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Recovery of Normal DNA Repair and Mutagenesis in Trichothiodystrophy Cells after Transduction of the *XPD* Human Gene¹

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ABSTRACT

To determine whether expression of the *XPD/ERCC2* repair gene in trichothiodystrophy (TTD) group D cells could restore mutagenesis characteristics of repair-proficient cells, we compared the UV mutagenesis of normal cells, TTD group D cells, and TTD group D cells retrovirally transduced by the wild-type *XPD/ERCC2* gene (TTD + ERCC2 cells). We first verified the expression of the XPD protein, correction of UV cell survival, and DNA repair ability of TTD + ERCC2 cells. UV-induced mutations were studied using the pR2 shuttle vector. The addition of the *XPD/ERCC2* gene in TTD cells led to a significant but partial decrease of mutation frequency compared with the parental TTD cells. Types of mutations of TTD + ERCC2 cells get closer to those observed in normal cells (i.e., a reduction of multiple mutations). New hotspots appeared and some disappeared in the complemented line, suggesting that hotspot distribution is particular to each cell line and cannot be correlated with the repair status of the cells. In conclusion, the expression of the *XPD/ERCC2* repair gene completely corrected UV hypersensitivity and almost all types of mutations of TTD group D cells, whereas hypermutagenesis was partially corrected.

INTRODUCTION

The NER³ system is the fundamental mechanism of cell protection against the effects of various types of DNA damage induced by genotoxic agents. Deficiency in NER has been associated with TTD and XP, two clinically distinct human syndromes inherited as an autosomal recessive trait (for review, see Refs. 1-3). In the XP syndrome, persistence of unrepaired DNA damage produced by exposure to UV light is associated with photophobia and an extremely high level of skin tumors in sun-exposed sites. In contrast to XP patients, TTD patients do not have an increased frequency of skin cancers (4, 5) and show sulfur-deficient brittle hair and mental and physical retardation (6). Approximately 50% of TTD patients exhibit photophobia and a marked sensitivity to sunlight. The clinical photosensitivity in TTD patients is usually associated with an increase of both cellular UV sensitivity (7, 8) and UV-induced mutability (9, 10). The NER defect of photosensitive TTD patients has been assigned to three genetic complementation groups. The large majority of cases (>90%) fall into the same group as XP-D and together represent the TTD/XP-D group (11-13). One family was found to belong to XP-B group (14) and another kindred constitutes a distinct NER deficient group, TTD-A (15).

In several TTD/XP-D patients, molecular analysis of the *XPD/ERCC2* gene showed point mutations and deletions leading to amino acid substitutions or to a truncated XPD protein (16-18). The *XPD/*

ERCC2 gene encodes a 760-amino acid protein with a predicted molecular weight of 86,900 (19). The purified XPD protein was shown to possess an ATP-dependent DNA 5'→3' helicase activity (20) and to be associated with the basic transcription factor II (TFIIH) complex (21).

The introduction of the functional *XPD/ERCC2* cDNA in TTD and XP-D cells, thanks to plasmidic (22-25) or retroviral vectors (26) led to the restoration of wild-type levels of UV sensitivity and DNA repair. Moreover, UV hypermutability of XP-D cells was partially corrected in two *XPD/ERCC2*-transformed XP-D cell lines after plasmid transfer (24).

To examine whether the complementation of TTD/XP-D cells with the wild-type *XPD/ERCC2* gene also concerned UV mutagenesis, we used a TTD/XP-D cell line previously characterized in our laboratory with respect to its UV-C mutagenesis properties (10). In the present study, this cell line was transduced with the LXPDSN retroviral vector carrying the wild-type *XPD/ERCC2* repair gene as described previously (26) and was called here the TTD + ERCC2 cell line. We first verified, after UV irradiation, the correction of UV hypersensitivity by colony-forming ability and the restoration of the wild-type DNA repair system by UDS. Then, the pR2 shuttle vector carrying the *lacZ'* target gene was used to compare UV-C mutagenesis characteristics of TTD/XP-D, TTD + ERCC2, and normal cell lines. We found that the expression of the *XPD/ERCC2* gene in the TTD/XP-D cells led to a mutation frequency showing intermediate values between the TTD parental line and the normal line. In addition, the types of mutations found in TTD + ERCC2 cell line were closer to those in normal cell line than to those in parental TTD cell line.

MATERIALS AND METHODS

Cells and Cell Transduction. Human fibroblasts were cultured in Eagle's MEM supplemented with 10% FCS, 2.5 μg/ml fungizone, and antibiotics (100 units/ml penicillin, 100 μg/ml streptomycin, and 0.25 μg/ml hygromycin B (Sigma) for the TTD and TTD + ERCC2 cell lines).

We used the human SV40-transformed MRC-5V1 DNA repair-proficient fibroblast line (27), provided by Dr C. Arlett (Brighton, England). The SV40 LT-antigen-transformed TTD1VI-LAS-KMT11 cloned fibroblast cell line (TTD/XP-D cells) has been established in our laboratory from a skin biopsy of a 9-month-old French TTD patient (10). The retrovirus producer cell line Ψ-CRIP-LXPDSN (26) was used to transfer the *XPD/ERCC2* cDNA into the TTD1VI-LAS-KMT11 cells. Preconfluent Ψ-CRIP-LXPDSN cells were grown for 24 h in fibroblast medium. This conditioned medium containing the virus was filtered through 0.22 μm nitrocellulose membrane and added to TTD1VI-LAS-KMT11 medium with 8 μg/ml polybrene (Sigma) for 48 h. Transduced cells were selected for 15 days with 1 mg/ml neomycin-analog G418 (Life Technologies, Inc.). G418-resistant cells were seeded in 10-cm Petri dishes to clonal density. All 12 isolated clones were UV resistant and were tested for their efficiency to replicate the pR2 shuttle vector, leading to the selection of the complemented TTD1VI-LAS-KMT11-D10 clone, called here the TTD + ERCC2 cell line. Mutagenesis assays on this cell line were performed during the month immediately following the G418-selection.

Cell Survival Study and Unscheduled DNA Synthesis. Cell survival after UV-C treatment was measured by the colony-forming ability procedure. Growing cells were UV irradiated under a germicidal lamp (254 nm) with a dose rate of 0.36 J · m⁻² · s⁻¹ and then plated at low density (100 cells per 10-cm Petri dish) to measure their colony-forming ability (28).

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³ The abbreviations used are: NER, nucleotide excision repair; TTD, trichothiodystrophy; XP, xeroderma pigmentosum; ERCC, excision repair cross complementing; UDS, unscheduled DNA synthesis.

Analysis of repair synthesis after UV-C irradiation was carried out by the UDS method, as described elsewhere (10).

Western Blotting. Western blot analysis was carried out using 50 μg of proteins as described previously (26). The monoclonal anti-XPD antibody, generously provided by Dr J. M. Egly (Strasbourg, France), was raised against amino acids 749–759 at the carboxy-terminal end of the human XPD protein. The intensities of the bands were quantitated using a Samba IPS 4.02 (Alcatel) bioimage analyser.

Plasmid UV Treatment, Transfection, and Mutational Assay. The pR2 shuttle vector contains the SV40 replication origin that allows its replication in human cells in the presence of the SV40 LT-antigen. The pR2 plasmid also harbors the pBR322 replication origin and the kanamycin resistance gene, which allows the selection of the pR2 plasmid in bacteria, and the 168-bp *lacZ'* gene, which is used as target gene for mutation analysis (10). The pR2 plasmid was irradiated with 254-nm UV light using a germicidal lamp at the dose rate of $2 \text{ J} \cdot \text{m}^{-2} \cdot \text{s}^{-1}$. Transfections of 5–10 μg , according to the cells, of UV-treated or untreated pR2 vectors into the three human cell lines were performed for 6 h using the calcium phosphate precipitation method (29). Five μg of unirradiated p205-KMT11 plasmid (30) were cotransfected with the pR2 plasmid into MRC-5V1 and TTD + ERCC2 fibroblasts. Cells were then incubated for 2–3 days, according to the ability of the cells to replicate the plasmid, in medium containing 100 μM ZnCl_2 and 1 μM CdCl_2 , which allows an overexpression of the SV40 LT-antigen gene carried by the p205-KMT11 vector and leads to a very efficient replication of the pR2 plasmid inside the human cells. TTD cells were not cotransfected with the p205KMT11 plasmid because these cells already contained the vector (10), and the pR2 plasmid was efficiently replicated.

Replicated plasmid DNA was recovered from the human cells by a small-scale alkaline lysis procedure (30), and the DNA preparations were then treated with *DpnI* restriction enzyme to degrade any input plasmid still carrying the bacterial methylation patterns. Rescued plasmids were used to transform the recombination-deficient *E. coli* DH5 α MCR (Bethesda Research Laboratories) plated on selective medium (31). The white or light blue bacterial colonies indicating an inactivated *lacZ'* gene were isolated as described previously (10). Sequence analysis of the *lacZ'* gene was performed by the chain elongation termination method on double-strand DNA using Sequenase 2.0 kits (United States Biochemical Corp.) using a specific primer.

Statistical Study. Differences in proportions were tested by the χ^2 test or by exact tests. A result was considered nonsignificant when its associated *P* value was above 0.05. Hotspot determination was carried out according to the Poisson law at a probability of less than 1%, as described previously (10).

RESULTS

Cell Characterization

XPD Protein. Expression of the XPD protein was monitored by Western blot hybridization in the TTD1VI-LAS-KMT11 fibroblasts (called here TTD cells) in the TTD1VI-LAS-KMT11-D10 fibroblast clone that contains the human wild-type DNA repair *XPD/ERCC2* gene (called here TTD + ERCC2 cells) and in MRC-5V1 repair-proficient human fibroblasts (normal cells; Fig. 1). The bands detected with the monoclonal anti-XPD antibody in proteins from normal and TTD + ERCC2 cells were located at the expected position of 87 kDa.

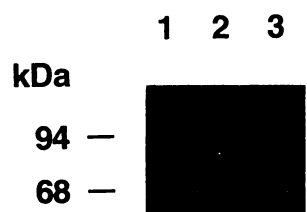


Fig. 1. Western blot analysis of the XPD/ERCC2 protein in normal, TTD, and TTD + ERCC2 cells. Fifty μg of protein were used for electrophoresis. The XPD protein was detected using a monoclonal antibody raised against the carboxy-terminal part of the protein. Lane 1, normal cells; Lane 2, TTD cells; Lane 3, TTD + ERCC2 cells. The unique band observed migrates at the expected value for the XPD protein.

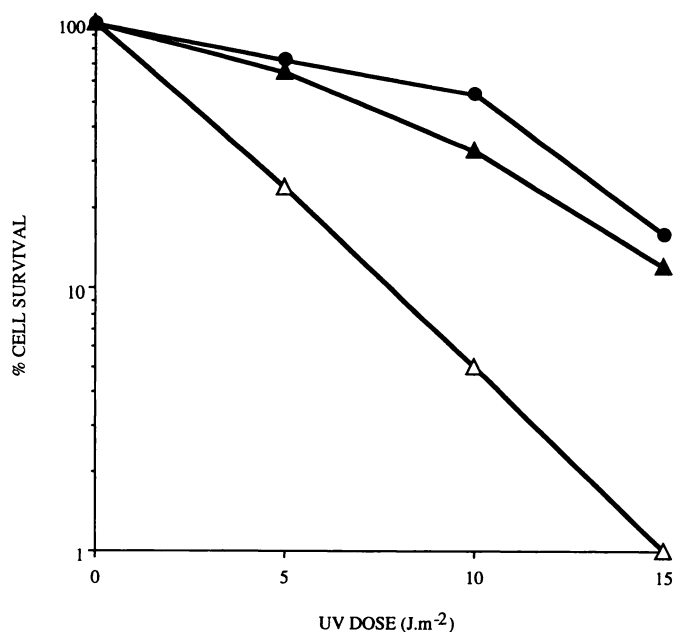


Fig. 2. UV-C cell survival of normal (●), TTD (Δ), and TTD + ERCC2 (▲) human cell lines. After UV irradiation of growing cells, UV cell survival was measured by colony-forming ability. The cloning efficiencies of the three unirradiated cell lines were 94, 85, and 81%, respectively.

Densitometric scans showed that the amount of the XPD protein isolated from TTD + ERCC2 cells was 2-fold higher than the amount isolated from normal cells. The protein was not detected in TTD fibroblasts.

Cell Survival and DNA Repair. Normal, TTD, and TTD + ERCC2 cells were tested for determining their colony-forming ability after UV irradiation (Fig. 2). We observed that the TTD + ERCC2 cell line showed an increased cell survival following UV irradiation compared with its parental TTD cell line and also a cell survival similar to normal cells.

The analysis of the DNA repair capacity of the TTD + ERCC2 cells performed by UDS was similar to that of normal cells and showed a striking higher level of DNA repair compared with TTD cells (Fig. 3). These results indicated that the TTD + ERCC2 cell line recovered the two essential properties of repair-proficient cells (*i.e.*, UV cell survival and DNA repair ability).

Plasmid Mutagenesis

Plasmid Mutation Frequency. The ratio of the number of bacterial colonies containing the inactivated *lacZ'* gene to the total number of bacterial colonies defined the mutation frequency on the *lacZ'* gene.

The spontaneous mutation frequency was low in the three cell lines, ranging from 0.8×10^{-4} to 7.9×10^{-4} (Table 1). However, these mutation frequencies were about 10-fold higher in the TTD + ERCC2 cell line and in the TTD cell line than in the normal cell line ($P < 0.05$). Thus, both the TTD + ERCC2 and TTD cell lines had a higher mutation background than the normal cell line.

Fig. 4 shows that the mutation frequency increased linearly with the UV dose (ranging from 100 to $1000 \text{ J} \cdot \text{m}^{-2}$) in all three cell lines. At all UV doses, the TTD + ERCC2 cell line showed a lower mutation frequency compared with the parental TTD cell line. However, the mutation frequency of the TTD + ERCC2 cells was still higher than for normal cells. For example, at a plasmid treatment with $1000 \text{ J} \cdot \text{m}^{-2}$, the mutation frequency of the TTD + ERCC2 cell line (58×10^{-4}) was significantly lower than for the TTD cell line

(130×10^{-4} ; $P = 0.0001$) and higher than for the normal cell line (26×10^{-4} ; $P = 0.0001$).

Mutation Analysis. Mutations inactivating the *lacZ'* gene were all characterized by DNA sequencing. We separated the mutated plasmids into two classes according to their nature, *i.e.*, a mutated plasmid containing point mutations on one hand and a mutated plasmid containing DNA rearrangements on the other hand. Point mutations include either one base (single mutation), two or three adjacent bases (tandem mutation), or several bases distant from each other (multiple mutation) or else frameshifts. DNA rearrangements were constituted of insertion, deletion or duplication of three or more nucleotides.

Two hundred six independent mutated plasmids from the TTD + ERCC2 cell line, 177 independent mutated plasmids from the TTD cell line, and 108 independent mutated plasmids from normal cell line were recovered. We found that the UV-induced mutated plasmids rescued from the three cell lines contained mostly point

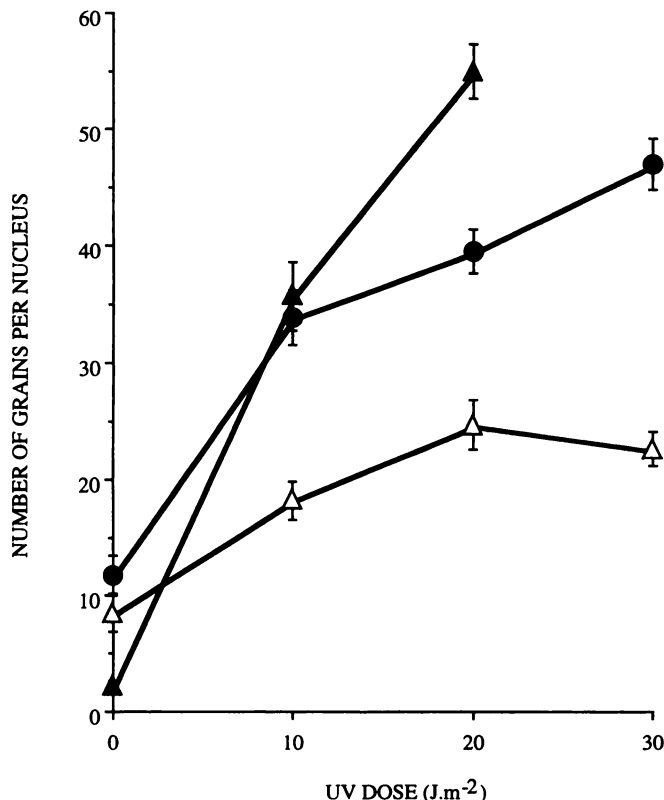


Fig. 3. Level of UDS induced by increased doses of UV-C in normal (●), TTD (Δ), and TTD + ERCC2 (▲) cell lines. UDS was quantified by the number of grains per nucleus as a function of UV dose. Bars, SE.

Table 1 Spontaneous frequency of mutated plasmids following replication of the untreated pR2 vector in human cell lines

The untreated pR2 shuttle vector was transfected into normal, TTD, and TTD + ERCC2 cell lines. Two to 3 days after replication, the plasmids recovered from human cells were used to transform indicator bacteria plated on kanamycin medium to measure mutations inactivating the *lacZ'* gene. The number of independent transfections was 5 for TTD + ERCC2, 19 for TTD, and 24 for the normal cell line.

Cell line	Total no.		
	Colonies	Mutants	Mutation frequency ($\times 10^4$)
Normal	26643	2	0.75
TTD + ERCC2	22550	13	5.76 ^a
TTD	13988	11	7.86 ^a

^a The value is statistically different from that found for normal cells, with $P < 0.05$ in Fisher's exact test. There is no statistical difference between TTD and TTD + ERCC2 cell lines in a χ^2 test.

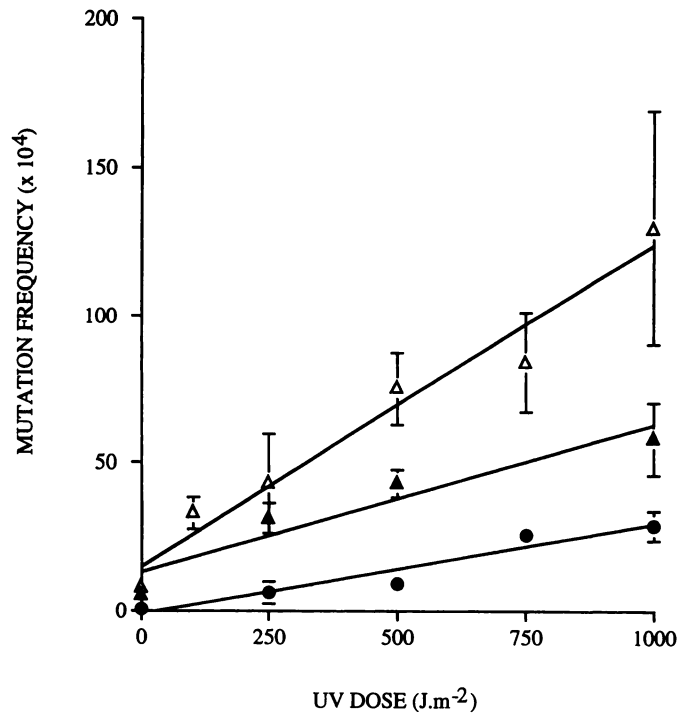


Fig. 4. Mutation frequency of UV-irradiated shuttle vector pR2 propagated in normal (●), TTD (Δ), and TTD + ERCC2 (▲) cell lines. The frequency corresponds to the number of mutants determined by their color (white or light blue) and, after sequencing, divided by the total number of kanamycin-resistant bacterial colonies. Data points, mean values from 5–24 independent transfection experiments; bars, SE. (Bars not shown are smaller than the symbols.)

mutations (91% for TTD + ERCC2 cells, 84% for TTD cells, and 97% for normal cells). In addition, a large part of the mutated plasmids showed single mutations (Fig. 5). Normal and TTD + ERCC2 cells had similar levels of mutated plasmids with single mutations (83 and 76% of the independent vectors with base substitutions, respectively), whereas TTD cells exhibited a significantly lower level of plasmids with single base substitutions (69%) than normal cells ($P = 0.01$; Fig. 5).

The proportion of multiple mutants was significantly higher in TTD cells (20%) compared with normal cells (8%; $P = 0.01$) and with TTD + ERCC2 cells (11%; $P = 0.01$; Fig. 5).

The types of single, tandem and multiple base substitutions are shown in Table 2. With all three cell lines, every type of base substitution was observed. A majority of transitions were found (69–78%), and most of them were G:C→A:T transitions (63–73%). A:T→C:G transversions were very rare for all three cell lines (1–3%). No significant difference between TTD and normal cells was found in the proportion of each type of transition and each type of transversion. The addition of the wild-type *XPD/ERCC2* gene into TTD cells did not change the type of UV-induced base substitution: no significant difference could be observed between TTD + ERCC2 cells and TTD cells or between TTD + ERCC2 cells and normal cells.

The CC→TT tandem base substitution, a signature of UV-induced lesions (32, 33), constituted a large part of tandem base substitutions induced by UV-C for all three cell lines: 50% of tandem substitutions were CC→TT for TTD + ERCC2 cells, 69% for TTD cells, and 64% for normal cells. No statistical difference was found between the three cell lines in a χ^2 test.

Table 3 shows the frequency of spontaneous or UV-induced mutants that contained DNA rearrangements. This type of mutation has been shown to be characteristic of TTD mutagenesis (10). For the three cell lines, no statistical difference was found between sponta-

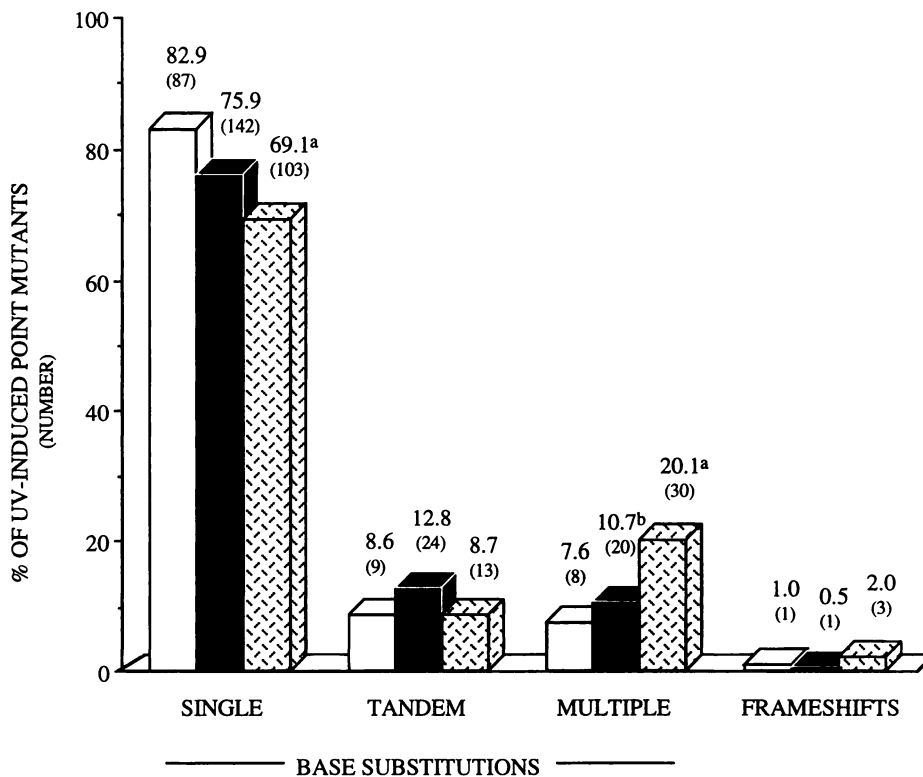


Fig. 5. Nature of independent UV-induced mutated plasmids containing point mutations after replication of the pR2 shuttle vector in normal (□), TTD + ERCC2 (■) and TTD (▨) cell lines. For normal and TTD + ERCC2 cells, the pR2 plasmid was treated with 250–1000 J · m⁻². For TTD cells, the pR2 plasmid was treated with 100–1000 J · m⁻². The numbers given at the top of the histogram columns correspond to the percentage of UV-induced plasmids with point mutations. The number of mutants is given in parentheses. *a*, different from normal cells; *P* = 0.01. *b*, different from TTD cells; *P* = 0.01.

Table 2 Types of base substitution mutations in UV-irradiated pR2 replicated in human cell lines

There are no statistical differences, for any type of base substitution mutations, between the three cell lines in a χ^2 test.

Mutation type	No. of mutations (%)		
	Normal ^a	TTD + ERCC2 ^a	TTD ^b
Transitions	97 (77.6)	166 (68.6)	153 (73.2)
G:C→A:T	91 (72.8)	153 (63.2)	145 (69.4)
A:T→G:C	6 (4.8)	13 (5.4)	8 (3.8)
Transversions	28 (22.4)	76 (31.4)	56 (26.8)
G:C→T:A	15 (12.0)	33 (13.6)	28 (13.4)
G:C→C:G	5 (4.0)	11 (4.6)	11 (5.3)
A:T→C:G	1 (0.8)	7 (2.9)	5 (2.4)
A:T→T:A	7 (5.6)	25 (10.3)	12 (5.7)
Total	125 (100)	242 (100)	209 (100)

^a Treatment, 250–1000 J · m⁻².

^b Treatment, 100–1000 J · m⁻².

neous and UV-induced frequency of rearranged mutants. This suggests that DNA rearrangements were not the consequence of UV irradiation. For the untreated or UV-treated pR2 plasmid, the frequency of mutants with rearrangements obtained with TTD and TTD + ERCC2 cells was at least 9-fold higher than that obtained with normal cells (*P* < 0.01).

Mutation Spectra. The distributions of all UV-induced base substitutions found in the *lacZ'* gene using TTD + ERCC2, TTD, and normal cell lines are shown in Fig. 6. We observed that almost all UV-induced base substitutions occurred at dipyrimidine sites (95% in the TTD + ERCC2 cells, 93% in the TTD cells, and 97% in normal cells).

The base substitutions were not scattered along the gene but formed several hotspots (Fig. 6, *light gray nucleotides*) that were determined by the Poisson probability distribution (*P* < 0.01%). We found 13 hotspots for TTD + ERCC2 cells, 9 hotspots for TTD cells, and 7 hotspots for normal cells. Five hotspots were common to the three cell lines; they are located at positions -11, 17, 43, 49, and 91. All of these hotspots did not share a common primary sequence.

In the TTD + ERCC2 cell line, most of the hotspots found only in TTD cells were still present (40, 71, and 95 bp). Hotspots at positions 79 and 92 were found in TTD + ERCC2 cells and in normal cells but not in TTD cells. Three new hotspots appeared in the TTD + ERCC2 cell line at positions -10, 94, and 127 (Fig. 6, *dark gray nucleotides*).

DISCUSSION

The TTD syndrome is an autosomal recessive disorder characterized by brittle hair with a reduced sulfur content. Clinical photosensitivity is present in about 50% of TTD patients but is not associated with an elevated frequency of cancers. The fibroblasts from these TTD patients exhibit features of hypersensitivity to killing by UV-C and reduced UDS similar to cells from patients with XP-D and UV-hypermutable (4, 10). The introduction of the wild-type *XPD/ERCC2* gene by a plasmidic vector into TTD/XP-D cells restored a normal colony-forming ability of cells following UV-C exposure and a normal DNA repair capacity as well (23).

Here, we showed that some mutagenesis characteristics of repair-proficient cells were restored in TTD/XP-D cells complemented by the wild-type *XPD/ERCC2* gene. We used a clone isolated from the human TTD1VI-LAS-KMT11 fibroblast cell line transduced with a retroviral vector carrying the wild-type *XPD/ERCC2* repair gene

Table 3 Frequency of mutated plasmids containing DNA rearrangements following replication of the untreated or UV-treated pR2 vector in human cell lines

Treatment		Normal ^a	TTD + ERCC2 ^a	TTD ^b
Untreated	Colonies	26643	22550	13988
	Mutants	1	8	9
	Frequency, ×10 ⁴	0.4	3.5	6.4
UV-treated	Colonies	107742	55323	18724
	Mutants	3	19	16
	Frequency, ×10 ⁴	0.3	3.4	8.5

^a The pR2 plasmid was treated with doses from 250 to 1000 J · m⁻².

^b pR2 plasmid was treated with doses from 100 to 1000 J · m⁻².

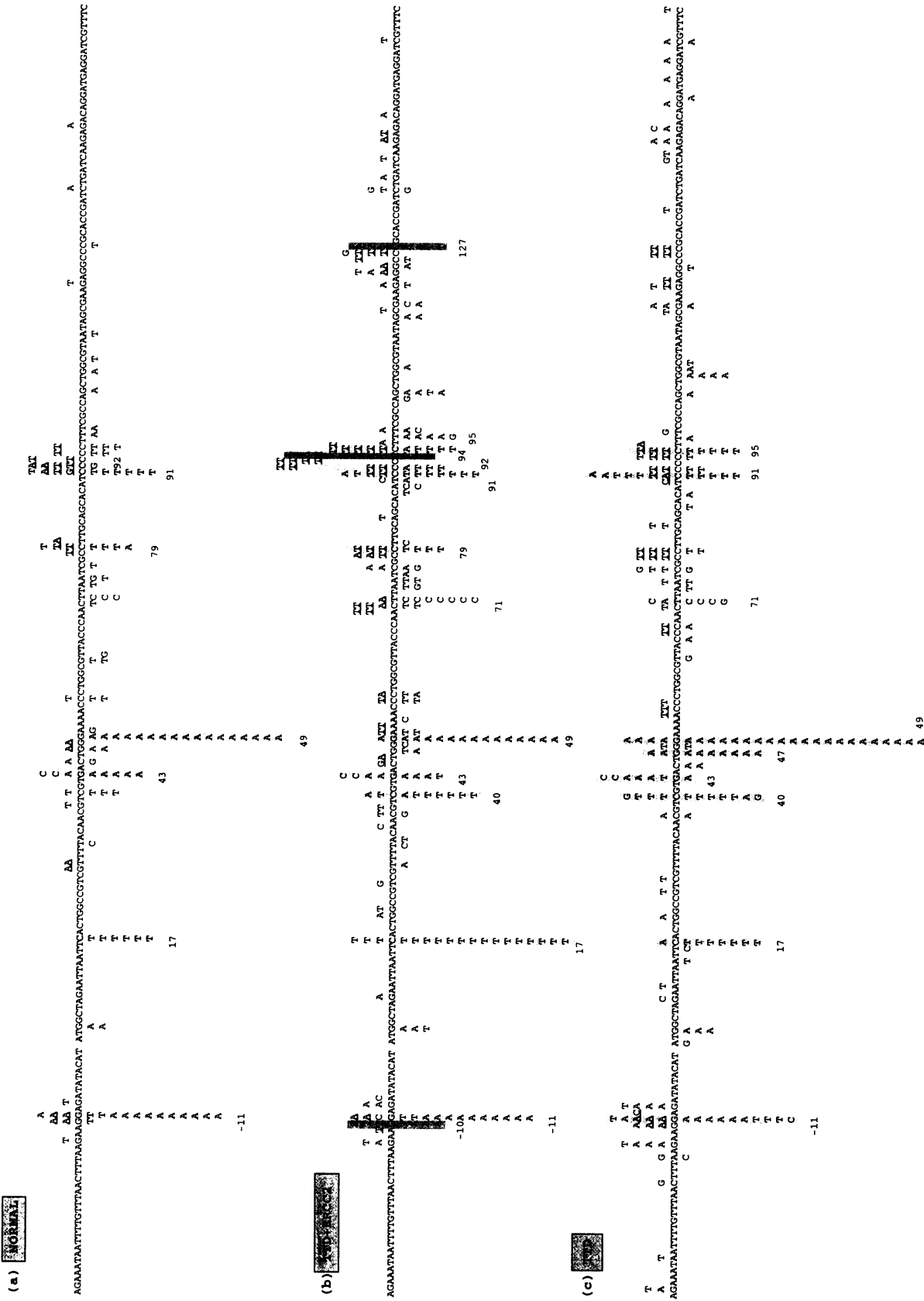


Fig. 6. Location of independent base substitutions on the coding strand of the *lacZ'* gene. Each letter represents a base substitution found in an independent mutated plasmid. Single substitutions are located below the wild-type sequences. Tandem (*underlined*) and multiple mutations are found above the wild-type sequences. The mutation hotspots, defined by the Poisson probability distribution ($P < 0.01$) are indicated in *light gray*. Dark gray nucleotides represent the mutation hotspots that are found in only the TTD + ERCC2 cell line, not in the two other cell lines. Numbers under the hotspots indicate the bp number. *a*, mutation spectrum of the *lacZ'* gene for UV-irradiated pR2 in the normal cell line. One hundred twenty-five base substitutions were found in 108 independent mutated plasmids. At least five mutations for a given nucleotide site were necessary to consider the site a hotspot. Seven hotspots were observed, at bp -11, 7, 43, 49, 79, 91, and 92. *b*, mutation spectrum of the *lacZ'* gene for UV-irradiated pR2 in the TTD + ERCC2 cell line. Two hundred forty-two base substitutions were found in 206 independent mutated plasmids. At least seven mutations for a given nucleotide site were necessary to consider the site a hotspot. Thirteen hotspots were observed, at bp -10, -11, 17, 40, 43, 49, 71, 79, 91, 92, 94, 95, and 127. *c*, mutation spectrum of the *lacZ'* gene for UV-irradiated pR2 in the TTD cell line. Two hundred nine substitution mutations were induced in 177 independent mutated plasmids. At least six mutations for a given nucleotide site were necessary to consider the site a hotspot. Nine hotspots were observed, at bp -11, 17, 40, 43, 47, 49, 71, 91, and 95.

(TTD + ERCC2 cells). This clone recovered a UV cell survival similar to that of normal cell line (Fig. 2), and the defect in UDS after UV treatment was corrected as compared with the parental TTD cell line (Fig. 3).

We showed that the XPD protein was expressed in the normal cell line and was mostly expressed in the TTD + ERCC2 cell line (Fig. 1). This result was also found by Carreau *et al.* (26), who obtained good signals corresponding to XPD protein, when using transduced G418-selected XP-D primary fibroblasts. These authors did not detect the expression of the XPD protein in repair-proficient untransformed primary skin fibroblasts, whereas in another study the protein could be detected in repair-proficient SV40-transformed fibroblasts (24). We failed to detect the XPD protein in the TTD cell line (Fig. 1). Takayama *et al.* (18) studied the nucleotide sequence of the *XPD/ERCC2* cDNA from the TTD1VI patient from whom the TTD cells we used derived (10). They showed that one allele of the gene exhibited a C→G transversion at bp 1459 leading to a Leu-461-to-Val substitution and a 45-bp deletion at bp 2224–2268 leading to a 15-amino acid deletion (amino acids 716–730) near the C-terminal part of the protein. The authors also found a point mutation on the other allele, which led to a protein having Arg-722 changed into Trp-722. Since the monoclonal antibody we used has been raised against amino acids 749–759 of the XPD protein, we can consider that in our TTD cell line, the protein expression was too low to be detected under our stringent conditions or that conformation of the mutated protein would not allow a good presentation of the epitope.

Recently, Cleaver *et al.* (34) showed that overexpression (about two-fold) of the *XPA* gene in XP-A cells or in repair-competent cells increased the UV survival of the cells to levels above those of normal cells. Thus, they concluded that a correlation exists between DNA repair capacity and level of XPA protein expression. However, a good correlation with UV cell survival but not with repair capacity has been shown in two XP-D clones expressing the XPD protein at different levels (24). In our case, the TTD + ERCC2 cell line showed a level of the XPD protein higher than in the normal cell line. However, the UV cell survival of the TTD + ERCC2 cells was not higher than that of normal cells, whereas the UDS in the TTD + ERCC2 cells was slightly increased at the UV dose of $20 \text{ J} \cdot \text{m}^{-2}$ (Figs. 2 and 3). Hence, overexpression of the XPD protein does not seem to induce a repair level higher than that observed in repair-proficient cells.

The sequence analysis of approximately 500 independent mutated plasmids from TTD + ERCC2, TTD, and repair-proficient cells revealed that the major UV-induced type of mutation was single base substitution (Fig. 5). This finding was already shown in numerous published works using either shuttle vectors or endogenous genes (for a review, see Ref. 35).

In the three cell lines, a large majority of G:C→A:T transitions (Table 2) were found, in agreement with earlier investigations, in which the G:C→A:T transition was the major type of UV-induced base substitution (for a review, see Ref. 35). Classes of UV-induced base substitutions were similar between TTD + ERCC2 and normal cell lines, as we already found between TTD and normal cells (10). Thus, the expression of the XPD protein in TTD cells did not change the proportion of base substitutions.

In previous works (10, 36), we noticed that UV mutagenesis of the TTD cell line was characterized, in part, by multiple base mutations. Here, we showed that expression of the wild-type XPD protein after addition of the *XPD/ERCC2* cDNA into the TTD cells led to a significant decrease of multiple mutants to a level similar to that of normal cells (Fig. 5). In turn, the level of single mutated plasmids that was lower in TTD cells (69%) than in normal cells (83%), was increased to a normal level (76%) after the introduction of the *XPD/ERCC2* repair gene. The process leading to multiple base substitu-

tions, which is still not understood, was overcome by the addition of the *XPD/ERCC2* gene. It is interesting to note that Gözükarar *et al.* (24) showed that the frequency of post-UV-mutated plasmids recovered with multiple base substitutions was lower in XP-D cells than in normal cells and remained low in the cell line transfected with the *XPD/ERCC2* gene.

The TTD + ERCC2 cells were characterized by a lower mutation frequency than the parental TTD cell line after UV irradiation (Fig. 4). Despite this result, the plasmid mutability of the TTD + ERCC2 cell line was not lowered to values similar to those of normal cells, as one could have expected from the expression of the wild-type XPD protein. The clonal G418 selection of the LXPSN-transduced cell line and mutagenesis assays performed immediately after this selection permit us to rule out the hypothesis that among the cells that replicate the pR2 plasmid, some may have lost the expression of the XPD protein. In addition, Quilliet *et al.* (37) recently showed the long-term reversion of DNA repair-deficient primary fibroblasts by the retroviral transduction of the *XPD/ERCC2* gene. We can suppose that the mutation frequency would be inherent in the TTD + ERCC2 and TTD parental cell lines. TTD and TTD + ERCC2 cells showed similar spontaneous mutation frequencies, 10-fold higher than the mutation frequency of normal cells (Table 1). Most of the spontaneous mutants contained DNA rearrangements, and we showed that mutants with DNA rearrangements obtained after UV irradiation could be due to spontaneous lesions, since their frequencies do not vary with the UV dose (Table 3). The level of spontaneous mutants was not decreased to that of normal cells in the TTD + ERCC2 cells (Table 3). Thus, the introduction of the *XPD/ERCC2* gene in the TTD cell line did not decrease the frequency of spontaneous mutations. This suggests that the intrinsic mutation background of TTD and TTD + ERCC2 cell lines should be independent of the NER system.

Concerning the distribution of mutation hotspots, Tornaletti and Pfeifer (38) suggested that hotspots could be generated by a slow DNA repair of lesions. In our case, the addition of the wild-type *XPD/ERCC2* repair gene in TTD cells failed to restore a pattern of mutation hotspot distribution similar to that observed in normal cells. This suggests that slow repair in TTD cells was not, or not only, the cause of mutation hotspots. This result was also found for XP-D (24) and XP-A cells (39). It is possible, as suggested by Seetharam *et al.* (40), that some differences in the distribution of the UV-induced mutation hotspots may be due to cellular factors.

In conclusion, the introduction of the wild-type *XPD/ERCC2* DNA repair gene in a TTD/XP-D cell line corrected most consequences of a DNA repair defect in the TTD cell line. It restored normal UV cell survival, normal DNA repair system, and, at least in part, mutagenesis characteristics of repair-proficient cells. Indeed, it allowed, qualitatively, the restoration of UV-induced mutations similar to those observed in normal cells. Quantitatively, the mutation frequency of the TTD + ERCC2 cell line was considerably lower than that of the TTD parental cell line, although it did not reach the normal range. It is possible that the parental TTD cell line, due to its state of transformation, had acquired intrinsic high mutagenic properties, which will lead to a still high mutation frequency found even after the correction of the DNA repair defect. Finally, the location of mutation hotspots found for the TTD + ERCC2 cells was unique to this cell line and not fully similar to that of the normal cell line we used as a control.

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REFERENCES

- Hoeijmakers, J. H. J. Human nucleotide excision repair syndromes: molecular clues to unexpected intricacies. *Eur. J. Cancer*, *30A*: 1912-1921, 1994.
- Kraemer, K. H., Levy, D. D., Parris, C. N., Gözükara, E. M., Moriawaki, S., Adelberg, S., and Seidman, M. M. Xeroderma pigmentosum and related disorders: examining the linkage between defective DNA repair and cancer. *J. Invest. Dermatol.*, *103*: 96-101, 1994.
- Ma, L., Hoeijmakers, J. H. J., and Van der Eb, A. J. Mammalian nucleotide excision repair. *Biochim. Biophys. Acta*, *1242*: 137-164, 1995.
- Sarasin, A. The paradox of DNA repair-deficient diseases. *The Cancer J.*, *4*: 233-237, 1991.
- Sary, A., and Sarasin, A. The genetic basis of xeroderma pigmentosum and trichothiodystrophy syndromes. *Cancer Surv.*, *26*: 55-171, 1996.
- Itin, P. H., and Pittelkow, M. R. Trichothiodystrophy: review of sulfur-deficient brittle hair syndromes and association with the ectodermal dysplasias. *J. Am. Acad. Dermatol.*, *22*: 705-717, 1990.
- Van Neste, D., Caulier, B., Thomas, P., and Vasseur, F. PIBIDS/Tay's syndrome and xeroderma pigmentosum. *J. Am. Acad. Dermatol.*, *12*: 372-373, 1985.
- Sarasin, A., Blanchet-Bardon, C., Renault, G., Lehmann, A., Arlett, C., and Dumez, Y. Prenatal diagnosis in a subset of trichothiodystrophy patients defective in DNA repair. *Br. J. Dermatol.*, *127*: 485-491, 1992.
- Lehmann, A. R., Arlett, C. F., Broughton, B. C., Harcourt, S. A., Steingrimsdottir, H., Stefanini, M., Malcolm, A., Taylor, R., Natarajan, A. T., Green, S., King, M. D., MacKie, R. M., Stephenson, J. B. P., and Tolmi, J. L. Trichothiodystrophy, a human DNA repair disorder with heterogeneity in cellular response to ultraviolet light. *Cancer Res.*, *48*: 6090-6096, 1988.
- Marionnet, C., Benoit, A., Benhamou, S., Sarasin, A., and Sary, A. Characteristics of UV-induced mutation spectra in human XP-D/ERCC2 gene-mutated xeroderma pigmentosum and trichothiodystrophy cells. *J. Mol. Biol.*, *252*: 550-562, 1995.
- Stefanini, M., Lagomarsini, P., Arlett, C. F., Borroni, C., Crovato, F., Trevisan, G., Cordone, G., and Nuzzo, F. Xeroderma pigmentosum (complementation group D) mutation is present in patients affected by trichothiodystrophy with photosensitivity. *Hum. Genet.*, *74*: 107-112, 1986.
- Broughton, B. C., Lehmann, A. L., Harcourt, S. A., Arlett, C. F., Sarasin, A., Kleijer, W. J., Beemer, F. A., Nairn, R., and Mitchell, D. L. Relationship between pyrimidine dimers, 6-4 photoproducts, repair synthesis and cell survival: studies using cells from patients with trichothiodystrophy. *Mutat. Res.*, *235*: 33-40, 1990.
- Stefanini, M., Lagomarsini, P., Giliani, S., Nardo, T., Botta, E., Peserico, A., Kleijer, W. J., Lehmann, A. R., and Sarasin, A. Genetic heterogeneity of the excision repair defect associated with trichothiodystrophy. *Carcinogenesis (Lond.)*, *14*: 1101-1105, 1993.
- Vermeulen, W., van Vuuren, A. J., Chipoulet, M., Schaeffer, L., Appeldoorn, E., Weeda, G., Jaspers, N. G. J., Priestley, A., Arlett, C. F., Lehmann, A. R., Stefanini, M., Mezzina, M., Sarasin, A., Bootsma, D., Egly, J.-M., and Hoeijmakers, J. H. J. Three unusual repair deficiencies associated with transcription factor BTF2 (TFIIH). Evidence for the existence of a transcription syndrome. *Cold Spring Harbor Sym. Quant. Biol.*, *LIX*: 317-329, 1994.
- Stefanini, M., Vermeulen, W., Weeda, G. A., Giliani, S., Nardo, T., Mezzina, M., Sarasin, A., Harper, J. I., Arlett, C. F., Hoeijmakers, J. H. J., and Lehmann, A. R. A new nucleotide excision repair gene associated with the disorder trichothiodystrophy. *Am. J. Hum. Genet.*, *53*: 817-821, 1993.
- Broughton, B. C., Steingrimsdottir, H., Weber, C. A., and Lehmann, A. R. Mutations in the xeroderma pigmentosum group D DNA repair/transcription gene in patients with trichothiodystrophy. *Nat. Genet.*, *7*: 189-194, 1994.
- Mondello, C., Nardo, T., Giliani, S., Arrand, J. E., Weber, C. A., Lehmann, A. R., Nuzzo, F., and Stefanini, M. Molecular analysis of the XPD gene in Italian families with patients affected by trichothiodystrophy and xeroderma pigmentosum group D. *Mutat. Res.*, *314*: 159-165, 1994.
- Takayama, K., Salazar, E. P., Broughton, B. C., Lehmann, A. R., Sarasin, A., Thompson, L. H., and Weber, C. A. Defects in the DNA repair and transcription gene ERCC2 (XPD) in trichothiodystrophy. *Am. J. Hum. Genet.*, *58*: 263-270, 1996.
- Weber, C. A., Salazar, E. P., Stewart, S. A., and Thompson, L. H. ERCC-2: cDNA cloning and molecular characterization of a human nucleotide excision repair gene with high homology to yeast RAD3. *EMBO J.*, *9*: 1437-1447, 1990.
- Sung, P., Bailly, V., Weber, C., Thompson, L. H., Prakash, L., and Prakash, S. Human xeroderma pigmentosum group D gene encodes a DNA helicase. *Nature (Lond.)*, *365*: 852-855, 1993.
- Schaeffer, L., Moncollin, V., Roy, R., Staub, A., Mezzina, M., Sarasin, A., Weeda, G., Hoeijmakers, J. H. J., and Egly, J.-M. The ERCC2/DNA repair protein is associated with the class II BTF2/TFIIH transcription factor. *EMBO J.*, *13*: 2388-2392, 1994.
- Fleijter, W. L., McDaniel, L. D., Johns, D., Friedberg, E. C., and Schultz, R. A. Correction of xeroderma pigmentosum complementation group D mutant cell phenotypes by chromosome and gene transfer: involvement of the human ERCC2 DNA repair gene. *Proc. Natl. Acad. Sci., USA*, *89*: 261-265, 1992.
- Mezzina, M., Eveno, E., Chevallier-Lagente, O., Benoit, A., Carreau, M., Vermeulen, W., Hoeijmakers, J. H. J., Stefanini, M., Lehmann, A. R., Weber, C. A., and Sarasin, A. Correction by the ERCC2 gene of UV-sensitivity and repair deficiency phenotype in a subset of trichothiodystrophy cells. *Carcinogenesis (Lond.)*, *15*: 1493-1498, 1994.
- Gözükara, E. M., Parris, C. N., Weber, C. A., Salazar, E. P., Seidman, M. M., Watkins, J. F., Prakash, L., and Kraemer, K. H. The human DNA repair gene, ERCC2 (XPD), corrects ultraviolet hypersensitivity and ultraviolet hypermutability of a shuttle vector replicated in xeroderma pigmentosum group D cells. *Cancer Res.*, *54*: 3837-3844, 1994.
- Carreau, M., Eveno, E., Quilliet, X., Chevallier-Lagente, O., Benoit, A., Tanganelli, B., Stefanini, M., Vermeulen, W., Hoeijmakers, J. H. J., Sarasin, A., and Mezzina, M. Development of a new and easy complementation assay for DNA repair deficient human syndromes. *Carcinogenesis (Lond.)*, *16*: 1003-1009, 1995.
- Carreau, M., Quilliet, X., Eveno, E., Salvetti, A., Danos, O., Heard, J. M., Mezzina, M., and Sarasin, A. Functional retroviral vector for a gene therapy of Xeroderma Pigmentosum group D patients. *Hum. Gene Ther.*, *6*: 1307-1316, 1995.
- Huschtscha, L. I., and Holliday, R. Limited and unlimited growth of SV40-transfected cells from human diploid MRC-5 fibroblasts. *J. Cell. Sci.*, *63*: 77-99, 1983.
- Lehmann, A. R., Kirk-Bell, S., Arlett, C. F., Harcourt, S. A., de Weerd-Kastelein, E. A., Keijzer, W., and Hall-Smith, P. Repair of ultraviolet light damage in a variety of human fibroblast cell strains. *Cancer Res.*, *37*: 904-910, 1977.
- Wigler, M., Pellicer, A., Silverstein, S., and Axel, R. Biochemical transfer of single-copy eucaryotic genes using total cellular DNA as donor. *Cell*, *14*: 725-731, 1978.
- Sary, A., and Sarasin, A. Simian virus 40 (SV40) large T antigen-dependent amplification of an Epstein-Barr virus-SV40 hybrid shuttle vector integrated into the human HeLa cell genome. *J. Gen. Virol.*, *73*: 1679-1685, 1992.
- Hanahan, D. Studies on transformation of *Escherichia coli* with plasmids. *J. Mol. Biol.*, *166*: 557-580, 1983.
- Bredberg, A., Kraemer, K. H., and Seidman, M. M. Restricted ultraviolet mutational spectrum in a shuttle vector propagated in xeroderma pigmentosum cells. *Proc. Natl. Acad. Sci., USA*, *83*: 8273-8277, 1986.
- Dumaz, N., Sary, A., Soussi, T., Daya-Grosjean, L., and Sarasin, A. Can we predict solar ultraviolet radiation as a causal event in human tumors by analysing the mutation spectra of the p53 gene? *Mutat. Res.*, *307*: 375-386, 1994.
- Cleaver, J. E., Charles, W. C., McDowell, M. L., Sadinski, W. J., and Mitchell, D. L. Overexpression of the XPA repair gene increases resistance to ultraviolet radiation in human cells by selective repair of DNA damage. *Cancer Res.*, *55*: 6152-6160, 1995.
- Sage, E. Distribution and repair of photolesions in DNA: genetic consequences and the role of sequence context. *Photochem. Photobiol.*, *57*: 163-174, 1993.
- Madzak, C., Armer, J., Sary, A., Daya-Grosjean, L., and Sarasin, A. UV-induced mutations in a shuttle vector replicated in repair deficient trichothiodystrophy cells differ with those in genetically-related cancer prone xeroderma pigmentosum. *Carcinogenesis (Lond.)*, *14*: 1255-1260, 1993.
- Quilliet, X., Chevallier-Lagente, O., Eveno, E., Stojkovic, T., Destée, A., Sarasin, A., and Mezzina, M. Long-term complementation of DNA repair deficient human primary fibroblasts by retroviral transduction of the XPD gene. *Mutat. Res.*, in press, 1996.
- Tomaletti, S., and Pfeifer, G. P. Slow repair of pyrimidine dimers at p53 mutation hotspots in skin cancer. *Science (Washington DC)*, *263*: 1436-1438, 1994.
- Levy, D. D., Saijo, M., Tanaka, K., and Kraemer, K. H. Expression of a transfected DNA repair gene (XPA) in xeroderma pigmentosum group A cells restores normal DNA repair and mutagenesis of UV-treated plasmids. *Carcinogenesis (Lond.)*, *16*: 1557-1563, 1995.
- Seetharam, S., Kraemer, K. H., Waters, H. L., and Seidman, M. M. Mutational hotspot variability in a ultraviolet-treated shuttle vector plasmid propagated in xeroderma pigmentosum and normal human lymphoblasts and fibroblasts. *J. Mol. Biol.*, *212*: 433-436, 1990.