Role of DNA Polymerase η in the UV Mutation Spectrum in Human Cells*

Received for publication, November 20, 2002, and in revised form, March 6, 2003
Published, JBC Papers in Press, March 18, 2003, DOI 10.1074/jbc.M211838200

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In humans, inactivation of the DNA polymerase η gene (pol η) results in sunlight sensitivity and causes the cancer-prone xeroderma pigmentosum variant syndrome (XP-V). Cells from XP-V individuals have a reduced capacity to replicate UV-damaged DNA and show hypermutability after UV exposure. Biochemical assays have demonstrated the ability of pol η to bypass cis-syn-cyclobutane thymine dimers, the most common lesion generated in DNA by UV. In most cases, this bypass is error-free. To determine the actual requirement of pol η in vivo, XP-V cells (XP30RO) were complemented by the wild type pol η gene. We have used two pol η-corrected clones to study the in vivo characteristics of mutations produced by DNA polymerases during DNA synthesis of UV-irradiated shuttle vectors transfected into human host cells, which had or had not been exposed previously to UV radiation. The functional complementation of XP-V cells by pol η reduced the mutation frequencies both at CG and TA base pairs and restored UV mutagenesis to a normal level. UV irradiation of host cells prior to transfection strongly increased the mutation frequency in undamaged vectors and, in addition, especially in the pol η-deficient XP30RO cells at 5'-TT sites in UV-irradiated plasmids. These results clearly show the protective role of pol η against UV-induced lesions and the activation by UV of pol η-independent mutagenic processes.

Solar UV induces lesions in genomic DNA. If not repaired by one of the error-free pathways, these lesions can give rise to mutations. In a large percentage of skin tumors, mutations in the p53 tumor suppressor gene are characterized by the “UV mutagenesis signature” namely C → T transitions at pyrimidine-pyrimidine sites and CC → TT tandem mutations (1, 2). The precise mechanism by which UV-induced damage results in mutations remains unclear. The frequency and the nature of mutation depends on the types of initial DNA damage, their potential to miscode during DNA replication, and the probability that specific enzymes act on a given type of damage. The mutational specificity of UV light correlates with the formation in DNA of the two predominant UV-induced lesions, the cis-syn cyclobutane pyrimidine dimer (CPD) and the pyrimidine-6-4-pyrimidone (6-4PP) photoproducts (3, 4). In naked DNA, a similar distribution of the main UV-induced photoproducts was obtained with either UVC (254 nm) or UVB (280–320 nm). In both cases, the T = T CPD is the most abundant photoproduct. The T = C CPD and the T(6-4)C damage are produced in similar yields, whereas the level of the T(6-4)T lesion and photoproducts at CT and CC sites remains much lower (5). These DNA lesions are normally repaired by the nucleotide excision repair system (6). The 6-4PPs are rapidly and efficiently removed by nucleotide excision repair within a few hours after cell irradiation, whereas the CPDs are repaired rather slowly and incompletely (7). Although individual unrepaired 6-4PPs are more mutagenic than CPDs (8, 9), CPDs are thought to be responsible for the majority of the premutagenic lesions in mammalian cells (10–12), probably because the CPDs are more frequent and repaired much less efficiently than the 6-4-PP lesions. Although T = T CPDs are relatively more frequent than CPD containing cytosine, UV-induced mutagenic events occur predominantly at 5'-TC-3' and 5'-CC-3' dipyrimidine sites, particularly in vivo in key genes found mutated in skin cancers. In most cases they are C → T transitions, arising from the misincorporation of an adenine opposite the 3'-C of 5'-TC-3' and 5'-CC-3' sites. The weak contribution of T = T dimers to the UV mutation spectrum in vivo could be explained by a specific and accurate DNA damage tolerance mechanism.

UV-induced DNA damage presents a strong block for the replication machinery, probably because of the inability of replicative DNA polymerases to deal with DNA distortions. Specialized DNA polymerases are temporarily required to perform translesion synthesis (TLS) (13–16). In bacteria, when DNA damage blocks the normal replication process, an “SOS” response is activated, and more than 20 genes are induced (17, 18). Among these genes, two DNA polymerases (DNA polymerases IV and V) are able to continue to synthesize DNA for a few bases across the damaged site and put an incorrect base opposite the lesion, producing a mutation. In yeast, three genes (REV1, REV3, and REV7) are required for most DNA damage-induced mutagenesis. Rev3 and Rev7 code for a specific mutagenic DNA polymerase activity (pol η) required for translesion synthesis and the Rev1 gene product is a dCMP transferase (19). In parallel to this error-prone activity, yeast cells have another specialized DNA polymerase, DNA polymerase η encoded by the RAD30 gene (pol η), that can perform translesion synthesis in a relatively error-free way (20). Most DNA polymerases associated with TLS belong to the Y family of DNA polymerases that have in common a highly distributive mode of DNA synthesis and copy undamaged DNA templates with low fidelity (21–23). Among TLS polymerases, human DNA polydimer; MER, mutation error rate; XP-V, xeroderma pigmentosum variant; BrdUrd, bromodeoxyuridine; pol η, polymerase η; 6-4PP, pyrimidine-6/4-pyrimidone; TLS, translesion synthesis.
merase η (pol η) is noteworthy for its ability to bypass T = T dimers and undamaged T-T residues in vitro with a similar efficiency and to insert predominantly two As opposite a T (24–26), suggesting that it is able to carry out error-free TLS past T = T CPDs in vivo.

The relevance of human pol η to mutagenesis in vivo is demonstrated by the existence of patients mutated in the human pol η gene (27, 28). These patients are affected by the variant form of xeroderma pigmentosum (XP-V), a rare, autosomal, recessive genetic syndrome with sun hypersensitivity associated with numerous skin abnormalities and a high level of early and multiple skin cancers on sun-exposed sites in the body (29, 30). The phenotype of XP-V cells includes UV hypersensitivity, especially in the presence of caffeine (31), and UV-hypermutability that is consistent with the high cancer proneness in XP-V patients (32–36). XP-V cells are proficient in nucleotide excision repair but are impaired in lesion bypass associated with DNA replication on damaged templates (37). Cell-free extracts of XP-V patients are unable to perform bypass through a T = T dimer (38–40), a T (6-4) C photoprod-uct (41), or other bulky DNA damage (42). Restoration of efficient DNA synthesis past a T = T dimer by the addition of pol η protein in cell-free extracts demonstrated the essential role of pol η in replication of this photoprotein in vitro (26). Complementation of UV + caffeine hypersensitivity after transfection of the human pol η cDNA into XP-V cells definitively assigned pol η as the gene responsible for the genetic defect in XP-V patients (43–45). Most mutations in the pol η gene in XP-V patients are heavily biased toward the N-terminal region and encode a pol η protein with either missense mutations or severe truncations that abolish both DNA polymerase and bypass activities (46). Complementation of the defect in XP-V cells required not only the N-terminal catalytic domain of pol η but also the C-terminal 120 amino acids containing a bipartite nuclear localization signal and a sequence needed for the relocalization into replication foci. Indeed, in normal cells, pol η is distributed most uniformly in the nucleoplasm in most cells, but following UV irradiation, it accumulates with proliferating cell nuclear antigen in intranuclear foci, which represent forks distributed most uniformly in the nucleoplasm in most cells, using SV40-transformed MRC5V1 (normal) and XP30RO human fibroblasts (47). The XP30RO cell line has a homozygous deletion near the 5′-extremity of the pol η gene leading to a severe truncation of the pol η protein (26, 46). Isolation of complemented cells by the wild type pol η gene has been described previously (43). Stable transformants isolated from transfection of cDNA.3geo-pol η plasmid or vector with no insert were grown in modified Eagle’s medium supplemented with 10% fetal bovine serum, fungizone (2.5 μg/ml), antibiotics (100 units/ml penicillin and 100 μg/ml streptomycin), and 100 μg/ml Zeocin (Invitrogen). MRC5V1 c1 and XP30RO c14 harboring the pCDNA.3geo (Invitrogen) plasmid without the pol η insert were utilized as control cell lines and are referred to MRC5 and XP30RO cells.

UV Survival Assay—Cells were plated at 100–1000 cells per 100-mm dishes and incubated for 24 h. They were then rinsed with phosphate-buffered saline and irradiated with 254 nm UV light using a germicidal lamp at a fluence rate of 0.24 J/m2/s. After 15 days of incubation in culture medium containing 75 μg/ml caffeine (Sigma), the colonies were fixed with methanol and stained with Giemsa, and colonies with >50 cells were counted. Three dishes were utilized for each dose.

Cell Cycle Analysis—Twenty-four hours after exposure to 0 or 7 J/m2 of UVC and caffeine (75 μg/ml) added to the medium, cells were pulsed with BrdUrd for 1 h, harvested, and stained for replicative DNA synthesis with a fluorescein isothiocyanate-conjugated anti-BrdUrd antibody and for DNA content with propidium iodide.

Mutagenesis Assay—The shuttle vector SV40-based pR2 carries the 300-bp bacterial lacZ′ gene and bacterial promoter as mutagenesis target, the kanamycin resistance gene, and the SV40 and bacterial replication origins (48). The p205-KMT11 plasmid carries the SV40 T-antigen gene required for replication of the pR2 plasmid (49). Both vectors were transfected together into the host cells. The RecA-deficient Escherichia coli DH5αMCR (Invitrogen) was used for screening of lacZ′ mutants among plasmid progeny.

Irradiation of plasmid DNA was performed with 254 nm UV light using a germicidal lamp at a fluence rate of 30 J/m2/s. Cells were irradiated at 7 J/m2 with 254 nm UV light using a germicidal lamp at a fluence rate of 0.24 J/m2/s.

Cells were seeded at a density of 5 × 104 per 100-mm dish and incubated for 24 h. Undamaged DNA (10 μg of plasmid DNA) or UV-exposed pR2 vectors (10 μg of plasmid DNA) were then cotransfected with the 5 μg of unirradiated p205-KMT11 plasmid into unirradiated or irradiated cells at 7 J/m2 just before DNA transfection by the polyethylenimine precipitation method (50). Cells were incubated for 4 days and then collected by trypsinization for extrachromosomal DNA extraction. At least three independent cell transfections were performed for each dose and for each cell line.

Plasmid DNA isolated from cells was purified by a small scale alkaline lysis method (51) and treated with DpnI restriction endonuclease to remove any unreplicated pR2. Rescued plasmid DNA was shuttled to competent E. coli DH5αMCR for selection of mutant vectors, and transformed colonies were plated on selective medium (48). The complementation between the lacZ′ gene carried by the pR2 vector and the truncated lacZ′ gene of DH5α bacteria gives rise to blue (wild type) or white/light blue (mutant) colonies. Selected colonies were then isolated and restreaked on the same medium to confirm the mutant phenotype.

Plasmid DNA was prepared by small scale alkaline lysis, and sequence analysis of the lacZ′ locus was performed with Big Dye terminator on an ABI Prism 377 DNA sequence.

Mutant frequency corresponds to the number of colonies containing mutated plasmid at the lacZ′ locus determined after DNA sequencing divided by the total number of bacterial colonies. Mutation error rate (MER) corresponds to the average of the frequency of each independent mutation calculated as the ratio of each independent mutation divided by the total number of bacterial colonies rescued from the cell transfection experiment in which the mutation was isolated. Identical mutations that occurred more than once among sequences analyzed from the same cell transfection were excluded, thus ensuring that each mutation is the result of one event of bypass process and not of several rounds of replication of one mutated plasmid during the 4-day postirradiation after DNA transfection. Although this will lead to a slight underestimation of the mutation frequency at hotspot sites, the number of individual transfections is sufficient enough to ensure that no major hot spots are missed.

Statistical Analysis—The significance of differences of percentages was assessed using χ2 or Fisher’s exact test with α = 0.05 as the significance level. The hypergeometric test was used to check the significant differences in the distribution of mutations between spectra (52, 53).
were isolated after transfection of pCDNA-pol η plasmid into pol η-deficient XP30RO cells as described previously (43). Among the stable XP30RO transfectants, we characterized two clones, XP30RO/pol η cl5 and XP30RO/pol η cl6 cells, for their resistance to UV irradiation in the presence of caffeine, which specifically enhances UV sensitivity of XP-V cells, thereby allowing a clear discrimination between XP-V and normal cells (31). The expression of wild type pol η protein, determined by Western blot analysis (Fig. 1) in the two complemented clones, is similar in XP30RO/pol η cl5 and normal MRC5 cells and slightly higher in XP30RO/pol η cl6 cells. As expected no pol η was detected in the parental XP30RO cells. The UV sensitivity of XP30RO/pol η cl5 and XP30RO/pol η cl6 clones was monitored by cell cycle analysis and cell survival after UVC irradiation and was compared with that of XP30RO and normal MRC5 fibroblasts, both transfected by vector without insert.

The distribution of cells in the G1, S, and G2/M phases of the cell cycle 24 h after UV + caffeine treatment are shown in Fig. 2A, and a quantitative assessment of cell cycle distribution is illustrated in Fig. 2B. In the absence of UV, the number of BrdUrd-labeled cells in S phase was similar in all cell lines (18–25%). UV irradiation and the addition of caffeine did not substantially change the proportion of BrdUrd-labeled XP30RO/pol η cl5 (28%), XP30RO/pol η cl6 (24%), or MRC5 cells (34%) in S phase 24 h after irradiation. In contrast, only a very small number of XP30RO cells (3%) incorporated BrdUrd. Examination of the distribution of DNA content in these cells (Fig. 2A, lower row, 2nd panel) shows that many of them were

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**Fig. 1.** 80 μg of total cell lysate from each of the cell lines was electrophoresed in 8% SDS-PAGE gels, immunoblotted, and probed with antibody generated in rabbits against full-length human pol η. The same filter was probed with mouse anti-proliferating cell nuclear antigen (PCNA) (PC10, Santa Cruz Biotechnology) as loading control.

**Fig. 2.** A cell cycle changes and cell survival in MRC5, XP30RO, XP30RO/pol η cl5, and XP30RO/pol η cl6 cells after exposure to UVC. A, FACS analysis of human fibroblasts expressing wild type pol η (MRC5), truncated pol η (XP30RO), or XP30RO cells stably transfected with pCDNA-pol η (XR30RO/pol η cl5 and XR30RO/pol η cl6). The G1, S, and G2/M phases of the cell cycle are shown on the top panel for unirradiated (0 J/m2) or UV-irradiated (7 J/m2) cells in the presence of caffeine. B, quantitative assessment of the percentage of cells undergoing DNA replication after exposure to 0 or 7 J/m2 of UVC and caffeine, evaluated from A. Open bars, 0 J/m2; closed bars, 7 J/m2. C, UV cell survival by colony forming ability. After UV irradiation, cells were incubated for 15 days in the presence of caffeine (75 μg/ml), fixed with methanol, and stained with Giemsa. Each point represents an average of three independent experiments. XP30RO (closed square); XP30RO/pol η cl5 (closed circle); XP30RO/pol η cl6 (open circle); MRC5 (closed diamond).
DNA. The survival DNA replication within XP30RO (closed square), XP30RO/pol η cl5 (closed circle), XP30RO/pol η cl6 (open circle), and MRC5 (closed diamond) cells as a function of UV dose to the plasmid DNA. Cells were untreated (A) or UV-irradiated with 7 J/m² (B) just before plasmid DNA transfection. Frequency at the lacZ locus corresponded to the number of colonies containing mutated plasmid determined after DNA sequencing divided by the total number of bacterial colonies. Data points, mean values from at least three independent cell transfection experiments; error bars, ± S.E.

blocked in S phase by the UV + caffeine treatment. Thus, pol η is able to prevent the prolonged delay in S phase after UV irradiation typical of XP-V cells.

The differential effect of UVC on XP30RO/pol η cl5, XP30RO/pol η cl6, and XP30RO cells was also demonstrated by examining cell survival after UV exposure and caffeine post-treatment with a colony forming assay (Fig. 2C). The survival curves of XP30RO/pol η cl5 and XP30RO/pol η cl6 cells were indistinguishable from that of normal MRC5 cells, whereas XP30RO cells were extremely sensitive to UV irradiation in the presence of caffeine (3% at 4 J/m²), as expected for XP-V cells. A similar result was obtained when viability was assessed by the ability of the cells to incorporate [3H]thymidine 2–4 days after exposure to different doses of UV in the presence of caffeine (46) (data not shown). The expression of pol η in XP30RO/pol η cl5 and XP30RO/pol η cl6 cells therefore results in the correction of UV sensitivity to normal levels.

The functional complementation of XP30RO/pol η cl5 and XP30RO/pol η cl6 cells compared with their parental counterpart XP30RO cells deficient in the expression of pol η gave us the opportunity to examine the outcome of the expression of pol η on UV mutagenesis in vivo.

Plasmid Mutagenesis—The SV40-based shuttle vector pR2 (48) was irradiated with different UV doses (500, 1000, or 2000 J/m²) and immediately transfected into XP30RO/pol η cl5, XP30RO/pol η cl6, XP30RO, and MRC5 cells. In some experiments the recipient cells were pre-irradiated with a UV dose of 7 J/m². Cells were further incubated for 4 days to allow DNA replication and mutation fixation to take place. The plasmid DNAs were recovered from human cells and shuttled into E. coli for mutant plasmid screening at the nonessential lacZ locus. Phenotypic selection of lacZ mutants followed by DNA sequencing enabled us to determine the mutant error rates and the sequence alterations.

Unirradiated plasmid DNA replication in unirradiated host cells (Fig. 3A) led to similar background mutant frequencies in the parental XP30RO cells (39 × 10⁻⁵) and in the corrected XP30RO/pol η cl5 and XP30RO/pol η cl6 cells (24 × 10⁻⁵ and 17 × 10⁻⁵ respectively). Mutant frequencies after replication of UV-irradiated shuttle vectors were increased significantly in all cells relative to the background levels. However, when UV-damaged plasmids replicated in XP30RO cells, there was a 5.1-fold increase in mutant frequencies from 39 × 10⁻⁵ to 200 × 10⁻⁵ (Student’s t test; p < 0.001), reflecting the cellular hypermutability of XP variant cells. The mutant error rates in XP30RO cells at all doses of plasmid irradiation were much higher than those in the XP30RO/pol η cl5, XP30RO/pol η cl6, and MRC5 cells (p < 0.001). The transfected wild type pol η gene thus complements the high UV-induced mutant frequency characteristic of XP-V cells.

After introduction of plasmid DNA into UV-exposed cells (7 J/m²) (Fig. 3B), there was an increase of the background frequencies in all cells, changing from 39 × 10⁻⁵ to 213 × 10⁻⁵ for XP30RO cells (5.5 times), from 24 × 10⁻⁵ to 165 × 10⁻⁵ for XP30RO/pol η cl5 cells (6.9 times), and from 17 × 10⁻⁵ to 87 × 10⁻⁵ for XP30RO/pol η cl6 cells (5.1 times). Since the global enhancement of the mutant error rate affected both pol η-deficient and proficient cells, the cellular process involved in this increase is independent of the cellular status of pol η. The UV-induced mutant frequency in XP30RO cells (787 × 10⁻⁵ and 565 × 10⁻⁵ at 1000 and 2000 J/m² doses of plasmid irradiation), however, remained substantially higher than the background mutant error rate (p < 0.02). In contrast, the mutant frequencies in XP30RO/pol η cl5 and XP30RO/pol η cl6 cells were not significantly different from the background frequencies. These data show that the expression of pol η in XP variant cells gives rise to a strong decrease in errors generated during replication of UV lesions.

Sequence Analysis—DNA sequence analysis of more than 300 independent undamaged and UV-induced mutants in the lacZ locus generated by replication of pR2 plasmids in unirradiated and in UV-exposed XP30RO, XP30RO/pol η cl5, and XP30RO/pol η cl6 cells led to the recovery of more than 500 independent mutations (Table I). Plasmids with base substitutions were the most frequent mutants for all treatment conditions. Only very few deletions or deletion/insertions and no frameshifts were recovered.

Type of Base Substitutions and Mutation Error Rates in Unirradiated Cells—The type of independent mutations identified from DNA sequence analysis of lacZ UV-induced mutants with single, multiple, and tandem base substitutions (taken here as two single base substitutions) is shown in Table II. To assess quantitatively the level of each type of base substitution for different cells and treatments, we determined the MER for each type of base substitutions. The MER corresponds to the actual mutation frequency for each type of mutation.

No significant difference in the qualitative distribution of the type of transitions and transversions (relative frequency, %) was found after replication of UV-treated DNA plasmids in the different unirradiated cells (Table II). The total MER in XP30RO cells (181 × 10⁻⁵) was significantly higher than those obtained in XP30RO/pol η cl5 cells (75 × 10⁻⁵) and in XP30RO/pol η cl6 cells (63 × 10⁻⁵) (p < 0.001). There was no significant difference in MER between the pol η-expressing clones and the normal MRC5 cells (79 × 10⁻⁵). As shown in Fig. 4 (left), the reduced MER in XP30RO/pol η cl5 and XP30RO/pol η cl6 cells relative to XP30RO cells was due to the decrease of mutation induction at both CG and TA base pairs, −2.5–(p < 0.001) and 2.8-fold (p < 0.02), respectively. Among mutations at CG base pairs, the CG → TA base substitutions were the most prevalent (Table II) and therefore contributed more to the reduction of MER in pol η-complemented clones than any other type of CG mutations (p < 0.001). For TA base pairs, it is especially the level of TA → CG transitions that was diminished, by 5.5-fold for XP30RO/pol η cl5 cells and 3.1-fold for XP30RO/pol η cl6 cells. These results show that pol η protects cells against UV-induced transitions at both CG and TA base pairs.

Type of Base Substitutions and Mutation Error Rates in UV-irradiated Cells—When the host cells were UV-exposed
before transfection with UV-damaged plasmids (Table II and Fig. 4, right), the total mutation error rate was again much higher in XP30RO cells than for the complemented clones (3.1 and 6.6 times, respectively) and the wild type clone (3.1 times).

The major effects of UV irradiation of host cells were a substantial increase in the mutation error rate for CG\textsuperscript{3}TA transitions in all cell lines (Table II), and a large increase in TA\textsuperscript{3}GC (7 times), TA\textsuperscript{3}AT (4 times), and CG\textsuperscript{3}AT (4 times) transversions that was found exclusively in the XP30RO cells. Complementation with pol\textsuperscript{\gamma}/H9257 protected the cells from this significant increase in transversions, which was much lower in both corrected clones and normal MRC5 cells. As found in unirradiated cells, the UV-treated XP30RO/pol\textsuperscript{\gamma}/H9257 cl5 cells displayed a reduction (2.8 times) of MER at CG base pairs compared with irradiated XP30RO cells, and an even stronger decrease (8.5 times) was observed in XP30RO/pol\textsuperscript{\gamma}/H9257 cl6 cells (Fig. 4, right). The difference between the corrected clones was largely attributable to the rate of CG\textsuperscript{3}TA base substitutions that was lower in XP30RO/pol\textsuperscript{\gamma} cl6 than in XP30RO/pol\textsuperscript{\gamma} cl5 and MRC5 normal cells (Table II). Some clonal variations during growth under antibiotic selection may occur when selecting for individual clones, and these can confer unknown specific advantages for a given clone. These variations could be associated with the greater protection against CG\textsuperscript{3}TA mutations in the XP30RO/pol\textsuperscript{\gamma} cl6 clone. On the other hand, the MERs at TA base pairs in irradiated pol\textsuperscript{\gamma}-complemented clones and in MRC5 cells were very similar and were substantially reduced compared with that in irradiated XP30RO cells (5 times). Taken together, these results demonstrate that the expression of the pol\textsuperscript{\gamma} in UV-irradiated XP-V cells also leads to a significant drop of mutations both at TA base pairs and CG base pairs.

### Table I

| UV rays on plasmids\(^b\) | XP30RO | XP30RO/pol\textsuperscript{\gamma} cl5 | XP30RO/pol\textsuperscript{\gamma} cl6 | MRC5 |
|--------------------------|--------|-------------------------------|--------------------------------|------|
| Independent mutant\(^c\) | 10     | 71                            | 5                              | 43   |

### Table II

| Unirradiated cells              | XP30RO | XP30RO/pol\textsuperscript{\gamma} cl5 | XP30RO/pol\textsuperscript{\gamma} cl6 | MRC5 |
|---------------------------------|--------|---------------------------------|---------------------------------|------|
| Transition                      |        | 67                              | 60                              | 112  |
| TA\textsuperscript{\gamma} / CG |        | 13                              | 12                              | 22   |
| Transversion                    |        | 12                              | 11                              | 20   |
| CG\textsuperscript{\gamma} / GC | 15     | 17                             | 15                              | 24   |
| TA\textsuperscript{\gamma} / GC | 2      | 2                              | 3                               | 2    |
| Total                           | 111    | 181                            | 131                             | 75   |

## References

- Human Polymerase\textsuperscript{\gamma} and Replication of UV Damage in Vivo
- Table I
- Table II
**Mutational Distribution**—The location of mutations along the lacZ' locus is presented in Fig. 5. UV-induced mutations were not scattered along the lacZ' locus and formed several hotspots (Fig. 5, light gray nucleotides) that were determined by the Poisson distribution \( p < 0.01 \) according to the total number of possible mutation sites (146 sites) shown in Fig. 5E. There was some overlap in the site specificity and the distribution by class of mutations. Mutations at some hotspots were absolutely specific, e.g., G at position 49 was mutated exclusively to A, whereas others were more variable, for example A at position 73 was mutated to C, G, and T in unirradiated XP30RO/pol η cl5 cells, reflecting a random base misincorporation during the mutagenic bypass process.

The comparison of hot spot distributions showed one common strong hot spot between spectra for all cell lines at position 49. If we exclude this common mutated sequence, comparative analysis of the base substitution localization revealed that the two spectra from XP30RO and XP30RO/pol η cl5 cells did not share the same distribution when cells were exposed to UV irradiation. Application of the Adams-Skopek test confirmed that the pairwise spectra comparison was significantly different \( p = 0.031 \). Likewise, there were significant variations when we compared the mutation spectra recovered either in unirradiated and irradiated XP30RO cells or in unirradiated and irradiated XP30RO/pol η cl5 cells \( p = 0.044 \) and \( p = 0.018 \), respectively. On the other hand, the comparison between the mutation patterns obtained from unirradiated XP30RO, XP30RO/pol η cl5, or XP30RO/pol η cl6 cells did not show a statistical difference between these distributions, as already observed for the distribution of the type of mutations (Table II).

To evaluate the signature of pol η-dependent DNA synthesis *in vivo*, we compared the site specificity of sequence alterations generated by replication of UV-irradiated vectors in the pol η-deficient XP30RO cells with those found in the XP30RO/pol η cl5 and cl6 and MRC5 cells. Knowing that mutations induced by UV occur essentially at the 3' site of 5'-pyrimidine/pyrimidine sequences, we determined the mutation error rates at 5'-TC, 5'-CC, 5'-TT, and 5'-CT sites (where underlined nucleotide was the mutated site), and we correlated them with the type of mutations in unirradiated and UV-irradiated cells (Fig. 6). As expected for UV mutation spectra, the highest levels of MER were found at 5'-TC and 5'-CC but also at 5'-TT for the XP30RO cells. Irrespective of the pol η status, the 3'-cytosines in a 5'-TC sequence context were the most mutagenic sites, and more than 78% of MER was due to the CG → TA transitions (Fig. 6A). A similar observation could be made for 5'-CC sites, except in UV-irradiated XP30RO cells, in which the CG → AT transitions contributed to 28% of MER. Thus, some misinsertions of T opposite the 3' of a 5'-CG site could explain the large increase in CG → AT transversions (4 times), only found in XP30RO cells after UV irradiation of the host cells. This result is consistent with previous UV mutagenesis studies on the HPRT gene in XP-V cells and cell extracts on lacZ' gene (33, 35) that pointed out a significantly higher frequency of mutants with CG → AT transversions. Likewise, for mutations at T residues, the type of mutations in UV-irradiated XP30RO cells differed strongly from those in pol η-positive cells, with TA → AT \((41 \times 10^{-5})\), TA → CG \((17 \times 10^{-5})\), and TA → GC \((13 \times 10^{-5})\) contributing to base substitutions at TT site (Fig. 6B). This result demonstrates that, after UV irradiation and in the absence of pol η, some DNA polymerases are able to carry out error-prone bypass of TT lesions by misincorporating a G, T, or C opposite the lesion. Therefore, the expression of pol η in normal cells enables them to avoid mutagenesis mainly at TT photoproducts and also to protect against CG → AT transversions at CC lesions.

**DISCUSSION**

To study the effect of the DNA polymerase η *in vivo*, we complemented XP30RO, a cell line isolated from an XP variant patient, characterized by inherited mutations leading to a very small truncated protein (26, 46). This cell line can be considered as a null cell for pol η. The expression of the wild type pol η driven by the cytomegalovirus promoter complements all the abnormal features of XP-V cells we tested. We isolated two stable functional pol η-complemented clones (cl5 and cl6) that exhibit normal UV survival in the presence of caffeine, normal cell cycle kinetics after UV irradiation, and normal level of mutagenesis induced by UV light.

The use of a shuttle vector system enabled us to study the mutation characteristics of various DNA polymerases involved in DNA synthesis of undamaged or UV-irradiated templates but also to study independently the consequences of UV irradiation of the host cells on replication of unirradiated or UV-irradiated plasmid templates. Our results provide *in vivo* evidence that pol η prevents error-prone bypass during replication of UV-damaged templates and affects the level of mutations during exogenous stress. The protection factor by pol η is around 2–8-fold for mutations at both TA and GC base pairs, although the mutation spectrum of the lacZ' target remains largely dominated by CG to TA transitions. The protection effect is less evident in untreated cells than in UV-irradiated cells, implying that pol η is the major non-mutagenic polymerase for all UV photoproducts.

It is interesting to consider which polymerases might be responsible for the error-prone bypass of UV damage in the...
absence of pol \( \eta \). One obvious candidate is DNA polymerase \( \iota \) (pol \( \iota \)), a human paralog of pol \( \eta \) (54, 55). Although not able to replicate past UV photoproducts on its own, in combination with pol \( \iota \) it is able to bypass both CPD and 6-4PP \textit{in vitro} (56, 57). Furthermore pol \( \iota \) preferentially inserts T or G opposite the 3'-T of a T = T CPD (58) consistent with the increased TA to AT and TA to CG mutations that we have found in XP-V cells and with the idea that pol \( \iota \), in association with other factors, may contribute to the induction of mutations that we observed in XP30RO cells.

UV irradiation of host cells prior to shuttle vector transfection strongly increases the mutation frequency on undamaged templates. This increase is independent of pol \( \eta \) because we found roughly the same level of mutations in the four cell lines. It suggests that UV insult to host cells can lead to abnormal replication of unirradiated vectors in a pol \( \eta \)-independent manner. The mechanism involved in this error-prone pathway is unknown but is unlike the one acting on UV-irradiated templates because the type of mutations are different with the two classes of templates. In brief, base substitutions, which are the predominant mutations generated after replication using undamaged templates, were distributed differently along the \( \text{lac}^Z \) gene in the unirradiated versus UV-irradiated cells. Because UV irradiation results in replication complexes stalling at sites of DNA damage in the genome, this might alter the balance of error-prone to error-free polymerases available for

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**Fig. 5. Localization of independent base substitutions on the \( \text{lac}^Z \) mutagenesis target.** For each mutational spectrum (A–D), single and tandem (underlined) mutations above the wild type sequence were generated by replication of UV-treated plasmid in unirradiated cells (No UV) and those below in UV-irradiated cells (UV). For a better visualization of the spectra, some portions to the left and right of the wild type sequence containing a few mutations scattered away from the hotspot-containing target sequences were not represented here but are included in the data analysis. The hotspot sequences of UV-induced mutations (dark gray nucleotides) were determined by the Poisson distribution with a probability less than 1% for each spectrum in comparison with a theoretical spectrum compiling all mutations described in this paper (E). This spectrum corresponds to the positions of mutations found in all UV-induced spectra obtained here (211 compiled mutations at 146 sites; 88% at dipyrimidine sites). A, XP30RO cells; No UV, 111 mutations at 55 sites; UV, 85 mutations at 61 sites; B, XP30RO/pol \( \eta \) cl5 cells; No UV, 131 mutations at 59 sites; UV, 40 mutations at 26 sites; C, XP30RO/pol \( \eta \) cl6 cells; No UV, 90 mutations at 51 sites; UV, 72 mutations at 47 sites; D, MRC5 cells; No UV, 65 mutations at 36 sites; UV, 44 mutations at 31 sites.
replicating extrachromosomal plasmids. It is possible that this high untargeted mutation rate could be linked either to a less accurate fidelity of classical replication polymerases or to the activation of a mutagenic polymerase different from pol $\eta$. Un-targeted mutagenesis on unirradiated plasmids may be the result of an error-prone damage tolerance that is activated by host cell damage, an observation that is reminiscent of the $dinB$ (pol IV)-dependent untargeted mutagenesis observed in phage-bacteria systems (59). Thus, a potential candidate for the UV-untargeted mutagenesis could be the product of the human homolog of DinB, DNA polymerase $\kappa$, which has relatively low fidelity and moderate processivity during copying of an undamaged DNA template (60). In vivo, an increase in point mutations in the $HPRT$ locus has been observed after transient expression of the mouse pol $\kappa$ cDNA in cultured mouse cells (61).

Irradiation of both shuttle vectors and host cells leads to the highest mutation frequency in the pol $\eta$-deficient line. Complementation by pol $\eta$ reduces strongly the mutation frequencies by 2–8-fold with a similar decrease for mutations at CG and TA base pairs to that in wild type cells. Although we did not find the same in vivo mutation spectrum for XP30RO cells as that previously described by Wang et al. (34, 35) for their own XP-V cells (XP115LO and XP4BE), our results performed with isogenic XP-V and pol $\eta$-complemented cells confirm their conclusion that XP-V cells are less likely than normal cells to incorporate dAMP and dGMP opposite UV photoproducts containing T and C, respectively. In pol $\eta$-complemented cells, as expected from biochemical experiments using T = T CPDs (24–26) and from UV-induced mutation spectra in mammalian cells, UV damage at TA base pairs is only weakly mutagenic (10–12). On the other hand, the requirement for pol $\eta$ in error-free bypass of UV lesions containing a C residue is consistent with a similar conclusion previously derived from genetic analysis in yeast (62). However, some substantial levels of shift from 5'-TT to 5'-TC sites and 5'-CC to 5'-CT sites occur, as revealed here in mutation spectra, even in pol $\eta$-proficient cells. As proposed recently (12), C to T mutations may result from “correct” bypass by pol $\eta$ of CPDs containing deaminated cytosine or 5-methylcytosine. Deamination of cytosine to uracil will cause the formation of 5'-TU dimers from 5'-TC dimers or 5'-CU dimers from 5'-CC dimers and leads to the incorporation of A during translesion synthesis by pol $\eta$ in wild type cells. Thus, pol $\eta$ may act as both an error-free and mutagenic DNA polymerase, especially after UV activation by relocalization at sites of DNA damage (43).

It is clear from this study that pol $\eta$ is able to protect us from UV-induced mutagenesis in vivo, consistent with the cancer-proneness of XP-V patients. As pol $\eta$ reduces the mutation frequency without substantially altering the mutation spectrum, one would not necessarily predict a difference in the mutation spectra in tumors. Indeed, studies of mutation spectra on the p53 gene in 18 non-melanoma skin cancers isolated from XP variant patients showed that types and distribution of the mutations were different from those found in tumors from excision repair-deficient XP individuals but similar to those isolated from DNA repair-proficient patients (63).

Acknowledgments—We thank Prof. J. C. Ehrhart for critical reading of the manuscript and C. Debaker and C. Pouvelle for technical assistance.

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