A temperature-sensitive disorder in basal transcription and DNA repair in humans

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The xeroderma pigmentosum group D (XPD) helicase subunit of TFIIH functions in DNA repair and transcription initiation[1,2]. Different mutations in *XPD* give rise to three ultraviolet-sensitive syndromes: the skin cancer-prone disorder xeroderma pigmentosum (XP), in which repair of ultraviolet damage is affected; and the severe neurodevelopmental conditions Cockayne syndrome (CS) and trichothiodystrophy (TTD). In the latter two, the basal transcription function of TFIIH is also presumed to be affected[3–5]. Here we report four unusual TTD patients with fever-dependent reversible deterioration of TTD features such as brittle hair. Cells from these patients show an *in vivo* temperature-sensitive defect of transcription and DNA repair due to thermo-instability of TFIIH. Our findings reveal the clinical consequences of impaired basal transcription and mutations in very fundamental processes in humans, which previously were only known in lower organisms.

The hallmark features[4,6] of TTD are brittle hair and nails and ichthyosis (scaling of the skin; Table 1), which are caused by reduced synthesis of cysteine-rich proteins that crosslink keratin filaments at the end of terminal differentiation (characteristic 'tiger-tail' pattern; Fig. 1*a*). TTD, XP and CS are caused by mutations in a single gene[7], *XPD*, encoding one of the helicases of the nine-subunit transcription/DNA repair complex TFIIH (refs. 1,2). The photo-sensitivity is accounted for by the defect in nucleotide excision repair (NER), the principal system for removal of DNA lesions caused by ultraviolet light and numerous chemicals[8,9]. The remainder (non-XP) manifestations of CS and TTD (Table 1) have been attributed to crippled basal transcription[3]. Indeed, TTD mice showed reduced transcript levels for crosslinking proteins in the ichthyotic skin[10]. But it is not known how an inborn defect in a general transcription factor leads to impaired transcription of specific genes in only particular organs.

TTD patient TTD1RO (ref. 11) had unusual additional features: during repeated episodes of pneumonia she lost all her (brittle) hair in one or two days, though it did regrow to the usual length after recovery (Fig. 1*b*,*c*). Concomitantly, her skin symptoms showed a transient but reversible deterioration (Table 1). The patient carries two unique point mutations in *XPD*: an R658C amino acid substitution in one allele and a G713R change in the other[12] (Fig. 2*d*,*e*). In screening various TTD cases, we detected the R658C mutation (Fig. 2*c*) in a female patient (TTD1DOD) of different ethnic background[13]. In conditions of high fever, she also exhibited unusual sudden hair loss and her signs of ataxia transiently aggravated (Table 1). An affected brother and a patient from a related family showed the same manifestations. Patient TTD1DOD was homozygous for the R658C mutation, as shown by single-strand conformation analysis and homozygosity for four common polymorphisms in the *XPD* coding region (Fig. 2*d*,*e*; ref. 14). Thus, the R658C change is the phenotype-determining allele ruling out indirect effects involving genetic background.

Primary fibroblasts of TTD1RO and TTD1DOD show a similar intermediate ultraviolet sensitivity compared with XP groups A, C and D and 30–40% residual ultraviolet-induced repair synthesis (Fig. *2a*,*b*), typical of a mild NER defect. To permit further analysis, we generated an SV40-transformed TTD1RO fibroblast line and used it to isolate two transfectants stably corrected with wild-type *XPD* cDNA (clones 13 and 20) as optimal wild-type controls. We confirmed conserved NER characteristics of TTD1RO-SV and restoration of repair in the corrected clones (Fig. 3*a*,*b*).

One interpretation of the fever-dependent clinical symptoms linked with the R658C substitution is that high temperature causes a transient aggravation of TFIIH dysfunction. Consequently, an even further reduction of crosslinking proteins would introduce a highly fragile point at the base of the hair resulting in immediate, complete hair loss. To test this possibility, we first determined the cellular content of XPD and other TFIIH subunits in TTD1RO-SV and corrected clones at 37 °C.

Immunoblot analysis of whole cell extracts indicated a 75–80% reduction of XPD level in TTD1RO-SV compared with MRC5-SV and corrected clones using other nuclear proteins as internal controls (Fig. 3*c*). We also saw a slight reduction in p62 and XPB. Introduction of wild-type *XPD* restored XPD and concomitantly p62 to normal levels (Fig. 3*c*,*d*). These results were

Table 1 • Clinical features of XPD syndromes

Clinical symptoms	TTD1RO/ TTD1DOD		PIBIDS	CS	XP-D	XP/CS
sun sensitivity	mild	+	+	+	++	+
pigmentation abnormalities	–	–	–	–	+	+
ichthyosis	+*	+	+	–	–	–
brittle hair and nails	+*	+*	+	–	–	–
skin cancer	–	–	–	–	++	+
caries	?	+	+	+	–	?
mental retardation	mild	mild	+	+	+	+
accelerated neurodegeneration	?	?	–	–	+	–
neurodysmyelination	?	?	±	+	–	+
cachectic dwarfism/ failure to thrive	+	mild	+	+	mild	+
impaired sexual development	?	?	+	+	–	+
ataxia	–	+*	±	–	±	+
recurrent infections	+	+	±	–	–	±

*Features more pronounced in episodes of fever. The main clinical features of three XPD syndromes: XP, CS combined with XP, and the photosensitive form of TTD designated PIBIDS (for photosensitivity, ichthyosis, brittle hair and nails, impaired intelligence, decreased fertility and short stature). Also indicated are the clinical manifestations of patients TTD1RO and TTD1DOD and the symptoms that changed during febrile episodes.

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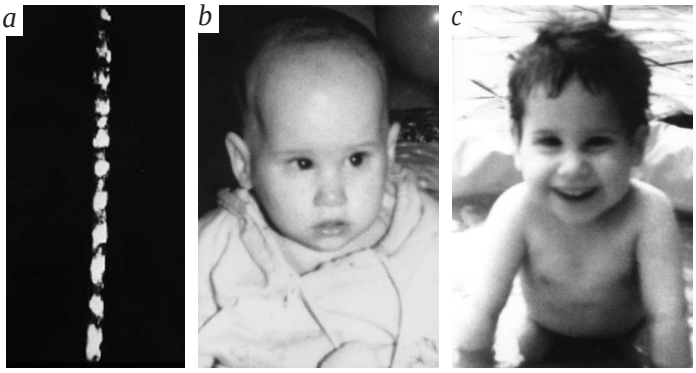

Fig. 1 Clinical characteristics of patients TTD1RO and TTD1DOD. ***a***, Alternating light and dark ('tiger tail') pattern of the hair-shaft of TTD patient TTD1DOD, as observed using polarizing light microscopy. This pattern is characteristic for the brittle hair of TTD, due to the reduced content of cysteine-rich matrix proteins that are involved in cross-linking of keratin filaments. ***b***, Photograph of TTD patient TTD1RO 10 days after pneumonia. ***c***, Same patient 10 weeks after she lost all hair.

obtained using different antibodies specific for distinct parts of XPD and different extraction protocols, indicating that XPD reduction is intrinsic to the mutation. To rule out *in vitro* artifacts, however, we developed a method to compare directly intracellular protein levels by immunofluorescence using a mixture of cells (discernable by bead-labeling) seeded on the same slide, excluding intersample variation. TTD1RO-SV cells (Fig. 3*d*) have strongly reduced XPD and slightly reduced p62 nuclear staining compared with MRC5-SV. p62 levels in TTD1RO-SV are restored by introduction of *XPD* (clone 13), extending the *in vitro* observations to the *in vivo* situation. These results are in line with our findings that the overall quantities of TFIIH are diminished in cells from classical TTD group D patients[15] (Fig. 4*f*) and TTD-A patients[16].

We investigated the effect of temperature by incubating TTD1RO-SV and controls at various temperatures for different periods and monitored basal transcription by *in vivo* incorporation of ^{3}H-uridine using liquid scintillation counting. We found that temperatures oscillating around 41 ºC yielded a clear-cut distinction between the mutant cells and both control transformants (Fig. 4*a*). Transcription in TTD1RO-SV dropped to approximately 30% at 32 hours and to approximately 10% at 48 hours after temperature shift, whereas both rescued clones maintained global transcription above 70% of the transcriptional level at 37 ºC. Thus, transcription in TTD1RO-SV is more sensitive to elevated temperatures than the same cells rescued with *XPD*. We next determined the *in vivo* rate of transcription in TTD1RO and C5RO (control) primary fibroblasts using ^{3}H-uridine incorporation detected by *in situ* autoradiography. At 37 ºC transcription of TTD1RO was

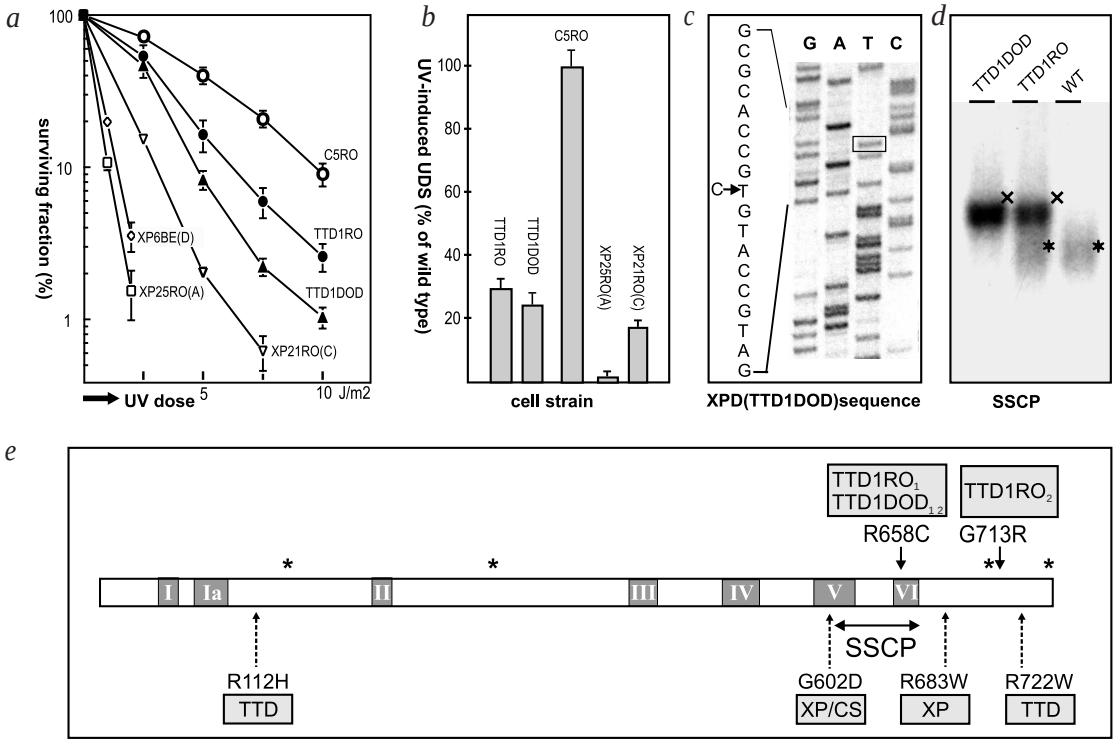

Fig. 2 DNA repair characteristics and XPD defect of TTD1RO and TTD1DOD. ***a***, Cellular sensitivity to ultraviolet light (survival assay) of TTD1RO and TTD1DOD primary fibroblasts compared with wild-type (C5RO) fibroblasts and three reference XP strains from groups A, C and D. The surviving fraction is measured by ^{3}H-thymidine incorporation, which reflects proliferative capacity. ***b***, Overall NER activity measured as ultraviolet-induced unscheduled DNA synthesis (UDS), normalized to wild-type C5RO and compared with XP strains from groups A and C. ***c***, Part of the *XPD* cDNA sequence analysis gel showing the C2050T transition (boxed) in the cDNA of patient TTD1DOD, which leads to an arginine-to-cysteine amino acid substitution in the XPD protein at position 658 (R658C) (*e*). ***d***, Single-strand conformation polymorphism analysis (SSCP) of RT–PCR cDNA fragment of *XPD* of patients TTD1DOD and TTD1RO containing the C2050T transition. Note the presence of the (diffuse) wild-type fragment in the RT–PCR products of TTD1RO (asterisk) indicating heterozygosity at this location in the gene and its absence in RT–PCR products of TTD1DOD. ***e***, Schematic presentation of the XP helicase and relevant mutations. Gray boxes, seven helicase motifs. Arrows above the protein indicate the mutant alleles found in the alleles of TTD1RO and TTD1DOD. Arrows below the protein indicate causative alleles for XP, XP/CS and classical TTD variants of XP-D patients. Asterisk, position of four common polymorphisms in XPD for which patient TTD1DOD is homozygous. The location of the *XPD* cDNA fragment used for the SSCP analysis in (*d*) is indicated.

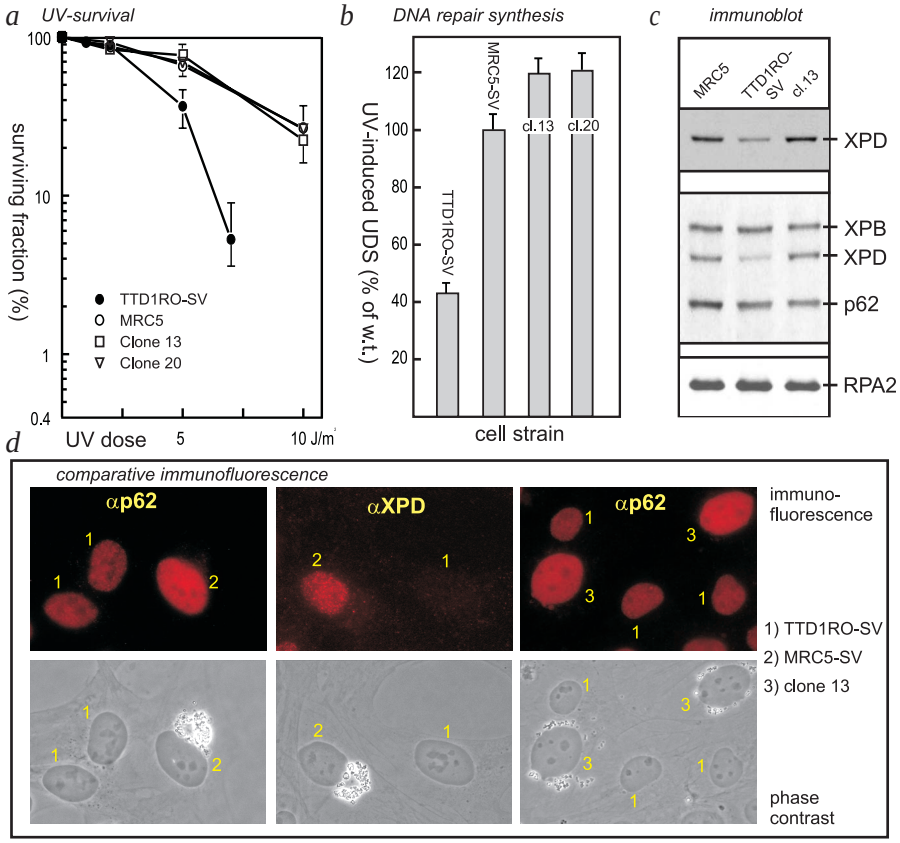

Fig. 3 DNA repair properties and TFIIH defect in TTD1RO-SV. ***a***, Cellular sensitivity to increasing ultraviolet dose of SV40-transformed TTD1RO fibroblasts (TTD1RO-SV) cells, repair-competent MRC5-SV cells and two TTD1RO-SV clones stably expressing wild-type *XPD* cDNA (clone 13 and clone 20). Ultraviolet survival was determined by ^{3}H-thymidine incorporation. ***b***, NER activity measured as ultraviolet-induced UDS of TTD1RO-SV cells normalized to wild-type MRC5-SV cells and compared with TTD1RO-SV transformants 13 and 20 stably expressing wild-type *XPD* cDNA. ***c***, Immunoblot analysis of whole cell extracts (10 μg) after SDS–PAGE (11% acrylamide) using monoclonal antibodies against XPB, XPD and the p62 subunits of TFIIH and against RPA2, the second subunit of the single strand binding protein replication protein A (RPA) used as a loading control. Top, a longer exposure of the same blot. Note that a lower amount of protein is loaded for clone 13 compared with the mutant cells (see RPA2 signal), masking the slightly higher signal for XPB and p62 in the clone 13 lane. Reduction in TFIIH was independent of the applied extraction procedure, ruling out degradation during extract preparation. ***d***, Comparative immunofluorescence of TTD1RO-SV (no beads, 1), MRC5-SV cells (2) and TTD1RO-SV corrected clone 13 (3), both labeled with latex beads for the presence of TFIIH subunits XPD, p62 protein, with antibody incubations. Top, immunofluorescent picture; bottom, phase contrast micrograph of the same cells.

slightly lower than that of C5RO (Fig. 4*b*). Normal C5RO fibroblasts incubated for 32 hours at 41 ºC (fluctuating) retained 70% of the transcription level at 37 ºC. Transcription in TTD1RO fell to 48% of the level at 37 ºC, confirming the findings with the SV40-transformed cells; however, the reduction in TTD1RO-SV was more pronounced, perhaps due to transformation-induced enhanced proliferation.

Upon incubation at 41 °C for 32 hours, overall NER activity of normal (C5RO) cells was reduced to approximately 75%, but dropped to less than 10% of the already reduced level at 37 °C in TTD1RO (Fig. 4*c*), demonstrating that NER in TTD1RO is even more temperature sensitive than transcription. To assess directly the effect of temperature on TFIIH, we examined TTD1RO and TTD1DOD primary fibroblasts for TFIIH levels by comparative immunofluorescence. Because XPD is barely detectable at 37 ºC (Fig. 3*d*), we focused on p62, a core subunit of TFIIH (Fig. 4*e*,*f*). p62 was slightly reduced at 37 ºC but strongly diminished at 41 ºC compared with neighboring normal cells (Fig. 4*e*), whereas cells from a classical TTD (group D) patient, TTD1BEL (Fig. 4*f*), failed to show a strong temperature-sensitive reduction of TFIIH. No difference was seen with XPA, another NER protein. We observed a similar temperature-dependent selective reduction of p62 in TTD1DOD fibroblasts (data not shown). These findings indicate that the R658C mutation in XPD confers thermolability to the other essential components of TFIIH. Elevated temperature also affects cellular viability (Fig. 4*d*), as expected with such dramatic effects on transcription.

Extensive clinical heterogeneity due to different mutations in *XPD* is apparent[4,6,17]. XP-type XPD patients are easily accounted for when only the NER function is affected. XP/CS and TTD classes of mutations in *XPD* are thought to derive from an additional crippled transcription[3]. This can occur in several ways: either by transcriptional insufficiency of specific genes for which TFIIH becomes rate limiting[5], or of its function in transcription-coupled repair, a system that is responsible for recovery of RNA synthesis after damage-induced transcriptional blockage[18–20]. In the latter scenario, transcription elongation becomes sensitive to endogenously induced DNA injuries, compromising transcription in a different manner. Finally, as shown here, a mutation may cause thermoinstability of XPD and subsequently of other TFIIH components. In classical TTD-type XPD patients, such as TTD1BEL, the content of XPD and other TFIIH subunits is constitutively reduced, as in the case of TTD1RO at 37 ºC (Fig. 4*f*). A less pronounced reduction in XPD was observed in XP/CS and XP-type XPD patients, consistent with earlier observations[15]. The notion that short-term TTD characteristics, such as hair and skin symptoms, are fever-dependent in patients with the R658C mutation indicates that the amount of TFIIH is critical for these clinical features. Short-term effects on other TTD and CS characteristics (such as retarded growth and neurodysmyelination) may occur, but are more difficult to determine. Alternatively, they may derive from the other consequences of mutant TFIIH.

Why does TFIIH instability mainly cause brittle hair, with no complications in many other tissues? We favor the following scenario. In most cells, continuous *de novo* synthesis of the complex may (partly) compensate for TFIIH instability. But in terminal differentiation of hair, nails and skin, the gene family of cysteine-

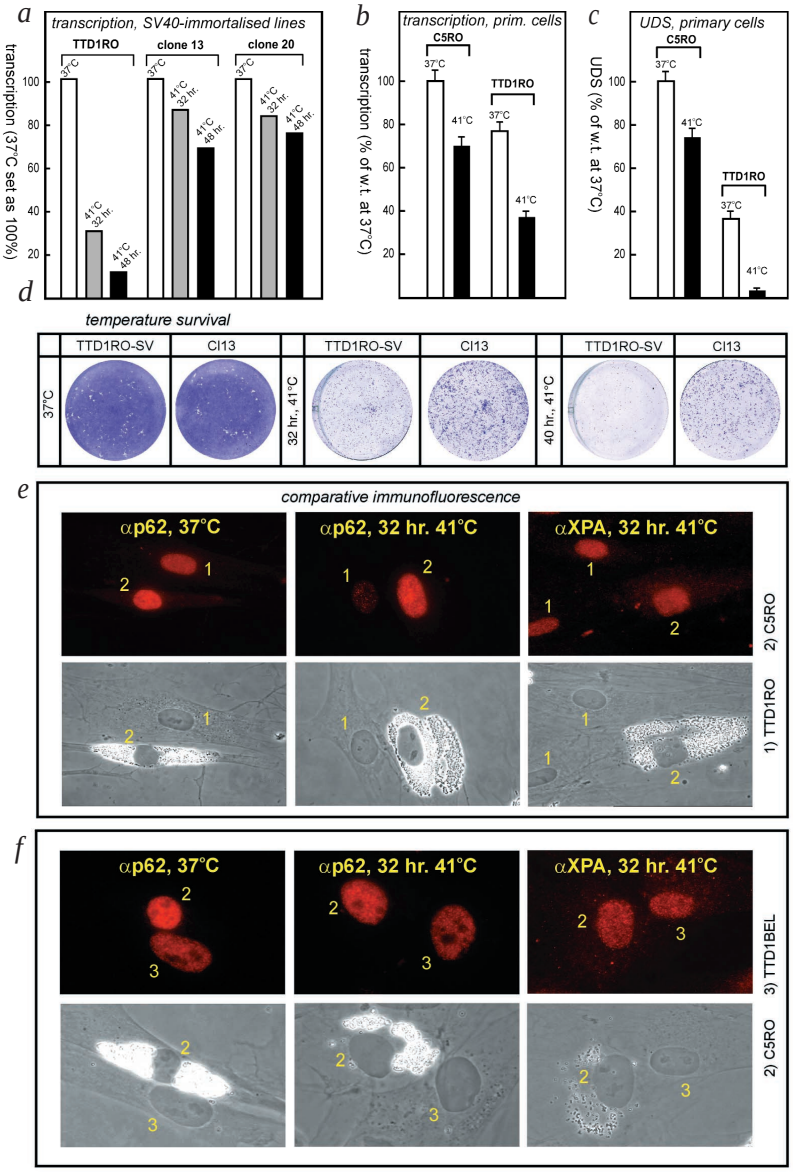

Fig. 4 Effect of temperature increase on transcription, repair, TFIIH and survival in TTD1RO cells. ***a***, Overall level of transcription (incorporation of ^{3}H-uridine) as measured by liquid scintillation counting at different temperatures in TTD1RO-SV and two corrected TTD1RO-SV clones stably transformed with the wild-type *XPD* cDNA (clone 13 and clone 20). The incorporation at 41 °C (fluctuating temperature, 32 h) is graphed relative to the level of transcription at 37 °C of the same cell line. ***b***, Overall level of transcription (incorporation of ^{3}H-Uridine) determined by *in situ* autoradiography at different temperatures in primary fibroblasts of TTD1RO and wild-type C5RO. All values normalized to the level of C5RO at 37 °C. ***c***, Residual NER activity determined by assay for ultraviolet-induced UDS at different temperatures in primary fibroblasts of TTD1RO and wild-type C5RO. All values normalized to the level of C5RO at 37 °C. ***d***, Colony-forming ability of TTD1RO-SV and the corrected transformant clone 13 containing wild-type *XPD* cDNA after 32 and 40 h incubation at (fluctuating) 41 °C. ***e***, Comparative immunofluorescence of primary fibroblasts of TTD1RO (1) and C5RO control fibroblasts (2) for the level of p62 at 37 °C, and p62 and XPA after 32 h at 41 °C (using fluctuating temperature). Top, immunofluorescence; bottom, phase contrast micrograph of the same cell. ***f***, Classical TTD, TTD1BEL (3) is compared with C5RO (2).

rich matrix proteins is the last to be transcribed at a very high rate: the encoded proteins constitute a major fraction of hair and ensure keratin-crosslinking before cell death[21]. In the case of destabilizing TTD mutations, TFIIH may be depleted before terminal differentiation is complete. As a consequence, crosslinking of keratin filaments is not finished, leading to incomplete (brittle) hair. This phenomenon becomes more pronounced in XPD-TTD patients carrying the R658C mutation, when high fever further destabilizes TFIIH.

In the human population, temperature-sensitive mutations with an associated feverish crisis have only been described sporadically[22,23]. The case presented here, however, shows that such mutations occur even in fundamental cellular process like transcription.

Methods

Cell lines, culture conditions and extract preparation. Primary fibroblasts (TTD1RO, TTD1DOD, TTD1BEL and XP6BE (all XP-D), C5RO (wild type), XP21RO (XP-C), XP25RO (XP-A)) and SV-40 transformed cells (TTD1RO-SV, MRC5 (wild type)) were both cultured under standard conditions at 37 ºC and 5% CO_2 in Ham's F-10 medium supplemented with 12% FCS and antibiotics, and a 1:1 mixture of F-10 and DMEM supplemented with 8% FCS and antibiotics, respectively.

We carried out SV-40 immortalization of TTD1RO primary fibroblasts as described[24]. Lipofectine-mediated gene transfer, according to the manufacturer's protocol (BRL), was used to isolate clones of TTD1RO-SV cells that stably express wild-type *XPD* inserted into the pCDNA3 (Invitrogen) expression vector containing the dominant selectable gene *neo*. Transfectants were selected by gentamycine (G418, 250 µg/ml, Gibco) resistance, 24 h after transfection, followed by triple ultraviolet-C irradiation of 8 J/m^2 with one-day intervals. Different ultraviolet-resistant clones were isolated and tested for their repair capacity.

Whole-cell extracts used for immunoblot analysis (according to standard procedures) were prepared as follows: sonication of cell pellets, boiling of cells in gel loading buffer, and Dignam and Manley-type of procedures, all in the presence of a cocktail of protease inhibitors.

Ultraviolet survival and survival after incubation at elevated temperature. We determined ultraviolet resistance by means of ^{3}H-thymidine incorporation (a measure of the number of proliferating cells) 5 d after treatment with the indicated ultraviolet doses[25]. To determine the temperature sensitivity, 10^4 cells (TTD1RO-SV and clone 13) were seeded 1 d before temperature shift into 6-well plates and grown at 37 ºC or 41 ºC (±0.6 ºC every ~15 min), respectively, for 32 h. After temperature shift, cells were grown for an additional 5 d under normal culture conditions at 37 ºC and subsequently fixed and stained with methanol/acetic acid/Coomassie brilliant blue.

Ultraviolet-induced unscheduled DNA synthesis. Cells were exposed to 15 J/m^2 ultraviolet-C light (254 nm), followed by a 2 h culturing in Ham's F-10 medium supplemented with 10% dialyzed FCS, antibiotics and 10 µCi/ml of ^{3}H-thymidine (Amersham; 120 Ci/mMol). Excess of radioactive nucleotides was removed by PBS washing and fixation. Incorporated ^{3}H-thymidine was visualized by microscopic autoradiography. Slides were dipped into a photographic emulsion (Ilford K2) and exposed for 3 d before photographic development. DNA repair synthesis was quantified by counting the autoradiographically-induced silver grains above the nuclei and comparing the mean number of grains (of at least 25 nuclei) with the number of grains above nuclei of repair-proficient cells assayed in parallel.

Overall transcription. General transcription within SV-40 immortalized cells was measured by ^{3}H-uridine incorporation in growing cells. Before temperature shifts, we seeded 10^{4} cells into 6-well plates and labeled them 1 d after plating with ^{14}C-thymidine (0.05 μCi/ml) during 24 h to provide a measure for the number of cells. Non-incorporated thymidine was removed and cells were differently placed at elevated temperature (41 °C±0.6 °C) during the indicated times. One hour after recovery at 37 °C, cells were pulse labeled for 1 h with 10 μCi/ml of ^{3}H-uridine. After extensive washing with PBS, cells were collected and lysed in 0.2 N NaOH (1 ml), 0.5 ml was added to 5 ml Hionic-fluor (Packard) liquid scintillation fluid and counted in a liquid scintillation analyzer (Tri-Carb 2100 TR, Packard). The ratio of ^{3}H counts over ^{14}C counts is a measure of the general transcription corrected for the number of cells.

The primary fibroblasts were subjected to a 1-h pulse labeling with ^{3}H-uridine (10 μCi/ml, Amersham: 50 Ci/mMol). Fixation, autoradiography and quantification were as described above for UDS.

Immunofluorescence. Primary fibroblasts C5RO and SV40-transformed MRC5 (wild-type control; NER-proficient) were labeled with 0.79 μm latex beads (Polybead Carboxylate Microspheres, Polysciences), whereas TTD1RO, TTD1RO-SV and TTD1BEL cells had no beads. Fibroblasts were cultured 2 d before mixing of the cells, in Ham's F-10 medium supplemented with 12% FCS and antibiotics (penicillin and streptomycin). Cells were thoroughly washed (3 times in PBS) before trypsinization to remove the non-incorporated beads and seeded in a 1:1 ratio on coverslips and cultured for 2 d. Cells were fixed with 2% paraformaldehyde and permeabilized by rinsing the cells 5 times during 5 min in PBS containing 0.1% Triton X-100, followed by washing with PBS^{+} (PBS + 0.15% glycine and 0.5% BSA). Antibody incubations were performed at RT during 2 h and were diluted in PBS^{+}. The different monoclonal antibodies were diluted as follows: anti-XPD (2F6), 2,000×; anti-p62 (3C9), 3,000×, and the polyclonal anti-XPA, 1,000×. After incubation coverslips were washed with PBS + 0.1% Triton X-100 (5×, 5 min), followed by the secondary antibody incubation, respectively: goat anti-mouse, Cy3-conjugated (The Jackson Laboratory) and goat anti-rabbit, Alexa 594-conjugated (Molecular Probes), both sera were diluted 800× in PBS^{+}. After the same wash procedure, coverslips were mounted in Vectashield mounting medium (Vector Laboratories) containing DAPI (1.5 μg/μl) and stored at –20 °C in the dark. Epifluorescent and phase-contrast images were produced on a Leitz Aristoplan microscope.

Acknowledgments

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