Proc. Natl. Acad. Sci. USA
Vol. 83, pp. 8273–8277, November 1986
Genetics

Restricted ultraviolet mutational spectrum in a shuttle vector propagated in xeroderma pigmentosum cells

(DNA repair/suppressor tRNA/ultraviolet carcinogenesis)

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Communicated by Aaron B. Lerner, July 7, 1986

ABSTRACT **A shuttle vector plasmid, pZ189, carrying a bacterial suppressor tRNA marker gene, was treated with ultraviolet radiation and propagated in cultured skin cells from a patient with the skin-cancer-prone, DNA repair-deficient disease xeroderma pigmentosum and in repair-proficient cells. After replication in the human cells, progeny plasmids were purified. Plasmid survival and mutations inactivating the marker gene were scored by transforming an indicator strain of *Escherichia coli* carrying a suppressible amber mutation in the β-galactosidase gene. Plasmid survival in the xeroderma pigmentosum cells was less than that of pZ189 harvested from repair-proficient human cells. The point-mutation frequency in the 150-base-pair tRNA marker gene increased up to 100-fold with ultraviolet dose. Sequence analysis of 150 mutant plasmids revealed that mutations were infrequent at potential thymine-thymine dimer sites. Ninety-three percent of the mutant plasmids from the xeroderma pigmentosum cells showed G·C→A·T transitions, compared to 73% in the normal cells ($P < 0.002$). There were significantly fewer transversions ($P < 0.002$) (especially G·C→T·A) and multiple base substitutions ($P < 0.00001$) than when pZ189 was passaged in repair-proficient cells. The subset of mutational changes that are common to ultraviolet-treated plasmids propagated in both repair-proficient and xeroderma pigmentosum skin cells may be associated with the development of ultraviolet-induced skin cancer in humans.**

Xeroderma pigmentosum is an autosomal recessive disorder marked by sun sensitivity and a >1000-fold-increased frequency of neoplasms in skin exposed to sunlight (1). This disorder is one of the best known examples of the relationship between exposure to a mutagenic agent and tumorigenesis. Cultured cells from individuals with xeroderma pigmentosum are hypersensitive to ultraviolet radiation and have a reduced capacity for DNA repair (2, 3). In particular, cells from the xeroderma pigmentosum complementation group A have a profound defect in DNA repair and are almost totally unable to excise ultraviolet photoproducts (2–4). Mutation data from xeroderma pigmentosum cells would be of considerable interest, particularly in light of our understanding of the role of mutagenesis in the activation of certain oncogenes (5).

To acquire this information, we used the shuttle vector plasmid pZ189 (6), which replicates in human and bacterial cells and which carries a small bacterial marker gene, the activity of which can be determined in a standard microbiological assay (Fig. 1). pZ189 contains the origin of replication and large tumor (T)-antigen gene from SV40, information for replication and maintenance in bacteria from pBR327, and the marker gene *supF*, which encodes a suppressor tRNA that suppresses an amber mutation in the β-galactosidase gene (*lacZ*) in an indicator strain of *Escherichia coli*. Bacterial colonies containing plasmids with mutant or wild-type suppressor tRNA genes can be identified by color (wild-type are blue, mutants are light blue or white). Plasmids with mutant tRNA genes can be isolated and the sequence of the 150-bp marker gene determined in a single operation (7). This (8) and other (9–11) vectors have been used to study ultraviolet mutagenesis in repair-proficient mammalian cells.

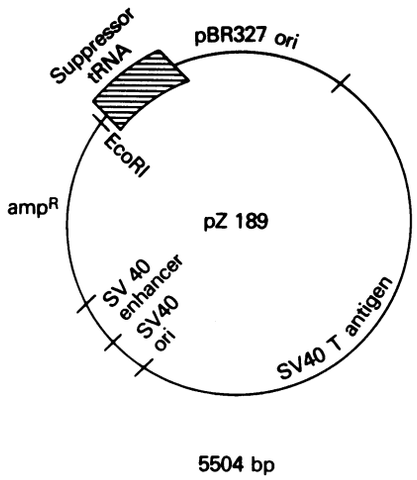

FIG. 1. Schematic diagram of plasmid pZ189, the shuttle vector used for mutagenesis studies. The plasmid contains simian virus 40 (SV40) sequences permitting growth in mammalian cells and pBR327 sequences enabling replication in bacteria. The approximately 150-base-pair (bp) suppressor tRNA marker sequence is flanked by sequences essential for survival of the plasmid in bacteria [pBR327 origin of replication and the ampicillin-resistance gene (amp^R)] thereby reducing the frequency of detection of large deletions in this region.

MATERIALS AND METHODS

Cells and Plasmid. The DNA repair-deficient, SV40-transformed, xeroderma pigmentosum cell line XP12BE(SV40) was derived from the skin of a xeroderma pigmentosum patient of complementation group A (4), who by age 20 has had 98 documented cutaneous basal-cell carcinomas. Fibroblasts from this patient have been shown to have <2% of normal DNA excision repair (4). The DNA repair-proficient SV40-transformed human fibroblast cell line GM0637(SV40) was derived from the skin of a healthy 18-year-old woman. Cell lines were obtained from the Institute for Medical Research (Camden, NJ) and grown in Dulbecco's modified Eagle's medium supplemented with 10% fetal bovine serum.

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Abbreviations: SV40, simian virus 40; bp, base pair(s).

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The plasmid used in these studies, pZ189 (6), was prepared under contract with Lofstrand Laboratories (Gaithersburg, MD), using standard techniques. Choramphenicol was not used to amplify the plasmid, in order to avoid possible RNA incorporation into the plasmid. The *E. coli* strain used to distinguish mutant and wild-type plasmids was MBM7070, a derivative of MC1061 [F⁻ *lacZamCA7020, lacY1, hsdR⁻, hsdM⁺, araD139, Δ(araABC-leu)7679, galU, galK, rpsL, thi*]. Colonies containing plasmids with wild-type tRNA genes were blue, and those with mutant tRNA genes were white or light blue on Luria–Bertani (LB) agar plates containing ampicillin (50 μg/ml), 5-bromo-4-chloro-3-indolyl β-D-galactoside (X-Gal), and isopropyl β-D-thiogalactoside (IPTG) (6).

Experimental Protocol. The pZ189 plasmid was treated with 254-nm ultraviolet radiation from a germicidal lamp, and 2×10^6 cells were transfected with 2–20 μg of plasmid DNA by a calcium phosphate precipitation procedure as described previously (12, 13). After 48 hr, the cells were extracted by the method of Hirt (14), treated with *Dpn* I to eliminate the input plasmid, which has methylated adenines typical of replication in bacteria (15), and the low molecular weight DNA was purified and introduced into competent MBM7070 cells. The bacteria were spread on LB agar plates containing ampicillin (50 μg/ml) and supplemented with X-Gal and IPTG (6). Colonies were scored, white and light-blue colonies were purified, and plasmids were isolated from 5-ml overnight cultures (6). The mutant plasmids were analyzed by agarose gel electrophoresis, and those chosen for sequence analysis were used in a primer-directed dideoxy procedure (7).

Statistical Analysis. Fisher's exact test for difference in proportions was used (16). The *P* values for a two-tailed test are presented.

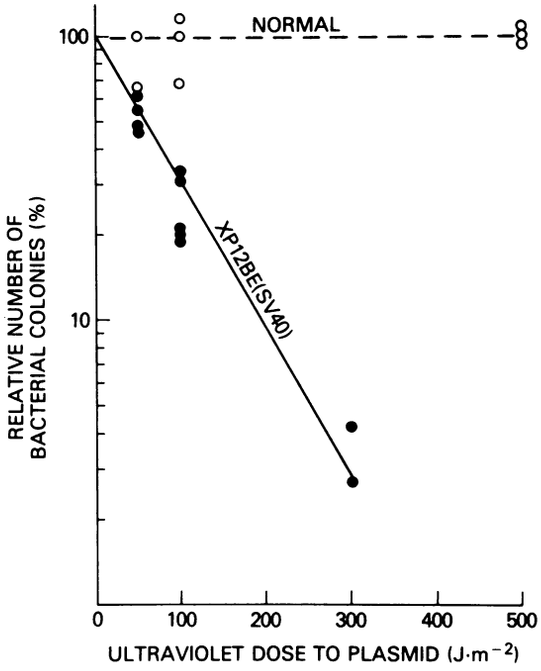

FIG. 2. Relative number of bacterial colonies observed after transformation by plasmids harvested from xeroderma pigmentosum and normal human cell lines transfected with ultraviolet-treated pZ189. The xeroderma pigmentosum line XP12BE(SV40) and the normal human fibroblast line GM0637(SV40) were transfected with ultraviolet (254-nm)-treated pZ189 (20 μg per 2×10^6 cells). Two days later the progeny plasmids were harvested and used to transform the indicator bacteria MBM7070.

RESULTS AND DISCUSSION

Plasmid Survival. pZ189 was treated with different doses of ultraviolet radiation and introduced into xeroderma pigmentosum and normal cells. Forty-eight hours after transfection, progeny plasmids were purified and used to transform indicator bacteria (6). In the xeroderma pigmentosum cells, survival of the transforming ability of pZ189 after exposure to 300 J/m² of ultraviolet radiation was reduced to about 3% of that of the untreated plasmid, whereas there was little or no effect on plasmid transforming ability in DNA repair-proficient human cells (Fig. 2). Southern blot hybridization (17) of the progeny DNA from extracts of the transfected xeroderma pigmentosum cells showed that the decline in transforming ability was related to the decline in the amount of form I (supercoiled) pZ189 DNA recovered as a function of ultraviolet dose. Similar analysis of pZ189 DNA recovered from the repair-proficient cells showed little variation with these ultraviolet doses (data not shown). These data are consistent with the results of cell-survival (2, 3, 18) and viral host-cell-reactivation (19–21) experiments with XP12BE and normal cells.

Plasmid Mutagenesis. We found only 8 white or light-blue colonies in more than 150,000 colonies examined after passage of the untreated plasmid through xeroderma pigmentosum cells (Table 1). Analysis of the mutant plasmids indicated that half contained deletions or insertions. Deletion/insertion mutagenesis of shuttle vector plasmids in mammalian cells has been described by several groups (22–25). Sequence analysis indicated that the other 4 mutant plasmids had single base-substitution mutations, a frequency of 2.5×10^{-5}, which was 1/15th that in the repair-proficient cells (40×10^{-5}) (Table 1). This lower spontaneous mutation frequency may be linked to the repair deficiency in xeroderma pigmentosum cells. However, variability of spontaneous mutation frequency of shuttle vector plasmids with different repair-proficient mammalian cell lines has been reported (26).

The frequency of mutant colonies increased as a linear function of the ultraviolet dose (Table 1). With the xeroderma pigmentosum cells, a dose of ultraviolet radiation that reduced plasmid transforming ability to about 30% (100 J/m²) increased the point-mutation frequency to more than 100 times the spontaneous level (Table 1). The frequency of deletion/insertion mutations declined following ultraviolet treatment.

Mutational Spectrum. Sequence analysis of 61 independent mutant plasmids recovered after passage of the ultraviolet-treated pZ189 through the xeroderma pigmentosum cells (Table 2, Fig. 3) revealed single base substitutions (47 plasmids at 10 sites) as well as tandem or closely spaced double mutations (12 plasmids including 10 additional sites). Only one mutant plasmid had multiple base substitutions (Table 2) (3 substitutions over 12 nucleotides at base pairs 163, 170, and 174). Analysis of 89 independent mutant plasmids recovered when ultraviolet-treated plasmid was passaged through the repair-proficient human cell line showed single base-substitution mutations at 16 sites (48 plasmids) and tandem mutations including 22 additional sites (16 plasmids) (Fig. 3). There was a significantly smaller proportion of mutant plasmids having single base substitutions ($P < 0.004$) and a greater proportion having multiple base substitutions ($P < 0.00001$) with the normal line (Table 2). The proportion of single, tandem, and multiple base substitutions in repair-proficient monkey cells (8) was similar to that in the repair-proficient human cells. In contrast to data from *E. coli* (27), frameshift mutations were infrequent in the human (Table 2) and monkey (8) cells.

Ninety-three percent of single or tandem base substitutions observed in the plasmids replicated in the xeroderma pigmentosum cells were G·C→A·T transitions (Table 3 and

Table 1. Analysis of bacterial colonies transformed by ultraviolet-treated shuttle vector pZ189 replicated in xeroderma pigmentosum (XP) or normal human cells

UV dose to plasmid, J/m²	No. of exps.*	Mutant colonies†, no./no. of colonies scored (frequency × 10³)	Mutant plasmids with altered gel mobility, no./no. tested	Sequenced mutants: No. with deletions‡	Sequenced mutants: No. with base substitutions	Base substitution mutant frequency§ × 10³
XP						
0	6	8/158,990 (0.05)	2/8	2	4	0.025
50	4	87/44,530 (2.0)	3/52	2	32	1.8
100	6	90/27,345 (3.3)	0/70	0	30	3.3
300	2	12/1,705 (7.0)	0/11	0	5	7.0
Normal						
0	5	39/54,700 (0.7)	10/36	4	22	0.4
100	3	26/10,645 (2.4)	1/22	0	21	2.3
500	3	67/10,515 (6.4)	0/40	0	40	6.4
1000	2	40/2,670 (15.0)	1/23	0	22	14.3

*Representative experiments.
†White or light-blue colonies.
‡From 3 to about 150 bp deleted.
§Assuming that the relative number of mutants with base substitutions was the same among the total number of mutants as in the fraction of mutants analyzed by agarose gel electrophoresis and sequencing.

Fig. 3). This is significantly greater than the 73% observed with the normal cells ($P < 0.002$). The frequency of the G·C→A·T transitions in the normal human cells was similar to that seen in CV-1 cells after *in vitro* plasmid treatment with ultraviolet radiation (8) and in another repair-proficient human cell line after intracellular plasmid exposure to ultraviolet radiation (9). There was one G·C→C·G transversion and three A·T→T·A transversions; two of these were in a double mutation separated by a single base (Fig. 3). Almost all of the G·C→A·T transitions were found in three strong hot spots at positions 156, 168, and 169. Lesser hot spots were seen at positions 123 and 155. With the repair-proficient cells, the major hot spot at position 156 was seen, as was the lesser hot spot at position 123. However, positions 168 and 169 were not seen as hot spots for transitions in the repair-proficient cells.

The mutational spectrum is a function of the relative probabilities of several events: a photoproduct might be correctly or incorrectly repaired, serve as a lethal barrier to replication, or be correctly or incorrectly copied during replication. This variety of possible consequences makes a simple interpretation of mutagenesis data difficult. The use of the xeroderma pigmentosum cells, in which excision repair of ultraviolet photoproducts does not occur, reduces the complexity of the analysis.

The appearance of a new ultraviolet G·C→A·T hot spot in DNA repair-deficient cells was noted previously in *E. coli* by Todd and Glickman (28). The sequence at that site, 5′ TCC 3′, is the same as at positions 167–169 in pZ189. The higher mutability at positions 168 and 169 in the ultraviolet-treated plasmids harvested from the xeroderma pigmentosum cells may be related to the formation of a premutagenic lesion at that site which is excised by the ultraviolet endonuclease in the repair-proficient cells.

A significantly lower frequency ($P < 0.002$) of transversion mutations was observed with the xeroderma pigmentosum cells (6%), in comparison to the normal cells (25%) among the single and tandem base-substitution mutations (Table 3). The G·C→T·A transversions represented 10% of the mutations with the normal cells but was not found with the xeroderma pigmentosum cells ($P < 0.01$). This difference in the transversion frequency suggests either that the xeroderma pigmentosum cells are deficient in an activity that is responsible for the transversion mutagenesis or that the opportunities for such an activity to function are reduced. If the only activity missing in these xeroderma pigmentosum cells is that involved in the excision of DNA lesions (2, 3), then it follows that the occurrence of transversions in normal cells is associated with excision repair. Miller (27) has discussed the problems posed by transversion mutations in *E. coli*.

Table 2. Mutations observed in ultraviolet-treated shuttle vector pZ189 replicated in xeroderma pigmentosum (XP) or normal repair-proficient human cells

	No. of plasmids with base changes: XP	No. of plasmids with base changes: Normal
Independent plasmids* sequenced	61	89
Point mutations		
Single base substitution	47	48
Tandem base substitutions†	12	16
Multiple base substitutions‡	1§	24
Insertions and deletions		
Single base insertion	0	2 [2]¶
Single or tandem base deletions	1	3 [2]¶

Ultraviolet dose was 50–300 J/m² for plasmids replicated in xeroderma pigmentosum cells and 100–5000 J/m² for those replicated in repair-proficient cells.
*From separate transfections or different mutations in the same transfection, including all experiments.
†Two base substitutions 0–2 bases apart, or 3 adjacent base substitutions.
‡Three to the maximum seen of 5 base substitutions, or 2 base substitutions more than 3 bases apart.
§Three base substitutions more than 2 bases apart.
¶Number of plasmids with insertion or deletion accompanied by 1 or 2 base substitutions.

Table 3. Types of single or tandem base substitutions in ultraviolet-treated pZ189 replicated in xeroderma pigmentosum (XP) or normal human cells

	No. of changes: XP	No. of changes: Normal
Transitions	67 (94%)	61 (75%)
G·C → A·T	66 (93%)	59 (73%)
A·T → G·C	1 (1%)	2 (2%)
Transversions	4 (6%)	20 (25%)
G·C → T·A	0	8 (10%)
G·C → C·G	1 (1%)	5 (6%)
A·T → T·A	3 (4%)	6 (8%)
A·T → C·G	0	1 (1%)

See Table 2.

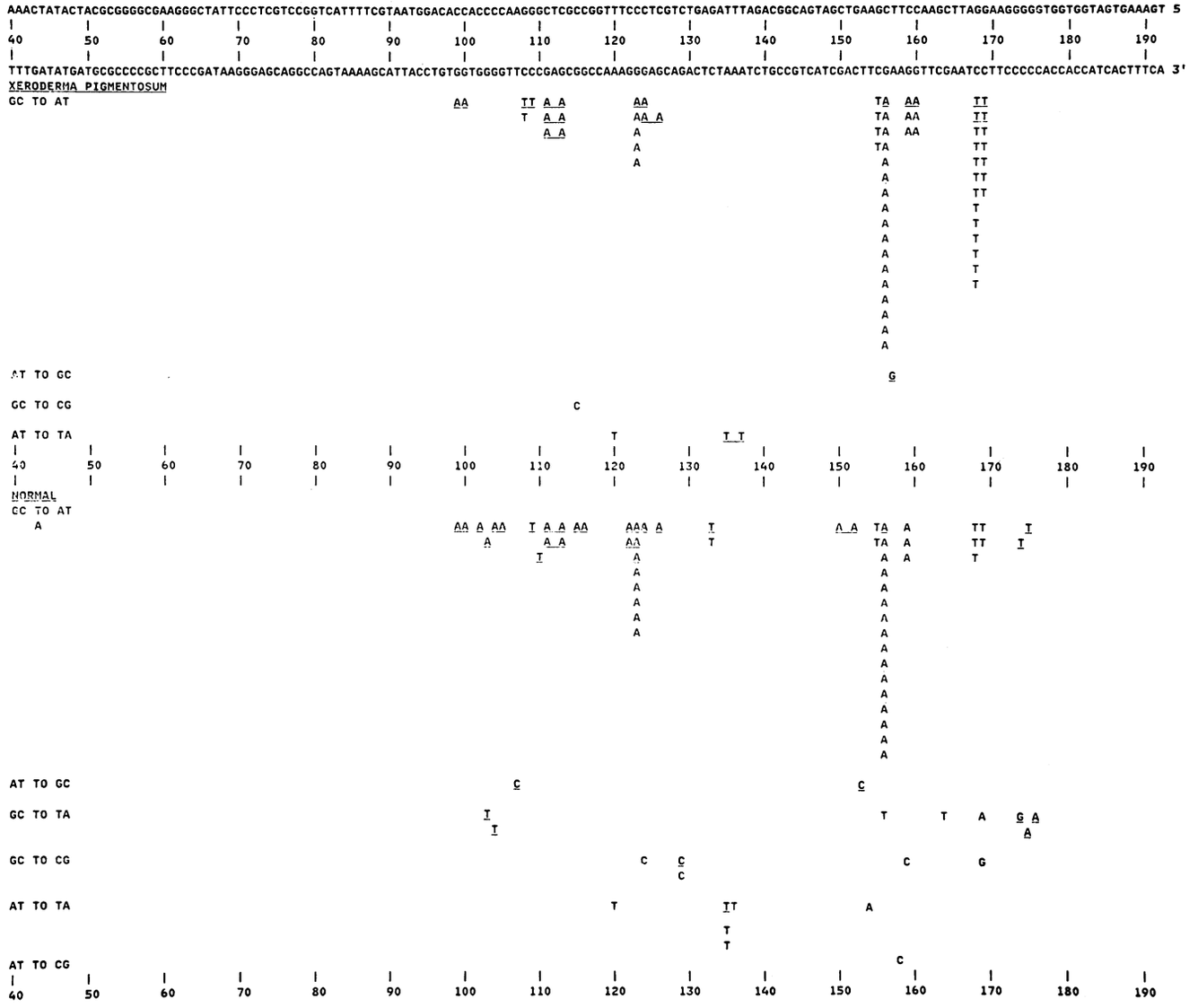

FIG. 3. Location and classification of independent single and tandem base-substitution mutations found in ultraviolet-treated plasmid pZ189 replicated in xeroderma pigmentosum cell line XP12BE(SV40) and repair-proficient line GM0637(SV40). The 152-bp sequence shown contains the marker gene including the suppressor tRNA sequence (base pairs 99–183), the pre-tRNA sequence (base pairs 59–98), and a portion of the promoter (base pairs 24–58). Base substitutions are indicated below the altered base pair as a change in the lower strand. Tandem or closely spaced base substitutions, deletions, or insertions are indicated by underlining. Mutations are listed by type of base substitution involved.

With both types of human cells, all the single base-substitution mutations observed in the ultraviolet-treated plasmid occurred at pyrimidine dinucleotide sites—i.e., sites of potential ultraviolet photoproducts (Fig. 3). With the xeroderma pigmentosum cells, except for the G·C→A·T transition at base pair 160, all of the single base substitutions could be interpreted as occurring at cytosines 3′ to a thymine or cytosine. There was only one single base substitution (T→A, position 120) at sites of potential thymine-thymine dimers, the major ultraviolet photoproduct (29), and only one of the closely spaced double mutations (TTT→ATA, positions 135–137) occurred at a probable thymine-thymine dimer site. With ultraviolet-treated pZ189 in the repair-proficient human cells, only 11% (5 of 45) of the single base-substitution mutations were A·T→T·A transversions, at four sites of potential thymine-thymine dimers (Fig. 3). Similar results were observed in repair-proficient monkey and human cells (8, 9). These results are consistent with data from prokaryotic studies (30, 31) and the analysis of Brash and Haseltine (29), which suggest that in addition to cyclobutane dimers, other photoproducts [such as the T-C and C-C pyrimidine-pyrimidone(6-4) photoproducts] may be premutagenic lesions.

The activity that generates the G·C→A·T transitions is present in both normal and xeroderma pigmentosum cells and is, therefore, unlikely to be related to DNA excision repair. Instead, the G·C→A·T mutagenesis probably occurs during replication of the ultraviolet-treated plasmid, since replication occurs in both cell lines. SV40 and derivative plasmids are replicated by cellular enzymes. It seems likely, then, that the mechanism of transition mutagenesis of ultraviolet-treated pZ189 and cellular DNA would be similar, if not identical. The dominance of G·C→A·T transitions and virtual absence of A·T→G·C transitions in our experiments is similar to the results in *E. coli* (27) and recalls observations made by Drake (32) and Howard and Tessman (33). Tessman (34) suggested that much ultraviolet transition mutagenesis could be explained by adenine insertion across from photoproducts during replication. *In vitro* and *in vivo* studies indicate that many polymerases preferentially insert adenine across from sites of DNA damage (35–37). (See also refs. 8, 27, 30 and 35 for further discussion.) Thus, in circumstances where thymine-thymine dimers were not lethal blocks to replica-

tion, they would not be mutagenic. T-C and C-T photoproducts would yield single base substitutions across from the cytosine. Only C-C photoproducts would yield tandem mutations. Our xeroderma pigmentosum data are consistent with the hypothesis that the major mutagenizing activity in the human cells adheres to this adenine-insertion rule. Since the selective advantage of this propensity is apparent (34), it may well have developed during the evolution of primitive forms of life, which were exposed to short-wavelength ultraviolet radiation.

Multiple-Change Mutant Plasmids. We found mutant plasmids with multiple base substitutions after transfection of the repair-proficient cells with ultraviolet-treated plasmid. However, there was no relationship between ultraviolet dose and the number of base substitutions in a given mutant gene. Thus they do not appear to be the result of multiple ultraviolet lesions, individually mutagenized, in the marker gene. Multiple-change mutants have been found in the bacteriophage M13 system (30) and the CV-1 study (8) and have been interpreted as evidence of an error-prone polymerase activity in those cells. Our observation that the frequency of this class of mutants is sharply reduced in the xeroderma pigmentosum cells (Table 2) suggests a linkage to excision repair. Perhaps the gaps generated during repair are occasionally filled by an error-prone polymerase. Somatic mutagenesis is an important feature in the generation of diversity of immunoglobulin genes, and an activity of this sort might be relevant to that problem (38, 39).

Relation to Skin Cancer. Patients with xeroderma pigmentosum experience at an increased frequency (1) the same types of ultraviolet-associated skin cancers (basal-cell and squamous-cell carcinomas) as are found in about 500,000 Caucasions annually in the United States (40). The mutations observed in ultraviolet-treated pZ189 propagated in cells derived from xeroderma pigmentosum skin are a subset of those seen upon propagation in cells derived from normal skin. The G·C→A·T transitions (at sites other than thymine-thymine dimer sites) present in the xeroderma pigmentosum spectrum may therefore be of greater importance in ultraviolet-induced cutaneous carcinogenesis than the transversions and multiple base-substitution mutations missing from this collection.

We thank Dr. R. Tarone for assistance in the statistical analysis of the data and Dr. D. Brash for stimulating discussions.

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