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The Cancer-free Phenotype in Trichothiodystrophy Is Unrelated to Its Repair Defect[1]

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ABSTRACT

The DNA repair-deficient genetic disorders xeroderma pigmentosum (XP) and trichothiodystrophy (TTD) can both result from mutations in the *XPD* gene, the sites of the mutations differing between the two disorders. The hallmarks of XP are multiple pigmentation changes in the skin and a greatly elevated frequency of skin cancers, characteristics that are not seen in TTD. XP-D and most TTD patients have reduced levels of DNA repair, but some recent reports have suggested that the repair deficiencies in TTD cells are milder than in XP-D cells. We reported recently that inhibition of intracellular adhesion molecule-1 (ICAM-1) expression by UVB irradiation was similar in normal and TTD cells but increased in XP-D cells, suggesting a correlation between ICAM-1 inhibition and cancer proneness. In the first part of the current work, we have extended these studies and found several other examples, including XP-G and Cockayne syndrome cells, in which increased ICAM-1 inhibition correlated with cancer proneness. However, we also discovered that a subset of TTD cells, in which arg112 in the NH_2-terminal region of the XPD protein is mutated to histidine, had an ICAM-1 response similar to that of XP-D cells. In the second part of the work, we have shown that TTD cells with this specific NH_2-terminal mutation are more sensitive to UV irradiation than other TTDs, most of which are mutated in the COOH-terminal region, and are indistinguishable from XP-D cells in cell killing, incision breaks, and repair of cyclobutane pyrimidine dimers. Because the clinical phenotypes of these patients do not obviously differ from those of TTDs with mutations at other sites, we conclude that the lack of skin abnormalities in TTD is independent of the defective cellular responses to UV. It is likely to result from a transcriptional defect, which prevents the skin abnormalities from being expressed.

INTRODUCTION

NER[5] is a highly conserved fundamental process by which organisms are able to remove many different types of damage from their DNA. Individuals afflicted with the disorder XP are defective in NER, and the defects in different patients have been assigned to seven different complementation groups, designated XP-A through G. The *XP* genes defined by these complementation groups have all been cloned, and their encoded products participate in different steps in the NER process. They comprise three proteins involved in damage recognition (XPA, XPC, and XPE), two helicases (XPB and XPD) and two nucleases (XPF and XPG; Refs. 1 and 2).

Cells from patients with CS and from many but not all individuals with TTD are also deficient in NER, but the clinical features of these disorders are quite distinct from those of XP. As well as being photosensitive, XP patients show a wide variety of pigmentation changes in sun-exposed areas of the skin and have an elevated incidence of skin cancer, which has been estimated to be ~2000 times greater than that in normal individuals (3). CS and TTD are, in contrast, cancer-free multisystem disorders. There are no reports of skin cancer in any TTD patient, nor do TTD patients show the pigmentation abnormalities that are the principal hallmark of XP (reviewed in Ref. 4). The discovery that, in most cases of NER-defective TTD, the defect was in the XP-D complementation group (5, 6) was therefore surprising, given the very marked differences in the clinical features of TTD and XP. A clue to the resolution of this paradox came with the finding that the XPB and XPD proteins are subunits of the transcription factor TFIIH, which has two distinct roles, in basal transcription and in NER (7). This raised the possibility that different mutations in these genes could affect NER and transcription differentially. It has been proposed that if such a mutation affected only NER, the outcome would be XP, whereas if transcription were subtly altered, the phenotype of TTD would result (8). Implicit in this hypothesis is the prediction that the site of the mutation determines the clinical phenotype. Extensive analyses of mutations in the *XPD* gene in XP and TTD patients in the XP-D complementation group have borne out this prediction (9).

Four TTD patients are known to contain a mutation resulting in the change of arg722→trp in the XPD protein (9), and a mutant mouse containing this single missense mutation has been generated (10). This mutant mouse showed many TTD-like features, including sulfur-deficient brittle hair, the major hallmark of this condition (10). These findings strongly support the hypothesis that the site of the mutation in the *XPD* gene determines the clinical phenotype and are consistent with the idea that the developmental defects in TTD result from subtle abnormalities in transcription. The mildness of the skin abnormalities in TTD relative to XP is not readily explained, however, by any of the above findings.

It has been proposed (11, 12) that apart from the repair deficiency, a defective immune response also contributes to the greatly elevated incidence of skin cancer in XP. There are several reports in the literature of immune deficiencies in XP patients, but the results have not been consistent (13, 14). Recently, we investigated the effect of UVB irradiation on the induction of ICAM-1 as a model immunological read-out system in cultured fibroblasts from normal, TTD, and XP patients (15). ICAM-1 is expressed in skin cells such as keratinocytes and Langerhans cells, where it interacts with leukocyte function-associated antigen 1, expressed on all leukocytes, and this interaction is essential for a large number of cell-mediated immune responses. When fibroblasts are stimulated with the proinflammatory cytokine IFN-γ, the expression of ICAM-1 on their surface is up-regulated. This up-regulation is inhibited by prior exposure of the cells to UVB. This inhibition is much greater in XP than in normal cells (16). In

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[5] The abbreviations used are: NER, nucleotide excision repair; XP, xeroderma pigmentosum; CS, Cockayne syndrome; TTD, trichothiodystrophy; ICAM-1, intracellular adhesion molecule 1; UDS, unscheduled DNA synthesis; GAPDH, glyceraldehyde phosphate dehydrogenase; ara-C, 1-β-D-arabinofuranosylcytosine; CPD, cyclobutane pyrimidine dimer.

contrast, when we examined three TTD cell strains with low, intermediate, and normal levels of NER as measured by UDS, the ICAM-1 suppression by UVB in all of them was similar to that in normal individuals (15). This raised the possibility that inhibition of the immune response after UV irradiation might be differentially affected in XP and TTD patients, and that the lack of skin pigmentation abnormalities and skin cancer in TTD individuals might be attributable to a normal ICAM-1 response in the skin.

To check the generality of the relationship between the skin symptoms and ICAM-1 response, in the first part of this report we describe an extension of our studies to include XP cells from different complementation groups, CS cells, and cells from additional TTD patients. Most mutations in the *XPD* gene in both XP and TTD individuals cause changes in the COOH-terminal third of the protein (9). An exception to this is a subgroup of TTD patients that are associated with the change arg112→his in the NH_2-terminal region. We report that cells from this group of patients have, like XP-D cells, a more pronounced ICAM-1 response than other TTD cell lines. In the second part of the report, we show that several other responses to UV in this subgroup are more severe than those of other TTDs and are indistinguishable from those of XP-Ds.

MATERIALS AND METHODS

Cell Culture. Primary human fibroblasts from normal and DNA repair-deficient individuals (Table 1) were cultured in Eagle's MEM (Life Technologies, Inc., Buckinghamshire, United Kingdom) containing 15% FCS and kept at 37°C in a humidified atmosphere with 5% CO_2. The TTD and XP-D cells are mutated at different loci in the *XPD* gene, as indicated in Fig. 1.

UV Irradiation. For irradiation, medium was removed, and cells were irradiated with the indicated doses of UVB from four Westinghouse FS20 sunlamps and UVC from a Phillips 6-W germicidal lamp, which are known to emit primarily in the UVB and UVC range, respectively. The UV output was measured by means of an IL1350 research photometer (International Light, Newburyport, MA).

ICAM-1 mRNA Detection. Detection of ICAM-1 mRNA after UVB irradiation has been described previously (15). In brief, immediately after UVB irradiation, cells were washed, cultured in medium, and stimulated with 500 units/ml recombinant human IFN-γ (Genzyme). After a 4-h incubation period, cells were harvested, total RNA was isolated, and IFN-γ-induced ICAM-1 mRNA expression was determined by semiquantitative differential reverse transcription-PCR (17). For estimation of similar amounts of cDNA used for PCR, samples were screened for expression of *GAPDH* as a "housekeeping" gene. The following primer oligonucleotides specific for ICAM-1 and GAPDH were used: ICAM-1, 5′-TGACCAGCCCAAGTTGTTGG-3′, 5′-ATCTCTCCTCACCAGCACCG-3′; and GAPDH, 5′-CCACCCATGGCAAATTCCATGGCA-3′, 5′-TCTAGACGGCAGGTCAGGTCCACC-3′. PCR products were separated either by ion-exchange chromatography and analyzed as described previously (15). Alternatively, they were separated electrophoretically in a 2% agarose gel, stained with the fluorescent dye Vistra green (Molecular Dynamics, Buckinghamshire, United Kingdom) at a dilution of 1 in 10^5 and quantified fluorimetrically with a Storm 840 Phosphorimager (Molecular Dynamics).

Cell Survival. The ability of cells to form colonies after UV irradiation was measured as described previously (18).

Single-Cell Gel Electrophoresis "Comet" Assay. A layer of 0.7% agarose in PBS was prepared on frosted microscope slides. Fibroblasts were trypsinized for 5 min with trypsin/0.4% EDTA at 37°C, resuspended in PBS, and mixed with an equal volume of previously melted low-melting-point agarose to give a final concentration of 4×10^5 cells/ml in 0.7% agarose. Fifty μl of cell/agarose suspension (containing 2×10^4 cells) were added on top of the previously prepared agarose layer on each slide, and a coverslip was placed over the cells. Slides were maintained on ice, and coverslips were removed for irradiation. Immediately after irradiation, cells were incubated at 37°C in the dark with 100 μM ara-C and 10 mM hydroxyurea to allow NER-mediated incisions to accumulate. The slides were subsequently immersed in lysis solution (2.5 M NaCl, 0.2 M NaOH, 100 mM EDTA-Na_2, 10 mM Tris base, 10% DMSO, and 1% Triton X-100, pH 10) for at least 1 h at 4°C. Slides were then electrophoresed in alkaline conditions (0.3 M NaOH, 1 mM EDTA-Na_2) at 20 V for 24 min. After electrophoresis, slides were washed with neutralizing buffer (0.4 M Tris-HCl, pH 7.5) and stained by the addition of 35 μl of ethidium bromide solution (20 μg/ml) onto the gel. Comet lengths were determined by fluorescence microscopy and measured using the Casys system (Synoptics, Cambridge, United Kingdom).

Removal of CPDs. For measurement of CPD removal, the comet assay was performed as described above with the following changes. After irradiation with 5 Jm^{-2} UVB, cells were overlaid with T4 endoV DNA glycosylase/AP lyase (T4endoV, 5–20 μg/ml; kindly provided by Applied Genetics, Freeport, NY) in T4 endoV buffer [10 mM Tris-HCl (pH 8.0), 10 mM EDTA, and 75 mM NaCl] for 1 h and kept at 37°C in a humidified atmosphere.

Human *XPD* Mutations in *Schizosaccharomyces pombe*. The construction of mutant *rad15* plasmids in the vector pRep81, containing the inducible *nmt* promoter, has been described in an earlier publication (9). *rad15* is the *S. pombe* homologue of *XPD*, and we have generated *rad15* mutations homologous to those found in human patients. One μg of plasmid was used to transform *rad15.P* cells. Transformants were selected by their growth in the absence of leucine. They were grown for 24 h in the absence of thiamine to induce the *nmt* promoter and plated in the continued absence of thiamine. Immediately after plating, the cells were exposed to different doses of UVC irradiation, and the number of colonies were counted after 4–5 days.

RESULTS

ICAM-1 Inhibition. ICAM-1 expression in human fibroblasts can be up-regulated by treatment with IFN-γ. This up-regulation is inhib-

Table 1 *Cell strains used*

Cell strain	Complementation group	Amino acid changes[a]	UDS (UVC) (% of normal)	D_{10} (UVC) (Jm^{-2})	D_{10} (UVB) (Jm^{-2})	Reference
1BR	Normal		100	14	350	
TTD1BEL	XP-D	R722W (R616P)	15	3.0	85	(23)
TTD2GL	XP-D	R112H (del aa[b] 488–493)	10	2.0	48	(22, 23)
TTD1LU	XP-D	ND	15	ND	ND	
TTD11PV	XP-D	R112H (del aa121–159)	12	0.5	ND	(24)
TTD1VI	XP-D	R722H (L461V; del aa 716–730)	30	2.5	63	(9, 37)
TTD9VI	XP-D	R112H	5	0.45	11	(30)
XP1BR	XP-D	R683W	25	1.6	34	(9)
XP16BR	XP-D	R683W (R616P)	25	0.8	26	(9)
XP125LO	XP-G		25	2.5	70	(38)
XP3BR	XP-G		2	1	ND	(21)
XP7NE	XP-F		25	12	350	
XP24BR	XP-F		5	3.5	110	
CS4BR	CS-B		(100)[c]	ND	30	(20)
CS5BR	CS-A		(100)[c]	4	200	(20)

[a] Patients were either homozygous for the indicated mutations or compound heterozygotes. In compound heterozygotes, the alterations shown in parentheses are changes found in the second *XPD* allele in these cell lines and shown or assumed to be lethal (9).

[b] del, deletion; aa, amino acid.

[c] CS-A and CS-B cells do not have defects in UDS, and although we have not measured UDS in these cells, we assume that it is normal.

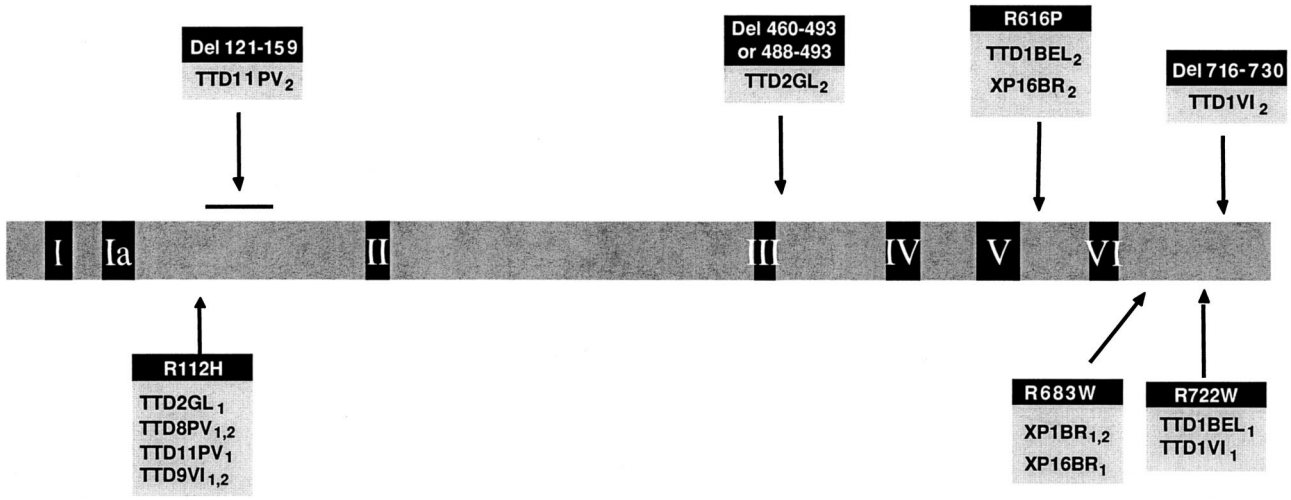

Fig. 1. The XPD protein, showing the seven helicase domains and the sites of the mutations in the cell strains used in this study. Mutations *above* the protein are null mutations completely destroying XPD function, whereas those shown *below* are the causative mutations (9).

ited by UVB. Our previous results suggested that there might be a correlation between the severity of the skin symptoms of NER-deficient individuals and the inhibition by UVB of ICAM-1 up-regulation. In this previous study, cells from three TTD individuals showed a normal UV response, whereas the inhibition in three XP-D cell lines was greater than that in normal cells (15). Here, we have extended our studies to include cells from other NER-deficient individuals. The cell lines that we have used are listed in Table 1.

We first investigated cells from patients with the cancer-free NER disorder, CS. At the cellular level, the cells of CS patients are sensitive to killing by UV irradiation, but NER in the bulk of the genome is normal. However, CS cells are specifically defective in transcription-coupled repair, a subpathway of NER, in which damage in the transcribed strand of active genes is removed rapidly (19). In CS cell lines from both known CS complementation groups (20), CS5BR (CS-A) and CS4BR (CS-B), the ICAM-1 response was close to normal (Fig. 2*A*). This result is consistent with our working hypothesis that a normal ICAM-1 response correlates with the lack of skin cancer and other pigmentation changes. If anything, there was less inhibition in the CS than in the normal cells, but the significance of this is unclear.

We had available in our collection of XP fibroblast cell lines cultures from two XP-G patients with quite different clinical characteristics. XP125LO is derived from an individual with very mild XP symptoms, but the cells are ~5-fold more sensitive than normal cells to killing by UVC or UVB, and UDS is severely reduced (~25% of normal). XP3BR, in contrast, is derived from a very severely affected patient, who died at age 18. The cells were >10-fold more sensitive to UV than normals, and UDS was barely detectable (21). Consistent with this disparity in clinical features, we found a normal ICAM-1 response in XP125LO but an increased inhibition in XP3BR (Fig. 2*B*).

In our previous work, we examined three TTD cell strains. The levels of NER vary in TTD cells (6, 22), and of the three cell lines examined previously, TTD4BR had normal NER; in TTD1BI, UDS was 50% of normal (22), whereas in TTD1BEL, UDS was only 15% of normal (6). We have extended these observations to include other repair-deficient TTDs with mutations at different sites in the *XPD* gene. In TTD1VI and TTD1LU, which have UDS levels of about 30 and 15%, respectively, the ICAM-1 response was normal, as with the other TTDs (Fig. 2*C*). However, we were surprised to discover that the responses of TTD2GL and TTD9VI were (at least in the lower dose range) similar to those found in the XP cells rather than showing a normal response, as found in the other TTD cells (Fig. 2*C*). This observation was reproducible in three separate experiments.

Finally, we measured the ICAM-1 response in a pair of XP-F cell strains, XP24BR and XP7NE. The former is derived from a patient with severe photosensitivity and numerous keratoses, whose cells display barely detectable UDS, whereas the latter is from a patient with very mild symptoms, no photodamage, and UDS ~25% of the normal level. Despite the mildness of the clinical features in the latter patient, the ICAM-1 response of both cell strains was characteristic of that of other XP cell lines (Fig. 2*D*).

We conclude from the experiments shown in Fig. 2 that, although the inhibition by UVB of ICAM-1 up-regulation correlated with the severity of clinical features in the skin in several instances, there were some important exceptions. In particular, the variation in responses of different TTD cell strains suggests that the ICAM-1 inhibition by UV cannot explain the phenotypic differences between XP and TTD patients.

TTD Cells with Severe ICAM-1 Response Are Mutated at the Same Site Near the NH_2 Terminus of the Protein. In view of the difference in the ICAM-1 responses of TTD2GL and TTD9VI from those of other TTDs, we decided to investigate the properties of these cell strains in more depth and to compare them with other TTD and XP-D cell strains to determine whether they were different in other responses to UV. Most of the mutations in the *XPD* gene in XP-D and TTD patients are located in the COOH-terminal third of the protein (9). In contrast, both TTD2GL and TTD9VI carry the same inactivating mutation in the *XPD* gene close to the NH_2 terminus, *i.e.*, arg112his (see Fig. 1). TTD9VI is homozygous for this mutation, whereas TTD2GL is functionally hemizygous, with the second allele being a null mutation (9, 23).

Cell Survival. All of the TTD fibroblast cultures used in the experiments of Fig. 2*C* had UDS levels in the 10–30% range, similar to those of many XP cell lines belonging to the XP-D complementation group. However, our cell survival assays showed that TTD9VI was dramatically more sensitive to both UVC and UVB than other TTD cell lines (Fig. 3). Furthermore, TTD11PV (Fig. 3*A*) and TTD8PV (results not shown), two other TTD cell strains mutated at arg112 (24), were also very hypersensitive. These cell lines were at least as sensitive to both UVC and UVB as the XP-D cell line XP16BR. (TTD2GL, also mutated at arg112, was somewhat less sensitive than the other three lines carrying this mutation, an observation for which we do not have a ready explanation). The other TTD

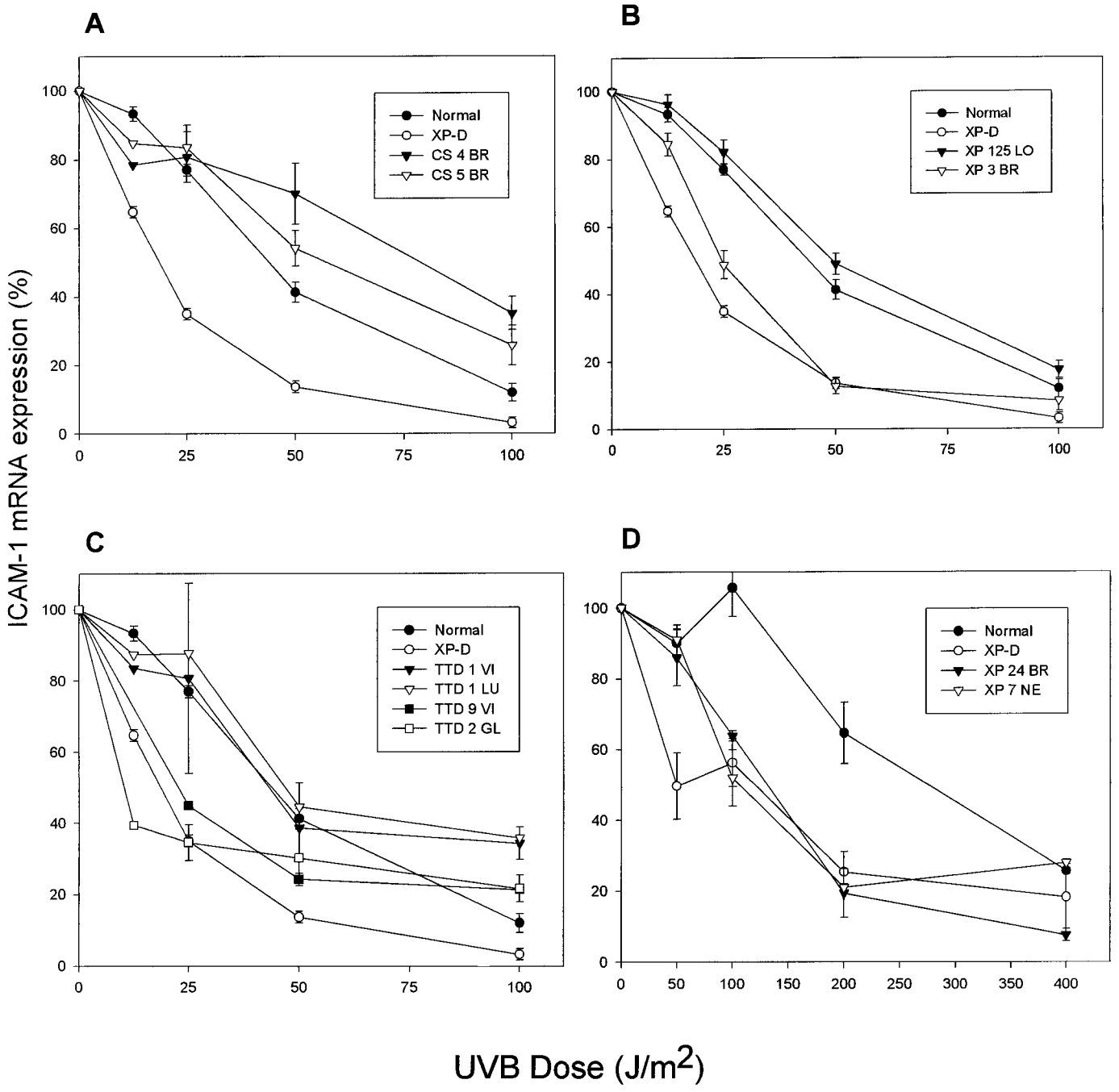

Fig. 2. Inhibition by UV irradiation of IFN-γ mediated up-regulation of ICAM-1 in different cell strains. In *A–C*, the mean responses of normal and XP-D cell strains are from our work published previously (15). *A*, CS cell strains CS4BR and CS5BR; *B*, XP-G strains XP125LO and XP3BR; *C*, four TTD cell strains; *D*, XP-F cell strains XP24BR and XP7NE. Results show means of three experiments; *bars*, SE. (Note that the experiments shown in *A–C* were carried out in Düsseldorf, whereas those shown in *D* and all other figures were performed in Brighton. The dosimetry of the UVB lamps are clearly different, accounting for the difference in the scales of the abscissae. The nature of the dose-response curves and the differences between XP and TTD cell strains were very similar with both lamps.)

cell lines were somewhat more resistant, with TTD1BEL being substantially more resistant than the XP-D cell lines. These data suggested that, within the TTD group, the increased ICAM-1 inhibition by UVB in strains carrying the arg112his mutation correlated with low cell survival after UV irradiation.

NER. In the following experiments, we compared different parameters of NER in TTD9VI (mutated at arg112his, sensitive ICAM-1 response, and low survival) with TTD1BEL (mutated at arg722trp, normal ICAM-1 response, and intermediate survival) and XP16BR, which like most XP-Ds is mutated at arg683trp (9) and has a sensitive ICAM-1 response and low survival.

The incision steps of NER are normally rate limiting, and incised intermediates are not readily observable after UV irradiation. If, however, hydroxyurea and ara-C are included in the postirradiation medium, the repair synthesis step of NER is inhibited, and incised intermediates accumulate (25) and can be assayed using the single-cell gel electrophoresis ("Comet") assay (26). Results using this assay with various cell lines are shown in Fig. 4*A*. Despite the similar UDS levels of the TTD strains, the incision dose-responses are quite different. In this low UVB dose range, incisions in TTD1BEL are introduced at about one-quarter of the frequency of those in normal cells. Similar results were obtained with TTD1VI (data not shown). In

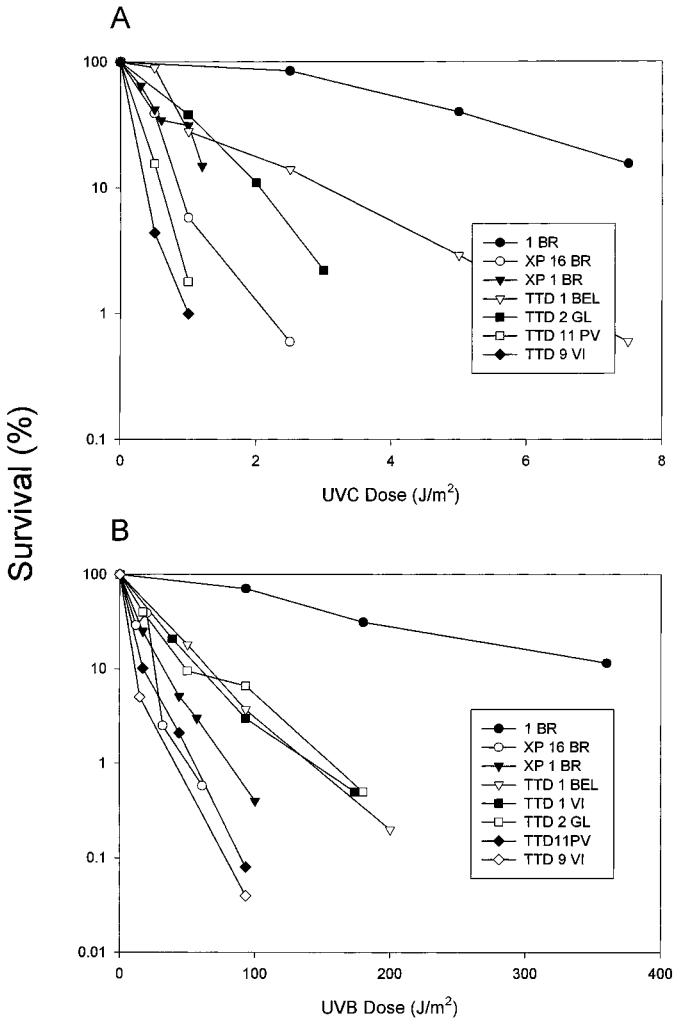

Fig. 3. Survival of different cell strains after exposure to UVC (*A*) or UVB (*B*) irradiation. The figures show the ability of the cell strains indicated to form colonies after exposure to irradiation.

contrast, in TTD9VI and in the XP-D cell strains XP16BR and XP1BR, incised intermediates are barely detectable. In earlier work, using a less sensitive assay, we showed that incision breaks were also undetectable in TTD2GL (cell line P2 in Ref. 22). These results show that TTD1BEL has a clear intermediate response using this assay, whereas TTD9VI and the XP-D lines have very similar low responses.

We have also used a modification of the Comet assay to measure the removal of CPDs from cellular DNA. Cells embedded in agarose were permeabilized and incubated with the T4 denV DNA glycosylase/AP lyase, which introduces nicks at the sites of CPDs. These nicks can be measured using the comet assay. This provides a method for measuring removal of CPDs at very low UV doses. We have used a UVB dose of 5 Jm^{-2}, which produces a similar yield of photoproducts to a UVC dose of only 0.25 Jm^{-2} (27). Note that this dose is one to two orders of magnitude lower than doses used in most other assays of NER. The data in Fig. 4*B* show that in TTD1BEL, the rate of removal of CPDs at this very low UV dose was close to that in normal cells, with 70% of the CPDs removed in 24 h. In contrast, there was substantially less repair in TTD9VI and XP16BR, with only 40% removal in 24 h. These results demonstrate clearly that the levels of NER in TTD cell strains containing the arg112his mutation are in fact considerably lower than in those mutated at other sites and similar to those in XP-D cells.

***rad15* Mutations in *S. pombe*.** To extend our observations correlating the site of the mutation in the *XPD* gene with the cellular defect, we have examined the effects of these mutations on the DNA repair phenotype in a simple system with a homogeneous genetic background. We have taken advantage of the high degree of conservation of the *XPD* gene in eukaryotes. The XPD protein is 55% identical to the Rad15 protein of *S. pombe* (28). We have described previously the construction of plasmids with mutations in the *rad15* gene corresponding to those found in several XP-D and TTD patients (9). We overexpressed the mutant *rad15* constructs in the UV-sensitive *rad15.P* mutant and measured their ability to restore UV resistance to *rad15.P*. Results are shown in Fig. 5. Overexpression of the wild-type construct completely restored sensitivity of the *rad15.P* mutation to that of wild-type *S. pombe* cells. The arg722trp mutation, corresponding to the inactivating mutation in patient TTD1BEL, also restored sensitivity to close to wild-type levels when overexpressed, as did the construct carrying arg683trp, found in many XP-D patients. In contrast, the arg112his construct conferred only partial restoration of UV resistance, and the construct carrying the mutation gly675arg, corresponding to the alteration found in patient XP8BR with the features of both XP and CS (29), is unable to confer any UV resistance. These

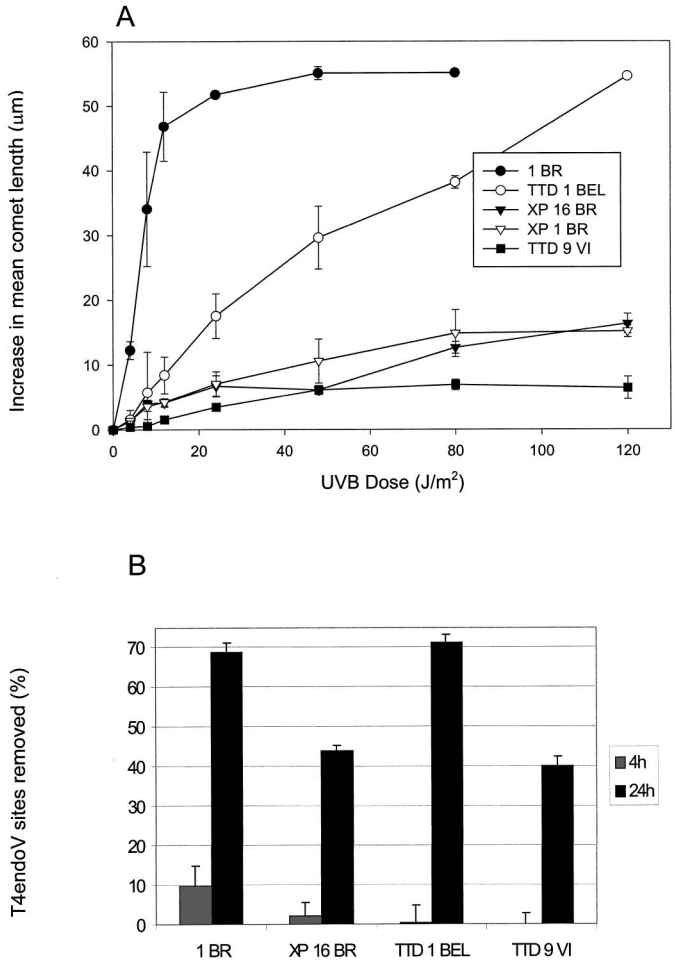

Fig. 4. *A*, incisions introduced during NER. Cell strains were exposed to different doses of UVB irradiation and incubated for 1 h in the presence of ara-C and hydroxyurea. After trypsinization, the cells were embedded in agarose and layered onto slides. The slides were electrophoresed under alkaline conditions and analyzed for comet lengths as described in "Materials and Methods." Results show the means of three experiments; *bars*, SE. *B*, T4 endonuclease-sensitive sites. After UVB irradiation with a dose of 5 Jm^{-2} and incubation for different times, cells were harvested and embedded in agarose as described above. The slides were incubated with T4 UV-endonuclease for 1 h before further processing. Results are means and ranges of two experiments. *Bars*, SE.

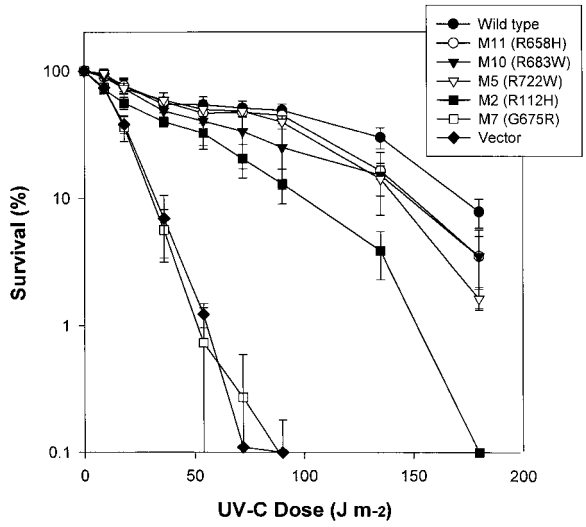

Fig. 5. *S. pombe rad15.P* cells were transformed with various mutant *rad15* constructs, cloned into the pRep81 plasmid containing the inducible *nmt* promoter. Under inducing conditions to overexpress the mutant *rad15* gene, cells were UV irradiated with different doses and plated out to measure UV survival. For clarity, the designations in the diagram refer to the equivalent changes in the XPD protein. In Rad15, the corresponding changes are R657H, R682W, R721W, R111H, G674R. Results show means of three experiments; *bars,* SD.

findings are consistent with our data on fibroblasts, showing that the arg112his mutation has more pronounced effects on DNA repair capacity than other mutations found in TTD patients, and results in a repair phenotype at least as severe as that of XP-D mutations. Note that the data of Fig. 5 show that, with the exception of the gly675arg mutation, all of the other mutations, including arg112his, permit some residual NER activity, because partial restoration of UV resistance is obtained when the mutant protein is overexpressed. The extreme sensitivity and unusual UV response of the XP/CS cell line carrying the gly675arg mutation will be the subject of a separate publication.

DISCUSSION

UV Responses in TTD Patients. The differences in clinical features between XP and TTD, despite apparently similar repair defects, pose one of the most tantalizing enigmas in the field of DNA repair. Although the developmental defects in TTD can be accounted for by specific subtle transcriptional defects (10), the mildness of the skin abnormalities in TTD remains unexplained. It has been suggested that thickening of the skin associated with ichthyosis, which is one of the characteristics of TTD, attenuates the effects of sunlight. Although this attenuation undoubtedly does occur, it is unlikely to account for the total absence of XP-like skin pigmentation changes in TTD.

It has also been suggested that the repair defect in TTD cells is milder than that in XP (10, 30). There are also several reports of other differences between XP and TTD cells in the responses to UV. Using an UV-irradiated shuttle vector transfected into XP and TTD cells, the induced mutation frequency in the target gene was similar in XP and TTD cells, but the mutation spectrum in the TTD cells was more like that in normal cells (31). In our earlier work (15), we demonstrated that the UVB-mediated inhibition of ICAM-1 expression was greater in XP than in TTD cells. This raised the possibility that as well as UV-induced mutations, immune depletion may also play a role in UV-induced carcinogenesis, a proposal that has been made on several previous occasions in different contexts (12–14, 32). We have extended the analysis of the ICAM-1 response in the current work and shown that ICAM-1 inhibition correlates with increased skin cancer susceptibility in several instances (Fig. 2, *A–C*) but not in others (Fig. 2, *C* and *D*).

UV Responses in TTD Patients Carrying the arg112his Mutation. One subset of TTD patients, containing mutations at arg112 of the XPD protein, showed an unambiguous XP-like abnormal ICAM-1 response (Fig. 2*C*). This finding does not, therefore, support our earlier suggestion that differences in the effects of UV on ICAM-1 expression in the skin can account for the different skin symptoms in XP and TTD patients. Our findings led us to evaluate in more detail the nature of the defect in this subset of TTDs mutated at arg112. It has been clear from the earliest work on DNA repair in TTD cells that the level of DNA repair varies substantially between TTD cell lines (22). Indeed, a significant proportion of TTD patients do not have a defect in NER (22, 33). In our earliest report on NER levels in TTD, we identified three categories, with normal, intermediate, and defective responses (22). Subsequent work from our own and other laboratories suggests that there is a continuum of repair activity between different TTD cell strains (6). Our current investigation has shown that even among the group with the lowest levels of NER as measured by UDS, there is heterogeneity. TTD cells containing the arg112his mutation are in fact more repair defective than TTDs mutated at any other site. We have found this to be true for ICAM-1 inhibition (Fig. 2*C*), cell killing (Fig. 3), incision activity (Fig. 4*A*), and CPD removal (Fig. 4*B*). Of the various parameters that we have measured in XP-D and TTD cells, ICAM-1 inhibition appeared to correlate best with the levels of NER measured by incisions and photoproduct removal. The greater sensitivity of the mutation at arg112 was corroborated in a yeast system, in which the effects of different mutations were examined in the same genetic background (Fig. 5).

The more marked defect in patients mutated at arg112 can indeed be noted retrospectively in other published work. The rate of removal of both CPDs and 6-4 photoproducts was markedly lower in arg112his mutants TTD9VI and TTD8PV than in a series of other TTD cell strains examined by Eveno *et al.* (30), although the authors did not comment on this difference. Nondividing cells from a series of Italian patients mutated at arg112 were more sensitive to cell killing than those mutated at other sites (24). The unique features of TTDs mutated at arg112, with repair defects at least as severe as those in XP-D cells by several criteria (cell killing, UDS, damage-induced incisions, and photoproduct removal), have very important implications. Any unifying hypothesis that attempts to explain the different clinical phenotypes in XP and TTD must be valid for all TTD patients. Because, among the TTD patients, only those containing the arg112his mutations have responses to UV as low as XP cells, conclusions drawn from differences in responses between XP and TTD cells that do not include an arg112his patient need to be reevaluated. Thus, for example, the differences in mutation spectra between XP-D and TTD cells did not include TTDs mutated at arg112 (31). Eveno *et al.* (30) suggested that repair of 6-4 photoproducts was normal in TTDs and reduced in XP-Ds. However, examination of their data shows in fact that in their study, in the two cell lines mutated at arg112, TTD9VI and TTD8PV, the kinetics of removal of 6-4 photoproducts was reduced and was very similar to that in the XP-D cell line. The TTD mouse generated by de Boer *et al.* (10) contained the arg722trp mutation, and the cells from this mouse were only mildly sensitive to UV. It would be interesting to compare the features of a mouse with the arg112his mutation.

Arg112his is the only relatively common mutation found in the NH_2-terminal region of the XPD protein. XPD is a 5′-3′ helicase subunit of transcription factor TFIIH. Available evidence on the yeast Rad3 protein, the homologue of XPD, suggests that the helicase activity is absolutely required for the NER function but is dispensable for the essential transcriptional function (34, 35). Recent work of Coin

et al. (36) has shown that the COOH-terminal part of XPD interacts with the p44 subunit of TFIIH, and this interaction greatly stimulates its helicase activity. Mutations such as arg722trp and arg683trp found in patients used in this study prevented this interaction and thereby reduced helicase activity of the TFIIH complex. arg112his was not tested in the study of Coin *et al.* (36), but being far removed from the COOH terminus, it is unlikely to affect the interaction with p44. arg112 is also not in one of the seven conserved helicase domains. Nevertheless, it is located in a run of six amino acids, S**R**KNLC (**R** is arg112), which are identical in seven species (*Saccharomyces cerevisiae*, *S. pombe*, *Drosophila melanogaster*, *Xiphophorus maculatus*, hamster, mouse, and human). This is part of a 16-amino acid sequence that is highly conserved (70% identity, 93% similarity) between these species, indicating that it fulfils a function vital for the DNA repair activity of the protein. At present, there is no information on the nature of this function, but it is not unreasonable to envisage that its effect on the activities of the TFIIH complex might be quite different from those of COOH-terminal mutations.

Transcriptional Defect and Skin Symptoms in TTD. We conclude from the above that fundamental differences in the clinical phenotypes of the skin of XP and TTD cannot be attributed to differences in cellular responses to UV irradiation, because all of the end points that have been studied thus far are indistinguishable in XP-D and TTD-arg112his cell lines. Furthermore, the severity of the clinical features of TTD patients does not correlate with their repair deficiencies. Patients with the arg112his mutation have, as described in this report, the most severe defect in repair, but in several instances, their clinical features are quite mild (24). We propose instead that it is the subtle transcriptional defects in TTD cells that prevent the unrepaired damage from generating the pigmentation changes and skin cancer seen in XP. Mutations in TTD but not in XP patients are thought to affect the transcriptional role of TFIIH, and the effects on transcription have been suggested to be the cause of the developmental defects in TTD (8, 10). We propose that the transcriptional deficiency in TTD would greatly reduce the transcription of one or more crucial genes, the overexpression of which is needed to generate the abnormal levels of melanogenesis associated with freckling and other types of hyperpigmentation in response to sunlight-induced DNA damage. This would reduce the excessive sunlight-induced pigmentation changes that are typical of XP. Note that there are no reports of an abnormal tanning response in TTD; therefore, it is likely that the normal process of melanogenesis is unaffected in TTD. With regard to the lack of skin cancers in TTD, we similarly propose that a critical gene or genes involved in the carcinogenic pathway would not be expressed at a sufficiently high level in TTD patients. This hypothesis proposes a subtle balance and interplay between the complex pathways of transcription, differentiation, and carcinogenesis. It would not be surprising if the outcome of this interplay differed between species; therefore, the finding that the TTD mutant mouse is, unlike TTD patients, somewhat susceptible to UVB-induced carcinogenesis (10, 39) is not inconsistent with our hypothesis.

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