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A Mutation in the *XPB/ERCC3* DNA Repair Transcription Gene, Associated with Trichothiodystrophy

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Summary

Trichothiodystrophy (TTD) is a rare, autosomal recessive disorder characterized by sulfur-deficient brittle hair and nails, mental retardation, impaired sexual development, and ichthyosis. Photosensitivity has been reported in ~50% of the cases, but no skin cancer is associated with TTD. Virtually all photosensitive TTD patients have a deficiency in the nucleotide excision repair (NER) of UV-induced DNA damage that is indistinguishable from that of xeroderma pigmentosum (XP) complementation group D (XP-D) patients. DNA repair defects in XP-D are associated with two additional, quite different diseases; XP, a sun-sensitive and cancer-prone repair disorder, and Cockayne syndrome (CS), a photosensitive condition characterized by physical and mental retardation and wizened facial appearance. One photosensitive TTD case constitutes a new repair-deficient complementation group, TTD-A. Remarkably, both TTD-A and XP-D defects are associated with subunits of TFIIH, a basal transcription factor with a second function in DNA repair. Thus, mutations in TFIIH components may, on top of a repair defect, also cause transcriptional insufficiency, which may explain part of the non-XP clinical features of TTD. Besides XPD and TTDA, the XPB gene product is also part of TFIIH. To date, three patients with the remarkable conjunction of XP and CS but not TTD have been assigned to XP complementation group B (XP-B). Here we present the characterization of the NER defect in two mild TTD patients (TTD6VI and TTD4VI) and confirm the assignment to X-PB. The causative mutation was found to be a single base substitution resulting in a missense mutation (T119P) in a region of the XPB protein completely conserved in yeast, *Drosophila*, mouse, and man. These findings define a third TTD complementation group, extend the clinical heterogeneity associated with XP-B, stress the exclusive relationship between TTD and mutations in subunits of repair/transcription factor TFIIH, and strongly support the concept of "transcription syndromes."

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Introduction

To counteract the deleterious effects of mutagenic and carcinogenic agents, all organisms are equipped with a sophisticated network of DNA repair systems that is essential for genetic stability. Nucleotide excision repair (NER)—one of the most studied repair pathways—removes a wide diversity of DNA lesions, including UV-induced lesions, in a multistep process (Hoeijmakers 1993).

Two subpathways are recognized in NER: a rapid preferential repair of the transcribed strand of active genes ("transcription-coupled repair") and the less efficient global genome repair process (Bohr 1991; Hanawalt et al. 1994). The importance of the NER system is highlighted by the clinical consequences of human hereditary diseases such as xeroderma pigmentosum (XP), an autosomal recessive condition displaying sun (i.e., UV) sensitivity, pigmentation abnormalities, accelerated neurodegeneration, and predisposition to skin cancer (Cleaver and Kraemer 1994). Cockayne syndrome (CS) and trichothiodystrophy (TTD) are two other NER-associated disorders with distinct clinical features. CS patients exhibit photosensitivity, severe mental and physical retardation, neurodysmyelination, impaired sexual development, skeletal abnormalities, and a wizened facial appearance. Remarkably, in contrast to XP, pigmentation abnormalities and sunlight-induced skin cancers are not seen (Lehmann 1987; Nance and Berry 1992). The major DNA repair defect in CS has been located in the transcription-coupled subpathway, whereas overall genome repair is unaffected (Venema et al. 1990). TTD patients have brittle hair and nails, because of reduced content of cysteine-rich matrix proteins, ichthyotic skin, and physical and mental retardation, and approximately half of the cases display photosensitivity, correlated with a NER defect. As in the situation of CS, no cases of skin cancer have been reported (Itin and Pittelkow 1990; Sarasin 1991; Stary and Sarasin 1996).

Complementation tests by cell fusion have demonstrated that these NER syndromes are genetically heterogeneous and comprise ≥10 complementation groups: 7 in XP (XP complementation groups A–G [XP-A–XP-G]), 2 in CS (CS-A and CS-B), and 2 in TTD (TTD-A and

XP-D) (reviewed by Hoeijmakers 1994). The finding of additional combined XP/CS patients falling into XP-B, XP-D, and XP-G indicates that there is considerable overlap and clinical heterogeneity within a subset of complementation groups.

Interestingly, photosensitive TTD patients have an NER deficiency indistinguishable from that in XP patients. To date, virtually all repair-deficient TTD patients have been assigned, by cell hybridization, to XP-D (Stefanini et al. 1986, 1993*a*; Nuzzo and Stefanini 1989). These observations are confirmed by the finding that microinjection and/or transfection with the *XPD/ERCC2* cDNA into cells of several unrelated TTD patients restores their DNA repair deficiency (Mezzina et al. 1994). Furthermore, mutations in the *XPD/ERCC2* gene have been detected in a number of TTD patients who previously had been assigned to XP-D (Broughton et al. 1994; Takayama et al. 1996). Thus there is considerable clinical heterogeneity associated with XP-D, harboring patients with XP, XP/CS, or TTD (Johnson and Squires 1992).

Recently, it was discovered that both the DNA repair helicases—XPD/ERCC2 and XPB/ERCC3—are in fact subunits of the basal transcription factor TFIIH (Schaeffer et al. 1993, 1994). Subsequently, it was shown that both ERCC2 and ERCC3, in the context of TFIIH, are directly involved in repair and transcription in vitro and in vivo (van Vuuren et al. 1994; Vermeulen et al. 1994*b*). These results show that both the XPD/ERCC2 gene product and XPB/ERCC3 gene product have a dual role in two distinct metabolic processes: DNA repair and transcription. In contrast to XPD, the XPB protein—which is part of the same TFIIH complex—has, so far, been associated only with combined XP/CS.

Recent somatic-cell fusion experiments have identified two additional TTD families, representing two other distinct TTD genetic entities. One cell strain (TTD1BR) has been assigned to an entirely new excision-deficient–repair complementation group (Stefanini et al. 1993*b*), whereas the other (TTD4VI and 6VI, from siblings) failed to complement XP-B cells, suggesting that the repair defect in this family resides in the *XPB/ERCC3* gene. Microinjection of *XPB/ERCC3* cDNA resulted in the correction of the repair defect, a result that is in agreement with the assignment by cell fusion experiments (Vermeulen et al. 1994*b*). Here we report the clinical description of these two new TTD siblings, their repair characteristics, the confirmation of the complementation assignment to XP-B, and the causative mutation in the *XPB/ERCC3* gene. These observations further extend the clinical complexity associated with XP-B and support the hypothesis of a relationship between different domains in both XPD/ERCC2 and XPB/ERCC3 gene products and the dual role in basic transcription and NER and the diverse clinical consequences of different mutations in these genes.

Material and Methods

Cell Strains and Culture Conditions

Skin biopsies were surgically obtained from unexposed parts of the patient's body. Primary skin fibroblasts were obtained from 1-mm^2 slices of biopsies, by a procedure described elsewhere (Cruickshank et al. 1960). Primary cell cultures were studied at passages 4–15. Transformed fibroblasts were established after transfection with plasmid pLASwt encoding the SV40 large T antigen, as described elsewhere (Daya-Grosjean et al. 1987). The cells used in the present study are listed in table 1. MRC5V1SV, XP1BR, and XP11BE cells were obtained from Drs. A. R. Lehmann and C. F. Arlett (Brighton, U.K.), and XP6BESV and XP4PALas cells were obtained from the NIH's National Institute of General Medical Sciences and A. Sarasin's laboratory, respectively. Lymphoblastoid cell lines were obtained from peripheral blood lymphocytes, immortalized with Epstein-Barr virus (EBV; B958 strain from G. Klein, Stockholm), by standard procedures (Miller and Lipman 1973). Fibroblast cell lines were grown in modified Eagle's medium containing 15% FCS and antibiotics (penicillin, streptomycin, and fungizone; Gibco). Lymphoblastoid cells were grown in RPMI medium supplemented with 20 mM glutamine, 10% FCS, and antibiotics. Routinely, 3×10^5 lymphoblastoid cells were seeded in 100 ml of medium on T75 culture flasks (Falcon), and every week the cell suspensions were diluted with fresh medium.

UV-Induced UDS

Cells were washed with PBS and were irradiated with UV-C at a fluence rate of 0.2 $J/m^2/s$. For unscheduled DNA synthesis (UDS) experiments, 5×10^4 primary fibroblasts were seeded onto two 2-cm^2 glass slides in tissue culture dishes. After 18–24 h, cells were cultured in medium containing 0.1% FCS, 10 mM hydroxyurea, and 100 μM fluorodeoxyuridine for 2 h prior to UV irradiation. After irradiation, cells were incubated in medium containing 10 μCi/ml of ^{3}H-TdR for 3 h and were chased for 1 h with medium containing 10 μM thymidine. Slides with radiolabeled cells were mounted and dipped in a photosensitive emulsion (Kodak). All other steps of the UDS procedure were performed as described elsewhere (Vermeulen et al. 1986; Sarasin et al. 1992). When untransformed fibroblasts were used, the medium was supplemented with 20% JB-clone fetal serum (Institut St. Jacques Boy SA, Reims).

UV Survival

To determine their colony- forming ability, cells were plated at densities varying from 1.5×10^3 to 3×10^3 per 90-mm dish. After attachment, cells were rinsed with PBS and were UV irradiated at various doses. The number of surviving colonies was counted in triplicate dishes.

Table 1

Cell Lines Used in Present Study

Phenotype	Cell Line(s)	
	Primary Diploid Fibroblasts	Transformed Fibroblasts
Wild type	MRC5, 20VI, 198VI, C5RO	MRC5V1SV, C5ROLas
Heterozygote TTD	TTDHF (father), TTDHM (mother)	
TTD/XPD	TTD2VI, TTD3VI	TTD2VILas
TTD/XPB[a]	TTD4VI, TTD6VI	TTD4VILas, TTD6VILas
XP-B	XP11BE, XPCS1BA	XPCS1BA(SV), 2BA(SV)
XP-D	XP1BR	XP6BESV
XP-C		XP4PALas

[a] For each category of fibroblast, the two cell lines are from two siblings.

Microneedle Injection

Plasmid cDNA (100 μg/ml) in PBS was injected into one of the nuclei of XPCS1BA homopolykaryons by glass microneedles, as described elsewhere (Vermeulen et al. 1994*b*). Injected cells on coverslips were cultured for 24 h to allow expression of the injected cDNA. Repair activity was determined after UV irradiation (15 J/m^2), [^{3}H]thymidine incubation (10 μCi/ml; specific activity 50 Ci/mM) fixation, and autoradiography. Grains above the nuclei represent a quantitive measure for NER activity.

Recovery of RNA Synthesis (RRS)

Routinely, 5×10^4 to 15×10^4 cells were seeded in duplicate wells in six-well tissue culture plates 24 h before UV irradiation with different doses. At the time points indicated, cell samples were labeled with 10 μCi/ml of [^{3}H]uridine for 1 h and were trypsinized and counted. Cells were lysed in the presence of 1% SDS, 1 M NaCl, 10 mM Tris-HCl pH 8.0, and 10 mM EDTA. Cell lysates were spotted onto 3MM Whatman paper, and radioactive acid-insoluble material was counted after trichloroacetic acid precipitation. Data were expressed, in terms of cpm/10^5 cells, as the percent of radioactivity in irradiated samples over the percent of radioactivity in unirradiated samples.

Plasmids, DNA Transfections, and Reactivation of UV-Irradiated pRSVL

Plasmids containing *ERCC1* (pSVL5), *XPA* (pSLM-XPA), *XPB/ERCC3* (pSVH3), *XPB/TTD6VI* (see below), *XPD/ERCC2* (pDE-ER2), and *XPC* (pREP-125) cDNAs were cloned into eukaryotic expression vectors driven by the SV40 early, CMV, or RSV promoters and were used for transient-expression studies. The wild-type *XPB/ERCC3* cDNA (pSVH3), driven by the SV40 early promoter, was used for microinjection into XP-B fibroblasts and has been described elsewhere (Weeda et al. 1990). The *XPB/ERCC3* cDNA harboring the TTD6VI mutation was constructed as follows: the 5′ part of pSVH3 was replaced by a 0.7-kb *Eco*RI/*Sac*I DNA fragment that was synthesized by means of PCR-amplified *XPB/ERCC3* cDNA derived from mRNA isolated from lymphoblastoid cells of patient TTD6VI. The mutant cDNA was subcloned in a pcDNA3 expression vector (Invitrogen), yielding pTTD6VI. Two independent PCR-amplified cDNA clones were sequenced to exclude PCR-induced mutations.

All plasmid DNAs were purified by alkaline lysis followed by two sequential CsCl gradients. DNA transfections were in part performed by a modification of the calcium phosphate procedure (Graham and van der Eb 1973). The DNA precipitate (1–10 μg) was mixed with 10^5–10^6 cells immediately after trypsinization in a final volume of 4 ml medium. The cell/DNA mixture was seeded in a 9.5-cm-diameter dish and incubated at 37°C for 15–20 h. Subsequently, cells were washed three times with PBS, and fresh medium was added.

For UV-irradiated plasmid-reactivation experiments, pRSVL (5 μg) DNA (expression plasmid containing the luciferase cDNA) was irradiated at 1,000 J/m^2 (as described elsewhere [Carreau et al. 1995]). For DNA transfection experiments, the calcium phosphate DNA precipitate was mixed with 10^5 cells and was seeded in six-well culture dishes in a final volume of 1.5 ml. At indicated time points after transfection, cells were harvested, and cell pellets were stored at −20°C. Lysis of cells and luciferase activity were measured by the Promega Luciferase system, according to the manufacturer's protocol. The luciferase activity for each sample was expressed as the percent of activity from cells transfected with irradiated DNA over the activity from cells transfected with unirradiated DNA.

RNA Isolation, DNA Amplification, and Mutation Detection

RNA was isolated by the LiCl/urea method (Auffray and Rougeon 1980). The RNA was used for preparing cDNA with *XPB/ERCC3*-specific primers (as described by Weeda et al. 1990). Amplification was performed by

30 cycles of, consecutively, 2 min denaturation at 95°C, 2 min annealing (temperature was dependent on the primers used), and 3 min extension at 72°C. The amplified DNA was purified and digested with the appropriate restriction enzyme and subsequently was cloned into M13mp18 vector for sequence analysis by the dideoxy-chain termination method (Sanger et al. 1977), by use of T7 DNA polymerase. The primer for mutation detection in cells of XP patient TTD6VI and of TTD4VI family members was p193, 5′TACAACTACCTGCCTACTCC-3′.

Results

Clinical Characterization of TTD6 and 4VI Patients

The index case (TTD6VI), a male born at term from first-cousin marriage (birth wt 3,110 g), was examined first at birth, when he was found to have congenital ichthyosis (collodion baby). The skin condition improved within 3 wk, leaving a mild ichthyosis of the trunk. TTD6VI was clinically evaluated regularly during his 1st year and then at the ages of 2, 4, 6, and 10 years. An electron-microscopy study showed the accumulation of intracellular lipid mortar and an increased number in the stratum corneum, corresponding to an increase in lamellar bodies in the stratum granulosum (Taïeb et al. 1987). A diagnosis of TTD was suspected at 3 years of age, on the basis of the following clinical manifestations: mild ichthyosis of the trunk, with involvement of scalp, palms, and soles; mild photosensitivity, noted during the summer of 1988; lack of second upper incisor; and hair growing normally but coarse and with a tiger-tail pattern under polarized light (van Neste et al. 1989). The diagnosis of TTD was confirmed by the analysis of hair amino acid content (J. M. Gillespie, Melbourne), showing an 11% decrease in cysteine residues (normal range 17%–17.8%) and a concomitant increase in lysine, aspartic acid, and leucine, reflecting a significantly lower content of cysteine-rich matrix proteins (van Neste et al. 1989). Other findings included normal intelligence and lower-limit growth (−2 SD), normal neurological examination, salt-and-pepper appearance of ophthalmic fundi, normal bone X-ray survey, negative phototesting (followed, however, by a finding of massive desquamation), and elevated (350 IU/liter) IgE levels. Except for the salt-and-pepper appearance of the fundi, there were no skin or CNS features that are associated with CS. The patient has one normal brother and a TTD sister (TTD4VI). She was born at term (birth wt 2,900 g) with a similar presentation as a collodion baby of favorable outcome. The diagnosis of TTD was confirmed by hair microscopy and biochemical analysis (cysteine content 8.33%). A systolic murmur was noted at 2 mo of age, and the rest of the examination was considered normal by her pediatrician. The relatively mild symptoms observed in this family compared with those in other TTD patients have led the clinician to describe them clinically as a TTD "variant" (van Neste et al. 1989). Both TTD6VI and TTD4VI are in good health, without physical and mental impairment, at the ages of 10 and 16 years, respectively.

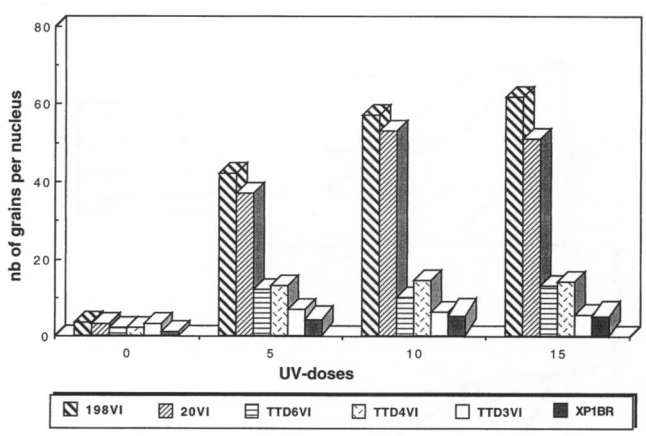

Figure 1 UDS levels of wild-type, TTD, and XP-D cells. Primary cultures were UV irradiated at indicated doses and processed for UDS. The histograms represent the average number of grains of ≥30 nuclei for each cell line: 198VI and 20VI (wild type), TTD6VI and TTD4VI (index cases), and TTD3VI (TTD/XP-D) and XP1BR (XP-D) cells.

Characterization of Cellular Responses to UV Irradiation

A preliminary study showed that the repair capacity (UV-induced UDS) of TTD6VI fibroblasts is reduced compared with that of normal repair-competent fibroblasts (Stefanini et al. 1993*a*). To fully characterize the DNA repair defect in both patients, UV-induced UDS and UV survival were compared with those in other TTD and XP patients. As shown in figure 1, TTD4VI cells and TTD6VI cells have 20%–30% residual UDS compared with that of wild-type 198VI and 20VI cells, but repair synthesis is slightly higher than that of TTD3VI cells (TTD/XP-D group) and is significantly higher than that of XP1BR (XP-D) cells. The colony-forming ability, after UV exposure, of primary and SV40-transformed TTD6VI fibroblasts, shown in figure 2, indicates that TTD6VI primary fibroblasts have a reduced UV survival compared with that of normal cells but have a still slightly higher survival than that of TTD2VI (TTD/XP-D) cells and a much higher survival than that in the XP1BR cells, at all UV doses tested (2–10 J/m^2). (The UV dose at which the relative survival is 37% [D37] is 10 J/m^2 for MRC5 wild-type cells and 6 J/m^2 for TTD6VI cells; see fig. 2*A*.) More-reduced cloning efficiencies have been found for two other TTD lines belonging to the TTD/XP-D group, such as TTD2VI cells (D37 is 4 J/m^2) and XP1BR (XP-D) cells (D37 is 2 J/m^2). Similar findings were made with the immortalized fibroblasts, showing a D37 value of 12 J/m^2 and 7 J/m^2

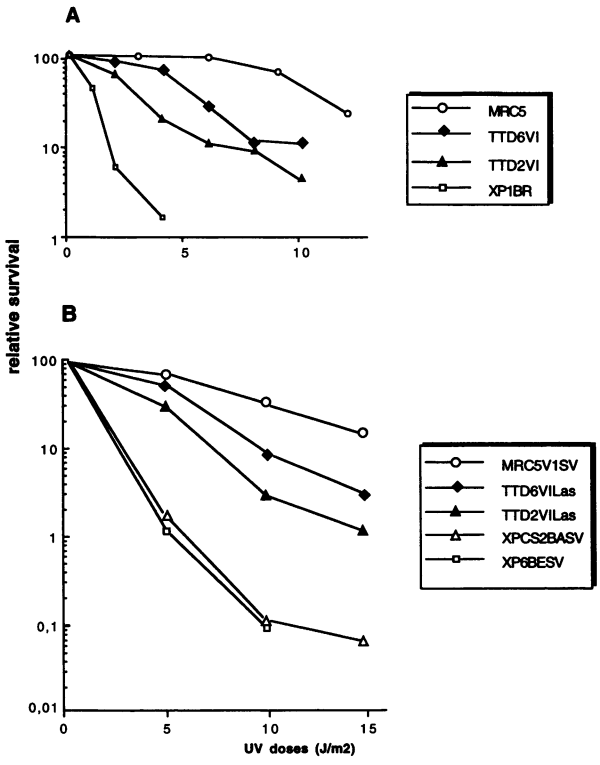

Figure 2 Effect of UV on cloning efficiency. Primary (*A*) and SV40-immortalized (*B*) fibroblasts were cultivated as described in Material and Methods and UV irradiated at the indicated doses. Survival was measured as described in Material and Methods, for MRC5 and MRC5VISV (wild type), TTD6VI and TTD6VILas (index case), XPCS2BASV (XP-B), TTD2VI and TTD2VILas (TTD/XP-D), and XP1BR and XP6BESV (XP-D) cells.

for MRC5V1 and TTD6VILas cells, respectively. TTD2VILas cells (TTD/XP-D) exhibit UV sensitivity similar to that seen in TTD6VILas cells (see fig. 2*B*), whereas XPCS2BASV cells (XP-B) exhibit UV sensitivity similar to that of XP6BESV (XP-D) cells (see fig. 2*B*). We further studied (1) the DNA repair characteristics of these TTD cells, by means of RRS after UV irradiation (fig. 3), and (2) the ability to reactivate a UV-irradiated plasmid containing the luciferase reporter gene (fig. 4). Figure 3 shows results obtained with primary diploid fibroblasts. In wild-type cells (MRC5 and C5RO), RNA synthesis is strongly repressed as a function of exposure to UV doses, and RRS occurs 9–24 h post irradiation, reaching the level of control cells. In contrast, in repair-deficient XP-B (XP11BE) cells, no RRS occurs over a period of 24 h post irradiation. TTD6VI cells exhibit levels of RRS similar to those seen in XB11BE fibroblasts (see fig. 3*A*). In figure 3*B* a typical dose-response inhibition of RRS, measured 24 h after UV irradiation, is presented. Heterozygote cells from TTD parents (TTDHF and TTDHM) exhibit a normal response as measured by this assay, compared with wild-type cells. Both TTD6VI and XP11BE show reduced RRS, as a function of UV doses.

The ability to recover expression of a UV-damaged reporter gene has been studied as a fourth biological end point. Wild-type, C5ROLas, MRC5V1SV, TTD6VILas, and repair-deficient cells belonging to XP-D (XP6BESV) were analyzed for reactivation of UV-irradiated plasmid pRSVL, harboring the luciferase gene. The maximum expression of luciferase was obtained 24–48 h after transfection (data not shown). However, in order to analyze the kinetics of expression of the UV-irradiated plasmid, transfected cells were harvested at different time points post transfection and were assayed for luciferase activity as described in Material and Methods. Results shown in figure 4 indicate that wild-type MRC5V1SV and C5ROLas cells efficiently reactivate UV-irradiated plasmid, since the relative expression level of the reporter gene reached a maximum of 70%–90% of the level in unirradiated cells at 24–48 h post transfection. In contrast, TTD6VILas cells showed reduced levels of expression (~30%, compared with unirradiated DNA), without increase within 6–8 h, indicating decreased repair of UV photoproducts in the luciferase

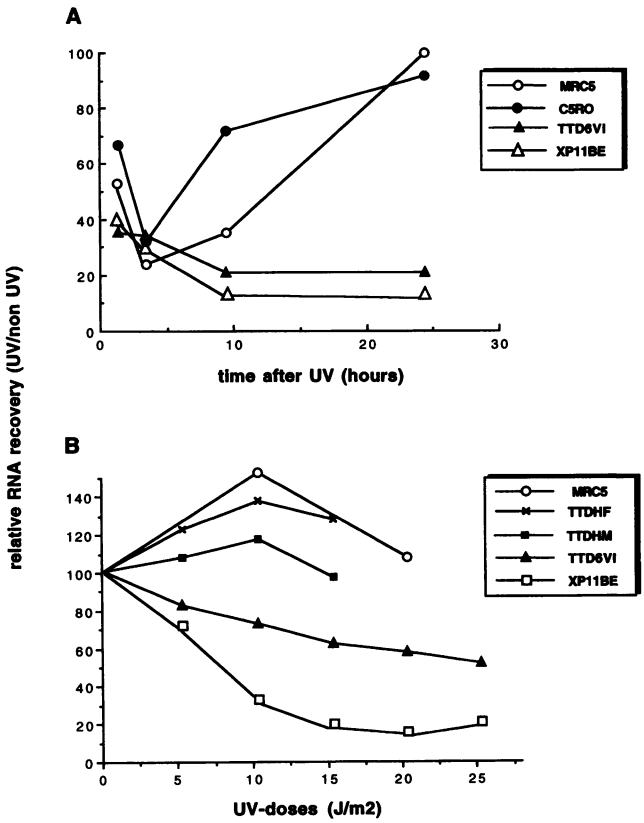

Figure 3 RRS. Cells were processed as described in Material and Methods. *A*, At the indicated times of post irradiation (20 J/m^2), cells (MRC5 and C5RO [wild type] and TTD6VI and XP11BE [XP-B]) were labeled with [^{3}H]uridine and harvested. *B*, MRC5, TTDHF, TTDHM, TTD6VI, and XP11BE cells were irradiated at indicated UV doses, incubated for 24 h, and harvested after 1 h labeling with [^{3}H]uridine.

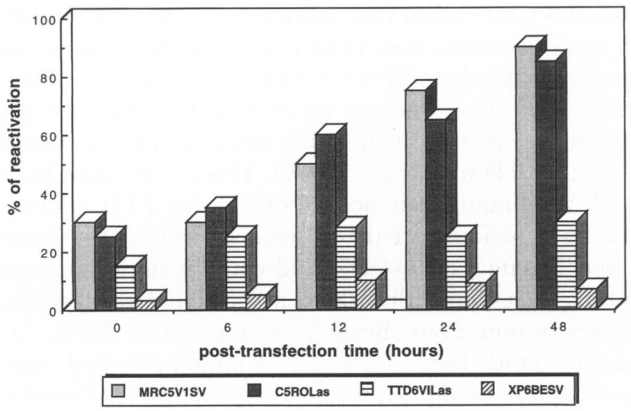

Figure 4 Kinetics of reactivation of UV-irradiated plasmid pRSVL. Cells (MRC5V1SV, C5ROLas, TTD6VILas, and XP6BESV) were transfected with plasmid pRSVL, irradiated at 1,000 J/m^2, harvested at the indicated time after transfection and luciferase activity was determined as specified in Material and Methods.

gene. Even-more-reduced levels of luciferase activity were detected in XP6BESV (XP-D) cells, which is in accordance with the UV survival and UDS results.

Correction of DNA Repair Deficiency in TTD6VI Cells by the XPB/ERCC3 *cDNA*

In order to study the effect of *XPB/ERCC3* on the reactivation properties of TTD6VI cells, UV-irradiated (1,000 J/m^2) reporter plasmid was cotransfected with cDNAs encoding the DNA repair genes *XPA, XPC, XPD/ERCC2, XPB/ERCC3,* and *ERCC1*. The results shown in figure 5 demonstrate that the reactivation rates in XP6BESV (XP-D) and XP4PALas (XP-C) cells have been corrected to wild-type levels, after introduction of *XPD/ERCC2* and *XPC* expression plasmids, respectively. C5ROLas cells reactivate plasmid to the extent

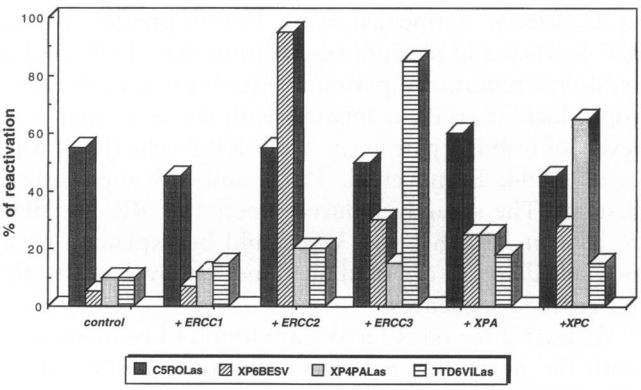

Figure 5 Correction of UV-reactivation defect. Cells were transfected with UV-irradiated (1,000 J/m^2) plasmid pRSVL alone (control) or cotransfected with a second plasmid harboring the NER repair gene as indicated. 48 h after transfection, cells (C5ROLas, XP6BESV, XP4PALas, and TTD6VILas) were harvested and luciferase activity was measured.

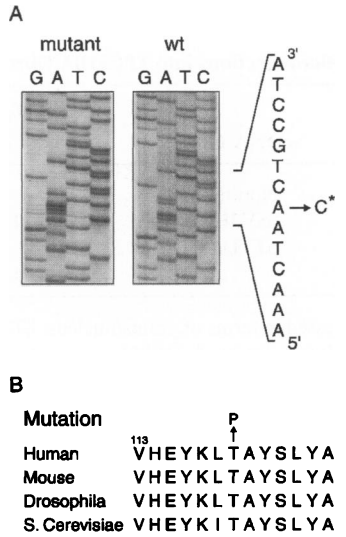

Figure 6 Nucleotide sequence analysis of the TTD6VI mutation and amino acid–sequence conservation of the mutated region in *XPB/ERCC3*. *A,* Nucleotide sequence of a part of the *XPB/ERCC3* cDNA of a wild-type (wt) cell, from HeLa and from patient TTD6VI (mutant). *B,* TTD6VI and 4VI mutations in a conserved region of the XPB/ERCC3 protein are shown. There is homology between the amino acid sequences of XPB/ERCC3, around position 119 of human, mouse, *Drosophila,* and *Saccharomyces cerevisiae*. The amino acid substitution into a proline is indicated by an arrow.

of 60%–85% (see figs. 4 and 5). When *XPB/ERCC3* was cotransfected into TTD6VILas cells, the extent of reactivation observed is 85%, indicating that the expression of *XPB* gene in these mutant cells restores the defect in reactivation of the actively transcribed luciferase gene to the wild-type level. These data clearly demonstrate that the repair defect is corrected by the *XPB/ERCC3* gene and confirm the complementation experiments by cell fusion and the microneedle injection of *XPB/ERCC3* cDNA into TTD6VI cells (Vermeulen et al. 1994*b*).

Determination of the XPB/ERCC3 *Mutation*

It is evident that full inactivation of the function of XPB/ERCC3 in basal transcription is lethal. Inactivating the yeast, *Drosophila,* and mouse homologues of *XPB/ERCC3* results in a lethal phenotype, suggesting that this is the case for man as well (Mounkes et al. 1992; Park et al. 1992; G. Weeda, unpublished observations). This implies that the presence of a gross alteration in the gene is rather unlikely. Sequence analysis of PCR-amplified mRNA of patient TTD6VI revealed a single base substitution (A→C transversion; see fig. 6*A* and *B*) in the 5′ part of the cDNA, resulting, at the protein level, in a threonine(T)-to-proline(P) substitution at amino acid residue 119. No other changes were observed in the remainder of the *XPB/ERCC3* cDNA of TTD6VI. The same mutation was found at the genomic

Table 2

TTD6VI cDNA Microinjections into XPCS1BA Fibroblasts

Cell Strain	cDNA Injected	Mean UDS ± SEM[a] (% of Control Value)
XPCS1BA	Noninjected	18 ± 2 (13)
XPCS1BA	pSV3H[b]	123 ± 6 (90)
XPCS1BA	pTTD6VI	54 ± 3 (40)
C5RO	...	136 ± 6 (100)

[a] Data are expressed in terms of grains/nucleus. UDS was performed as described by Vermeulen et al. (1986).

[b] *ERCC3* cDNA (wild type).

level and in TTD4VI by reverse-transcriptase–PCR and dot-blot analysis with a mutant-specific oligonucleotide (data not shown). Both the unaffected brother and the parents exhibited both mutant and wild-type sequences at the 119 codon (data not shown).

To assess the effect of the TTD6VI mutation in NER, a cDNA construct carrying the TTD6VI mutation was microinjected into XPCS1BA fibroblasts. As shown in table 2, a significant increase in UDS—albeit not to the level seen in normal fibroblasts—was observed (from 13% to ~40% of wild type) with this mutant cDNA, whereas injection of *XPB/ERCC3* cDNA containing either the more severe XP11BE or the XPCS1BA mutation did not enhance UDS (Vermeulen et al. 1994*a*). These experiments clearly indicate that the TTD6VI mutation partially inactivates the *XPB/ERCC3* repair function.

Discussion

Complementation analysis by cell fusion has assigned virtually all photosensitive TTD patients to XP-D, with the exception of TTD1BR (TTD-A). Here we present the clinical, cellular, and molecular description of two TTD siblings (TDD6VI and TTD4VI) falling within the extremely rare XP-B complementation group.

A comparison of the three other XP-B cases all displaying combined XP/CS already had revealed a remarkable clinical heterogeneity: the two XPCS1BA and XPCS2BA brothers are clinically much less affected than the first described XP11BE patient (Robbins et al. 1974; Scott et al. 1993; Vermeulen et al. 1994*a*). XPCS1BA and XPCS2BA show less severe physical and mental retardation, a much longer life span, and, most important, absence of skin cancer at advanced age, whereas XP11BE had multiple skin lesions in her teenage years. However, the corresponding fibroblasts indicated the same low level (<10% of the normal level) of UDS, a complete absence of cyclobutane pyrimidine dimer removal, and a low UV survival in colony-forming assays (Scott et al. 1993). TTD6VI has an intermediate repair defect, on the basis of data on UV survival, UDS and RNA synthesis recovery experiments, and UV-irradiated plasmid reactivation (see below). Yet, clinically the XP-B cases described here exhibit extra TTD features not present in the three XP/CS patients in XP-B known hitherto. Thus these findings point to a disconnection between the severity of the NER defect and the presence of extra TTD features in TT6VI. This is consistent both with the finding that nonphotosensitive TTD patients have a genetic defect that is independent of XP mutations (Stefanini et al. 1987) and with the notion that, in XP-D, each type of disorder is associated with a specific subset of mutations (Broughton et al. 1994, 1995; Takayama et al. 1995, 1996; M. Stefanini, personal communication). Similarly, we find here that the TTD mutation in XPB is distinct from the two mutations known to be responsible for the XP/CS cases in this group.

Our results define the causative mutation in the *XPB/ERCC3* gene, responsible for the phenotype of the TTD6VI and TTD4VI patients, as a single base substitution resulting in an amino acid change of threonine 119 to a proline. In the remainder of the cDNA, no other changes are found, and both wild-type and mutant sequences are detected in the unaffected children and parents, a finding that agrees well with the consanguinity in this family. The threonine (T119) resides in a region to which no specific functional domain has been assigned. However, as shown in figure 6*B*, this residue is not changed during eukaryotic evolution. This indicates that this portion of the protein has an important function that cannot tolerate changes. The NER defect in TTD6VI reveals that this domain is at least in part implicated in the repair function of the protein. The *XPB/ERCC3* mutations in patients XPCS1BA and XPCS2BA and in patient XP11BE (an F99S substitution and a frameshift at position 740, respectively) have a similar, much more severe effect on NER. The characteristics of the NER defect in the TTD individuals presented here include intermediate levels of cellular DNA repair properties (20%–30% of normal levels of UDS) (Stefanini et al. 1993*a*; Vermeulen et al. 1994*b*; present study), UV survival and plasmid reactivation (figs. 1–4), and a wild-type removal of pyrimidine (6-4) pyrimidone photoproducts (6-4PP), compared with the extremely low levels of 6-4PP repair in the other XP-B cells (Galloway et al. 1994; Eveno et al. 1995; authors' unpublished results). The severely reduced kinetics of RRS, similar to that in XP11BE (fig. 3*A*), could be explained by a possible defect in cyclobutane dimer removal in actively transcribed sequences.

At least three NER factors are found to be associated with the nucleotide excision repair/transcription complex TFIIH. Both XPB/ERCC3 (Schaeffer et al. 1993) and XPD/ERCC2 (Schaeffer et al. 1994; Vermeulen et al. 1994*b*) and the TTDA factor (not yet identified) reside in this complex. In this context it is relevant to consider a number of striking molecular and clinical parallels between *XPB/ERCC3* and *XPD/ERCC2*. Both

genes are essential and encode DNA helicases with similar size and degree of sequence conservation but with opposite directionality of DNA unwinding. Clinically, both are associated with NER complementation groups that display exceptional heterogeneity, including XP in combination with CS and—as shown here—TTD. In addition, the analysis of the patients investigated thus far supports the idea that the causative mutations have not been associated with different diseases. Thus our findings further extend the parallels between XP-B and XP-D. On the other hand, molecular and clinical differences between both genes also are observed. First, XP-D occurs much more frequently than XP-B. This correlates well with both the number of mutations tolerated by the respective genes and the degree to which the enzymatic function can be affected before the stage of inviability is reached. Mutation analysis of the genes in yeast and mammals has shown that the helicase function of XPD is not critical for transcription and is not even totally indispensable for NER in mammals (G. S. Winkler, J. H. J. Hoeijmakers, and G. Weeda, unpublished results), whereas inactivation of the XPB helicase is essential for both transcription and repair (Park et al. 1992). Second, within XP-D also, a category of patients with only XP symptoms is found that is lacking in XP-B. This might be due to the rarity of XP-B, because, on theoretical grounds, one would predict the occurrence of this class of patients in XP-B as well.

The spectrum of diseases linked with TFIIH is heterogeneous, including seemingly unrelated symptoms, such as photosensitivity, brittle hair, neurodysmyelination, impaired sexual development, ichthyosis, and dental caries. In view of the dual functionality of TFIIH in repair and transcription, it is conceivable that a mutation in a subunit can affect either one or both of its functions. On this basis, we elsewhere have proposed a tentative model for the etiology of the defects in CS and TTD and related disorders (Bootsma and Hoeijmakers 1993).

Inactivating only the NER function of TFIIH results in an XP phenotype that is observed in classical XP patients of XP-D (and in other non-TFIIH XP complementation groups). When, in addition, the transcription function is subtly affected, the photosensitive form of combined XP/CS and TTD is found. Theoretically, mutations causing a viable transcription problem without NER impairment are predicted to exist as well (Vermeulen et al. 1994*b;* Eveno et al. 1995). This prediction provides a plausible explanation for the molecular defect in the category of nonphotosensitive TTD patients and in a recently identified class of patients with typical CS features but without either sun sensitivity or a measurable repair defect (authors' unpublished results)

However, when the non-XP symptoms in CS and TTD are due to a crippled TFIIH function in basal transcription, how can we rationalize the differences between CS and TTD—that is, the additional presence of brittle hair and nails in TTD? It is possible that mutations in TFIIH subunits influence the stability of the complex, as well as its direct function in repair and/or in transcription. Particularly for an intricate complex such as TFIIH, made up of at least nine subunits, it easily can be envisioned that mutations cause conformational changes whereby the molecule no longer fits well within the complex. We favor the idea that reduced stability of TFIIH may cause the typical non-CS TTD features, which mainly affect terminally differentiating cells in the skin. When these cells are exhausted for TFIIH prior to completion of their terminal differentiation, transcription of the last genes in the differentiation program is impaired. In this view, a general malfunction of the transcriptional role of TFIIH would be the cause of the CS hallmarks. In this regard, it is worth noting that the "CS-component" in the spectrum of clinical features of TTD4VI and TTD6VI is less prominent, compared with that in the other XP/CS patients in XP-B. This would mean that the T119P mutation found in TTD4VI and TTD6VI not only moderately affects the transcription function of TFIIH (as it does the repair function) but also has an effect on the stability of the complex. Obviously, biochemical experiments using TFIIH carrying different mutations should provide molecular proof for this interpretation. Such experiments in mutant mice and in in vitro cultured cells are ongoing. In conclusion, the findings reported here define a third TTD complementation group, extend the clinical heterogeneity within XP-B, and strongly support the concept of "transcription syndromes."

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