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Genetics

Photoproduct frequency is not the major determinant of UV base substitution hot spots or cold spots in human cells

(UV carcinogenesis/DNA structure/xeroderma pigmentosum/DNA repair/shuttle vector)

DOUGLAS E. BRASH*†, SARASWATHY SEETHARAM‡, KENNETH H. KRAEMER†‡, MICHAEL M. SEIDMAN†§, AND ANDERS BREDBERG‡¶

*Laboratory of Human Carcinogenesis and ‡Laboratory of Molecular Carcinogenesis, National Cancer Institute, Bethesda, MD 20892; and §Otsuka Pharmaceutical Co., Rockville, MD 20854

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ABSTRACT **The role of UV radiation-induced photoproducts in initiating base substitution mutations in human cells was examined by measuring photoproduct frequency distributions and mutations in a *supF* tRNA gene on a shuttle vector plasmid transfected into DNA repair-deficient cells (xeroderma pigmentosum, complementation group A) and into normal cells. Frequencies of cyclobutane dimers and pyrimidine-pyrimidone (6-4) photoproducts varied by as much as 80-fold at different dipyrimidine sites within the gene. All transition mutations occurred at dipyrimidine sites, predominantly at cytosine, with a 17-fold variation in mutation frequency between different sites. Removal of >99% of the cyclobutane dimers by *in vitro* photoreactivation before transfection reduced the mutation frequency while preserving the mutation distribution, indicating that (*i*) cytosine-containing cyclobutane dimers were the major mutagenic lesions at these sites and (*ii*) cytosine-containing non-cyclobutane dimer photoproducts were also mutagenic lesions. However, at individual dipyrimidine sites neither the frequency of cyclobutane dimers nor the frequency of pyrimidine-pyrimidone (6-4) photoproducts correlated with the mutation frequency, even in the absence of excision repair. Mutation hot spots occurred at sites with low or high frequency of photoproduct formation and mutation cold spots occurred at sites with many photoproducts. These results suggest that although photoproducts are required for UV mutagenesis, the prominence of most mutation hot spots and cold spots is primarily determined by DNA structural features rather than by the frequency of DNA photoproducts.**

The majority of base substitution mutations, whether chemically or physically induced or spontaneous, appear to be located at DNA lesions (1, 2). In the case of UV radiation, the lesions appear to be dipyrimidine photoproducts that initiate G·C to A·T transitions (2–8). It has long been anticipated that identification of the mutagenic UV photoproduct in human cells would provide a missing link in the etiology of sunlight-related human skin cancers, in oncogene activation by point mutations (9), and in the pathogenesis of xeroderma pigmentosum, a human disease with defective photoproduct excision and markedly elevated skin cancer incidence (10). For example, the lesion distribution might reveal why different carcinogens activate different protooncogenes even in the same tissue type (11).

Recently it has become possible to measure mutation spectra in mammalian cells following treatment of DNA with UV or other DNA-damaging agents by using shuttle vector plasmids (5–8). We have now measured the frequencies of two UV photoproducts implicated in mutagenesis, the cyclobutane dimer and the pyrimidine-pyrimidone (6-4) photoproduct (refs. 3 and 4 and references therein), in a shuttle vector *supF* tRNA gene (6). The vector was treated with UV *in vitro* and the photoproduct frequency was measured. The vector was transfected into repair-deficient and normal human cells, and the number of mutations at different sites was determined (6). By *in vitro* photoreactivation of cyclobutane dimers prior to transfection, we determined that cyclobutane dimers and non-cyclobutane dimer photoproducts contributed to base substitution mutations. The frequency of these photoproducts at mutated sites did not correlate with the mutation frequency at the same sites, even in the absence of excision repair.

MATERIALS AND METHODS

Site-Specific Determination of Cyclobutane Dimers and (6-4) Photoproducts. Form I plasmid pZ189 was restriction digested and end labeled following UV treatment, so the photoproduct frequencies measured were identical to those transfected into the cells. The DNA was 3′ end labeled by filling in the unique *Eco*RI site (top strand) or the base pair 239 *Xho* II site (bottom strand). To detect the presence of cyclobutane dimers and pyrimidine-pyrimidone (6-4) photoproducts at individual base pairs, end-labeled DNA was incubated with T4 dimer-specific endonuclease (a gift from A. Ganesan, Stanford University) or 1 M piperidine, respectively (12). Samples were electrophoresed and quantitated as described (12) or quantitated by scanning the autoradiograms with an LKB Ultroscan laser densitometer using known radioactivity standards. The variation of measurements of replicate samples was <20%.

Plasmid Treatment and Mutagenesis. pZ189 (6) was treated with 254-nm UV radiation from a germicidal lamp as described (13, 14). *Escherichia coli* DNA photolyase (12) (a gift from A. Sancar, University of North Carolina) was used as described (14) to monomerize pyrimidine dimers but not (6-4) photoproducts (12). The extent of photoreactivation was measured by the T4 endonuclease V assay on supercoiled DNA (13) and on defined sequence DNA (12). Simian virus 40 (SV40)-transformed xeroderma pigmentosum (XP12Be) and normal (GM0637) cell lines were obtained from the Institute for Medical Research (Camden, NJ), and grown as described (13, 14). XP12Be fibroblasts have been shown to have <2% of normal DNA excision repair (15). Mutation spectra were determined as described (6, 13).

RESULTS

Photoproduct Frequency and Mutation Frequency. The frequency of UV photoproducts was measured at 58 of the 72

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Abbreviation: SV40, simian virus 40.

†To whom reprint requests should be addressed.
¶Present address: Department of Medical Microbiology, Malmo General Hospital, University of Lund, Malmo, Sweden.

dipyrimidines in the 126-base-pair region of the *supF* tRNA gene in which all base substitution mutations were found. Fig. 1 shows the distribution of cyclobutane pyrimidine dimers and pyrimidine-pyrimidone (6-4) photoproducts in representative regions of the *supF* gene, including the sites of mutation hot spots (Fig. 1 *a–c*).

Table 1 shows the quantitation of photoproduct frequencies from gels such as in Fig. 1. The frequency of molecules with a dipyrimidine photoproduct at a measured site (after exposure to UV radiation at 1000 J/m^2) ranged from 0.1% to 7.9% for cyclobutane dimers and from 0% to 1.8% for (6-4) photoproducts. These values are comparable to those found in the *E. coli lacI* gene (3), with a somewhat higher upper limit for cyclobutane dimers. The photoproduct frequency distribution in *supF* followed the same rules previously determined in the *lacI* gene (3): cyclobutane dimers were more frequent than (6-4) photoproducts at most sites; at some sites (6-4) photoproducts were more frequent than cyclobutane dimers; the frequency of cyclobutane dimers was in the order TT > TC = CT > CC; the frequency of (6-4) photoproducts was TC > CC; no (6-4) photoproducts were detected at CT or TT sites. UV photoproducts were more frequent at the 3′ end of pyrimidine runs.

The UV-treated pZ189 was passaged through repair-proficient and repair-deficient human cells and the mutation spectra were determined (6). Transitions constituted 94% of the single and tandem base substitution mutations with the XP-A cells and 75% with the repair-proficient cells. For this reason we have focused on the analysis of transition mutations; their distribution is shown in Fig. 2. With the XP-A cells there were hot spots (i.e., sites at which >10% of the single base substitution mutations occurred) at base pairs 156, 168, and 169. With the normal cells, hot spots at base pairs 156 and 123 were prominent. All isolated single base substitution transition mutations were of the G·C to A·T type and occurred at dipyrimidines. The specificity of G·C to A·T transition mutagenesis for dipyrimidine sequences implies that these mutations were initiated at photoproducts (2). Most of the mutations occurred at cytosines that were 3′ to another pyrimidine or could be interpreted as such. However, mutations at a cytosine that was unambiguously 5′ to another pyrimidine were occasionally found (base pair 160 in the XP-A cells, Fig. 2).

To assess the relation between photoproducts and mutations, we plotted (Fig. 3) the frequencies of cyclobutane dimers and pyrimidine-pyrimidone (6-4) photoproducts (Table 1) versus the number of single or tandem transition mutations found at each site (Fig. 2). Data were available for 58 mutants at 26 sites with the normal cells and 64 mutants at 14 sites with the XP12BE (SV40) cells. There was a clear lack of correlation between these photoproduct frequencies and the mutation frequencies at the same sites (coefficient of determination, $r^2 < 0.2$ for each of the four comparisons). Whether cyclobutane dimers or (6-4) photoproducts were plotted, with normal cells or with XP-A, low lesion frequencies were often associated with high mutation frequencies and high lesion frequencies were often associated with low mutation frequencies.

Photoreactivation. To identify the mutagenic photoproduct, we treated the UV-damaged plasmid with photoreactivating enzyme *in vitro* before transfection. Photoreactivation left no detectable cyclobutane dimers (Fig. 1*c*), while preserving pyrimidine-pyrimidone (6-4) photoproducts (12). Since photoproducts present at a frequency of 0.08% were detectable and cyclobutane dimers were initially present at levels up to nearly 8%, photoreactivation reduced the cyclobutane frequency by a factor of at least 100. The same result was obtained in an assay that measured loss of the supercoiled form (14).

With XP-A cells, the overall mutation frequency in the *supF* gene after photoreactivation was reduced by a factor of 10, indicating that cyclobutane dimers were essential to generation of 90% of the mutations. This result also excludes deaminated cytosine-containing dimers as major mutagenic lesions. In the absence of cyclobutane dimers, mutations were still recovered (Fig. 2), indicating that nonphotoreactivatable lesions were also mutagenic and were associated with about 10% of the mutations. The same hot spots were prominent in the photoreactivated spectrum, implying that the fraction of mutations at individual hot spots due to cyclobutane dimers was also ≈90%.

Since the transition mutations remaining after photoreactivation occurred at cytosine-containing dipyrimidines (Fig.

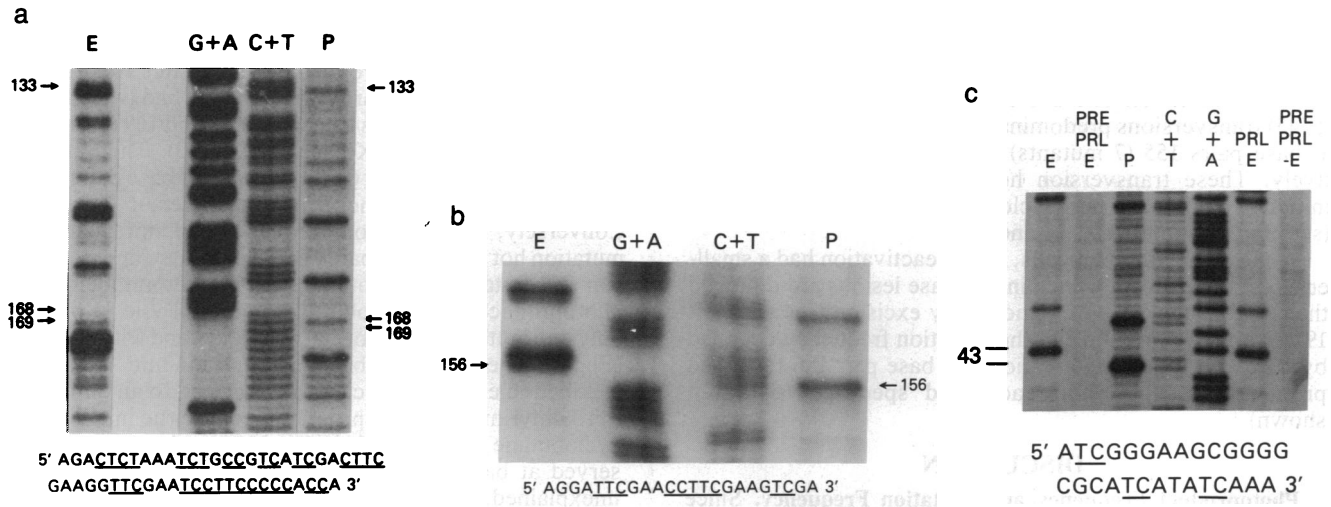

FIG. 1. Distribution of cyclobutane pyrimidine dimers and pyrimidine-pyrimidone (6-4) photoproducts in the *supF* gene. (*a*) Lower strand, bases 128–180 (top to bottom). (*b*) Upper strand, bases 170–148. (*c*) Upper strand, bases 67–40. E, cyclobutane dimer-specific T4 endonuclease treatment; G+A, guanine plus cytosine Maxam–Gilbert sequencing reaction; C+T, cytosine plus thymine Maxam–Gilbert sequencing reaction; P, piperidine treatment to reveal (6-4) photoproducts; PRE, *E. coli* DNA photolyase treatment; PRL, 405-nm photoreactivating light treatment. Autoradiogram of sequencing gel. Underlined sequences indicate locations of adjacent pyrimidines appearing as prominent bands in the photoproduct lanes. The intensity of a band reflects the frequency of scissions at the corresponding base and hence the frequency of photoproducts at that site in the population of irradiated DNA molecules. Cyclobutane dimer bands migrate about 1 base slower than the 5′ base of the dimer. Pyrimidine-pyrimidone (6-4) photoproduct bases comigrate with the 3′ cytosine of the (6-4) photoproduct.

Table 1. Frequency of cyclobutane dimers and pyrimidine-pyrimidone (6-4) photoproducts at 58 sites in the *supF* gene

Base pairs*	Bases	Transition mutants†	% dimers	% (6-4)	Base pairs*	Bases	Transition mutants†	% dimers	% (6-4)
44–43	T-C	0-S	1.75	1.78	137–136	T-T	S-D	1.91	—
49–48	T-C	0-0	1.15	1.07	151–150	T-C	0-S	0.10	0.02
66–65	T-C	0-S	0.53	0.39	152–153	C-T	0-D	0.23	—
69–68	T-T	0-0	7.93	—	153–154	T-T	D-0	1.18	—
73–72	T-C	0-0	2.04	1.67	154–155	T-C	0-S	1.18	0.64
82–81	C-T	0-0	0.31	−0.04	157–156	T-C	D-S	1.58	1.09
85–84	T-T	0-0	5.31	—	158–157	T-T	0-D	2.95	—
86–85	T-T	0-0	5.65	—	159–158	C-T	S-0	0.81	—
87–86	T-T	0-0	5.49	—	160–159	C-C	S-S	0.10	0.02
100–99	C-C	S-S	0.60	0.26	161–162	T-T	0-0	0.73	—
103–102	C-C	D-D	0.67	0.38	162–163	T-C	0-0	0.28	0.68
104–103	C-C	D-D	0.29	0.12	165–164	T-C	0-D	0.88	0.70
105-104	C-C	S-D	0.32	0.08	166–165	T-T	0-D	2.22	—
112–111	T-C	S-S	0.65	0.43	167–168	T-C	0-S	0.28	0.17
113–112	C-T	S-S	0.14	—	168–169	C-C	S-S	0.40	0.07
116–115	C-C	D-S	0.11	0.03	169–170	C-T	S-D	0.40	—
120–119	T-T	S-0	4.36	—	170–171	T-T	D-D	3.91	—
121–120	T-T	0-S	4.61	—	171–172	T-C	D-S	1.9	0.60
122–121	C-T	S-0	0.87	—	172–173	C-C	S-0	0.43	0.15
123–122	C-C	S-S	0.48	0.07	173–174	C-C	0-D	0.34	0.15
124–123	C-C	D-S	0.48	0.20	174–175	C-C	D-S	—	0.15
125–124	T-C	D-D	1.21	0.70	175–176	C-C	S-0	0.50	0.18
126–125	C-T	D-D	0.31	—	178–179	C-C	S-0	0.54	0.125
129–128	C-T	S-0	0.67	—	181–182	C-C	0-0	0.44	0.12
130–129	T-C	0-S	0.29	0.03	184–185	T-C	0-0	0.40	0.50
131–132	C-T	0-0	0.38	—	187–188	C-T	0-0	0.27	—
132–133	T-C	0-S	0.76	0.17	188–189	T-T	0-0	1.75	—
133–134	C-T	S-S	0.76	—	189–190	T-T	0-0	4.8	—
136–135	T-T	D-S	5.90		190–191	T-C	0-0	2.00	1.28

*Dipyrimidine sites on both strands listed as 5′ pyrimidine–3′ pyrimidine (see Fig. 2).
†Transition mutation found to result in inactivation of *supF* tRNA function at indicated base; 0 = none found; S = isolated single transition mutation found; D = part of a tandem double mutation found (refs. 6, 8, and 16; unpublished observations).

2), a dipyrimidine photoproduct is implicated. One candidate for such a lesion is the pyrimidine-pyrimidone (6-4) photoproduct. Thus, cyclobutane dimers and non-cyclobutane dimer photoproducts each generate G·C to A·T transition mutations in UV-damaged DNA replicated in human cells.

The proportion of transversion mutations increased from 6% (4/71) (ref. 6) to 36% (16/45) after photoreactivation ($P < 0.0003$), resulting in a proportion of types of transitions and transversions that was not significantly different than with the normal line (6). The G·C to C·G (8/45) and A·T and T·A (5/45) transversions predominated, with prominent hot spots at base pairs 155 (7 mutants) and 120 (5 mutants), respectively. These transversion hot spots were thus probably initiated mainly by non-cyclobutane dimer photoproducts (see, for example, refs. 17 and 18).

In excision-proficient cells, photoreactivation had a smaller effect, as expected since in this case lesions are removed that would otherwise be removed by excision (see also ref. 19). With the normal cells, the mutation frequency declined by 50% after photoreactivation and base pair 156 remained prominent in the photoreactivated spectrum (data not shown).

DISCUSSION

Photoproduct Frequency and Mutation Frequency. Since mutation frequency was a linear function of UV dose to the shuttle vector plasmid (6), photoproducts were required for UV mutagenesis. However, the photoproduct frequencies measured at mutation sites in the UV-treated plasmid pZ189 did not correlate with the mutation frequencies at the same sites (Fig. 3). Recently, a similar conclusion was reached in a comparison of the spectra of mutations with sites of UV-induced polymerase terminations (8). In the present study neither quantitation of cyclobutane dimers and (6-4) photoproducts nor identification of the mutagenic lesions resolved the discrepancy between the photoproduct frequency and the mutation frequency.

A low photoproduct frequency did not prevent the occurrence of a mutation hotspot. Although the principal mutagenic lesion at base pairs 168 and 169 was identified as the cyclobutane dimer, the frequency of cyclobutane pyrimidine dimers and (6-4) photoproducts at these sites was not large (Table 1). Indeed, the nearby base pair 133 had twice as many of these photoproducts as base pairs 168 and 169 (Table 1) yet had no mutations with XP-A cells (Fig. 2). This difference cannot be ascribed to low dimer excision at base pairs 168 and 169 because this cell line is unable to repair dimers (13). Conversely, a high lesion frequency did not guarantee a mutation hot spot. Base pair 43 on the same strand as the base pair 156 mutation hot spot had even greater numbers of (6-4) photoproducts and cyclobutane dimers (Table 1) but made only one mutation with normal cells and none with XP-A cells (Fig. 2). The difference between sites 156 and 43 cannot be ascribed to excision nor can it be ascribed to an unmeasured third dipyrimidine photoproduct, because the inability of the cyclobutane dimers and (6-4) photoproducts actually observed at base pair 43 to initiate mutations would remain unexplained.

Our results accentuate a previous result in the *E. coli lacI* gene: though the UV mutation frequency at many hot spots was correlated with photoproduct frequency, the strongest nonsense mutation hot spot in the *lacI* gene, site A23, was markedly more mutagenic than could be accounted for by the photoproduct frequency (3). In human cells, the same result was obtained since the distribution of mutations in the *lacI* gene closely resembled that in *E. coli* (5). In addition, though

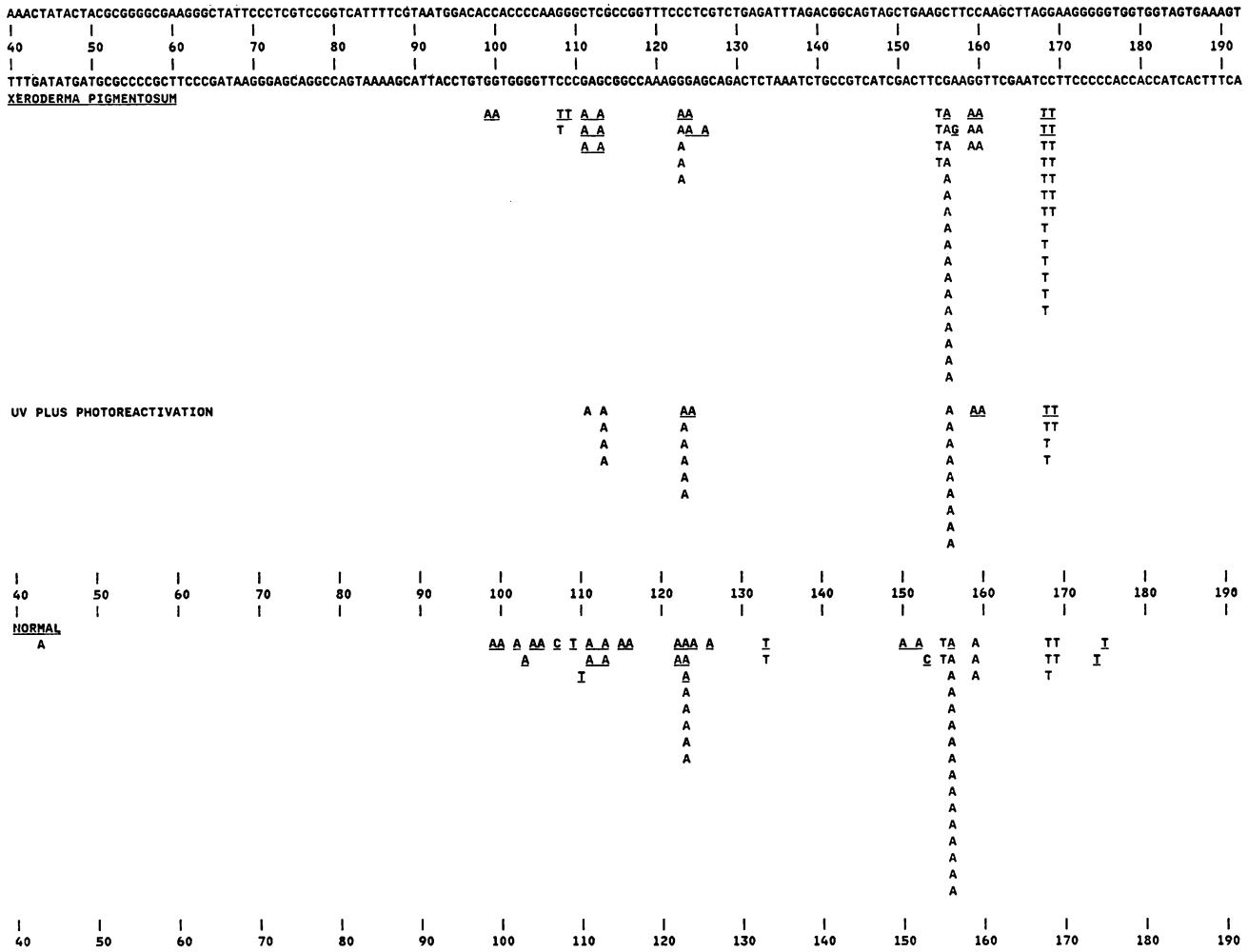

FIG. 2. Location of transition mutations found in pZ189 after UV treatment or UV treatment followed by *in vitro* photoreactivation and propagation in xeroderma pigmentosum cell line XP12BE (SV40). The 152-base-pair sequence shown contains the marker *supF* gene (base pairs 99–183), the pre-tRNA sequence (base pairs 59–98), and a portion of the promoter sequence (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 are indicated by underlining. Nonphotoreactivated mutants from the repair-proficient cell line GM0637 (SV40) and from XP12BE (SV40) are modified from ref. 6.

one of the two strongest UV missense mutation hot spots in *lacI* (base pair 174) (ref. 2) had a high frequency of cyclobutane dimers and (6-4) photoproducts, the other (base pair 75) had low frequencies of both lesions (D.E.B., unpublished). Thus, two genes in mammalian and bacterial systems contain highly mutation-prone loci at which UV mutation frequencies do not correlate with lesion frequencies. In the *lacI* gene, these sites coexist with less prominent hot spots at which mutation frequency and lesion frequency do correlate.

Pass/Fail Options. The lack of correlation of mutation frequency with photoproduct frequency at different sites implies that the slope of the linear UV dose–response varies from site to site. One can envision at least two mechanisms having a profound influence on site-specific mutation frequency independent of photoproduct frequency.

The first acts at the step of polymerase readthrough opposite a lesion: the correctness of such readthrough would be disfavored at base pairs 156, 168, and 169 and favored at base pairs 43 and 124. An example of a readthrough mechanism is the "A rule," in which adenine insertion opposite a noninstructional lesion prevents mutations at thymine, despite the high frequency of cyclobutane dimers directly measured at TT sites (reviewed in ref. 6; see also ref. 20), and generates the observed G·C to A·T transitions at cytosine. Readthrough does not readily explain the site-specific frequency of G·C to A·T transitions, because at site 43 or 124 fortuitously correct insertion of guanine opposite damaged cytosine would be required for two sterically dissimilar photoproducts.

We prefer a second alternative, acting at the step of DNA replication block, applicable to all of these sites: photoproducts tend to block DNA replication more rarely at certain base pairs than at others, so that mutations at these sites are less frequently lost to termination. We suggest a scenario in which, when the replication complex encounters a modified base, it will either pass contiguously—with some proportion of the pass events being mutagenic during readthrough—or fail to continue replication—resulting in lethality. This decision is made even in the absence of altered bases, resulting in a low proportion of spontaneous mutations and terminations. The pass/fail ratio will be a function of presently unknown rules that lie in the DNA sequence. Mutation hot spots will occur at photoproduct hot spots in regions of the gene with typical pass/fail ratios. Base pair 156 in *supF* fulfills this criterion, as do many hot spots in *lacI* (3). However, an additional class of mutation hot spots would be expected to occur at DNA sites where the pass/fail ratio is atypically large. Although lesion frequency might be small, a larger fraction of decision events would be pass events rather than terminations, thereby slightly increasing the number of replicated molecules recovered but substantially increasing the number of replicated molecules recovered that carry a mutation at this site. Points above the dashed line in Fig. 3, such as base pairs 123, 168, and 169 in *supF* (and site A23 in

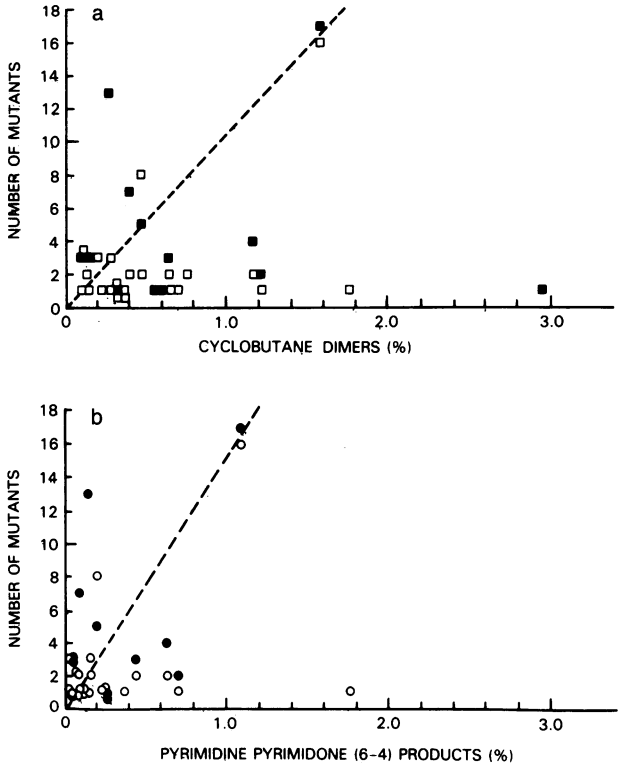

FIG. 3. Relationship of cyclobutane dimer and pyrimidine-pyrimidone (6-4) photoproduct frequency to the number of transition mutants found in pZ189 propagated in xeroderma pigmentosum [XP12BE (SV40)] or repair-proficient [GM0637 (SV40)] human cells. (*a*) Cyclobutane dimer frequency versus number of transition mutants. (*b*) Pyrimidine-pyrimidone (6-4) photoproduct frequency versus number of transition mutants. ● and ■, xeroderma pigmentosum cells; ○ and □, repair-proficient cells. Mutations were assigned to the 3′ base of a photoproduct where possible. Attribution of mutations to the 5′ base did not alter the results. The dashed line in each graph indicates a theoretical linear relationship between photoproduct frequency and mutation frequency assuming that the mutations at base pair 156 are proportional to the photoproduct frequency.

lacI), appear to be such sites. [These scenarios do not specify whether the mutation is made after the pass event, concomitant with it (error-prone reactivation), or prior to it (disclosure).] The lack of correlation between photoproduct frequency and mutation frequency at these sites implies that the effect of the increase in pass/fail ratio outweighed the effect of photoproduct frequency—i.e., that these sites were pass-determined hot spots rather than photoproduct-determined hot spots. A final class of sites falling below the dashed line in Fig. 3, such as base pairs 43 and 124 in *supF*, may have atypically low pass/fail ratios so that even large photoproduct frequencies do not produce appreciable numbers of mutations because of the increased frequency of terminations.

Are these observations limited to UV base substitution mutagenesis? In an elegant study (21, 22) Fuchs and coworkers compared the spectrum of acetylaminofluorine (AAF)-induced frameshift mutations in *E. coli* with the spectrum of sites at which T4 DNA polymerase exonuclease activity was blocked by AAF adducts. Finding little correlation, they proposed that AAF frameshift mutations occurred at mutation-prone sequences, outside of which the DNA adduct structure was lethal. Mutation-prone sequences correspond to pass sites. The similarity of these results for a guanine-modifying agent to the present results with UV suggests that the lack of correlation of lesion frequency with mutation frequency may be a general feature of DNA lesion-induced mutagenesis.

DNA Secondary Structure. Any explanation of the present data requires that DNA structural features differ between hot spots and non-hot spots. Hairpin loops in DNA calculated from the thermodynamics of RNA folding have been suggested to predispose to UV mutation (23), but sites 123, 156, 168, and 169 do not lie in such loops (16). Other sequence-specific parameters include twist angle, roll angle, propeller twist, and torsion angle (24, 25). DNA bends at AT tracts (26), which occur 5′ to some UV mutation hot spots (3). DNA polymerases terminate *in vitro* replication spontaneously at specific sequences (27). In these cases mutation spectra for different DNA-damaging agents would be similar, as has been observed for certain UV and 4-nitroquinoline-1-oxide base substitutions (1). In addition, DNA modifications and chemical adducts can disproportionately alter DNA structure at specific sites (28); mutation hot spots would then differ between agents (1).

Conclusion. We anticipate that, in a particular cell type, the mutation frequency is determined by a DNA structural feature and the lesion frequency. The stronger determinant of transition mutagenesis appears to be the DNA structural feature. If UV photoproducts are neither qualitatively nor quantitatively the strongest determinant of UV transition mutations in human cells, the search for the identity of mutagenic physical or chemical DNA lesions may be of less importance to the activation of oncogenes than characterization of sequence-specific alterations in DNA structure.

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