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Determination of the Spectrum of Mutations Induced by Defined-Wavelength Solar UVB (313-nm) Radiation in Mammalian Cells by Use of a Shuttle Vector

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Mutations induced by UVB (313-nm) radiation, a wavelength in the region of peak effectiveness for sunlight-induced skin cancer in humans, have been analyzed at the sequence level in simian cells by using a plasmid shuttle vector (pZ189). We find that significant differences exist between the types of mutations induced by this solar wavelength and those induced by nonsolar UVC (254-nm) radiation. Compared with 254-nm radiation, 313-nm radiation induces more deletions and insertions in the region sequenced. In addition, although the types of base substitutions induced by the two wavelengths are broadly similar (in both cases, the majority of changes occur at G-C base pairs and the G-C to A-T transition is predominant), an analysis of the distribution of these base changes within the *supF* gene following irradiation at 313 nm reveals additional hot spots for mutation not seen after irradiation at 254 nm. These hot spots are shown to arise predominantly at sites of mutations involving multiple base changes, a class of mutations which arises more frequently at the longer solar wavelength. Lastly, we observe that most of the sites at which mutational hot spots arise after both UVC and UVB irradiation of the shuttle vector are also sites at which mutations arise spontaneously. Thus, a common mechanism may be involved in determining the site specificity of mutations, in which the DNA structure may be a more important determinant than the positions of DNA photoproducts.

Solar UV radiation is a potent environmental carcinogen. Epidemiological studies suggest that exposure to sunlight is responsible for at least 90% of nonmelanoma skin cancer in humans (Causes and effects of of stratospheric ozone reduction: an update, National Academy Press, Washington, D.C., 1982). Recent studies of oncogene activation in chemically induced animal tumors have provided strong evidence that point mutations play a fundamental role in the initiation of oncogenesis (1, 28). Therefore, to gain an understanding of the molecular mechanisms involved in solar carcinogenesis it is of fundamental interest to determine the mutagenic specificity of sunlight at the DNA sequence level in mammalian cells.

The important wavelengths with respect to the carcinogenic potential of sunlight are believed to lie in the UVB (290- to 320-nm) region of the solar spectrum (22). However, to date, studies of UV-induced mutagenesis in both procaryotes (6) and mammalian cells, where recently developed shuttle vector systems have been used (12, 14), have been confined to UVC radiation at 254 nm, a wavelength which does not reach the surface of the earth since it is absorbed by the ozone layer.

It has been demonstrated that the spectra of lesions induced in DNA by UVB and UVC radiations can differ markedly. The Cyt<>Thy/Thy<>Thy pyrimidine dimer ratio is increased at 313 nm relative to 254 nm (9), and UVB radiation apparently induces high levels of radiomimetic damage such as thymine glycols and DNA strand breaks (5, 17). Important differences are also found when the effects of UVB and UVC radiations are compared at the biological level. Recent studies in this laboratory have shown (i) that UVB radiation, unlike UVC radiation, may induce a large sector of lethal damage via the intermediacy of active oxygen species (24) and (ii) that following UVB (313-nm) irradiation of human cells, the ratio of ouabain-resistant mutants to thioguanine-resistant mutants is 10 times higher than at 254 nm, indicating that a unique type of premutagenic lesion is induced at the longer wavelength (23). The abovementioned studies all strongly suggest that distinct types of genetic damage may be induced by solar UVB radiation and that this damage may be of biological significance.

In this study, we have used a simian virus 40-based shuttle vector (pZ189) which contains a bacterial suppressor tRNA gene as the target for mutagenesis (21) to analyze the mutations generated during passage of UVB (313-nm)-irradiated vector DNA in simian (CV-1) cells. The mutation spectrum obtained has been compared with the spectra of mutations induced by UVC (254-nm) irradiation of vector DNA and with the spectra of mutations which arise spontaneously during passage of this shuttle vector in CV-1 cells.

MATERIALS AND METHODS

Cells and plasmids. African green monkey kidney cells (CV-1) were grown in Earle minimal essential medium supplemented with 15% fetal calf serum (both Seromed) at 37°C in a 5% CO_2 incubator. The shuttle vector plasmid used in these studies, pZ189 (21), was prepared by using standard techniques. The indicator strain used to distinguish between mutant and wild-type plasmids was *Escherichia coli* MBM7070 [F^- *lacZ*(Am) *CA7020 lacY1 hsdR hsdM* Δ(*araABC-leu*)*7679 galU galK rpsL thi*]. In the presence of IPTG (isopropyl-β-D-thiogalactoside), an inducer of the *lac* operon, and X-Gal (5-bromo-4-chloro-3-indolyl-β-D-galactoside), a synthetic substrate for β-galactosidase, MBM7070 gives rise to blue colonies only if it contains an active *supF* suppressor tRNA gene. If the *supF* gene is carrying an inactivating mutation, then MBM7070 forms white or occasionally light blue colonies.

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UV treatment and transfection. Vector DNA at a concentration of 1 μg/ml in Tris-buffered saline (pH 7.5) was irradiated at room temperature with either germicidal UVC (254-nm) radiation or monochromatic UVB (313-nm) radiation. Details of light sources, filtration, and dosimetry have been described previously (25). CV-1 cells at about 50% confluence were transfected with pZ189 DNA (50 ng per 9-cm petri dish) by using the DEAE-dextran method (15). After 48 h, vector DNA was extracted from the cells by using the method of Hirt (13) as modified by McMaster et al. (16).

Mutant selection and characterization. The indicator strain MBM7070 was transformed with vector DNA by using the simple procedure for preparation of frozen competent cells described by Hanahan (10). Bacteria were then plated on Luria broth plates with ampicillin (50 μg/ml) and spread with X-Gal (2 mg) and IPTG (12 mg) to select for transformants and to distinguish colonies carrying mutant plasmids. White and light blue colonies were picked and streaked on Luria broth plates by using the same selection for confirmation of phenotype and stored as stabs in soft Luria broth agar containing ampicillin (50 μg/ml) until analysis. Using a similar procedure, Hauser et al. (12) established that all mutations arise in the mammalian cells and not in the bacterial host.

Plasmid DNA was prepared for agarose gel electrophoresis and DNA sequencing by the alkaline lysis procedure of Birnboim and Doly (2) as modified by Sahli et al. (19). Plasmid DNA was then run on 0.8% agarose gels to distinguish between plasmids carrying deletions or insertions and those which migrated with a mobility indistinguishable from that of the wild type. The latter plasmids were subjected to sequence analysis. Where any doubt existed as to the migration of a particular vector DNA, it was analyzed by sequencing, and in the subsequent quantitation of deletions and insertions, no distinction was made between those detected by agarose gel electrophoresis or by sequencing. The tRNA region of the pZ189 vector was sequenced by using a modification of the Sanger dideoxyribonucleotide procedure which uses a strand-specific pBR322 *Eco*RI primer and avian myeloblastosis virus reverse transcriptase to sequence directly from the double-stranded plasmid DNA (27).

RESULTS

Induction of *supF* mutants by UVB (313-nm) radiation in CV-1 cells. Vector DNA (pZ189) either untreated or irradiated with monochromatic UVB (313-nm) or UVC (254-nm) radiation was transfected into CV-1 cells and allowed to replicate. Progeny plasmids were then rescued and introduced into *E. coli* MBM7070 to identify those carrying a mutation in the *supF* gene. The results of the transfection experiments are summarized in Table 1, and the induced mutation frequencies are plotted as a function of UV fluence for the two radiation treatments in Fig. 1. Passage of untreated vector DNA in CV-1 cells resulted in a spontaneous *supF* mutation frequency of 0.051% or 5.1×10^{-4} (Table 1), a value in the same range as that previously reported for this vector in simian cells (12). Irradiation of vector DNA with UVB (313-nm) radiation at fluences above 5.0×10^5 J m^{-2} prior to transfection resulted in a roughly linear increase in mutation frequency, and at a fluence of 2×10^6 J m^{-2} the level of induced mutants was increased approximately 30-fold over the spontaneous value (Table 1).

Figure 1 has been plotted such that the scale at the two wavelengths corresponds to fluence levels which induce similar levels of pyrimidine dimer photoproducts and which are approximately equitoxic to both the plasmid and mammalian cells (i.e., a difference of $\approx 10^3$ between the two wavelengths; 17, 18; and Fig. 1). From this comparison, it is clear that UVB (313-nm) radiation is less mutagenic per dimer or per lethal event by a factor of about 3 than UVC (254-nm) radiation in this shuttle vector system. This might imply that pyrimidine dimers are not the major lesion responsible for induced mutation at both wavelengths or that there is an interaction between two classes of lesion (which could include the pyrimidine dimer) which modulates the mutation frequency at one wavelength but not the other. It should also be noted that the mutation frequency we have observed after irradiation of the plasmid in vitro at 313 nm may be lower than the value that would be found in vivo, where a sector of UVB-induced damage arises indirectly via the intermediacy of active oxygen species (24).

Sequence comparison of spontaneous and induced mutations. Following isolation of white and light blue mutant

TABLE 1. Mutagenesis of unirradiated or UV (254- and 313-nm)-irradiated pZ189 shuttle vector in simian (CV-1) cells

Radiation treatment (J m^{-2}) (wavelength)	No. of independent transfections	Total colonies examined	No. of mutant colonies scored	% Mutants
None	18	215,426	111	0.051
5×10^5 (313 nm)	19	75,185	203	0.270
1×10^6 (313 nm)	5	20,711	135	0.652
2×10^6 (313 nm)	7	13,766	193	1.40
5×10^2 (254 nm)	8	23,709	172	0.725
1×10^3 (254 nm)	9	20,260	373	1.84

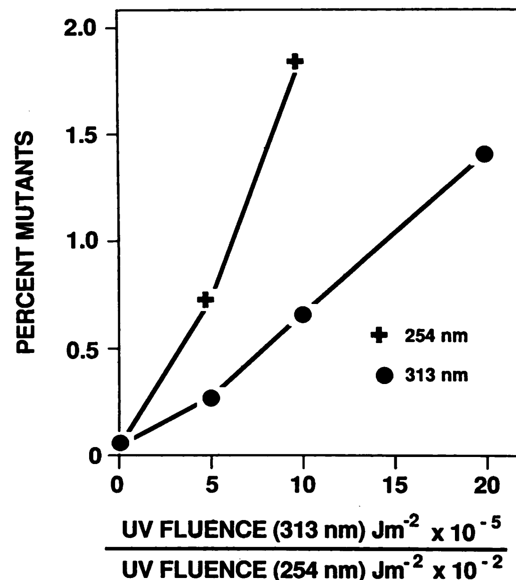


FIG. 1. Fluence response curves for induction of mutations in pZ189 by UVC (254-nm) and UVB (313-nm) radiation. The figure has been plotted so that the scale at the two wavelengths corresponds to fluence levels which induce similar levels of pyrimidine dimer photoproducts and which are approximately equitoxic to the plasmid. Fluences of 5×10^2 J m^{-2} at 254 nm or 5×10^5 J m^{-2} at 313 nm inactivated approximately 20% of the plasmid population. Fluences of 10^3 J m^{-2} at 254 nm or 10^6 J m^{-2} at 313 nm inactivated approximately 65% of the plasmid population.

TABLE 2. Sequence alterations in spontaneous, UVB-, and UVC-induced mutants

Radiation treatment (J m^{-2}) (wavelength)	Total no. analyzed	No. (%) of mutants analyzed with:					
		Deletions or insertions[a]	Single changes		Two changes		Three or more changes
			One base	Tandem double base	<15 bases apart	>15 bases apart	
None	72	36 (50)	14 (19.5)	3 (4)	4 (5.5)	4 (5.5)	11 (15.5)
5×10^5 (313 nm)	97	44 (45)	33 (34)	3 (3)	5 (5)	3 (3)	10 (10)
1×10^6 (313 nm)	51	21 (41)	11 (21.5)	1 (2)	6 (12)	1 (2)	11 (21.5)
2×10^6 (313 nm)	78	37 (47)	19 (24)	4 (5)	4 (5)	3 (4)	14 (18)
5×10^2 (254 nm)	67	23 (34)	23 (34)	8 (12)	4 (6)	6 (9)	3 (4)
1×10^3 (254 nm)	76	16 (21)	30 (39.5)	10 (13)	4 (5)	9 (12)	11 (14.5)

[a] Where a sequence contained both a small deletion and one or more point mutations, these were treated as separate events.

colonies in the experiments described above, vector DNAs were prepared from individual clones and analyzed on agarose gels to distinguish those with gross DNA alterations (either deletions or insertions). Vector DNAs which appeared to migrate with normal mobilities were then subjected to sequence analysis. The coding sequence of the mature tRNA lies between positions 99 and 183, and sequence analysis extended from position 47 to position 190. Mutants which occurred more than once amongst sequences analyzed from a single transfection were excluded, thus ensuring that the mutants were of independent origin. Although this will lead to a slight underestimation of the mutation frequency at hot spot sites, the number of individual transfections analyzed is sufficient to ensure that no major hot spots will be missed (Table 1).

The characteristics of the sequence alterations in both the spontaneous and UV-induced mutants are shown in Table 2. Of the spontaneous mutants analyzed, 50% contained either deletions or insertions in the region of the tRNA coding sequence. Over half of the remainder (26.5%) contained multiple mutations (two or more changes), and 23.5% contained single mutations, either single-base or tandem double-base changes.

The mutations generated by UVB (313-nm) radiation differed from those induced by UVC (254-nm) radiation in three respects. (i) The proportion of mutants containing deletions or insertions was consistently higher following UVB irradiation of vector DNA and was close to the spontaneous value (45% compared with 50%). In contrast, the level of these gross alterations that resulted from UVC irradiation of the plasmid appeared to decline with increase in UV fluence being only 21% following irradiation with 10^3 J m^{-2}, the highest UVC fluence used. (ii) The number of UVB-induced mutants which contained three or more changes within the sequence analyzed was consistently higher than the value obtained at 254 nm (15.5% compared with 9.8%). (iii) The number of mutations involving single changes within the tRNA coding sequence was lower after UVB irradiation than after UVC irradiation (31% at 313 nm compared with 49% at 254 nm). This was particularly marked in the case of tandem double-base changes which constituted only 3.5% of mutations induced by UVB radiation compared with 12.6% at 254 nm.

Despite the differences in classes of mutations that are apparent from Table 2, the analysis of the individual base changes shown in Table 3 indicates that the types of base changes are very similar at the UVC (254-nm) and the UVB (313-nm) wavelengths. In both cases, the G-C to A-T transition predominates, accounting for over 60% of the UV-induced mutations. When the two possible transversions are added, changes at G-C base pairs make up about 90% of all base substitutions seen at both wavelengths. The remaining 10% of base substitutions include both the A-T to G-C transition and the two possible transversions.

Figure 2 displays the complete spectrum of base changes along the sequenced portion of the vector DNA found in spontaneous (A), UVC-induced (B), and UVB-induced (C) mutants. The distribution of base changes in the spontaneous and UVC-induced mutants is basically similar to that reported previously (12). Most of the major mutation hot spots induced by radiation at 254 nm also appear in the spectrum of UVB-induced mutations (positions 111, 156, and 168). However, two lesser UVC hot spots (at positions 113 and 169) are underrepresented in the UVB spectrum. More striking is the observation that additional hot spots not seen in the UVC spectrum appear at 313 nm, notably at positions 118, 122, 139, 149, and 155 and, to a lesser degree, at positions 143 and 146. It is clear from Table 3 that a difference in the types of base changes induced by 313 nm relative to 254 nm cannot be responsible for the appearance of these new hot spots. Therefore, we set out to determine whether these might reflect differences in the classes of mutations induced by UVC and UVB radiations. As shown in Table 2, UVB radiation induces fewer single changes within the region sequenced and correspondingly more multiple (3 or more changes) mutations. Figure 3 shows only the disposition of mutations involving multiple changes found in spontaneous (A), UVC-induced (B), and UVB-induced (C) mutants. Comparison of these spectra at 254 and 313 nm clearly shows that multiple mutations are responsible for generating the additional hot spots seen in the UVB spectrum in Fig. 2. The only exception is the 313-nm-induced hotspot at position 122, which arises largely as a consequence of single changes unique to this wavelength.

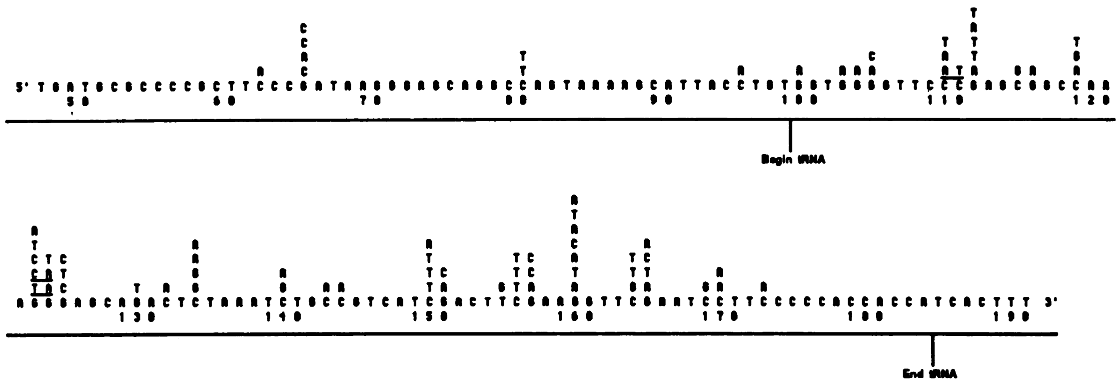
DISCUSSION

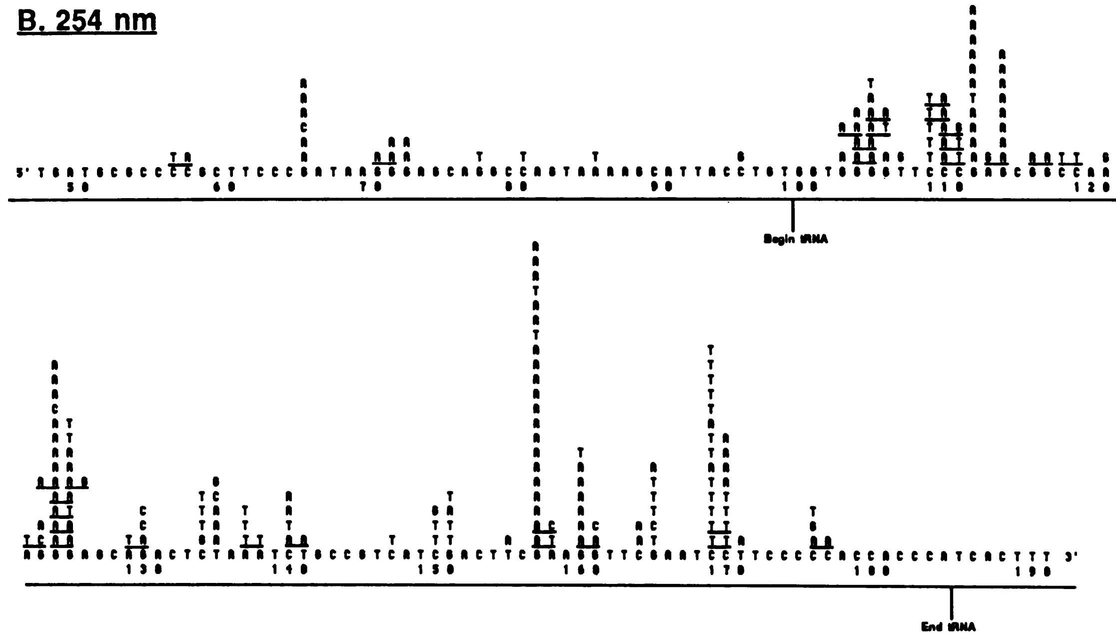
The sequence specificity of UVC (254-nm)-induced mutations for G-C base pairs and the predominance of the G-C to

TABLE 3. Base changes in spontaneous, UVC-, and UVB-induced mutants

Base change	No. (%) of mutants observed		
	Control	254 nm	313 nm
G-C to A-T	32 (40)	127 (64.5)	169 (62)
G-C to T-A	25 (32)	36 (18)	48 (17)
G-C to C-G	21 (27)	12 (6)	29 (11)
A-T to G-C	0	4 (2)	8 (3)
A-T to C-G	1 (1)	3 (1.5)	3 (1)
A-T to T-A	0	15 (8)	16 (6)

A. SPONTANEOUS





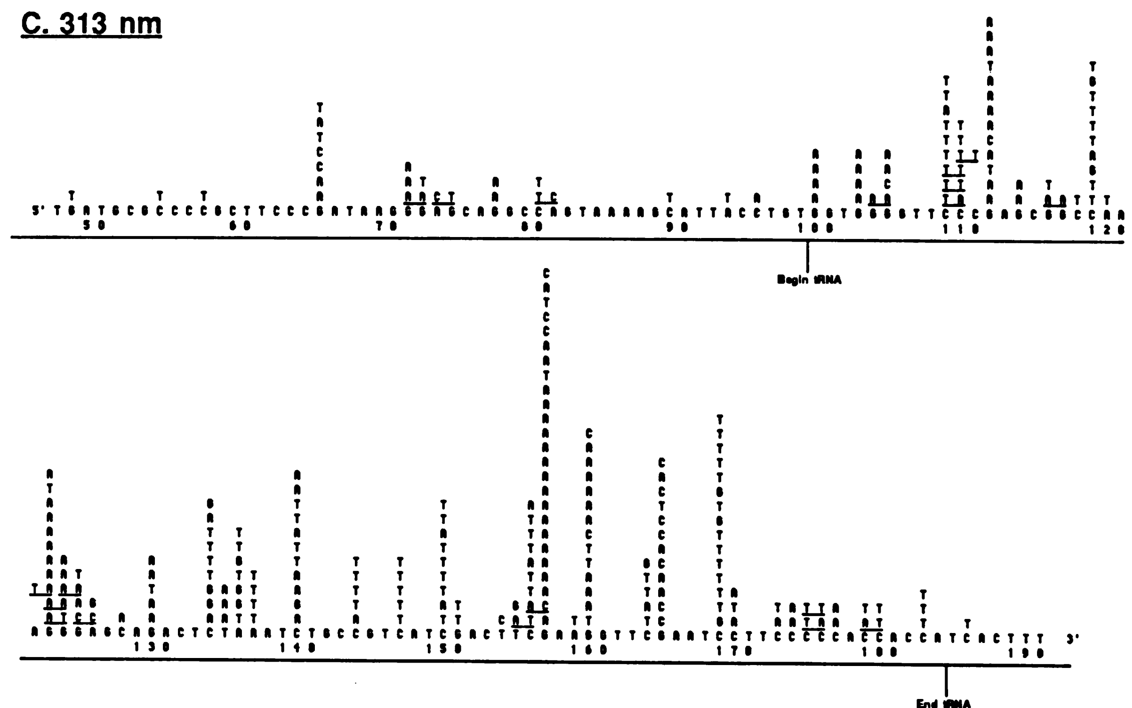
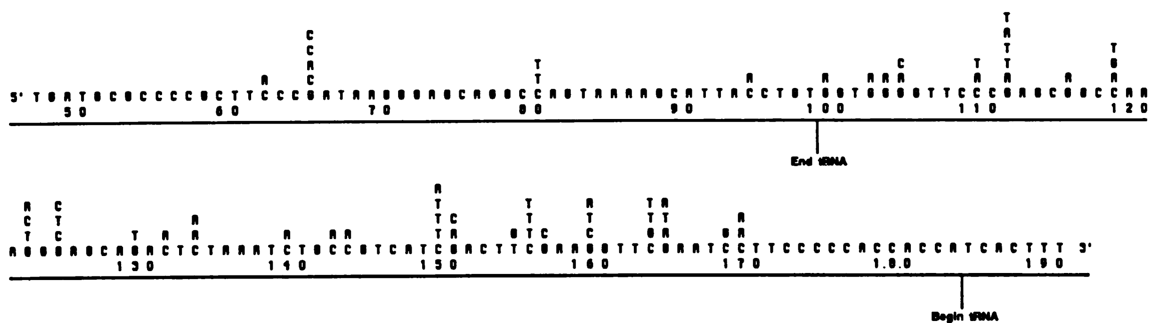
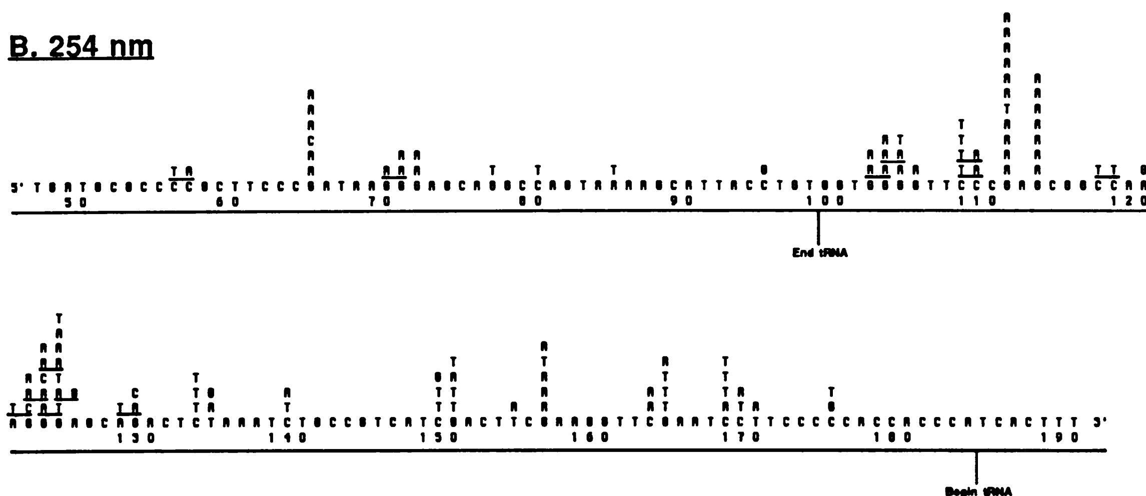


FIG. 2. The complete spectrum of DNA sequence changes found in spontaneous (A), UVC-induced (B), and UVB-induced (C) mutants. Tandem double-base changes are underlined.





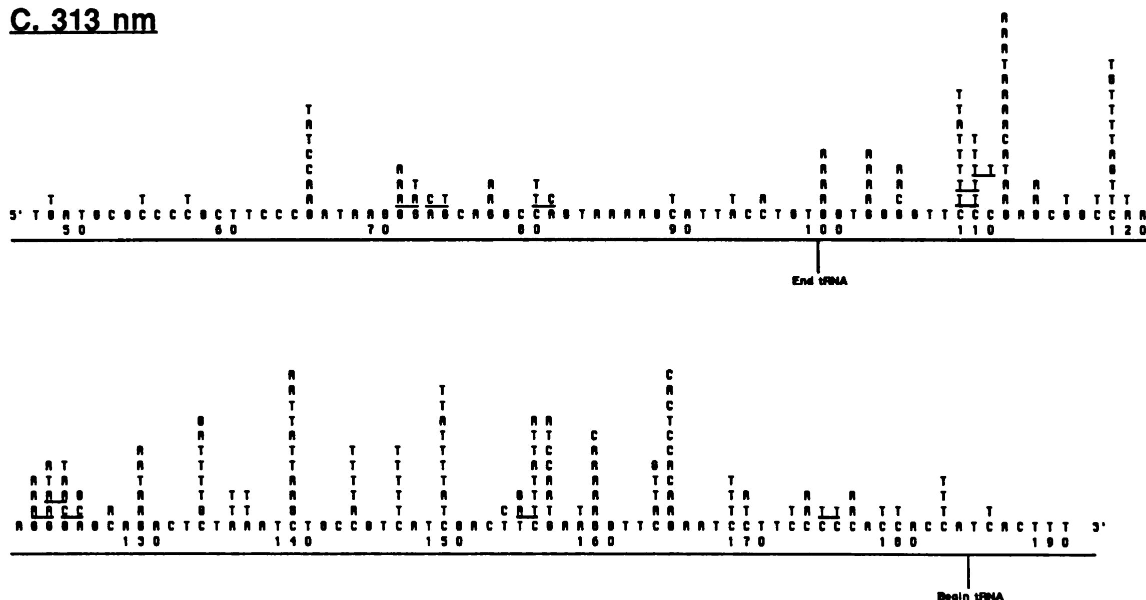


FIG. 3. Spectrum of DNA sequence changes which results from multiple changes (two or more base substitutions in a given sequence analyzed) found in spontaneous (A), UVC-induced (B), and UVB-induced (C) mutants. Tandem double-base changes are underlined.

A-T transition have been reported previously with mammalian cells in experiments with both the pZ189 vector (4, 12) and a simian virus 40-based shuttle vector carrying the bacterial *lacI* gene (14). Our data obtained at 313 nm clearly indicate that the UVB wavelengths involved in solar carcinogenesis will lead to a pattern of base changes similar to that observed after the shorter nonsolar wavelength. Thus, an important implication of our work is that in studies of the

effects of UV radiation on gene activity and, in particular, on the role of UV-induced point mutations in the activation of cellular proto-oncogenes, it should be possible to extrapolate from results obtained by using germicidal UVC at 254 nm to the situation where cells or tissues are exposed to solar UVB radiation.

It should be pointed out that the predominance of changes at G-C base pairs among UVC- and UVB-induced base substitutions is not a feature which in itself distinguishes a UV-induced mutant from one which has arisen spontaneously. On the contrary, UV-induced mutants are marked overall by a relaxation in specificity towards G-C base pairs when compared with spontaneous mutants (Table 3). In a recent analysis of spontaneous mutations occurring in pZ189 vector DNA during passage through CV-1 cells, Hauser et al. (11) found that 73 of 74 base changes involved G-C base pairs. An analysis of the sites of these mutations revealed a striking sequence specificity, since almost all (63 out of 74) of the changes occurred in the right-hand base pair of the two sequences 5′-TC-3′:3′-AG-5′ and 5′-CC-3′:3′-GG-5′. Despite differences in the actual distribution of base changes along the *supF* sequence among spontaneous mutations analyzed in the present study and in that of Hauser et al. (11), both the prevalence of changes at G-C base pairs (78 of 79) and the sequence specificity of these changes (74 of 79) are conserved (Table 3 and Fig. 1A). Furthermore, if we examine the sequence specificity of mutations arising at G-C base pairs among both the UVC- and UVB-induced mutants, the same preference for 5′-Pyr-C-3′ sequences was observed (163 of 175 at 254 nm and 238 of 246 at 313 nm). This is largely the result of the fact that despite being distributed more evenly throughout the sequenced region than are the UV-induced mutations, the spontaneous base changes are still occurring at most of the sites where UV-induced hot spots arise. This would suggest that a large number of the UV-induced mutants may be induced by much the same mechanism as the base changes that arise spontaneously and that DNA structure may be a more important determinant of mutation hot spots than are the positions of DNA photoproducts.

Hauser et al. (11) suggest that the spontaneous point mutations might arise as a result of DNA replication infidelity occurring during repair of the transfected DNA which has been damaged by cellular nucleases. If so, then a similar loss of replication fidelity provoked by the presence of UV-induced damage in the template might also give rise to the same sequence specificity for the UV-induced mutations. Support for this hypothesis is provided by recent studies in which UVC-damaged pZ189 DNA was passed through both excision repair-proficient and -deficient (xeroderma pigmentosum) human cells. Not only was the spontaneous mutation frequency much lower in the repair-deficient cells than in the repair-proficient cells, but very few mutants containing multiple changes (two or more) within the region of the *supF* gene were recovered from the repair-deficient cells compared with the repair-proficient cells (2% compared with 20 to 30%) (4, 20). In our study, multiple mutations (two or more changes) accounted for a large proportion of the base substitutions observed, particularly after 313-nm radiation (195 out of 273, or 71.4%), and it is particularly evident that these multiple mutations are induced by the radiation treatment and not simply contaminating spontaneous mutants scored among the UVB set (Table 2). The mechanism of mutation induction suggested by Siedman et al. (20) to account for the induction of these multiple mutations—that is, that they are the result of gap filling by an error-prone DNA polymerase activity during repair—may account for the apparent lack of correlation observed between mutation hot spots and the positions at which UV photoproducts are formed (3).

The results of the study by Seidman et al. (20) may also provide an explanation for the differences in the types of mutations produced by UVC and UVB radiations observed in our study (Table 2). Seidman et al. found that they could restore the ability of the xeroderma pigmentosum cells to induce multiple mutations into a UVC-damaged vector if, prior to transfection, a single-strand nick was introduced into the plasmid just upstream of the *supF* gene. UVB (313-nm) radiation, unlike UVC radiation, is known to cause a significant yield of single-strand breaks in DNA (≈1 for every 44 pyrimidine dimers induced; 17), and it is conceivable that the additional presence of these lesions in the UVB-irradiated vector is responsible for the increased number of multiple mutations which we observed.

An important consideration in evaluating mutation studies which use shuttle vectors is the degree to which the mutation spectrum obtained reflects that which would be induced in a chromosomal gene. One inherent limitation of the shuttle vector system used here is its inability to detect the kinds of gross DNA sequence changes which might result from chromosomal deletions and translocations. Recent evidence would suggest that such changes are likely to form at least part of the mutation spectrum induced by chemical and physical mutagens including UVC (254-nm) radiation (26). However, the data obtained so far in studies of induced point mutation at endogenous chromosomal loci in mammalian cells show a good correlation with data obtained by using shuttle vector systems. Spontaneous mutations analyzed in the endogenous adenine phosphoribosyl transferase (*aprt*) locus of Chinese hamster ovary cells (7) show the same predominance of G-C to A-T transitions (80%) seen in both the present study and previous analyses with shuttle vectors (11, 14). Furthermore, UVC-induced mutations analyzed at this same locus resemble those seen in shuttle vector systems, with the G-C to A-T transition also predominant (8). Interestingly, as is noted in the present study (Fig. 2A, B, and C), a striking resemblance was also seen in the distribution of UVC-induced mutants and the spontaneous mutants, supporting the idea that DNA structure rather than the actual distribution of photoproducts plays a significant role in mutation fixation in mammalian cells.

In conclusion, we have defined the spectrum of mutations that can be induced in a specific gene by an environmentally relevant solar UVB wavelength. We have discovered a similarity between the types of base changes which are induced by UVB radiation when compared with a more widely studied germicidal UVC wavelength. However, differences do exist in the specific sites at which the base substitutions occur when the distributions of mutation hot spots are compared at the two wavelengths. These differences may be attributable to the different spectrum of premutagenic lesions induced in DNA at the longer solar wavelength.

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