# Evidence that in Xeroderma Pigmentosum Variant Cells, which Lack DNA Polymerase η, DNA Polymerase ι Causes the Very High Frequency and Unique Spectrum of UV-Induced Mutations

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## Abstract

**Xeroderma pigmentosum variant (XPV) patients have normal DNA excision repair, yet are predisposed to develop sunlight-induced cancer. They exhibit a 25-fold higher than normal frequency of UV-induced mutations and very unusual kinds (spectrum), mainly transversions. The primary defect in XPV cells is the lack of functional DNA polymerase (Pol) η, the translesion synthesis DNA polymerase that readily inserts adenine nucleotides opposite photoproducts involving thymine. The high frequency and striking difference in kinds of UV-induced mutations in XPV cells strongly suggest that, in the absence of Pol η, an abnormally error-prone polymerase substitutes. *In vitro* replication studies of Pol ι show that it replicates past 5′T-T3′ and 5′T-U3′ cyclobutane pyrimidine dimers, incorporating G or T nucleotides opposite the 3′ nucleotide. To test the hypothesis that Pol ι causes the high frequency and abnormal spectrum of UV-induced mutations in XPV cells, we identified an unlimited lifespan XPV cell line expressing two forms of Pol ι, whose frequency of UV-induced mutations is twice that of XPV cells expressing one form. We eliminated expression of one form and compared the parental cells and derivatives for the frequency and kinds of UV-induced mutations. All exhibited similar sensitivity to the cytotoxicity of $UV_{(254\ nm)}$, and the kinds of mutations induced were identical, but the frequency of mutations induced in the derivatives was reduced to ≤50% that of the parent. These data strongly support the hypothesis that in cells lacking Pol η, Pol ι is responsible for the high frequency and abnormal spectrum of UV-induced mutations, and ultimately their malignant transformation.** [Cancer Res 2007;67(7):3018–26]

## Introduction

DNA lesions remaining in DNA when the replication fork encounters them can interrupt replication; however, human cells possess highly specialized DNA replication polymerases capable of replicating past fork-blocking lesions (i.e., translesion synthesis; ref. 1). DNA polymerase (Pol) η is unique among the Y-family polymerases in its ability to bypass T-T cyclobutane pyrimidine dimers (CPD) readily (2, 3), incorporating adenine nucleotides (4), an action that is error-free for thymine nucleotides but would result in cytosine to thymine transitions commonly observed (5). Lack of nucleotide excision repair of UV photoproducts by the majority of xeroderma pigmentosum (XP) patients (6) explains why their cells are abnormally sensitive to DNA damage–induced mutations, leading to cancer. However, XP variant (XPV) patients exhibit a normal rate of excision repair (7), and are only slightly more sensitive to UV-induced cytotoxicity (8); however, they are significantly more sensitive than normal to its mutagenic effects (8–10). The kinds of mutations (i.e., spectrum) induced by UV in XPV cells differ significantly from those induced in normal human cells or cells from excision repair–deficient XP patients. The majority are C → A or T → A transversions, rather than C → T and T → C transitions (10). This higher frequency and altered spectrum of UV-induced mutations was also observed in a target gene of UV-irradiated plasmids replicated in cells from XPV patients, compared with what was found when the plasmid replicated in normal human cells (11). A similar high frequency and unusual spectrum of UV-induced mutations was found when replication-competent cell-free extracts from XPV cells were used to replicate a target gene, compared with such extracts from normal cells (12).

Masutani et al. (13), using complementation by proteins in extracts from normal human cells to eliminate the defect in extracts from XPV cells, determined that the variant form of XP results from the lack of functional Pol η, a polymerase that accurately inserts adenine nucleotides opposite thymine bases in CPDs, a fact also shown by Johnson et al. (14). The lack of Pol η explains the increased frequency of mutations involving thymidine in XPV cells. However, the mere absence of Pol η cannot explain the abnormal kinds of mutations induced by UV in the hypoxanthine phosphoribosyltransferase (*HPRT*) gene of XPV cells (i.e., ~63% transversions rather than the 12% induced in normal human cells; 10). Such bias indicates that XPV cells make prominent use of a polymerase (15) that preferentially misinserts nucleotides opposite photoproducts, a property of Pol ι.

Woodgate and colleagues showed that Pol ι is highly distributive, inaccurate, and lacking in intrinsic exonuclease activity. Its misincorporation specificity is unique in that it incorporates incorrect nucleotides even more efficiently than correct ones (15–18). *In vitro* replication studies reveal that, compared with that of Pol η, Pol ι–dependent bypass of T-T CPDs is inefficient (16), with G and T misincorporations opposite the 3′ base of the CPD occurring in a sequence context-dependent manner (17). Recent *in vitro* replication studies indicate that Pol ι also exhibits a similar pattern of misincorporations opposite a T-U dimer (18). If deamination of cytosine into uracil were to occur in cells, such misincorporations

**Note:** Supplementary data for this article are available at Cancer Research Online (http://cancerres.aacrjournals.org/).

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by Pol ι would be either error-prone or error-free. Misincorporation of T opposite the deaminated cytosine (U) leads to C → A transversions, whereas misincorporation of G opposite U, in the next round of semiconservative DNA synthesis, restores a G:C base pair, decreasing C → T transitions (18).

Our study tested the hypothesis that in cells devoid of Pol η, Pol ι is responsible for the observed high frequency and abnormal kinds of UV-induced mutations. To do so, we used infinite life span, diploid XPV cell strain XP115LO.hTERT. We discovered that this strain expresses two forms of Pol ι, one migrating to the usual position, ~80 kDa, and the other migrating somewhat slower. (We found that fibroblast cells from normal donor VE45 express these same two forms of Pol ι.) Western blotting revealed that our antisense construct only eliminated expression of the slower-migrating form of Pol ι. Comparative studies using parent cell strain, XP115LO, two vector control strains, and two derivative cell strains of XP115LO in which expression of Pol ι had been reduced by ~50% revealed that the latter cell strains are not more sensitive than their parent to the cytotoxic effect of UV, but their frequency of UV-induced mutations of Pol ι decreased to ≤50% that of the parent and a vector control. The kinds of mutations induced by UV in these derivative strains, expressing 50% as much Pol ι as their parent, are virtually identical to the spectrum induced in the parent. These data strongly support the hypothesis that in the absence of Pol η, Pol ι substitutes for Pol η and causes the very high frequency and abnormal spectrum of UV-induced mutations found in XPV cells (10).

## Materials and Methods

**Cells and cell culture.** Finite life span human fibroblast strain VE45, derived from a normal adult (19), and foreskin-derived fibroblast lines generated in the Carcinogenesis Laboratory were routinely cultured in Eagle's MEM, supplemented as described (20) and supplemented calf serum (Hyclone, Logan, UT; 10% v/v). XP4BE (CRL1162) and human embryonic kidney cell line 293 (HEK293) were obtained from the American Type Culture Collection (Manassas, VA). The finite life span XP115LO variant cells are from the National Institute of General Medical Sciences Cell Repository (GM02359). The GM02359-hTERT infinite life span cell line was derived in the laboratory of Roger Schultz (University of Texas Southwestern, Dallas, TX). XP5KA, finite life span fibroblasts from XPV patients, were provided by Masami Watanabe (Kyoto University Research Reactor Institute, Osaka, Japan). For culturing XPV cells, this same medium was used, but calf serum was replaced with 13% v/v FCS (Hyclone, Logan, UT). The latter was also used at 10% v/v for HEK293 and BL-2 cell lines (21). BL-2 cell lines were grown in RPMI 1640 medium (Invitrogen, Carlsbad, CA).

**Transfection.** LipofectAMINE was used according to the manufacturer's protocol (Invitrogen). After 48 h, transfectants were selected in medium containing the appropriate antibiotic. Medium was renewed every 3 to 4 days. When drug-resistant colonies formed, they were isolated, expanded in that medium, and stored frozen until used. Transient transfection was carried out using Fugene-6 (Roche, Indianapolis, IN) according to the manufacturer's instructions.

**Western blot analysis.** Whole-cell lysates were prepared using standard techniques. Unless indicated, 50 μg of total protein were analyzed for each cell strain. A peptide spanning Pol ι amino acids 1 to 24 (MELADVG-AAASSQGVHDQVLPTPN) was conjugated to keyhole limpet hemocyanin (KLH) and used to immunize rabbits to generate the $NH_2$-terminal anti-Pol ι polyclonal antibody. That antibody was affinity-purified using a glutathione *S*-transferase–tagged Pol ι fusion protein. A peptide spanning Pol ι amino acids 701 to 715 (AEWKRTGSDFHIGHK) was similarly conjugated to KLH and used to generate the COOH-terminal anti–Pol ι polyclonal antibody (22). The monoclonal Pol ι antibody was from Abnova (Taipei, Taiwan). β-Actin antibody was from Sigma (St. Louis, MO).

**Preparation of feeder layers.** To increase the cloning efficiency of derivative clones 10 and 40, populations derived from several thioguanine-resistant mutant clones arising spontaneously in the parental strain were irradiated on ice with 30 Gy, and plated at $6 \times 10^3/cm^2$ as a feeder layer.

**Assay of cytotoxicity.** Unless noted, asynchronously growing cells were plated in culture medium at cloning density (100–500 cells/100-mm-diameter dish) as described (20), and allowed 14 to 16 h to attach. At the time of UV irradiation, the medium was removed and cells were washed twice with PBS, irradiated, given fresh culture medium, and allowed to form colonies. After 24 h, and again after 7 days, the medium was replaced with fresh medium. When colonies formed (~14 days later) they were fixed, stained, and counted. The percentage of surviving cells was determined by comparing the cloning efficiency of the irradiated cells with that of their sham-irradiated control cells.

**Cell cycle synchronization.** Cells were plated at $0.5 \times 10^6$ per 150-mm-diameter dish. After 24 h, the medium was replaced with medium containing 60 μmol/L Lovastatin (Sigma). After 24 h, it was changed to medium containing mevalonic acid (6 mmol/L) and aphidicolin (2 μg/mL; Sigma) to stop the cells at the $G_1$-S border. After 24 h, the cells were washed twice with PBS at room temperature and supplied with fresh culture medium.

**Assaying the frequency of UV-induced *HPRT* mutations.** The methods have been described (20). Briefly, for synchronized cells, at the time the cells were beginning to replicate their *HPRT* gene (ref. 23; i.e., 1.5 h after being released from the $G_1$-S border), the medium was aspirated from the dishes and the cells were washed twice with PBS, irradiated, and given fresh medium. Medium was renewed after 24 h. Four days later, the cells for each dose were pooled, counted, plated into fresh culture medium at a lower density, and allowed to continue replicating to deplete their preexisting supply of HPRT. Eight days later, each population was detached, pooled, and counted, and $0.8 \times 10^6$ cells were selected in medium containing 40 μmol/L 6-thioguanine at a density of 500 cells/$cm^2$ for loss of functional HPRT. Cells from each dose were also plated at cloning density in nonselective medium to determine the cloning efficiency of cells plated in selective medium. Fresh selection medium was given after 1 week. After 7 more days, colonies of thioguanine-resistant cells were fixed and stained, and the frequency of mutants was calculated from the thioguanine-resistant clones, divided by the total number of cells plated, and corrected for cloning efficiency. Induced frequencies were calculated by subtracting the background frequency in the untreated controls, which was low.

**Sequencing the *HPRT* cDNA of thioguanine-resistant mutants.** The methods used have been described in detail (24) by using primers designated by Li et al. (25). *HPRT* cDNA was amplified directly from mRNA in each lysate, purified using the QIAquick PCR purification kit (Qiagen, Valencia, CA), and the sequence of the gene in each clone was analyzed by Lark Technologies (Houston, TX) or by Michigan State University Genomic Technology Support Facility.

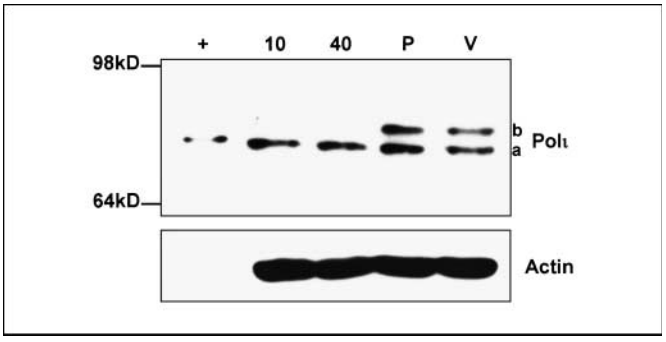


**Figure 1.** Western blot analysis of the expression of Pol ι protein in the parental cell strain (*P*), its derivative cell strain transfected with an empty vector (*V*), and two derivatives (clones 10 and 40). The lane labeled (+) contains 1 μg of protein from a lysate prepared from HEK293, transiently transfected with a plasmid encoding the human *POLI* gene. The antibody was prepared from the COOH-terminal of Pol ι. *a,* the faster-migrating form of Pol ι; *b,* the slower-migrating form; β-Actin was used as the loading control.

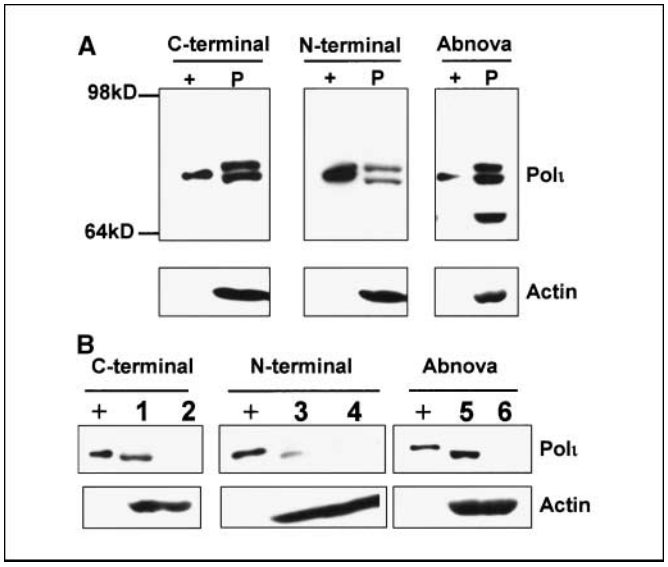


**Figure 2.** Evidence that both the slower-migrating and the faster-migrating bands are Pol ι. *A,* Western blot analysis of the expression of Pol ι protein using a COOH-terminal Pol ι–specific antibody, an $NH_2$-terminal Pol ι–specific antibody, and a monoclonal Pol ι–specific antibody from Abnova. The lane labeled (+) is the same as shown in Fig. 1. The lanes labeled P contain 100 μg of protein from a lysate of the parental XP115LO cell line. Actin was used as loading control. *B,* Western blot analysis to test three different Pol ι antibodies (a COOH-terminal Pol ι-specific antibody, an $NH_2$-terminal Pol ι-specific antibody, and a monoclonal Pol ι antibody from Abnova) using cell lysates from the parental BL2 (*POLI*$^{+/+}$) and BL2 *POLI*$^{-/-}$ cell strains. The lanes labeled (+) are as in Fig. 1; lanes 1, 3, and 5 contain 200 μg of protein from a lysate of the parental BL2 (*POLI*$^{+/+}$) cell line; lanes 2, 4, and 6 contain 200 μg of protein from a lysate of the BL2 (*POLI*$^{-/-}$) cell line. β-Actin was used as the loading control.

## Results

**Generation of XPV cells with a decreased level of Pol ι.** Cell strain XP115LO.hTERT has an unlimited life span in culture, a diploid karyotype, and grows well in culture with high cloning efficiency. To generate cell clones expressing lower levels of Pol ι, we transfected this strain with a tetracycline transactivator (t-TAk) construct, under the control of the TetP promoter (Tet Off). A clone, XP115LO.t-TAk.M, with a high cloning efficiency, a low background frequency of thioguanine-resistant colonies, and a UV-induced frequency of *HPRT* mutants similar to that of its precursor, was transfected with a plasmid designed to express antisense *POLI* RNA, constructed by inserting a 1,482-bp *POLI* fragment (nucleotides 56–1,538) in an antisense orientation into plasmid pTet/Bsd, which contains a gene coding for blasticidin (*Bsd*) resistance for use in stable transfections, and places the gene of interest under the control of the TetP promoter. Because the parental strain XP115LO.t-TAk.M proved not to grow well if t-TAk was expressed, tetracycline was always included in the medium. An empty vector was transfected into the parent strain to generate vector control cell strains. *Bsd*-resistant transfectants were isolated and screened by reverse transcription-PCR for expression of antisense *POLI* RNA. Whole-cell lysates from clones found to express *POLI* antisense RNA, from the parental strain, and several vector control were analyzed by Western blotting for expression of Pol ι, using a polyclonal antibody specific for the COOH-terminal region of Pol ι (22). Of the 45 antisense-transfected clones, two, designated clones 10 and 40, exhibited a reduced level of Pol ι (Fig. 1). The vector control strain expressed Pol ι at the same level as the parent (Fig. 1).

**Two forms of Pol ι in XP115LO cells.** The parental strain and vector control express two forms of Pol ι protein. Antisense-expressing clones 10 and 40 do not express the slower-migrating form (*b*). Recombinant Pol ι generated by transient expression of full-length Pol ι in HEK293 cells was used as a positive control (+) in these assays. To confirm that the two forms of the protein (*a* and *b*) correspond to Pol ι, we did Western blot analysis of lysates from the parent, and a positive control (+) prepared in HEK293 cells, using three Pol ι antibodies, two polyclonal rabbit antibodies raised to peptides from the $NH_2$- or COOH-terminal of Pol ι, and a commercially available mouse monoclonal antibody generated using the COOH-terminal 100 amino acid of Pol ι. All three recognized the two forms of Pol ι (Fig. 2*A*). Note that the control protein (+), migrated slightly slower than Pol ι (*a*). (This slightly altered migration of proteins prepared using HEK293 cell is not uncommon.) Cell lysates prepared from human BL-2 and BL-2 *POLI*$^{-/-}$ cell lines (21) were also used to test the COOH-terminal, $NH_2$-terminal, and monoclonal antibodies. As shown in Fig. 2*B*, all three recognized Pol ι in the BL-2 cells (*lanes 1, 3*, and *5*). As expected, no Pol ι was detected in the BL-2 *POLI*$^{-/-}$ cells (*lanes 2, 4*, and *6*). In summary, cell strain XP115LO was found to express two forms of Pol ι, and our antisense construct only eliminated expression of the slower-migrating common form.

**Expression of Pol ι protein in various human cell strains.** To determine whether strains from XPV patients, other than XP115LO cells, also express two forms of Pol ι protein (Figs. 1 and 2), we analyzed finite and infinite life span strains, including finite life span cell strain XP115LO (Fig. 3). The latter, like its hTERT-expressing derivative, expresses both forms of Pol ι, whereas cells from the other three XPV patients express only the common faster-migrating form of Pol ι. Note that finite life span cell line VE45, from a normal donor (19), also expresses these two forms. Thus, in contrast to the majority of human cell lines, a subset expresses two forms of Pol ι.

**Evidence that the level of Pol ι has no effect on UV cytotoxicity.** Clones 10 and 40 were compared with their parent and two vector controls for sensitivity to the cytotoxic effect of UV. The top panels of Fig. 4 show there was little difference in survival between these strains.

**Evidence that Pol ι is responsible for the abnormally high frequency of mutations induced by UV in XPV cells.** Flow cytometry analysis indicated that the parent and clones 10 and 40 are diploid. Chromosome analysis showed they have only one X

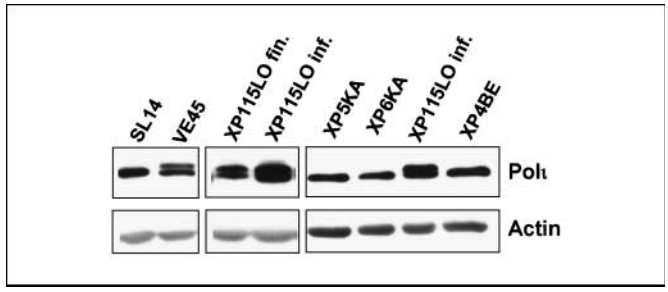


**Figure 3.** Western blot analysis of the forms of Pol ι protein in a series of finite and infinite life span human cell lines. *Lanes 1* and *2,* lysates from finite life span diploid skin fibroblasts from normal donors. Note that the cells from donor VE45 express both forms of Pol ι, characteristic of cells from XPV115LO. *Lanes 3, 5, 6,* and *8,* lysates from finite life span, diploid skin fibroblasts derived from XPV patients. *Lanes 4* and *7,* independent lysates from XP115LO cells that acquired an unlimited life span. β-Actin was used as loading control.

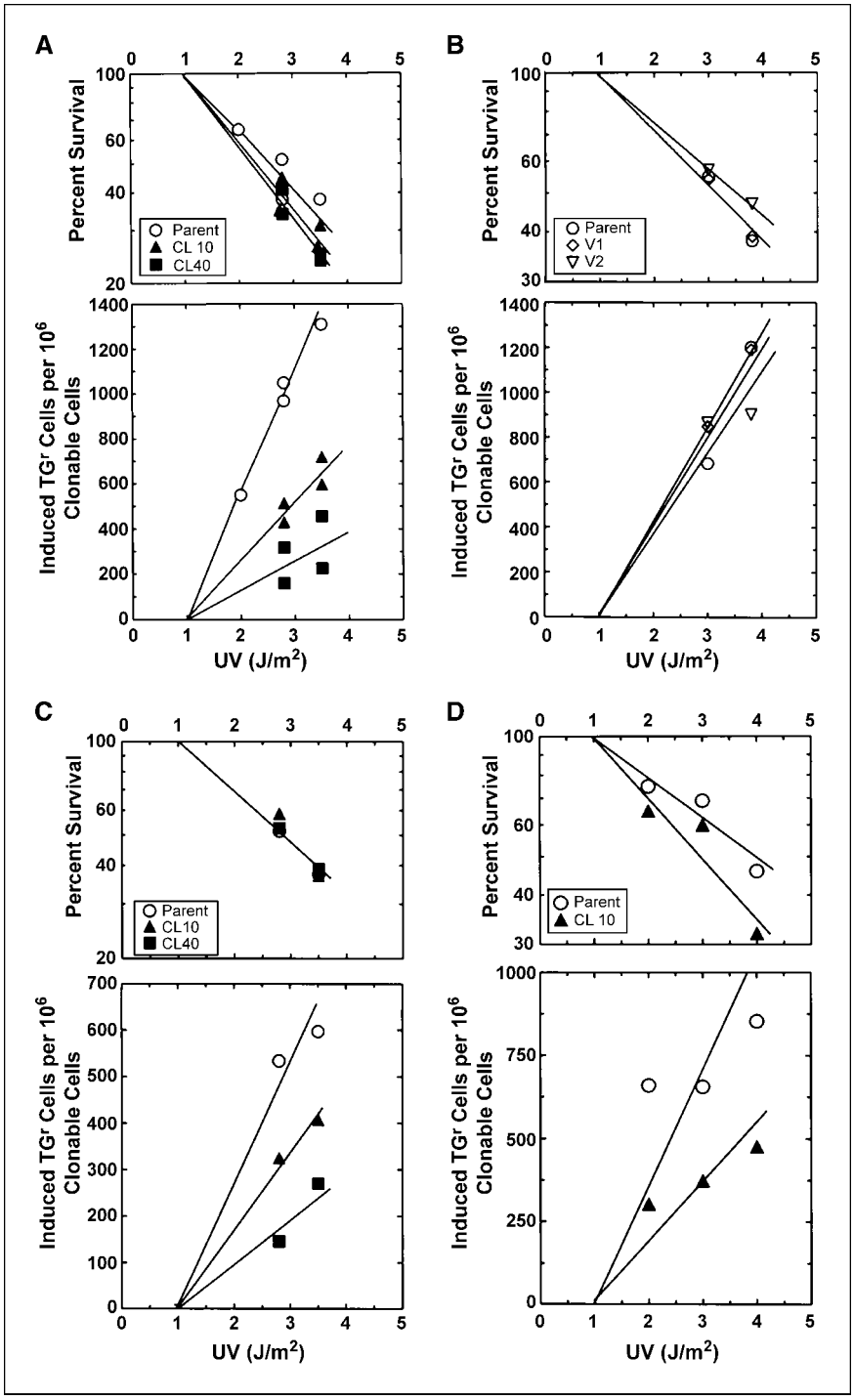


**Figure 4.** Evidence that Pol ι is responsible for the very high mutation frequency (*bottom*) induced by UV in human cells that lack Pol η, but does not significantly alter their sensitivity to its cytotoxic effect (*top*). *A,* strain XP115LO.hTERT and its two derivatives with reduced levels of Pol ι were synchronized by being released from a $G_1$-S block, irradiated 1.5 h later, and assayed for survival and mutagenicity. *B,* vector controls and the parental cells were similarly synchronized and UV irradiated. *C,* results from an experiment similar to that of (*A*), but using a less optimal serum, showing essentially the same relationships as found in (*A*). *D,* cells growing asynchronously, but with conditions designed to ensure they replicated at the same rate. The frequencies of UV-induced mutations have been corrected for the cloning efficiency of the cells assayed at the time of thioguanine selection, which ranged from 34% to 60%. Feeder layers were used with clones 10 and 40 to increase their cloning efficiency to that of their parent. The lines represent the least-square lines for the data. The fact that doses <1 J/m$^2$ did not cause loss of colony-forming ability or mutagenicity reflects the fact that XPV cells actively carry out nucleotide excision repair.

chromosome. Clones 10 and 40 replicate somewhat slower than their parent. Therefore, to minimize the possibility that these two strains would have more time than their parent to excise UV photoproducts before their replication fork encounters lesions in the *HPRT* gene, we synchronized all three strains at the $G_1$-S border, released them, and irradiated them 1.5 h later (i.e., just as they began replicating their *HPRT* gene). Under such conditions, a dose of 2.8 J/m$^2$ given to normal human cells which express both Pol η and Pol ι at the normal level, would lower their survival to 90%, and induce a mutant frequency of $\sim 20 \times 10^{-6}$ (unpublished data). As shown in Fig. 4*A*, at a dose of 2.8 J/m$^2$, the parent strain, XP115LO, exhibited a survival of 48% and a mutant frequency of $1{,}000 \times 10^{-6}$; clone 10, which expresses only 50% as much Pol ι, showed a 39% survival and a frequency of $500 \times 10^{-6}$. No such difference in survival or frequency of mutations was found between the parent and its two vector controls (Fig. 4*B*).

In the experiment shown in Fig. 4*C*, synchronized populations of the three cell strains used in Fig. 4*A* were compared, but the serum used did not promote optimal growth. This explains why the relationship between the parent and clones 10 and 40 are similar to that shown in Fig. 4*A*; however, the frequency of mutations in all three are lower (e.g., 2.8 J/m$^2$ reduced survival to 50% as before,

but induced only $550 \times 10^{-6}$ mutants in the parent strain and only $300 \times 10^{-6}$ mutants in clone 10).

Figure 4*D* compares XP115LO and clone 10, grown in the same batch of FCS as used in Fig. 4*A*; however, this time, we used cells that were growing asynchronously. Such cells could not be irradiated just at their *HPRT* gene was replicating. Therefore, we adjusted the percentage serum used so that both strains grew at the same rate (data not shown). Survival of the two populations irradiated in asynchronous growth (i.e., 63% and 52% at a dose of 2.8 J/m$^2$) was similar to that shown in Fig. 4*A*. Note that, once again, the relationship between the frequency of UV-induced mutations in the parent and clone 10 remain the same. As predicted (26), such results show that (*a*) the frequency of induced mutations is dependent on time available for excision repair before replication of the specific target gene, but cell survival is not and (*b*) the frequency of UV-induced mutations in human cells that lack Pol η (i.e., XPV cells) correlates with their level of expression of Pol ι.

Because the cells used in Fig. 4*A* to *C* to assay the cytotoxicity of UV were growing asynchronously, whereas those used for assaying mutations were synchronized, we carried out another experiment to determine whether synchronized populations, irradiated 1.5 h after release from the $G_1$-S block (i.e., just as their *HPRT* gene is replicated), would exhibit the same relative percentage survival of colony-forming ability as was found in Fig. 4*A*. Duplicate sets of the parent and clone 10 cells were synchronized as before, washed, released, and UV-irradiated as usual, 1.5 h after release from the $G_1$-S block. After irradiation, each set of cells was picked up in culture medium, diluted, and assayed for survival of the colony-forming ability. The percentage survival results (shown in the Supplementary Figure) were very similar to those in Fig. 4*A*. These data indicate that cell survival is not determined by the amount of time before a specific target gene (e.g., *HPRT*) is replicated. The results of these four mutagenesis experiments, taken together with the Western blot analysis (Fig. 1), indicate that in the absence of Pol η, the level of Pol ι expressed plays a critical role in error-prone translesion synthesis past UV-photoproducts *in vivo*.

As further evidence that the UV-induced mutation frequency is directly related to the level of expression of Pol ι in cells lacking Pol η, we also compared the frequency of mutations induced in the finite life span and unlimited (infinite) life span XP115LO cells, both of which express twice the normal level of Pol ι, with that induced in finite life span XPV cell line XP5KA, which, as shown in Fig. 3, expresses the normal level of Pol ι. As predicted, all three populations showed similar sensitivity to the cytotoxic effect of UV, but the frequency of mutations induced in the XP5KA cells was only ~50% of that induced in both sets of XP115LO cells tested (data not shown).

**Evidence that the kinds of mutations (spectra) induced by UV in XPV cells result from Pol ι–dependent translesion synthesis.** Table 1 compares the kinds of UV-induced *HPRT* mutations induced in 249 independent thioguanine-resistant mutant clones derived from multiple experiments involving cell strains/lines expressing or lacking Pol η and expressing Pol ι at the



**Table 1.** Kinds of mutations induced by $UV_{(254nm)}$ in the *HPRT* gene of human cells that differ in expression of Pol ι or Pol η or induced in a target gene (*lacZα*) by replication-competent cell-free extracts from such cells

| | Cells expressing Pol η and Pol ι | | | Cells devoid of Pol η, but expressing Pol ι | | | | | |
|---|---|---|---|---|---|---|---|---|---|
| | Intact cells | | Cell extract | Intact cells | | Cell extract | Intact cells (expressing hTERT) | | |
| | XP12BE | | | XP5KA | XP4BE | XP115LO | XP115LO | XP115LO | XP115LO |
| | Excision minus | Normal | Normal | Variant | Variant | SV40 strain variant | Parent variant | Clone 10 variant | Clone 40 variant |
| Pol η | 100% | 100% | 100% | 0% | 0% | 0% | 0% | 0% | 0% |
| Pol ι | 100% | 100% | 100% | 100% | 100% | 200% | 200% | 100% | 100% |
| Transversions | 6 (27.2%) | 8 (12.9%) | 6 (11.3%) | 14 (70.0%) | 16 (55.0%) | 21 (63.7%) | 48 (63.2%) | 17 (70.0%) | 11 (68.8%) |
| T → A | 1 (4.5%) | 2 (3.3%) | 1 (1.9%) | 5 (25.0%) | 3 (10.3%) | 8 (24.3%) | 21 (27.7%) | 8 (33.0%) | 6 (37.5%) |
| C → A | 2 (9.1%) | 1 (1.6%) | 3 (5.7%) | 9 (45.0%) | 9 (31.0%) | 13 (39.4%) | 20 (26.3%) | 6 (25.0%) | 4 (25.0%) |
| T → G | 2 (9.1%) | 0 | 0 | 0 | 3 (10.3%) | 0 | 7 (9.2%) | 1 (4.0%) | 1 (6.3%) |
| C → G | 1 (4.5%) | 5 (8.2%) | 2 (3.7%) | 0 | 1 (3.4%) | 0 | 0 | 2 (8.0%) | 0 |
| Transitions | 16 (72.8%) | 54 (87.1%) | 47 (88.7%) | 6 (30.0%) | 13 (45.0%) | 12 (36.3%) | 28 (36.8%) | 7 (30.0%) | 5 (31.2%) |
| C → T | 15 (68.2%) | 37 (59.7%) | 43 (81.1%) | 4 (20.0%) | 7 (24.0%) | 11 (33.3%) | 19 (25.0%) | 3 (13.0%) | 1 (6.2%) |
| T → C | 1 (4.6%) | 17 (27.4%) | 4 (7.6%) | 2 (10.0%) | 6 (21.0%) | 1 (3.0%) | 9 (11.8%) | 4 (17.0%) | 4 (25.0%) |
| | 22 | 62 | 53 | 20 | 29 | 33 | 76 | 24 | 16 |

NOTE: Of the 335 independent mutations shown here, 163 are taken from published reports. Data from excision repair–deficient complementation group A cells, XP12BE, are from ref. 5; 26 of the 62 data derived using normal human skin fibroblasts (column 2) were reported in ref. 5 or 10; 53 data obtained using replication-competent cell-free extracts from HeLa cells and from foreskin-derived human fibroblast cell line MSU1.2 and 33 using extract from an SV40-immortalized XP115LO variant cell line are taken from ref. 12. The 172 additional new data (i.e., 36 mutations induced using normal human cells; 20 from XPV cell line XP5KA cells; 76 from infinite life span parental 115LO cells; 24 from its derivative, clone 10; and 16 from its derivative, clone 40) were obtained in the present study.

**Table 2.** Kinds and sequence context of independent *HPRT* mutations induced by $UV_{(254nm)}$ radiation in early S phase of synchronized cells that lack Pol η (XPV) and differ in their levels of Pol ι

| XP4BE cells* and parental XP115LO cells | | | | XP115LO-derived clones 10 and 40* (Pol ι levels ~50%) | | | | |
|---|---|---|---|---|---|---|---|---|
| Position | Mutation | Photoproduct † | Strand ‡ | Insertion§ | Position | Mutation | Photoproduct † | Strand ‡ |
| 523 | T → A | C **TT** AT | T | **T** | 386 | T → A | CA **TT** C | T |
| 523 | T → A | C **TT** AT | T | | 62 | T → A | T **TT** AT | NT |
| 82 | T → A | A **TT** AT | NT | | 82 | T → A | A **TT** AT | NT |
| 82 | T → A | A **TT** AT | NT | | 82* | T → A | A **TT** AT | NT |
| 62 | T → A | T **TT** AT | NT | | 125 | T → A | AA **TT** A | NT |
| 297 | T → A | T **TT** AT | NT | | 297 | T → A | T **TT** AT | NT |
| 297 | T → A | T **TT** AT | NT | | 297* | T → A | T **TT** AT | NT |
| 541 | T → A | GA **TT** T | NT | | 605 | T → A | T **TT** GA | NT |
| 605* | T → A | T **TT** GA | NT | | 605 | T → A | T **TT** GA | NT |
| 323 | T → A | GG **TC** A | T | | 605* | T → A | T **TT** GA | NT |
| 296 | T → A | T **TTT** A | NT | | 49* | T → A | G **TT** AT | NT |
| 542 | T → A | A **TTT** G | NT | | 605* | T → A | T **TT** GA | NT |
| 604* | T → A | A **TTT** G | NT | | | | | |
| 643* | T → A | T **TTT** G | T | | 296 | T → A | T **TTT** A | NT |
| 223 | T → A | T **CTT** T | NT | | 146* | T → A | T **CTT** G | NT |
| 40* | C → A | T **TC** AT | T | **T** | | | | |
| 40 | C → A | T **TC** AT | T | | | | | |
| 88 | C → A | C **TC** AG | T | | | | | |
| 229 | C → A | G **TC** AG | T | | 40 | C → A | T **TC** AT | T |
| 412 | C → A | G **TC** AA | T | | 412 | C → A | G **TC** AA | T |
| 412 | C → A | G **TC** AA | T | | 606 | C → A | T **TC** AA | T |
| 509* | C → A | T **TC** GT | T | | 40* | C → A | T **TC** AT | T |
| 606 | C → A | T **TC** AA | T | | 606* | C → A | T **TC** AA | T |
| 606 | C → A | T **TC** AA | T | | 628* | C → A | T **TC** AC | T |
| 606* | C → A | T **TC** AA | T | | | | | |
| 606* | C → A | T **TC** AA | T | | 97 | C → A | T **TCC** A | T |
| 119 | C → A | G **TC** CA | T | | | | | |
| 154 | C → A | A **TCT** C | T | | | | | |
| 292 | C → A | A **TCT** A | T | | | | | |
| 553 | C → A | G **TCT** G | T | | 222 | C → A | T **TCT** T | NT |
| 118* | C → A | T **CC** AT | T | | | | | |
| 134* | C → A | A **CC** TG | T | | 3 | C → A | G **CC** AT | T |
| 517 | C → A | T **CC** AA | T | | | | | |
| 517 | C → A | T **CC** AA | T | | | | | |
| 197 | C → A | A **ACA** G | T | | | | | |
| 562 | C → A | A **ACA** A | T | | 617 | C → A | C **ACA** A | T |
| 617 | C → A | C **ACA** A | T | | | | | |
| 479 | T → G | GG **TC** G | NT | **C** | | | | |
| 62 | T → G | T **TT** AT | NT | | 296 | T → G | T **TTT** A | NT |
| 82 | T → G | A **TT** AT | NT | | 296* | T → G | T **TTT** A | NT |
| 221 | T → G | A **TT** CT | NT | | | | | |
| 417* | T → G | A **CT** GG | NT | | | | | |
| 436* | T → G | C **TTT** G | NT | | 509 | C → G | T **TC** GT | NT |
| 294/295* | TT → GG | A **TTT** T | NT | | 551 | C → G | T **CC** AG | T |
| 576/577 | CC → TT | C **CC** TT | NT | **A** | | | | |
| 399/400 | CC → TT | T **CC** AC | T | | | | | |
| 112/113 | CC → TT | T **CC** TC | NT | | | | | |
| 464 | C → T | T **CC** AA | NT | | 464 | C → T | T **CC** AA | NT |
| 464 | C → T | T **CC** AA | NT | | 464* | C → T | T **CC** AA | NT |
| 464* | C → T | T **CC** AA | NT | | | | | |
| 464* | C → T | T **CC** AA | NT | | | | | |
| 464* | C → T | T **CC** AA | NT | | | | | |
| 355 | C → T | T **CC** AC | T | | | | | |
| 506* | C → T | C **CC** AC | T | | | | | |
| 463 | C → T | A **TCC** A | NT | | 544 | C → T | T **TC** AA | T |

(*Continued on the following page*)

**Table 2.** Kinds and sequence context of independent *HPRT* mutations induced by $UV_{(254nm)}$ radiation in early S phase of synchronized cells that lack Pol η (XPV) and differ in their levels of Pol ι (Cont'd)

| XP4BE cells* and parental XP115LO cells | | | | XP115LO-derived clones 10 and 40* (Pol ι levels ~50%) | | | | |
|---|---|---|---|---|---|---|---|---|
| Position | Mutation | Photoproduct† | Strand‡ | Insertion§ | Position | Mutation | Photoproduct† | Strand‡ |
| 635 | C → T | T **TCC** A | T | | 400 | C → T | T **TCC** A | T |
| 601* | C → T | A **TCC** C | NT | | | | | |
| 74 | C → T | A **CCT** A | NT | | | | | |
| 145 | C → T | G **TCT** T | NT | | | | | |
| 196 | T → C | T **CT** GT | NT | **G** | | | | |
| 205* | T → C | C **TT** GA | T | | 437 | T → C | T **TT** GC | NT |
| 392* | T → C | C **TT** GA | NT | | 616 | T → C | T **TT** GT | NT |
| 392 | T → C | C **TT** GA | NT | | 616 | T → C | T **TT** GT | NT |
| 488 | T → C | C **TT** GC | NT | | 616* | T → C | T **TT** GT | NT |
| 374* | T → C | T **TT** AA | NT | | 616* | T → C | T **TT** GT | NT |
| 410 | T → C | AA **TT** G | NT | | 386* | T → C | CA **TT** C | T |
| 221 | T → C | A **TTC** T | NT | | 596 | T → C | C **TTC** A | NT |
| 296* | T → C | T **TTT** A | NT | | | | | |
| 296* | T → C | T **TTT** A | NT | | 146* | T → C | T **CTT** G | NT |
| 295* | T → C | A **TTT** T | NT | | | | | |

*Mutations in XP4BE and clone 40.

† Altered nucleotides are underlined; nucleotides are written 5′ to 3′.

‡ In *HPRT*, the transcribed strand serves as the template for leading strand synthesis.

§ The inserted nucleotide is indicated by the bold initial.

normal level or at a 2-fold higher level. (If two thioguanine-resistant clones derived from the same UV-irradiated population contained the same mutation, only one was used for determining the spectrum.) The parental cell strain, XP115LO (column 7), and its derivatives, clones 10 and 40 (columns 8 and 9), and also the XPV 5KA cells (column 4) were synchronized at the $G_1$-S border, released, and UV-irradiated just as their *HPRT* gene began replicating. Clones 10 and 40 and XP5KA express 50% as much Pol ι as their parental XP115LO cells. Their percentages of each kind of mutation are very similar and also similar to those found previously (10) using XP4BE variant cells (column 5), released from confluence, and irradiated in early S-phase just as their *HPRT* gene was being replicated. Also analyzed were 86 *lacZα* mutants induced by cell-free replication-competent extracts from such cells lines/strains expressing 100% Pol η and Pol ι, or no Pol η, but 200% Pol ι (columns 3 and 6). In the latter assay, the origin of replication of the target gene, *lacZα*, can be reversed. Approximately 60% of the mutations in parent XP115LO and its two derivative clones and those generated by the extracts from XPV cells were C → A or T → A transversions. These percentages contrast starkly with the average of 8.7% found in the cells and cell extracts expressing functional Pol η (columns 1–3).

Table 2 shows the nature and context of 114 unequivocally independent UV-induced mutations derived from XPV cells. It also specifies the strand of the *HPRT* gene that contained the photoproduct and indicates the nature of the nucleotide mis-inserted opposite the photoproduct involved. The background frequency in each of the mock-treated control populations of the cell strains used for obtaining these data was >30-fold lower than that of the induced frequency. Therefore, the vast majority of the mutations analyzed represent UV-induced changes. Table 2 also shows that 97% of the C → A transversions were derived from photoproducts located on the transcribed strand. In contrast, the vast majority of the other mutations, including T → A transversions, were derived from photoproducts located on the nontranscribed strand. The origin of replication of the *HPRT* gene is now known to be located between exons 1 and 2. Therefore, the transcribed strand of the *HPRT* gene is the rapidly replicated (leading) strand. In contrast, the majority of the C → T and T → C transitions and the T → A transversions observed in the XPV cells occurred when the photoproduct was located in the lagging strand (Table 2).

Maher and colleagues also previously found such extreme bias for C → A transversions in the leading strand of the *lacZα* gene, using replication-competent extracts derived from XP4BE cells (12). In that study, two plasmids were used, each with the origin of replication located at an opposite end of the target gene. When the leading strand being replicated more rapidly was reversed, the location of C → A transversions were also found to be reversed (i.e., located in the opposite strand), which now was the leading strand (12). No such strand bias was seen using cell extracts from two non-XPV normal human cell strains, expressing both Pol η and Pol ι (10, 12).

The data from our present study indicate clearly that in human cells lacking Pol η, reduction in the amount of Pol ι results in a decrease in the overall frequency of UV-induced mutants, but does not change the spectrum of mutations. These data strongly support the hypothesis that in the absence of Pol η, Pol ι is responsible for the high frequency and abnormal spectrum of mutations induced by UV.

## Discussion

These results, which compare XPV cells devoid of Pol η and differing in their expression of Pol ι, strongly suggest that XPV patients are predisposed to sunlight-induced cancer because they carry out translesion synthesis past UV-induced fork-blocking photoproducts using an exceptionally error-prone Y-family polymerase, Pol ι. The

data in Fig. 4, and the Supplementary Figure, together with those of Fig. 3, indicate that the frequency of mutations induced in cell lines devoid of Pol η (XPV cells) is closely correlated with their relative level of expression of Pol ι. Such cells with the highest frequency of mutations express the highest level of Pol ι.

*In vitro* replication experiments have shown that human Pol ι misinserts T or G nucleotides opposite the 3′T of T-T CPDs and T-U CPDs more frequently than it inserts the correct nucleotide (16, 18). Our results in Table 2 show that misinsertion of T opposite the 3′T of T-T CPDs generates T → A transversions. Such transversions occur at a very high frequency in UV-irradiated XPV cells or their extracts (Table 1), but are rarely found in cells from normal donors (column 2) or their extracts (column 3) or in those of excision repair–deficient group A cells. We also observed a striking decrease in the percentage of C → T transitions in XPV cells, compared with that found in normal human fibroblasts or classic XP cells (column 1). In normal cells, Pol η might readily incorporate adenine nucleotides opposite the 3′U of T-U CPDs, resulting in C → T transitions (4). Our results strongly suggest that in the absence of functional Pol η, Pol ι substitutes for Pol η and incorporates G or T nucleotides opposite 3′U of T-U CPDs more frequently than the correct nucleotide, A. If misincorporation of G nucleotides opposite 3′U occurred, this would not result in a permanent mutation because in the next round of semiconservative synthesis, a G:C base pair would be restored. This explains why the relative percentage of C → T transitions induced in XPV cells is greatly decreased compared with that seen with excision-repair deficient cells, normal cells, and normal cell extracts (Table 1, columns 1–3). Conversely, misincorporation of T opposite U would lead to an increase in the relative percentage of C → A transversions, as observed using XPV cells (Table 1, columns 4–9).

Recent research by Dumstorf et al. (27) using mouse cells, which unlike human cells only carry out transcription-coupled repair, also showed a significant increase in UV-induced base substitutions, between wild-type mouse cells and Pol $\eta^{-/-}$/Pol $\iota^{+/+}$ mouse cells not only in frequency, but also in strand distribution. These investigators also showed that mice lacking Pol η, but possessing Pol ι, were more sensitive to UV-induced skin cancer than wild-type mice. Such a result would explain why XPV patients, devoid of Pol η, but expressing the normal amount of Pol ι (i.e., 500%), develop skin cancer (7). A recent report by Ohkumo et al. (28) using mice showed that Pol $\eta^{-/-}$ /Pol $\iota^{+/-}$ develop skin tumors early.

As shown in Fig. 3, human cells express two forms of Pol ι. The majority express a protein that corresponds in size to that expected of the 715 amino acid Pol ι protein. However, a subset (e.g., XP115LO and VE45) also express a slower-migrating form. Our data indicate that both forms of Pol ι are functionally active. In our effort to eliminate expression of Pol ι protein in XP115LO cells using an antisense construct, we found that our construct only eliminated the slower-migrating form. To be effective, antisense must achieve close interaction with its targeted mRNA (29). mRNAs that differ by just a few nucleotides produce secondary structures that differ significantly. Because the disrupting action of antisense derives from its ability to base pair with its target mRNA, finding that only one form of protein was eliminated indicates that two different Pol ι mRNAs exist in these cells and that the structure of the mRNA encoding the slower-migrating form of Pol ι is more accessible to our construct. We carefully determined (data not shown) that the slower-migrating form is not the result of the use of an upstream in-frame ATG start codon. Our extensive attempts to eliminate expression of both forms of Pol ι using ribozymes and siRNAs against *POLI* were not effective.

Recent studies show that in UV-irradiated cells, foci formation involving Pol ι partially depends on Pol η because the number of cells with Pol ι foci drops ~3-fold in XPV cells (22). Nevertheless, such reduced ability of Pol ι to form foci in cells lacking Pol η cannot be used as an argument against the hypothesis that Pol ι is responsible for the increased mutation frequency and abnormal spectrum observed in XPV cells (30), because use of Pol ι to bypass photoproducts is extremely error-prone. In cells that express both Pol η and Pol ι (i.e., normal human cells), it is unlikely that Pol ι is able to compete with the far superior CPD-bypassing ability of Pol η. However, in cells that lack Pol η (i.e., XPV cells), there is no competition between Pol η and Pol ι for foci formation, and the cells that do form foci would be sufficient to generate the 25-fold higher (see Fig. 4*A*) frequency of mutations found in XPV cells with the normal amount of expression of Pol ι and the 50-fold higher frequency found in XP115LO, which express twice as much Pol ι. It also explains the striking difference in the kinds of substitutions (spectrum) observed in the present study and in the studies summarized in Tables 1 and 2.

In conclusion, our data in Figs. 1 and 4*A* highlight the very critical contribution of Pol η in protecting human cells from the potentially mutagenic effects of UV radiation, and, therefore, also from its potentially carcinogenic effects, but show that in the absence of Pol η, translesion synthesis past photoproducts is facilitated by very error-prone Pol ι. Our data strongly support the hypothesis that Pol ι is the enzyme responsible for the greatly increased frequency of mutations observed in XPV cells, and ultimately their increased risk of sunlight-induced cancer.

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