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Evidence from Mutation Spectra that the UV Hypermutability of Xeroderma Pigmentosum Variant Cells Reflects Abnormal, Error-Prone Replication on a Template Containing Photoproducts

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Xeroderma pigmentosum (XP) variant patients are genetically predisposed to sunlight-induced skin cancer. Fibroblasts derived from these patients are extremely sensitive to the mutagenic effect of UV radiation and are abnormally slow in replicating DNA containing UV-induced photoproducts. However, unlike cells from the majority of XP patients, XP variant cells have a normal or nearly normal rate of nucleotide excision repair of such damage. To determine whether their UV hypermutability reflected a slower rate of excision of photoproducts specifically during early S phase when the target gene for mutations, i.e., the hypoxanthine (guanine) phosphoribosyltransferase gene (*HPRT*), is replicated, we synchronized diploid populations of normal and XP variant fibroblasts, irradiated them in early S phase, and compared the rate of loss of cyclobutane pyrimidine dimers and 6-4 pyrimidine-pyrimidones from DNA during S phase. There was no difference. Both removed 94% of the 6-4 pyrimidine-pyrimidones within 8 h and 40% of the dimers within 11 h. There was also no difference between the two cell lines in the rate of repair during G_1 phase. To determine whether the hypermutability resulted from abnormal error-prone replication of DNA containing photoproducts, we determined the spectra of mutations induced in the coding region of the *HPRT* gene of XP variant cells irradiated in early S and G_1 phases and compared them with those found in normal cells. The majority of the mutations in both types of cells were base substitutions, but the two types of cells differed significantly from each other in the kinds of substitutions observed either in mutants from S phase ($P < 0.01$) or from G_1 phase ($P = 0.03$). In the variant cells, the substitutions were mainly transversions (58% in S, 73% in G_1). In normal cells, transversions were much rarer (8% in S, 24% in G_1; $P < 0.001$ for S, $P < 0.01$ for G_1). In the normal cells irradiated in S, the majority of the substitutions were G·C→A·T, and most involved CC photoproducts in the transcribed strand. In the variant cells irradiated in S, substitutions involving cytosine in the transcribed strand were G·C→T·A transversions exclusively. G·C→A·T transitions made up a much smaller fraction of the substitutions than in normal cells ($P < 0.02$), and all of them involved photoproducts located in the nontranscribed strand. The data strongly suggest that XP variant cells are much less likely than normal cells to incorporate either dAMP or dGMP opposite the pyrimidines involved in photoproducts. This would account for their significantly higher frequency of mutants and might explain their abnormal delay in replicating a UV-damaged template.

Xeroderma pigmentosum (XP) variant patients inherit a predisposition to sunlight-induced skin cancer and develop the same clinical characteristics of the disease as do classic nucleotide excision repair-deficient XP patients (26). However, in contrast to the cells from the classic XP patients, fibroblasts derived from XP variant patients are reported to excise UV photoproducts at a normal or near-normal rate (5, 21, 26, 35). Maher et al. (15) and Myhr et al. (22) showed that such cells 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 (≥5-times-steeper slope). If, as evidence suggests, mutations are causally involved in carcinogenesis, such hypermutability can help explain the genetic predisposition of XP variant patients to skin cancer on sunlight-exposed parts of the body. However, a fundamental question still remains: what mechanism(s) is responsible for the UV hypermutability of XP variant cells?

The hypermutability cannot be accounted for by error-prone excision repair because when synchronized populations of XP variant cells were irradiated at various times prior to S phase to allow different lengths of time for excision repair before DNA replication, the mutant frequency decreased as a function of time for repair until it reached background levels (32). One possible explanation for the UV hypermutability in the variant cells is that their replication fork encounters more photoproducts than does that of normal cells. This would be the case if the slightly lower rate of excision repair sometimes reported for XP variants reflects a significantly slower rate specifically during S phase. A second possible explanation for the hypermutability is that

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the number of unexcised photoproducts is the same for both types of cells, but the replication complex of the XP variant cells is defective and less likely than that of normal cells to incorporate the correct nucleotide opposite the photoproduct during S-phase replication.

This second hypothesis is supported by the results of several groups of investigators who showed that XP variant cells replicate DNA containing UV photoproducts with greater difficulty than do normal cells (2, 6, 11, 13, 23, 29). For example, Boyer et al. (2) reported that for a given dose of UV, normal and XP variant cells receive the same number of UV lesions, but the variant cells are three to four times more sensitive to inhibition of daughter strand growth. An average of 5.1 cyclobutane pyrimidine dimers (CPD) per replicon was needed to inhibit DNA strand growth in normal cells, but only 1.4 were needed for XP variant cells. Similarly, van Zeeland and Filon (29) showed that within 15 min after irradiation, the size of nascent DNA in normal human fibroblasts is greater than the interdimer distance and continues to increase in size at the same rate during the next few hours, so that within 4 h it is 14 times the interdimer distance. However, in the XP variant cells, no increase in length occurs during the first 15 min, and for the next few hours, the rate of increase is six times slower than normal. These data are consistent with the replication forks of XP variant cells being blocked at photoproducts longer than normal cells are and taking much longer for transdimer synthesis. Additional data supporting the second hypothesis come from our recent finding that the kinds of mutations induced when a UV-irradiated plasmid is allowed to replicate in XP variant cells differ significantly from those seen with normal cells (31).

To determine whether there was a difference between the two cell types in the rate of excision repair during S phase, we synchronized normal and XP variant cells, irradiated them at the onset of S phase, harvested them immediately or after various hours of time for excision repair, and analyzed them for rate of loss of photoproducts, using antibodies specific for CPD or 6-4 pyrimidine-pyrimidones (6-4s). We found no difference in the rate of excision of either photoproduct. To determine whether there was a difference between the two cell types in the kinds of mutations induced, we determined the spectrum of *HPRT* mutations in the XP variant and compared the results with those from normal cells. The kinds of base substitutions in the two cell types differed significantly, strongly supporting the hypothesis of abnormal error-prone replication bypass of photoproducts by the XP variant cells.

MATERIALS AND METHODS

Cells and media. Normal fibroblasts, designated NFSL89, were explanted from the foreskin of a normal newborn as described previously (17). XP variant cells (XP4BE; CRL 1162) were obtained from the American Type Culture Collection (Rockville, Md.). Cells were routinely cultured in Eagle's minimal essential medium containing 10% fetal bovine serum. For selection of thioguanine (TG)-resistant cells, this medium was supplemented with 40 μM TG. For thymidine incorporation experiments, the medium was supplemented with [^{3}H]thymidine (5 μCi/ml of medium, 78.5 Ci/mmol; New England Nuclear, Dupont, Wilmington, Del.).

Determining onset of S phase in synchronized cells. Cells were driven into the G_0 state by density inhibition as described previously (12) and stimulated to enter the cell cycle by being plated in fresh culture medium at a density of

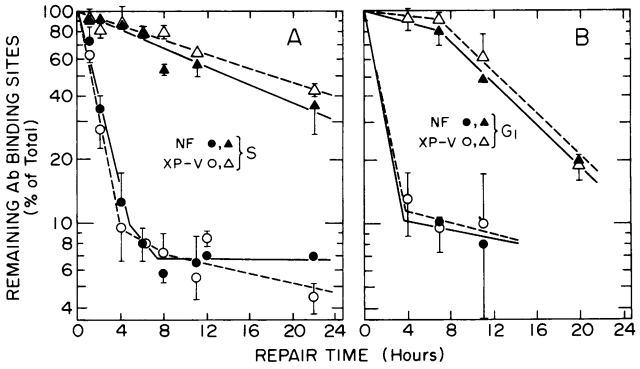

FIG. 1. Rate of repair of 6-4 photoproducts and CPD by XP variant cells and normal cells during S phase (A) or G_1 phase (B). XP variant cells (XP-V; open symbols) and normal cells (NF; closed symbols) were irradiated with UV light at 6 J/m^2, harvested immediately, or incubated for the indicated time before their DNA was assayed for the number of binding sites of antibodies (Ab) against 6-4s (circles) or CPD (triangles). The data were obtained from two separate experiments; each point is the average of at least two separate assays. The error bars are calculated as the square root of the sample variance in separate experiments.

10^4 cells per cm^2. The time of onset of S phase following release from G_0, as well as the length of S phase, was determined by measuring the incorporation of 15-min pulses of [^{3}H]thymidine into acid-insoluble material as described previously (9).

Exposure to UV light and determination of cytotoxicity and mutant frequency. Cells were released from the G_0 state and plated at 10^4 cells per cm^2 for mutagenicity studies and at cloning densities for cytotoxicity determination. One hour after the cells began S phase (17 h after release from G_0) or in early G_1 phase (6 h after release), the culture medium was aspirated, and the cells were washed with phosphate-buffered saline, irradiated with UV (254 nm) as described previously (24), and refed with culture medium. Cytotoxicity was determined from the decrease in the cells' colony-forming ability. The target cells for mutation analysis were allowed an 8-day expression period and then selected for TG resistance as described previously (17). When clones developed 14 days later, they were isolated and *HPRT* cDNA was amplified directly from the original clones. The mutant frequency was determined from the number of TG-resistant clones per 10^6 clonable cells as described previously (17).

Amplification of *HPRT* cDNA and DNA sequencing. First-strand cDNA was synthesized directly from mRNA in lysates of 100 to 500 cells, using the polymerase chain reaction as described previously (34). The amplified *HPRT* coding region was sequenced under the conditions recommended by the manufacturer (protocols for DNA sequencing with Sequenase version 2.0, U.S. Biochemical, Cleveland, Ohio). The three primers used were those described in reference 34. This reference also gives the concentration of three dideoxy-chain termination mixes. For the fourth, i.e., tube T, the concentration was 150 μM each deoxynucleoside triphosphate–3 μM ddTTP.

Measurement of the rate of repair of specific photoproducts. XP variant and normal cells in exponential growth were plated at a density of ~7 × 10^3/cm^2, i.e., one-eighth of their density at confluence, and allowed to undergo three population doublings in medium containing [^{14}C]thymidine (0.02 μCi/ml, 59 mCi/mmol; New England Nuclear, Dupont) to

TABLE 1. Kinds and locations of mutations induced in the coding region of the *HPRT* gene in XP variant cells (XP4BE) irradiated in early S phase

Mutant[a]	Base substitution		Type of mutation	Surrounding sequence[b]	Amino acid change	Premutagenic photoproduct[c]	Strand containing photoproduct[d]
	Position	Exon					
Base substitutions involving cytosine							
VS16[e]	145	3	G·C→A·T	CGT CTT GCT	Leu→Phe	TC or CT	NT
VS66	151	3	G·C→A·T	GCT CGA GAT	Arg→stop	TC	NT
VS58	464	6	G·C→A·T	AAT CCA AAG	Pro→Leu	CC	NT
VS70	464	6	G·C→A·T	AAT CCA AAG	Pro→Leu	CC	NT
VS77	464	6	G·C→A·T	AAT CCA AAG	Pro→Leu	CC	NT
VS78	506	7	G·C→A·T	ACC CCA CGA	Pro→Leu	CC	NT
VS11[e]	601	8	G·C→A·T	AGG GAT TTG	Asp→Asn	TC or CC	T
VS1	115	2	G·C→C·G	CCT CAT GGA	His→Asp	TC	NT
VS73	40	2	G·C→T·A	GAT GAA CCA	Glu→stop	TC	T
VS62[e]	118	2	G·C→T·A	CAT GGA CTA	Gly→stop	CC	T
VS23	134	2	G·C→T·A	GAC AGgtaa	Arg→Met	CC or CT	T
VS68	196 197 tandem	3	A·T→T·A G·C→T·A	CTC TGT GTG	Cys→Ile[f]	ACA[f]	T
VS59	509	7	G·C→T·A	CCA CGA AGT	Arg→Leu	TC	T
VS33	562	8	G·C→T·A	TTT GTT GTA	Val→Phe	ACA	T
VS17	606	8	G·C→T·A	GAT TTG AAT	Leu→Phe	TC	T
VS42	606	8	G·C→T·A	GAT TTG AAT	Leu→Phe	TC	T
VS9	617	9	G·C→T·A	GTT TGT GTC	Cys→Phe	ACA	T
Base substitutions involving thymine							
VS31	205	3	A·T→G·C	CTC AAG GGG	Lys→Glu	TT	T
VS16[e]	295	3	A·T→G·C	GAT TTT ATC	Phe→Leu	TTTT	NT
VS6	296	3	A·T→G·C	GAT TTT ATC	Phe→Ser	TTTT	NT
VS61	296	3	A·T→G·C	GAT TTT ATC	Phe→Ser	TTTT	NT
VS76[e]	374	4	A·T→G·C	ACT TTA ACT	Leu→Ser	TTT	NT
VS48	392	5	A·T→G·C	GTC TTG ATT	Leu→Ser	TT	NT
VS75	294 295 tandem	3	A·T→C·G A·T→C·G	GTA GAT TTT GAT TTT ATC	Asp→Glu Phe→Val	TTTT	NT
VS62[e]	417	6	A·T→C·G	GAC ACT GGC	No change	CT	NT
VS64	436	6	A·T→C·G	ACT TTG CTT	Leu→Val	TTT	NT
VS26	604	8	A·T→T·A	GAT TTG AAT	Leu→Met	TTT	NT
VS76[e]	605	8	A·T→T·A	GAT TTG AAT	Leu→stop	TTT	NT
VS30	643	9	A·T→T·A	GCA AAA TAC	Lys→stop	TTTT	T

[a] In addition to the independent mutants listed, another mutant, VS4, had an A·T→G·C base substitution at position 314 causing a Tyr-to-Cys amino acid change. However, there was no discernible photoproduct at that site.

[b] Sequence of the nontranscribed strand. The sequence is shown in a 5′-to-3′ orientation. The lowercase letters represent the sequence in an intron. The sites of substitution are underlined.

[c] The sequence shown is from the appropriate strand and is listed in a 5′-to-3′ orientation. The site of substitution is underlined.

[d] T, transcribed; NT, nontranscribed.

[e] Contained more than one mutation, nontandem.

[f] Mutant VS68 also had an A·T→T·A base substitution at position 196, where an ACA photoproduct was located. This resulted in the Cys-to-Ile amino acid change.

label the DNA. When they reached confluence, the cells were refed with fresh unlabeled medium each day for 3 days and then held at confluence in the absence of mitogens for 3 more days to achieve the G_0 state. The cells were released from G_0 and irradiated with 6 J/m^2 in early G_1 or S phase. Following irradiation, the cells were either harvested immediately or incubated for various periods of time up to 22 h. Any DNA replication occurring during this period did not influence the measurement of repair rates since the DNA samples were normalized to equal amounts of parental (^{14}C-labeled) DNA. The DNA was isolated and assayed as described previously (20) for the presence of CPD and 6-4s, using polyclonal antibodies that specifically recognized either of these photoproducts. The radioimmunoassay consisted of 2 μg of heat-denatured parental (prelabeled) DNA from the human cells and 10 pg of ^{32}P-labeled UV-irradiated pBR322 plasmid DNA competing for CPD-specific antibody-binding sites or 6-4-specific antibody-binding sites. To deter-

mine the amount of excision repair that had occurred, the extent of inhibition of ^{32}P-bound antibodies by the human DNA was converted to percent of antibody sites remaining, using a standard curve.

RESULTS

Rate of loss of photoproducts in synchronized populations of normal and XP variant cells. To test the hypothesis that during S phase, XP variant cells excise UV photoproducts at a slower rate than do normal cells, we synchronized large populations of both types of cells by release from the density-inhibited G_0 state and irradiated them with 6 J/m^2 17 h after release at the onset of S phase. (The time of onset of S in the two types of cells plated at 10^4 cells per cm^2 was verified.) Irradiated cells were harvested immediately or after various hours of incubation, and the DNA was assayed for the rate of removal of CPD and 6-4s, using antibodies specific for these photoproducts. (For purposes of comparison, we also measured the rate of removal of photoproducts during G_1 phase.) There was no difference between the XP variant and the normal cells in the rate of repair of either photoproducts during either S phase (Fig. 1A) or G_1 phase (Fig. 1B). Both types of cells exhibited very rapid repair of 6-4s during S phase; i.e., >90% were removed within 6 h. The rate of CPD during S or G_1 phase was significantly slower than that of 6-4s, i.e., only 40% removed within 11 h, but there was no significant difference between the two types of cells. For both cell lines, the extent of removal of CPD after 20 h was somewhat greater during G_1 phase than during S phase.

Comparative study of the spectrum of mutations induced by UV. To test the hypothesis that the UV hypermutability of XP variant cells reflects an abnormally error-prone replication complex, we determined the spectrum of mutations induced by 4 J/m^2 in synchronized populations of XP variant cells irradiated in early S phase (17 h after release from G_0) or in early G_1 phase (6 h after release) and compared the results with what we had obtained previously (18) with normal cells under similar conditions. To facilitate analysis of unequivocally independent mutants, we plated the synchronized XP variant cells into a series of individual dishes (eight populations for cells to be irradiated in early S phase and 11 populations for those to be irradiated in early G_1). In addition, there was a set of unirradiated control cells. The survival of the cells irradiated in early S was 19% of the unirradiated control; that of the cells irradiated in G_1 was 23%. The frequencies of TG-resistant mutants averaged $680 \times 10^{-6} \pm 260 \times 10^{-6}$ for the S-phase cells and $220 \times 10^{-6} \pm 150 \times 10^{-6}$ for the G_1-phase cells; the background frequency averaged $18 \times 10^{-6} \pm 14 \times 10^{-6}$. The large variance in these values reflects the fact that rather than using very large populations of pooled cells as we do when our purpose is to determine frequencies, we used a series of smaller populations that were deliberately kept separate from each other to avoid analyzing siblings.

(i) Mutations found in cells irradiated in S phase. The results of our analysis of 37 unequivocally independent mutants from XP variant cells irradiated in early S phase are shown in Tables 1 and 2. Eleven (30%) appeared to have a splice site mutation. One of these (VS11) also had a base substitution. Of the other 26 mutants, 21 contained only a single base substitution, 2 contained tandem base substitutions, and 3 had two substitutions, nontandem. For 28 of the substitutions, the premutagenic lesion could be assigned to a dipyrimidine. In three cases, the substitution involved a cytosine flanked by two adenines. Such ACA sites represent rare UV photoproducts (1).

TABLE 2. Mutants with putative splice site mutations

Mutation induced in coding region of *HPRT* gene in:	Mutant	Missing exon
XP4BE cells in early S phase	VS34	2
	VS14	4
	VS53	5
	VS7	8
	VS11[a]	8
	VS20	8
	VS32	8
	VS56	8
	VS79	8
	VS60	8
	VS37	18 bp missing from 1st part of exon 9, 610–627
Normal cells in early S phase	NUS2[b]	4
	NUS30	4
	NUS18[b]	5
	NUS17[b]	5
	NUS22[b]	7
	NUS11[b]	7
	NUS10[b]	8
	NUS12[b]	8
	NUS20[b]	10-bp deletion, 536–545 very near 5′ end of exon 8
XP4BE cells in early G_1 phase	VG92	4
	VG98	4
	VG106	7
	VG61	8
	VG96	8
	VG107	8
	VG24	21 bp missing from 1st part of exon 8, 533–553
	VG91	21 bp missing from 1st part of exon 8, 533–553

[a] Contained more than one mutation, nontandem.
[b] Reported previously by McGregor et al. (18).

McGregor et al. (18) reported data obtained by sequencing 22 independent mutants from normal cells irradiated in early S phase. They found 8 (36%) with putative splice site mutations and 14 with base substitutions. The total number of base substitutions that they analyzed was 19. To increase that number before trying to compare the mutation spectra of XP variants and normal cells, we analyzed eight additional independent mutants derived from those earlier experiments and obtained eight more base substitutions, along with a putative splice site mutation. These additional data, along with those reported by McGregor et al. (18), are shown in Tables 2 and 3.

The kinds of base substitutions induced in the two types of cells differed significantly ($P = 0.001$ by the chi-square test). In the XP variant cells, 45% (14 of 31) of the base substitutions involved A·T, and 12 of these 14 (86%) were targeted to TT dipyrimidines, with 10 of 12 located in runs of T's. In the normal cells, only 28% (7 of 25) of the base substitutions involved A·T, with no more than 5 targeted to TT dipyrimidines and only 2 of these TT dipyrimidines located in runs of T's. The most significant difference between the variant cells and the normal cells was the distribution of G·C→A·T

TABLE 3. Kinds and locations of mutations induced in the coding region of the *HPRT* gene of normal human cells irradiated in early S phase

Mutant[a]	Base substitution		Type of mutation	Surrounding sequence[b]	Amino acid change	Premutagenic photoproduct[c]	Strand containing photoproduct[d]
	Position	Exon					
Base substitutions involving cytosine							
NUS23	103	2	G·C→A·T	AGG GTG TTT	Val→Met	CC	T
NUS24	173	3	G·C→A·T	ATG GGA GGC	Gly→Glu	TC or CC	T
NUS21	208 tandem 209	3	G·C→A·T G·C→A·T	AAG GGG GGC	Gly→Lys	CCCCCC	T
NUS27[e]	390	5	G·C→A·T	AAT GTC TTG	No change		NT
NUS16	463 tandem 464	6	G·C→A·T G·C→A·T	AAT CCA AAG	Pro→Leu	TCC	NT
NUS14	471	6	G·C→A·T	AAG ATG GTC	Met→Ile	CC	T
NUS7	500 tandem 501	7	G·C→A·T G·C→A·T	AAA AGG ACC	Arg→Lys	TCCT	T
NUS4	505	7	G·C→A·T	ACC CCA CGA	Pro→Ser	CCCC	NT
NUS28	509	7	G·C→A·T	CCA CGA AGT	Arg→Gln	TC	T
NUS1[f]	568	8	G·C→A·T	GTA GGA TAT	Gly→Arg	CC or CT	T
NUS29	569	8	G·C→A·T	GTA GGA TAT	Gly→Glu	TC or CC	T
NUS5	599 tandem 600	8	G·C G·C→A·T	TTC AGG GAT	Frameshift	CCCT	T
NUS3	601	8	G·C→A·T	AGG GAT TTG	Asp→Asn	TC or CC	T
NUS9	447	6	G·C→C·G	TCC TTG GTC	Leu→Phe	CC	T
NUS6	600	8	G·C→C·G	TTC AGG GAT	Arg→Ser	CCC	T
Base substitutions involving thymine							
NUS8	122	2	A·T→G·C	GGA CTA ATT	Leu→Pro	CT	NT
NUS1[f]	123	2	A·T→G·C	GGA CTA ATT	No change	TT	T
NUS26	294	3	A·T→G·C	GTA GAT TTT	No change	TTTT	NT
NUS27[e]	392	5	A·T→G·C	GTC TTG ATT	Leu→Ser	TT	NT
NUS19	498 tandem 499	7	A·T→G·C A·T→G·C	GTG AAA AGG GTG AAA AGG	No change Arg→Gly	CCTTTT	T
NUS15	596	8	A·T→G·C	TAC TTC AGG	Phe→Ser	TT or TC	NT

[a] The mutants with identifying numbers lower than 23 were reported previously by McGregor et al. (18). They are included along with information from additional independent mutants from the present study. In addition to the independent mutants listed, two other mutants, NUS13 and NUS25, had an A·T→C·G or A·T→T·A base substitution at position 84 or 200, respectively. These substitutions caused a Tyr-to-stop codon change or a Val-to-Glu amino acid change, respectively. However, there was no discernible photoproduct at either site.

[b–d] See Table 1, footnotes *b* to *d*.

[e] This mutant also contained an A·T→G·C base substitution at position 392. The change at position 390 may have resulted from the TT photoproduct at positions 391 and 392.

[f] See Table 1, footnote *e*.

transitions. Such transitions made up the majority (64%) of the substitutions in the normal cells, and virtually all of them were targeted to photoproducts located in the transcribed strand. In contrast, G·C→A·T transitions made up a much smaller fraction of the substitutions in the variant cells ($P < 0.02$), and all of them were targeted to photoproducts in the nontranscribed strand. Substitutions involving cytosine in the transcribed strand were exclusively G·C→T·A transversions. In the variant cells, 57% (17 of 30) of the substitutions were transversions, compared with only 8% (2 of 25) in the normal cells ($P < 0.001$). In addition, the types of transversions differed significantly.

(ii) Mutations found in cells irradiated in early G_1 to allow time for repair. We also analyzed 24 independent mutants from the XP variant cells irradiated in early G_1 phase (Tables 2 and 4). Eight of the twenty-four (33%) appeared to have a splice site mutation. Fifteen of the other sixteen contained single base substitutions; one contained a tandem base substitution. All but two of the pyrimidines involved in these substitutions were located adjacent to another pyrimidine. The kinds of base substitutions that we found in XP variant cells from G_1 phase (Table 4) differed significantly from what was reported in reference 18 for normal cells similarly irradiated ($P = 0.03$ by the chi-square test). In the XP variant cells, only 27% (4 of 15) of the substitutions were transitions, compared with 76% (13 of 17) in the normal cells ($P < 0.01$). Only 13% (2 of 15) of the substitutions in the variant cells were G·C→A·T transitions, compared with 47% (8 of 17) in the normal cells ($P < 0.02$). Table 5 shows the distribution of base substitutions found in XP variants and normal cells irradiated in G_1 phase and in S phase.

Strand distribution of the premutagenic lesions in the two types of cells. Knowledge of the kinds of photoproducts induced by UV (1, 8) allowed us to infer from the sequence data the strand in which the photoproducts that resulted in the observed mutations, i.e., the premutagenic lesions, were located in the gene. These are listed in the last columns of Tables 1, 3, and 4, and the totals are compared in Table 6. In the mutants derived from XP variant cells irradiated in S phase, the 29 premutagenic lesions were distributed 41% transcribed strand:59% nontranscribed strand. In the mutants derived from normal cells irradiated in S, the 21

TABLE 4. Kinds and locations of mutations induced in the coding region of the *HPRT* gene in XP variant cells irradiated in earlyG_1 phase

Mutant[a]	Base substitution		Type of mutation	Surrounding sequence[b]	Amino acid change	Premutagenic photoproduct[c]	Strand containing photoproduct[d]
	Position	Exon					
Base substitutions involving cytosine							
VG36	145	3	G·C→A·T	CGT CTT GCT	Leu→Phe	TC or CT	NT
VG13	151	3	G·C→A·T	GCT CGA GAT	Arg→stop	TC	NT
VG80	96	2	G·C→T·A	GAT TTG GAA	Leu→Phe	CC	T
VG104[e]	195	3	G·C→T·A	GCC CTC TGT	Leu→Pro[e]	CTCT	NT
VG122	209	3	G·C→T·A	AAG GGG GGC	Gly→Val	CCC	T
VG111	325	4	G·C→T·A	GAC CAG TCA	Gln→Lys	CC	NT
VG21	529	7	G·C→T·A	CCA GAC TTT	Asp→Tyr	TC or CT	T
VG15	580	8	G·C→T·A	CTT GAC TAT	Asp→Tyr	TC	T
VS56	628	9	G·C→T·A	AGT GAA ACT	Glu→stop	TC	T
Base substitutions involving thymine							
VG104[e]	194	3	A·T→G·C	GCC CTC TGT	Leu→Pro[e]	CTCT	T
VG84	488	7	A·T→G·C	AGC TTG CTG	Leu→Ser	CT or TT	NT
VG10	203	3	A·T→C·G	GTG CTC AAG	Leu→Arg	CT or TC	NT
VG89	344	4	A·T→C·G	ATA AAA GTA	Lys→Thr	TTTT	T
VG53	92	2	A·T→T·A	GAG GAT TTG	Asp→Val	TC	T
VG79	295	3	A·T→T·A	GAT TTT ATC	Phe→Ile	TTTT	NT

[a] In addition to the independent mutants listed, one mutant, VG4, had an A·T→T·A base substitution at position 109, causing an Ile-to-Phe amino acid change, and another mutant, VG117, had an A·T→T·A substitution at position 407, causing an Ile-to-Lys amino acid change. However, there was no discernible photoproduct at either site.

[b–d] See Table 1, footnotes *b* to *d*.

[e] This mutant contains a tandem mutation.

premutagenic lesions were distributed 67% transcribed strand:33% nontranscribed strand. The chance that the strand distributions in the two types of cells are equal is $P = 0.08$ by the chi-square test. In the normal cells irradiated in G_1 phase to allow at least 6 h for excision repair prior to S phase, the strand distribution was 20%:80%. In the XP variant cells irradiated in G_1 and allowed even more time for excision repair prior to S phase, the distribution was 50%:50%.

DISCUSSION

The data in Table 5, comparing XP variant and normal cells, especially those from cells irradiated in early S phase so that there would be little or no time for excision repair before replication of the *HPRT* gene (9), support the hypothesis that the significantly higher frequency of mutants in the XP variant cells reflects an abnormally error-prone replication complex bypassing unexcised lesions. The hypermutability of the XP variant cells cannot be explained merely by assuming that their *HPRT* gene contains more unexcised photoproducts than remain in normal cells. If that were the explanation, the kinds of base substitution and the strand distribution of the premutagenic photoproducts in the XP variant cells should be similar to those seen in excision repair-deficient from group A (18). Instead, they differ significantly ($P = 0.03$). The data suggest that the replication complex of XP variant cells is less likely than that of normal cells to incorporate dAMP and dGMP opposite photoproducts during replication.

Evidence that the normal cells exhibit preferential incorporation of dAMP opposite photoproducts is the high proportion of G·C→A·T substitutions, i.e., 64% in the cells irradiated in S phase, and the very low proportion of substitutions involving thymine (28%). The preference for G·C→A·T transitions among UV-induced base substitutions has also been seen in excision repair-deficient XP cells (18) as well as in many other studies of UV-induced mutations, including the *hprt* locus of an excision repair-deficient hamster strain (30), the *aprt* locus of CHO cells (7), and muta-

TABLE 5. Types of base substitutions induced in the coding region of the *HPRT* gene in XP variant cells and normal cells irradiated in early G_1 and early S phases

Type of base substitution	No. of substitutions observed[a]			
	Cells irradiated in G_1 phase		Cells irradiated in S phase	
	XP variant	Normal[b]	XP variant	Normal[c]
Transitions	(27)	(76)	(43)	(92)
G·C→A·T	2 (13)	8 (47)	7 (23)	16 (64)
A·T→G·C	2 (13)	5 (29)	6 (19)	7 (28)
Transversions	(73)	(24)	(58)	(8)
G·C→C·G	0 (0)	1 (6)	1 (3)	2 (8)
G·C→T·A	7 (47)	1 (6)	9 (29)	0 (0)
A·T→C·G	2 (13)	0 (0)	4 (13)	0 (0)
A·T→T·A	2 (13)	2 (12)	3 (10)	0 (0)
Total	15	17	30	25

[a] Numbers in parentheses are percentages of total base substitutions.

[b] Data are from McGregor et al. (18) and are included for ease of comparison.

[c] Values include data reported by McGregor et al. (18) along with those obtained in this study.

TABLE 6. Strand distribution[a] of the premutagenic lesions responsible for the mutations observed in XP variant and normal cells irradiated in early S and early G_1 phases

Strand	Strand distribution of the premutagenic lesions[b]			
	Cells irradiated in S phase		Cells irradiated in G_1 phase	
	XP variant	Normal	XP variant	Normal[c]
Transcribed	12 (41)	14 (67)	7 (50)	3 (20)
Nontranscribed	17 (59)	7 (33)	7 (50)	12 (80)
Total	29	21	14	15

[a] Strand assignments were made on the basis of which strand contained the photoproduct that presumably resulted in the observed mutation (see Tables 1, 3, and 4).

[b] Numbers in parentheses are percentages.

[c] Data are taken from McGregor et al. (18) and are included for purposes of comparison.

tions induced on a shuttle vector in simian virus 40-transformed fibroblasts from normal donors and from classic XP patients (3, 7, 33), and has been attributed to preferential incorporation of dAMP opposite a photoproduct (10, 14, 25). In both normal cells and repair-deficient XP cells, the vast majority of these G·C→A·T transitions are targeted to photoproducts located in the transcribed strand. In the variant cells, almost none (one of nine) were. Instead, all of the substitutions involving cytosine in the transcribed strand were G·C→T·A transversions. Very few such transversions are seen in normal cells. The XP variant cells showed a high proportion of substitutions involving thymine (14 of 31 [45%]), and 86% (12 of 14) of these resulted from photoproducts that had to have consisted of a TT dipyrimidine. The vast majority of these involved photoproducts that were located in the nontranscribed strand. Less frequent incorporation of dAMP opposite UV photoproducts by the XP variant cells would result in a significantly higher frequency of mutants, since thymine is the base most frequently involved in UV photoproducts (8).

The data in Table 5 for the cells irradiated in early S phase also suggest that the XP variant cells are less likely than normal cells to incorporate dGMP opposite UV photoproducts. Transversions made up a much higher proportion of the substitutions in the XP variant cells than in the normal cells (57% in XP variant cells, 8% in normal cells), implying that XP variant cells are less likely than normal cells to incorporate purines opposite pyrimidines involved in photoproducts. If the XP variant cells were to incorporate dAMP less frequently, as discussed above, but incorporated dGMP at the normal frequency, G·C→T·A transversions would be very rare instead of occurring at a rate of 30%. Moreover, substitutions involving thymine should constitute a very large proportion of the total substitutions observed. They did not. Instead, they were only 45%, suggesting less frequent incorporation of dGMP as well as dAMP. Less frequent incorporation of dGMP opposite UV photoproducts by the XP variant cells would also contribute to their significantly higher frequency of UV-induced mutants.

The majority of the substitutions seen in normal cells irradiated in S phase are G·C→A·T transitions from lesions in the transcribed strand (Table 3). If XP variant cells are less likely than normal cells to incorporate purines opposite photoproducts, the strand distribution of the pyrimidines involved in the observed mutations from S-phase cells should differ from normal. Table 6 shows that this is the case. The ratio of base substitutions involving G·C and A·T base pairs was 55:45 in XP variant cells irradiated in early S phase. Therefore, it is not surprising that the pyrimidines involved in the mutations observed in the XP variant cells irradiated in early S phase were evenly distributed between both strands, viz., 41% transcribed and 59% nontranscribed. The corresponding ratios in the normal cells are 72:28 and 67%:33%.

The strand distribution of the premutagenic lesions in mutants derived from the normal cells irradiated with 6 J/m^2 in mid-G_1 phase (6 h prior to S) differed from that seen in normal cells irradiated in S phase, i.e., 20:80 versus 67:33 ($P < 0.01$) (Table 6). As suggested previously (18), this difference in strand distribution can be attributed to transcription-coupled, strand-specific repair of photoproducts in the transcribed strand during the 6-h repair period (19). In the XP variant cells irradiated with 4 J/m^2 and allowed ≥11 h for repair before S phase there was no such difference in the strand distribution (50:50 versus 41:59). This similar strand distribution between the XP variant cells irradiated in S and G_1 phases could reflect a lack of strand-specific repair in the *HPRT* gene of the variant cells. However, the overall rate of excision of CPD and 6-4s in XP variant cells was equal to that in the normal cells, and Mayne and Lehmann (16) showed that the rate of recovery of RNA synthesis in XP variant cells after UV irradiation is the same as in normal cells. The latter data imply that preferential repair and probably strand-specific repair also occurs in XP variant cells. A more likely explanation for the similar strand distribution in the XP variant cells irradiated in S and G_1 phases is that during the 11 or more h available for repair before S phase, the XP variant cells irradiated in G_1 removed the majority of the photoproducts from both strands. If so, among the mutants that we recovered, the distribution of premutagenic lesions (pyrimidines involved in a mutation) might well reflect the original distribution, i.e., that seen in mutants recovered from the S-phase cells that had little or no time for repair. Removal of photoproducts preferentially from the transcribed strand would be obscured. In the study by McGregor et al. (18), the normal cells had less time for repair before the onset of S phase, and therefore, the preferential removal of lesions from the transcribed strand could be detected.

In summary, our data strongly suggest that the significant difference between XP variant and normal cells in the kinds of base substitutions induced by UV results from XP variant cells being less likely than normal cells to incorporate dAMP and dGMP opposite unexcised photoproducts during replication. Unlike normal cells, the variant cells incorporate dTMP opposite cytosine-containing photoproducts located in the transcribed strand and incorporate dAMP opposite such lesions mainly in the nontranscribed strand. The mechanisms responsible for this infrequent incorporation of dAMP and dGMP (nucleotide pool imbalances in the XP variant, abnormal binding affinity between nucleotides and the replication complex, etc.) can best be investigated in an in vitro replication fidelity assay such as that employed by Robert and Kunkel (27) or by Carty et al. (4). An error-prone replication process would account for the XP variant cells' UV hypermutability. If the replication complex was stalled because of an inability to incorporate purines opposite photoproducts, this might account for the XP variant cells' abnormal delay in producing nascent DNA of a size greater than the interdimer length.

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