutational class induced by UVA (up to 50%) and (*ii*) the incidence of T → G transversions is significantly lower in UVB- vs. SSL-exposed cells (9% vs. 25%, $P < 0.05$; Table 1). Thus, T → G may constitute a unique molecular fingerprint for mutations induced by UVA in rodent cells, which could ultimately prove useful for assessing the genotoxic role of this wavelength region in human photocarcinogenesis.

It is also noteworthy that while tandem double CC → TT mutations are clearly a hallmark of SSL mutagenesis at the CHO *aprt* locus (25%; Table 1), as well as at the p53 locus in human skin tumors (15, 16), these events are much less prevalent in UVB-irradiated cells (only 5%; $P < 0.05$). The overall data therefore indicate that not only does UVA influence a significant sector of solar mutagenesis in rodent cells but also, as corollary, that the direct effects of UVB alone do not make as important a contribution as one might predict based on the relatively powerful mutagenic potential of this wavelength region. Indeed, our findings highlight the need for caution when employing UVC, as well as "environmentally relevant" UVB, as models for investigating mechanisms of sunlight-induced mutagenesis and carcinogenesis.

One potential caveat concerns the difference between the UVB content of the source depicted in Fig. 1*A* ($\lambda > 290$ nm) vs. that of SSL ($\lambda \geq 310$ nm), since the shorter UVB wavelengths are considerably more efficiently absorbed by DNA. It is therefore important to ensure that the apparent role of UVA in solar mutagenesis has not been overestimated due to any effective lack of UVB in the filtered SSL source. We note that wavelengths in the UVB and UVA regions contributed 4 $kJ \cdot m^{-2}$ and 40 $kJ \cdot m^{-2}$, respectively, to the total SSL dose of 500 $kJ \cdot m^{-2}$ used for the collection of *aprt*$^-$ mutants. CPD induction, expressed as T4 endonuclease V-sensitive sites (22), was determined under various conditions *in vitro* using a plasmid relaxation assay (23). These experiments were performed in another laboratory (E. Sage, Institut Curie, Paris; personal communication), but with identical model lamps and glass filters. The number of endonuclease-sensitive sites per plasmid following irradiation with 500 $kJ \cdot m^{-2}$ of SSL and 4 $kJ \cdot m^{-2}$ of UVB was 0.4 and >4.0, respectively. No CPD were detected after exposure to 40 $kJ \cdot m^{-2}$ of UVA. Therefore, given the ≈10-fold increase that would be predicted for CPD induction by UVB vs. SSL (18), it appears that virtually all of the highly premutagenic dipyrimidine photoproducts induced by SSL were generated by the UVB component, as is the case for natural sunlight.

The identity of the premutagenic photoproduct responsible for T → G mutations in UVA/SSL-irradiated cells remains to be established. We suggest either (*i*) CPDs, which are detectable at low levels in UVA-irradiated cells (24), or (*ii*) an unidentified, UVA-specific, nondimer lesion. In the latter case, UVA would contribute directly to the genotoxic burden imposed by sunlight by inducing a unique photoproduct causing T → G mutations. Conversely, if CPDs are in fact implicated, then UVA as a component of broad-spectrum sunlight would act by influencing mutation fixation opposite thymine-containing cyclobutane dimers generated orders of magnitude more efficiently by the UVB region. Although our data do not clearly distinguish these possibilities, they nonetheless favor a role for CPD in UVA mutagenesis (Fig. 2). (*i*) The majority of UVA-induced *aprt*$^-$ mutations are located at dipyrimidine sequences. (Only five events were targeted at isolated purines/pyrimidines, among which three were also observed in the spontaneous mutant collection.) (*ii*) The recovery of three CC → TT "signature" mutations at positions 241–242 indicates the importance of CC CPDs in the formation of UVA-induced mutational hotspots. (*iii*) Tandem double TT → GG mutations were recovered only in UVA- and SSL-exposed cells, which, by analogy with the situation for CC → TT, may be diagnostic for TT CPDs. (*iv*) An extremely prominent UVA-induced hotspot was observed at TT 243–244. T → G events are recovered opposite both nucleotides at this site, suggesting the involvement of a TT dipyrimidine photoproduct, where each base has significant miscoding potential. We believe that the TT CPD would be involved rather than the TT pyrimidine-pyrimidone(6-4) photoproduct or its Dewar valence isomer, as the latter are relatively rare in UV-irradiated cells (25). Since reactive oxygen species (ROS) have been implicated in the genotoxic effects of sunlight (3), it should be noted that the above arguments favoring the involvement of CPD in UVA mutagenesis also dispute a role for the highly mutagenic oxidative intermediate 8-oxo-dGTP, the only potential candidate to our knowledge for a nondimer photoproduct that specifically causes T → G transversions (26). In addition, although CC → TT mutations have been recovered on plasmid DNA after exposure *in vitro* to ROS followed by passage through bacteria (27), this has yet to be demonstrated for a mammalian chromosomal gene.

A striking feature of solar UV mutagenesis at the CHO *aprt* locus is revealed in the distribution of mutations, as 60% of the entire collection is attributable to just 12 dipyrimidine sites located within four pyrimidine runs (Fig. 2). This represents only a small proportion of the available genetic target (28), demonstrating that the clustering of events is not an artifact of phenotypic selection bias. It is also remarkable that the unique characteristics of mutation spectra induced by each UV wavelength region are manifested within a single mutation-prone pyrimidine run (CCTCCTTCC 238–246) that includes 31% of all events (Fig. 2). This particular sequence constitutes a hotspot for dipyrimidine photoproduct formation following irradiation with UVC and SSL *in vitro* (19, 29) and appears to be preferentially hypermutable by UV light, since mutants induced by a wide variety of other mutagens are only rarely recovered there (28). We note that 67% of DNA sequence alterations recovered at the p53 locus from human nonmelanoma tumors are also clustered within a very limited portion of the effective genetic target (16). In this case, the nonrandom distribution of mutations has been attributed to the frequency of damage as well as the rate of nucleotide excision repair for CPDs at individual sites (30, 31).

The creation here of a comprehensive data base for mutations induced by defined regions of the solar wavelength spectrum constitutes a fundamental step toward elucidating the mechanism of sunlight mutagenesis in mammalian cells as well as the genotoxic basis of multistage photocarcinogenesis. Ultimately, it remains to be established whether the T → G fingerprint for UVA mutagenesis at the CHO *aprt* locus also applies to mutated oncogenes/tumor suppressor genes in transformed human melanocytes *in vivo*. In addition, although T → G transversions were not observed at the p53 locus in human basal and squamous cell carcinomas, it is possible that these mutations will be recovered from such tumors appearing in subsequent generations. This would reflect the long latency period for nonmelanoma skin cancer, coupled with the prevalent "abuse" of UVB-specific sunscreens, which has only recently led to massive increases in human exposure to UVA.

We thank Drs. Evelyne Sage, Claude Perreault, and Alasdair Gordon for valuable discussions. This work was funded by The Cancer Research Society, Inc. (Canada).

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