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Genetics

Xeroderma pigmentosum variant cells are less likely than normal cells to incorporate dAMP opposite photoproducts during replication of UV-irradiated plasmids

(UV mutagenesis/*supF* gene/error-prone replication)

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ABSTRACT **Xeroderma pigmentosum (XP) variant patients show the clinical characteristics of the disease, with increased frequencies of skin cancer, but their cells have a normal, or nearly normal, rate of nucleotide excision repair of UV-induced DNA damage and are only slightly more sensitive than normal cells to the cytotoxic effect of UV radiation. However, they are significantly more sensitive to its mutagenic effect. To examine the mechanisms responsible for this hypermutability, we transfected an XP variant cell line with a UV-irradiated (at 254 nm) shuttle vector carrying the *supF* gene as a target for mutations, allowed replication of the plasmid, determined the frequency and spectrum of mutations induced, and compared the results with those obtained previously when irradiated plasmids carrying the same target gene replicated in a normal cell line [Bredberg, A., Kraemer, K. H. & Seidman, M. M. (1986) *Proc. Natl. Acad. Sci. USA* 83, 8273–8277]. The frequency of mutants increased linearly with dose, but with a slope 5 times steeper than that seen with normal cells. Sequence analysis of the *supF* gene showed that 52 of 53 independent mutants generated in the XP variant cells contained base substitutions, with 62 of 64 of the substitutions involving a dipyrimidine. Twenty-eight percent of the mutations involved A·T base pairs, with the majority found at position 136, the middle of a run of three A·T base pairs. (In the normal cells, this value was only 11%.) If the rate of excision of lesions from *supF* in the two cell lines is equal, our data suggest that XP variant cells are less likely than normal cells to incorporate dAMP opposite bases involved in photoproducts. If such incorporation also occurs during replication of chromosomal DNA, this could account for the hypermutability of XP variant cells with UV irradiation.**

It is now widely recognized that the transformation of normal cells into tumorigenic cells is a multistep process, and substantial evidence indicates that mutations play a fundamental role in cellular transformation and carcinogenesis, as well as in many inheritable diseases and developmental anomalies (1, 2). However, our understanding of the factors and influences governing the formation of these changes in gene structure is considerably less advanced. Cells isolated from patients with the rare autosomal recessive disorder xeroderma pigmentosum (XP) present a unique model system for investigating DNA repair and mutagenesis in human cells. In the present study, we made use of a shuttle vector assay to investigate the kinds of mutations induced when a UV-irradiated plasmid replicated in cells derived from the class of XP patients called XP variants in order to provide clues to the mechanisms responsible for the hypermutagenic effect of UV radiation on these cells.

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XP variants inherit the characteristic predisposition to sunlight-induced skin cancer, but unlike the majority of XP patients, their cells do not exhibit a significant deficiency in the rate of nucleotide excision repair of endogenous UV-induced DNA damage, including both cyclobutane pyrimidine dimers (3–8) and pyrimidine-pyrimidone(6-4) photoproducts (7, 8). Cells from XP variant patients have an abnormality in the manner in which DNA replicates on a template containing UV lesions (9–11) and an inability to convert a very minor UV photoproduct to an excisable lesion (12). They are only slightly more sensitive than cells from normal donors to the cytotoxic effect of UV but are significantly more sensitive to its mutagenic action (13–15). However, the molecular mechanism(s) responsible for the abnormal sensitivity of XP variant cells to UV-induced mutations has not been explained.

To examine this question, we UV-irradiated a shuttle vector, pS189 (16), carrying the *supF* gene as the target for mutations and transfected the plasmids into a simian virus 40-transformed XP variant cell line (XP-V) where they could be replicated by the human cell polymerase(s). The progeny plasmids were analyzed for the frequency of *supF* mutants and the kinds of mutations and their location in the gene was determined. The results were compared with those reported by Bredberg *et al.* (17) who used the same assay in a repair-proficient cell line from a normal donor. We found a dose-dependent decrease in yield of replicated plasmids and a corresponding increase in the frequency of *supF* mutants. The slope of the mutant frequency curve was 5 times steeper than that seen with the normal cells. Sequence analysis of the *supF* gene from 53 mutant plasmids indicated that an abnormally high proportion of the base substitutions involved A·T base pairs, with many at a unique "hot spot," position 136, in the middle of a run of three A·T base pairs.

MATERIALS AND METHODS

Cells and Plasmid. The XP-V cell line, a simian virus 40-immortalized derivative of XP cell line GM2359, was kindly provided by Roger Schultz (University of Maryland, Baltimore). The cells were grown in modified MCDB-110 medium (18) prepared with Earle's salts and supplemented with 10% (vol/vol) fetal calf serum (GIBCO) and antibiotics. The ampicillin-sensitive indicator bacterial host was *Escherichia coli* SY204, carrying an amber mutation in the β-galactosidase gene and in the tryptophan gene (19). The 5337-base-pair (bp) shuttle vector pS189 (16), a deletion derivative of pZ189 (20), contains the tyrosine amber suppressor tRNA gene (*supF*) flanked by the gene for ampicillin resistance and the bacterial origin of replication (16). It also contains an

Abbreviation: XP, xeroderma pigmentosum.
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origin of replication that facilitates its replication in mammalian cells.

UV Irradiation. The plasmid DNA was diluted with Tris/EDTA (10 mM Tris·HCl, pH 7.5/1 mM EDTA) to 50 μg/ml immediately before irradiation and 1 ml was pipetted into a sterile plastic 60-mm tissue culture dish that had been placed on ice. The plasmids were irradiated with the indicated doses of UV from an unfiltered germicidal lamp at a dose rate of 2.5 $J \cdot m^{-2} \cdot sec^{-1}$, then precipitated with ice-cold ethanol, redissolved in Tris/EDTA at a concentration of 500 μg/ml, and stored at −20°C until used.

Transfection and Rescue of Replicated Plasmids. The procedures used for calcium phosphate coprecipitation transfection were essentially as described (21) but with the density of the cells increased from 1×10^4 cells per cm^2 to 3×10^4 cells per cm^2 (1.5×10^6 cells per 100-mm dish) and with the amount of plasmid per dish increased from 6 μg to 40 μg. The cells were harvested after the transfection, and progeny plasmids were extracted as described (22). To distinguish between independent mutants with identical mutations and putative siblings derived from a single event, progeny plasmids obtained from each dish of cells were maintained and assayed separately. Prior to bacterial transformation, the plasmids were treated with *Dpn* I to digest any DNA that still had the bacterial methylation pattern to ensure that the purified DNA was derived from plasmids that had replicated in the human cells.

Bacterial Transformation and Mutant Characterization. The techniques used were essentially as described (21). Briefly, progeny plasmids were assayed for mutant *supF* genes by transforming SY204 bacterial cells to ampicillin resistance and selecting on agar plates containing ampicillin, an indicator dye, and isopropyl β-D-thiogalactoside. On this medium, bacterial transformants containing plasmids with a mutant *supF* gene form light blue or white colonies; those with a wild-type *supF* gene form blue colonies. Mutant colonies were restreaked on these agar plates and on plates lacking tryptophan to confirm the phenotype, and then the plasmids were amplified, purified using a small-scale alkaline lysis procedure (23), and analyzed by electrophoresis on 0.8% agarose gels for altered DNA mobility. Plasmids without evidence of gross alterations were sequenced as described (21).

RESULTS

Yield of Plasmids and Mutation Frequency. Before beginning the study, we found we could increase the yield of plasmid DNA obtained from the XP-V cells 60-fold by increasing the amount of plasmid DNA per transfection from 6 μg to 40 μg and by increasing the cell density to 3×10^4 cells per cm^2. As shown in Fig. 1*A*, UV-irradiation caused a dose-dependent decrease in yield of replicated plasmids from XP-V cells, a decrease that was not found by Bredberg *et al.* (17) using GM637 cells from a normal donor as their host cells. The yield after a dose of 200 J/m^2 was 33% of the unirradiated control.

There was a corresponding dose-dependent increase in the frequency of *supF* mutants (Fig. 1*B*), reaching 330×10^{-4} with a background of 3×10^{-4} at a dose of 500 J/m^2. The slope of the mutant frequency curve was 5 times steeper than that found previously (17) using the GM637 cell line as host. Table 1 gives the number of plasmids analyzed and their characterization.

Spectrum of Mutations Produced in the *supF* Gene of UV-Irradiated Plasmids That Replicated in XP-V Cells. DNA sequence analysis (Fig. 2) of 53 equivocally independent mutants from passage of the UV-irradiated plasmids through the XP-V cells revealed 64 base substitutions at 27 sites and showed that 52 of 53 mutants contained base substitutions.

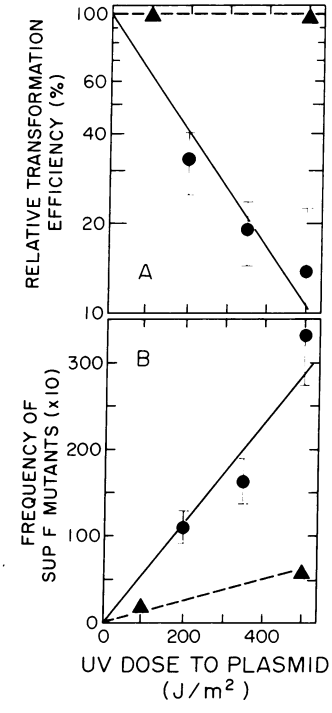

FIG. 1. (*A*) Yield of plasmids after replication in XP-V cells (●), as estimated from the relative frequency of transformation of bacteria to ampicillin resistance, as a function of the UV dose to the plasmids. (*B*) Frequency of *supF* mutants induced in plasmids replicated in XP-V cells (●) as a function of UV dose to the plasmid. Comparable data for plasmids that replicated in GM637 cells (▲), from a normal donor are shown. These latter data, taken from Bredberg *et al.* (17), are reproduced for comparison.

The one plasmid with a rearrangement in the *supF* gene came from a plasmid preparation that received 200 J/m^2. As noted in Table 2, the majority of mutants (41/53) contained only a single base substitution. Three of 53 mutants had tandem substitutions; 6 of 53 had two base substitutions, but not located in tandem; 1 had three separate base substitutions; and 1 contained a complex mutation. All except two base substitutions (sites 101 and 114) were found at sites of adjacent pyrimidines. The A·T → T·A transversion at site 101 was part of a tandem substitution, and the G·C → T·A transversion at site 114 was part of the complex triple mutant. All 41 single base substitutions could have occurred at the 3′ side of the photoproduct, but 14 of 41 definitely occurred there; i.e., those located at sites 134, 135, 155, and 156. In the mutant with base substitutions at positions 122 and 126, the mutated site at position 126 was found at the 5′ side of a dipyrimidine. Two other mutants had two base substitutions (i.e., at sites 136 and 152 and at sites 149 and 156) that could not be explained by the presence of two photoproducts in one plasmid, since the dipyrimidines at those positions are located on opposite strands.

Table 3 compares the types of base-pair substitutions observed in the *supF* gene of UV-irradiated plasmids passaged through XP-V cells with those found using GM637 cells. In both cases, the major class of base-pair substitution was the G·C → A·T transition, but the frequency of this change with plasmids from XP-V cells was much lower than from GM637 cells. In plasmids from XP-V cells, transversions made up 47% of the substitutions in the *supF* gene, a frequency twice as high as that observed with GM637 cells. In addition, the types of transversions differed; i.e., plasmids from XP-V cells had twice the frequency of G·C → T·A transversions, and base-pair substitutions involving A·T base pairs occurred at a frequency of 28% with XP-V cells

Table 1. Analysis of mutants obtained by transformation of *E. coli* with progeny of UV-irradiated pS189 generated during replication in XP-V cells

UV dose to plasmid, J/m²	No. human cell transfection experiments	No. *supF* mutants/no. transformants*	Frequency of *supF* mutants ($\times 10^4$)	No. plasmids with altered gel mobility/ no. examined†	Total no. *supF* genes sequenced	Characterization of mutants: No. with rearrangements	Characterization of mutants: No. with point mutations‡	Mutants with point mutations per 10⁴§
0	32	9/29,566	3	0/9	0	ND	ND	ND
200	5	35/3,072	114	0/35	18	1	17	109
350	5	26/1,566	166	0/26	14	0	14	166
500	5	34/1,017	334	0/34	21	0	21	334

ND, not determined.

*White or light blue colonies were restreaked on plates lacking tryptophan to ensure that the inability of the cell to metabolize 5-bromo-4-chloro-3-indolyl β-D-galactoside resulted from inactivation of the *supF* gene.

†Alteration visible on agarose gel (>150 bp).

‡Substitution, deletion, or insertion of 1 or 2 bp.

§Calculated from fraction of mutants with point mutations times the observed frequency (column 4). The fraction of mutants with point mutations is the number in column 8 divided by the number in column 6 plus those mutants showing altered gel mobility (numerator, column 5).

compared to 11% with GM637 cells. These differences are statistically significant ($P < 0.01$). Sixty-seven percent of these A·T substitutions occurred at TTT sites (at positions 120, 135, and 136).

Mutational Hot Spots for UV-Induced Mutations. As shown in Fig. 2, there were five prominent hot spots (i.e., positions 123, 136, 155, 156, and 169). It should be noted that 12 bp intervene between positions 123 and 136 and between positions 156 and 169. Except for site 136, each hot spot involved a G·C base pair. Site 136, which is located in the middle of a run of three A·T base pairs, was the strongest hot spot, with 11% of the total base substitutions found there. This hot spot is unique to plasmids replicated in XP-V cells; i.e., to our knowledge, it has not previously been found with UV-irradiated *supF* genes that have been replicated in mammalian cells (17, 24, 25). All the mutations at this site were transversions, whereas those at the other four hot spots were mainly transitions.

DISCUSSION

Because the frequency of *supF* mutants in our study was so much higher than background, we are quite certain that the mutations resulted from UV radiation. The increase in frequency was linearly related to dose as predicted for mutations resulting from single UV-induced photoproducts, and, in all except two of the 64 base substitutions observed, the pyrimidine involved was adjacent to another pyrimidine. The exception at position 101, which involved a tandem mutation 101-102, may have been caused by a rare UV lesion consisting of a purine flanked by pyrimidines, i.e., CAC (26). The other exception, at position 114, was part of a complex triple mutant with two base substitutions and one insertion. It is not easy to imagine the origin of this complex set of changes. However, if they were triggered by photoproducts at positions 111-112 and/or 112-113, it should be noted that in no case was dAMP incorporated.

Even the mutations we designate as multiple base substitutions in Table 2 appear to be targeted to UV photoproducts for the following reasons. (*i*) The frequency of plasmids that contained multiple base substitutions was directly related to the dose. (*ii*) The proportion of mutant plasmids containing multiple base substitutions increased as a function of dose [i.e., 11% (2/18) at 200 J/m²; 14% (2/14) at 350 J/m²; 19% (4/21) at 500 J/m²]. (*iii*) The distribution of types of base substitutions in the mutants with multiple base substitutions was almost the same as that in mutants with single or tandem base substitutions (e.g., 44% vs. 45% for G·C → A·T; 7% vs. 8% for A·T → G·C, etc.). [The latter results differ significantly from those of Seidman *et al.* (27), who found more G·C → T·A transversions and fewer G·C → A·T transitions with the plasmids with multiple mutations than with the plasmids with

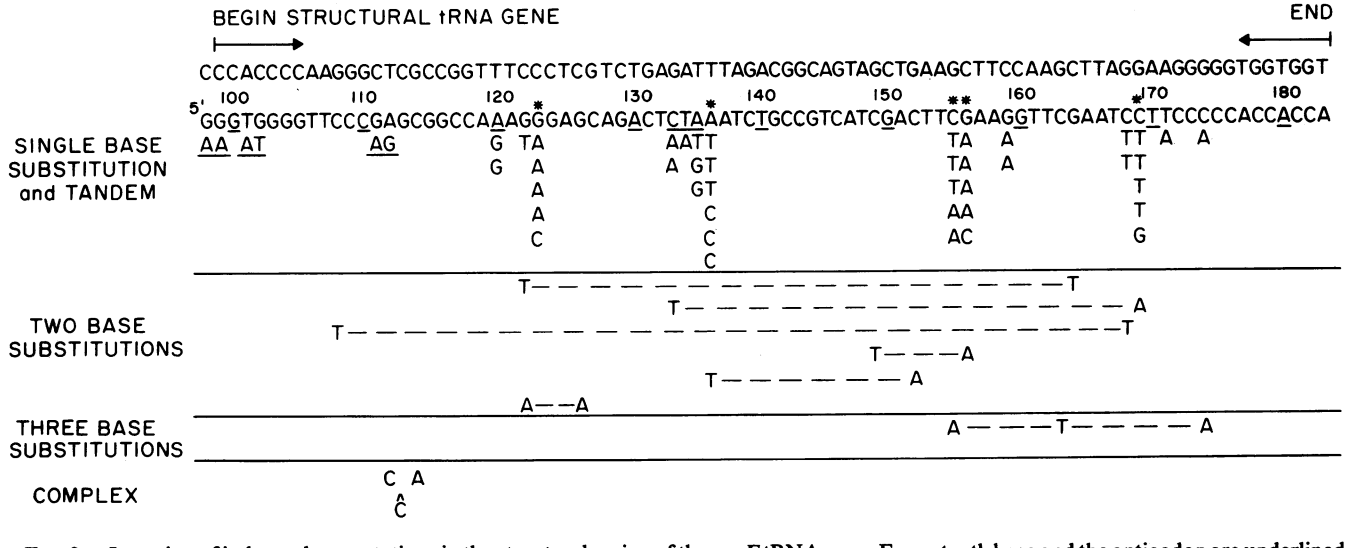

FIG. 2. Location of independent mutations in the structural region of the *supF* tRNA gene. Every tenth base and the anticodon are underlined. The mutations observed in the progeny of the irradiated plasmids are placed below the sequence. The mutations underlined represent tandem mutations. In the classes of two base substitutions and three base substitutions, the mutations connected by the dashed line represent the individual mutants with multiple base substitutions. Caret shows the location of an inserted cytosine. Asterisks indicate the prominent hot spots.

Table 2. Analysis of sequence alterations generated in the *supF* gene by replication of UV-irradiated plasmids in XP-V and GM637 cells

Sequence alteration(s)	No. plasmids with base changes	
	XP-V	GM637*
Single base substitution	41 (77)	44 (49)
Tandem base substitutions	3 (6)	16 (18)
Multiple base substitutions	8 (15)	28 (31)
Two base substitutions		
≤15 bases apart	3	—†
35–59 bases apart	3	—†
Three base substitutions	1	—†
Complex‡	1	4
Insertion and deletion§	0	1 (1)
Gross rearrangement	1 (2)	0
Total	53	89

Data in parentheses are percent of total mutants.
*Data obtained in cell GM637 are from Bredberg *et al.* (17) and are shown here to allow easy comparison.
†Data not available.
‡Plasmids with insertion or deletion accompanied by one or two base substitutions.
§Plasmids contain insertion or deletion only.

single or tandem base substitutions.] (*iv*) In three of our six mutants involving two base-pair substitutions, the distance between the substitutions was at least 35 bp, suggesting that two photoproducts were involved. Two photoproducts in a single plasmid is clearly possible since Hauser *et al.* (24) estimates that, under the experimental conditions used for the present study, the mean number of photoproducts per *supF* gene induced by 500 J/m^2 is one, and a Poisson distribution predicts that approximately 35% of the *supF* genes will receive more than one photoproduct. Even the two mutations located close to each other (sites 122 and 126) could have resulted from two independent photoproducts. In the other two mutants with two nontandem base substitutions the mutations cannot be explained by two photoproducts in a single plasmid since the dipyrimidines that would have been involved are located in opposite strands (i.e., sites 136 and 154 and sites 149 and 156). Such mutations could arise if a second base change occurred spontaneously during replication of a plasmid containing a targeted base substitution. Another possible explanation is that recombination between two plasmids carrying *supF* mutations occurred during replication in the host cells. A third possibility, and one proposed by Seidman *et al.* (27), is that the multiple base substitutions are the result of an error-prone polymerase that gains access to the DNA by excision repair incision breaks.

Since we and Bredberg *et al.* (17) used very similar protocols for irradiating our plasmid DNA, it is unlikely that our plasmids received a higher dose of UV and carried more lesions unless the dosimetries were not comparable. Another possible explanation for the higher frequency of mutants in plasmids from XP-V cells than in those from GM637 cells is that the rate of excision repair of the photoproducts in the plasmids was significantly slower in XP-V cells than in GM637. However, XP variant cells are reported to excise UV-induced lesions from their endogenous DNA at virtually the same rate as normal cells (3–8). Wood and colleagues (28) have found that, in contrast to their earlier observations (29), cell-free extracts from XP variant cell lines can exhibit a normal rate of excision of UV photoproducts from DNA plasmids. Nevertheless, if the higher frequency merely reflected a higher number of unexcised lesions in the *supF* gene, the kinds of mutations observed in plasmids from XP-V and GM637 cells should have been very similar. They were not. It is not likely that the excision process itself introduces mutations, since Watanabe *et al.* (30) showed that if XP variant cells are synchronized and irradiated at various times prior to DNA replication of the *HPRT* gene during S phase, the frequency of 6-thioguanine-resistant mutants decreases with time after irradiation before S phase. If the cells are prevented from replicating for 24 hr after UV, the mutant frequency is decreased to background levels. No such decrease occurs if the cells are incapable or virtually incapable of excision repair (31).

Table 3. Types of base pair substitutions generated in the *supF* gene by replication of UV-irradiated plasmids in XP-V and GM637 cells

Base pair substitution	No. mutations observed	
	XP-V	GM637*
Transition	(53)	(75)
G·C → A·T	29 (45)	59 (73)
A·T → G·C	5 (8)	2 (2)
Transversion	(47)	(25)
G·C → T·A	14 (22)	8 (10)
G·C → C·G	3 (5)	5 (6)
A·T → T·A	9 (14)	6 (8)
A·T → C·G	4 (6)	1 (1)
Total	64	81

Data in parentheses are percent of total mutations.
*Data obtained in cell GM637 are from Bredberg *et al.* (17) and are shown here to allow easy comparison.

To explain the hypermutability of the XP variant cells, Watanabe *et al.* (30) suggested that the process the XP variant cells use to replicate past unexcised UV photoproducts differs from that of normal cells; i.e., either the XP variant cells use a more "error prone" process or the normal cells use a more "error-free" process. These investigators could not distinguish between these two possibilities, but in view of the findings of Cleaver and associates (32–34) on the increased blocking effect UV photoproducts have on DNA initiation and chain elongation in the variant compared to normal cells, Watanabe *et al.* (30) suggested that "some process unavailable to the XP variant cells is operating in the normal cells."

The results of the present study comparing the types of base-pair substitutions observed in the *supF* gene of plasmids derived from XP-V and GM637 cells support this hypothesis. The data suggest that the polymerase(s) of the XP-V cells is less likely than that of GM637 cells to incorporate dAMP opposite bases involved in UV photoproducts during DNA replication. This is because there was a significantly lower frequency of G·C → A·T transitions, 45% compared to the 73% observed with the GM637 cells. Many investigators (35–37) suggest that the preference for the G·C → A·T transitions among UV-induced mutations results from dAMP being preferentially incorporated by the DNA polymerase opposite a noninstructive lesion. In addition there was a significantly higher frequency of A·T base-pair substitutions, 28% compared to 11% (Table 3), an occurrence that would result if the polymerase failed to incorporate dAMP opposite photoproducts involving thymidine. Note that the strongest hot spot in the *supF* spectrum from XP-V cells occurred at position 136, which necessarily involved a T·T photoproduct. There were no mutations at site 136 in plasmids from GM637 cells, and none of the hot spots found in plasmids from GM637 cells involved A·T base pairs (17). If during replication of their endogenous genome, XP variant cells also are less likely than normal human cells to incorporate dAMP opposite bases involved in UV photoproducts, this would contribute to their hypermutability with UV radiation.

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