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Molecular and Cellular Analysis of the DNA Repair Defect in a Patient in Xeroderma Pigmentosum Complementation Group D Who Has the Clinical Features of Xeroderma Pigmentosum and Cockayne Syndrome

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Summary

Xeroderma pigmentosum (XP) and Cockayne syndrome (CS) are quite distinct genetic disorders that are associated with defects in excision repair of UV-induced DNA damage. A few patients have been described previously with the clinical features of both disorders. In this paper we describe an individual in this category who has unusual cellular responses to UV light. We show that his cultured fibroblasts and lymphocytes are extremely sensitive to irradiation with UV-C, despite a level of nucleotide excision repair that is 30%–40% that of normal cells. The deficiency is assigned to the XP-D complementation group, and we have identified two causative mutations in the *XPD* gene: a gly→arg change at amino acid 675 in the allele inherited from the patient's mother and a −1 frameshift at amino acid 669 in the allele inherited from his father. These mutations are in the C-terminal 20% of the 760-amino-acid XPD protein, in a region where we have recently identified several mutations in patients with trichothiodystrophy.

Introduction

Deficiencies in excision repair of DNA damage produced by exposure to UV light are associated with three human genetic disorders: xeroderma pigmentosum (XP), Cockayne syndrome (CS), and trichothiodystrophy (TTD). XP patients are extremely sensitive to sunlight-induced skin damage, including an elevated incidence of skin cancers, and in some cases they have associated neurological abnormalities (Cleaver and Kraemer 1989).

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Individuals with CS have cachectic dwarfism, severe mental retardation, microcephaly, skeletal and retinal abnormalities, sun sensitivity, but no cancers (Nance and Berry 1992; Lehmann et al. 1993). TTD patients have sulfur-deficient brittle hair, ichthyosis, physical and mental retardation, abnormal facies, and photosensitivity, in many but not all cases; but the individuals are, again, not cancer prone (Lehmann 1987; Itin and Pittelkow 1990). In cells from most XP individuals there is a defect in the ability to remove ultraviolet-induced damage from DNA. Cell-fusion studies have identified seven complementation groups (XP-A to XP-G) associated with defective excision repair in XP, and there is also an eighth group, comprising the XP variants, with normal excision repair but a defect in daughter-strand repair. Cells from some patients with TTD have a normal response to UV irradiation, but a large proportion of patients (20 of 28 cases reported in the literature; e.g., see Stefanini et al. 1993*a*) have a defect similar to that in XP. The defect in the majority of repair-deficient TTD patients has been assigned to the XP-D complementation group (Stefanini et al. 1993*a*). Recently, however, we have demonstrated that one TTD patient falls into an entirely new complementation group, designated "TTD-A" (Stefanini et al. 1993*b*), and that another one is in the XP-B group (Vermeulen et al., in press). The products of the *XPD, XPB,* and *TTDA* genes, in addition to their roles in DNA repair, are subunits of the transcription factor TFIIH (Schaeffer et al. 1993, 1994; Drapkin et al. 1994; Vermeulen et al., in press). CS cells are UV sensitive, but, in contrast to XP and TTD cells, excision repair of UV damage in the bulk of the genome is similar to that in normal cells (Mayne et al. 1982). The defect in CS cells is confined to an inability to effect the rapid and preferential repair of cyclobutane dimers in active genes (Venema et al. 1990). Two complementation groups (CS-A and CS-B) have been identified (Lehmann 1982).

The clinical features of XP and CS are quite distinct, but a few patients have been described in the literature who have characteristics of both disorders. The XP-B group comprises only three families. The patients in two of these

families have the features of both XP and CS (Robbins et al. 1974; Scott et al. 1993). Two patients in the XP-G group, XPCS1LV and XPCS2LV, also have the features of both XP and CS (Vermeulen et al. 1993), although other XP patients in this group do not appear to have features of CS. The XP-D group is large and heterogeneous, consisting of patients either with XP of differing severity or with TTD (Johnson and Squires 1992). One patient in this group, who has features of both XP and CS (Lafforet and Dupuy 1978), was originally assigned to XP complementation group H (Moshell et al. 1983), but subsequent studies showed that the defect in this individual was in fact in the XP-D group (Johnson et al. 1989; Vermeulen et al. 1991). We now report on a second XP/CS patient in the XP-D group. We have carried out extensive DNA repair studies on this individual, and in addition we have identified the causative mutations in the predisposing *XPD* gene.

Patient, Material, and Methods

Clinical Description of Patient

The male patient was born at term after an uneventful pregnancy, with height (50 cm), weight (2.5 kg), and head circumference (32 cm) all below the 3d percentile. Cataracts were noted at birth and were removed at age 1 mo. At age 16 mo he had made no developmental progress (height 70 cm, weight 5.4 kg, and head circumference 39 cm), and he had feeding difficulties and severe failure to thrive. He had a wizened facial appearance, with enophthalmos. There was pigmentation of the exposed surfaces of the skin, with freckling and thin hair. The patient was sensitive to sunlight, with blistering on minimal sun exposure. There were no actinic keratoses or skin cancers. Skin histology showed changes characteristic of a photosensitive dermatitis. The patient had aphakia, nystagmus, and pigmentary retinopathy; spasticity in all limbs, with brisk tendon reflexes; and cryptorchidism. His neurological development was globally delayed at the 2-mo level.

Further investigations showed normal chromosomes, metabolic screen, skeletal survey, visual evoked responses, brain-stem auditory-evoked responses, and nerve conduction studies. Electroencephalography was of low voltage, and electroretinography was of small amplitude and poorly formed. Computed-tomography brain scan showed calcification in the basal ganglia and white matter of frontal and parietal lobes. Magnetic-resonance imaging of the brain showed both mild ventricular dilatation with delayed myelination throughout the hemispheric white matter tracts and areas of increased signal in the periventricular region, particularly adjacent to the anterior horns of the ventricles.

Over the following 12 mo the patient made no progress; he did not gain weight nor did his head circumference increase, and his neurological state was unchanged. He died from pneumonia at age $2\frac{1}{2}$ years. Many of his clinical features were characteristic of CS (Nance and Berry 1992; Lehmann et al. 1993), with additional cutaneous features of XP.

Table 1

Cell Strains

Clinical Features	Complementation Group	Strain Designation[a]
Normal		1BR, (42), (102)
XP	XP-A	XP4LO
	XP-C	XP6BR, XP12RO
	XP-D	XP3NE, XP1NE (309), XPJCLO, XP107LO (98)
CS	CS-A	CS6BR
	CS (unknown group)	CS3BR
XP/CS	XP-D	XP8BR (316), XP-CS-2

[a] Designations of fibroblast strains, or (in parentheses) lymphocyte donors.

Cell Culture and DNA Repair

All procedures for growth of human fibroblasts, measurement of cell survival in fibroblasts (Cole and Arlett 1984) and lymphocytes (Arlett et al. 1993), repair synthesis (Smith and Hanawalt 1976), RNA synthesis after UV irradiation (Lehmann et al. 1985), and complementation (Vermeulen et al. 1991; Stefanini et al. 1992) have been described in detail in earlier work. Cell strains used are listed in table 1.

Molecular Analysis

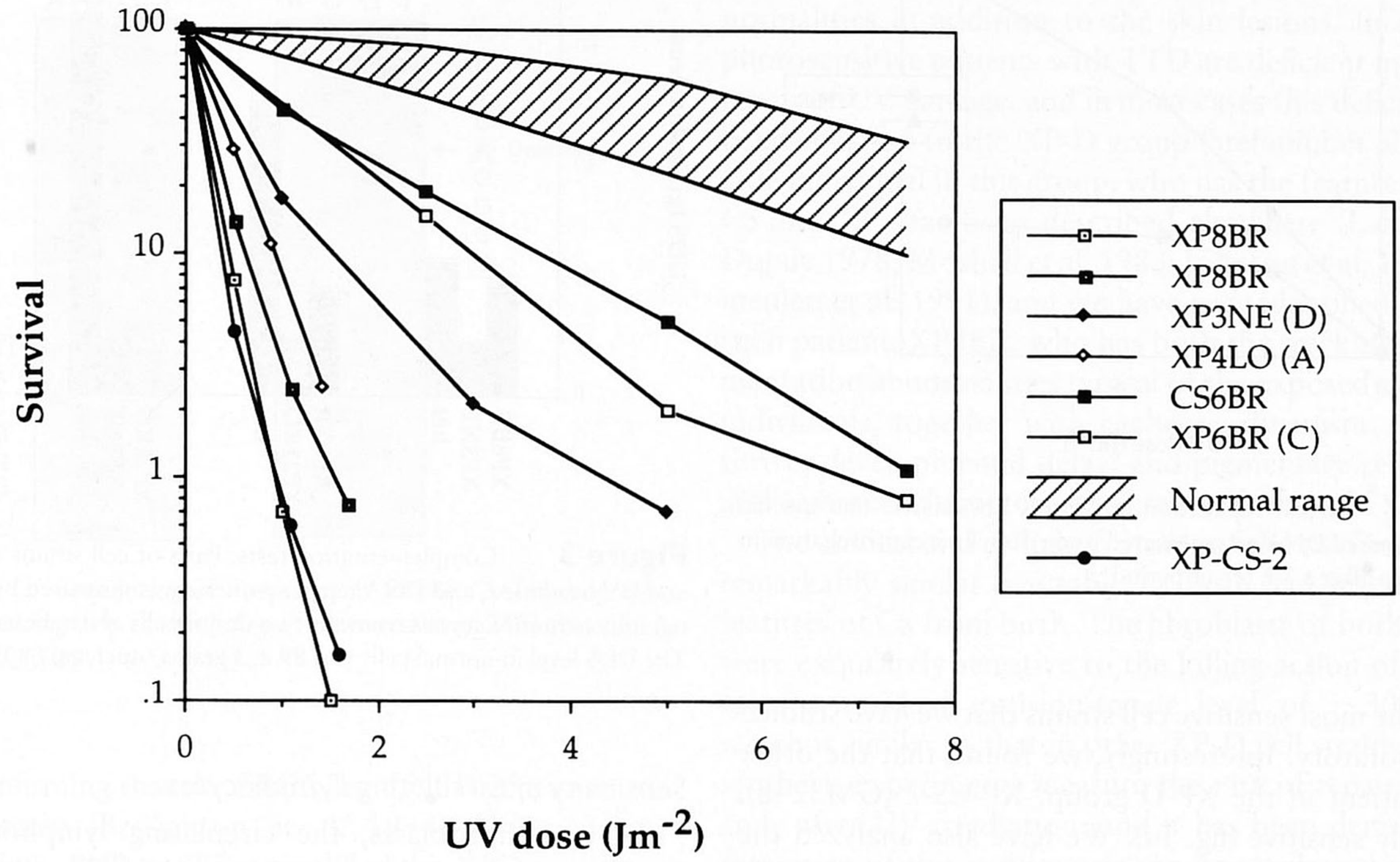
The procedures were similar to those described by Broughton et al. (1994). RNA was extracted from XP8BR cells and was reverse-transcribed into cDNA, using Superscript II (Pharmacia) for 1 h. The *XPD* gene was amplified in four separate fragments as described earlier (Broughton et al. 1994). The products were sequenced directly, using the ΔTaq cycle sequencing kit (USB). In addition, the PCR products were cloned into a "T-vector" (Kovalic et al. 1991), and the inserts in individual clones were sequenced using standard dideoxynucleotide procedures with Sequenase II (USB). PCR on genomic DNA was carried out using primers BB28, GTCCCCTACGTCTACACACAGAGCCG; and BB29, CGGTGGAAGGGCTGTGCCAT.

Results

Response to UV-C of Cultured Fibroblasts

The colony-forming ability of XP8BR cells after UV-C irradiation was compared with that of other cell strains from XP donors in different complementation groups and with that of cell strains from CS donors. The sensitivity was as great as that in XP cell strains from group A and exceeded that of cell strains from other groups (fig. 1*a*). It

a



b

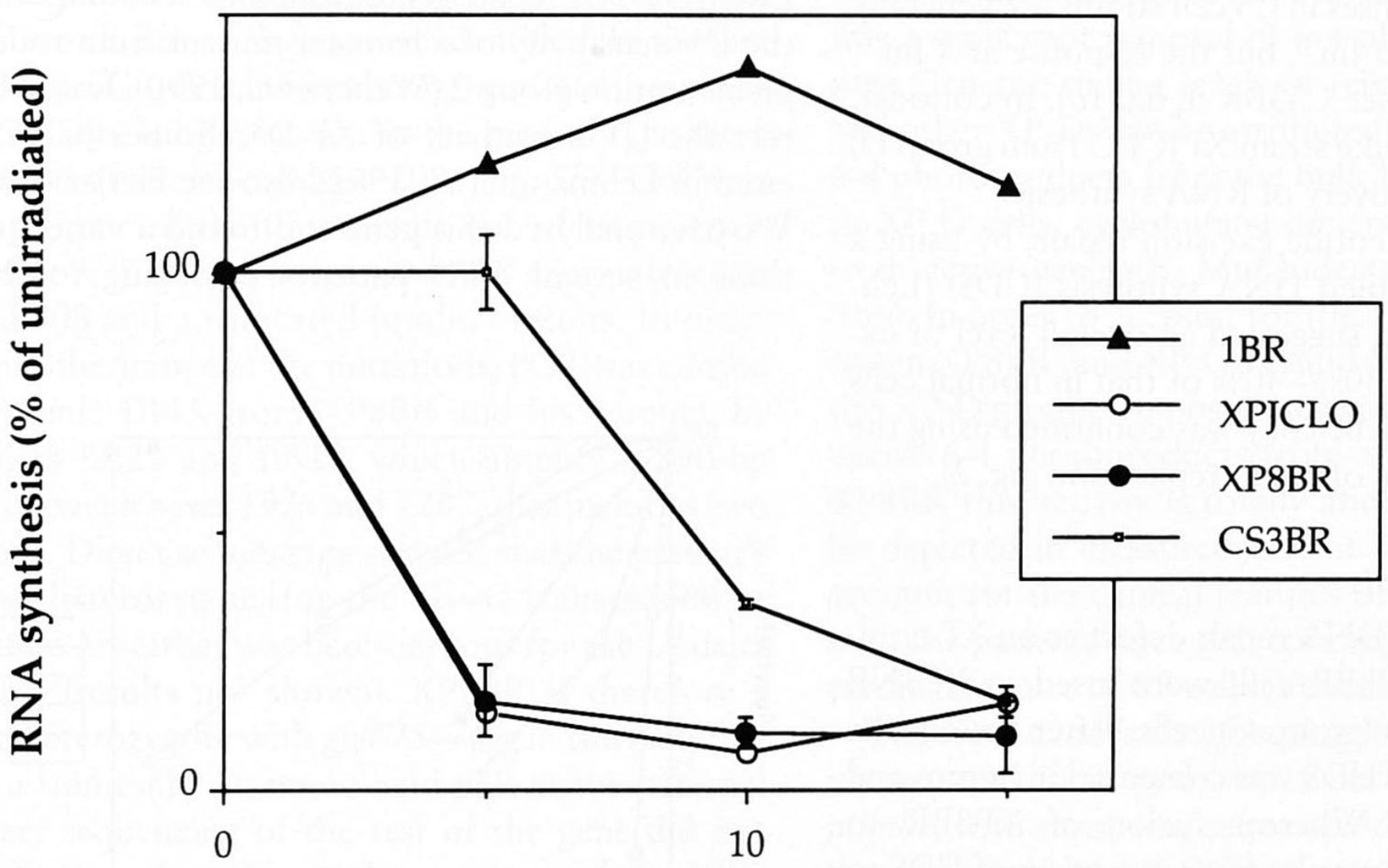


Figure 1 *a*, Survival of UV-C-irradiated fibroblasts. Two separate experiments with XP8BR are shown. *b*, RNA synthesis in UV-irradiated fibroblasts. The data represent the relative incorporation of ^{3}H-uridine into RNA 24 h after UV irradiation.

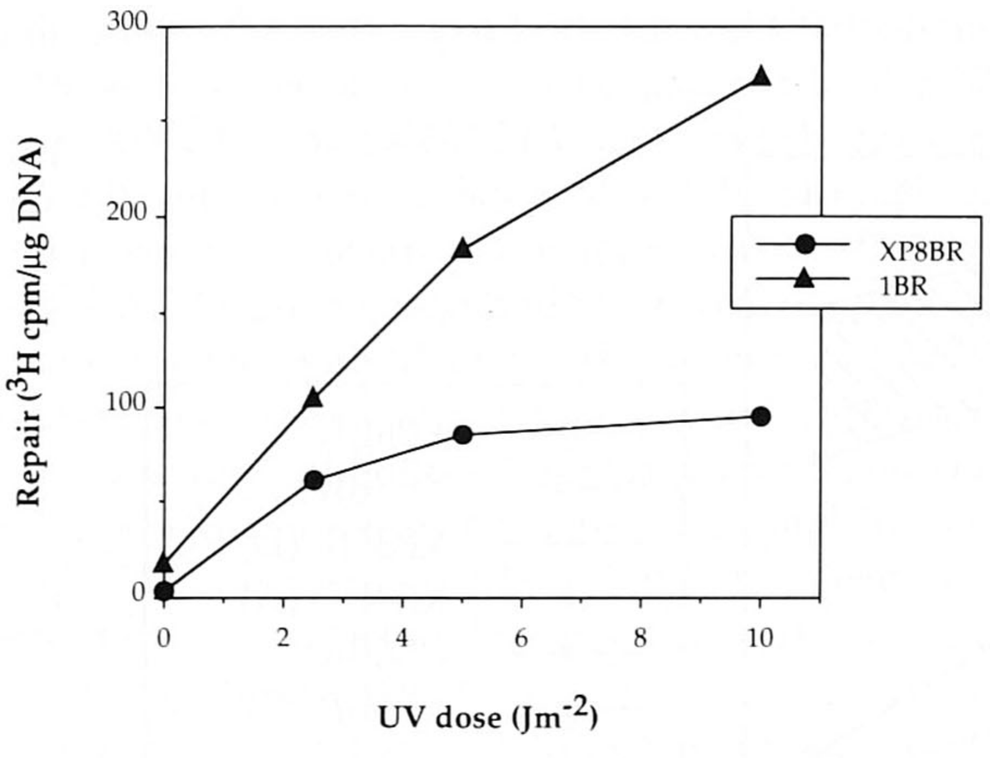


Figure 2 Repair replication in XP8BR fibroblasts. The specific activity of repaired DNA was measured using isopyknic centrifugation in CsCl gradients after a 3-h repair period.

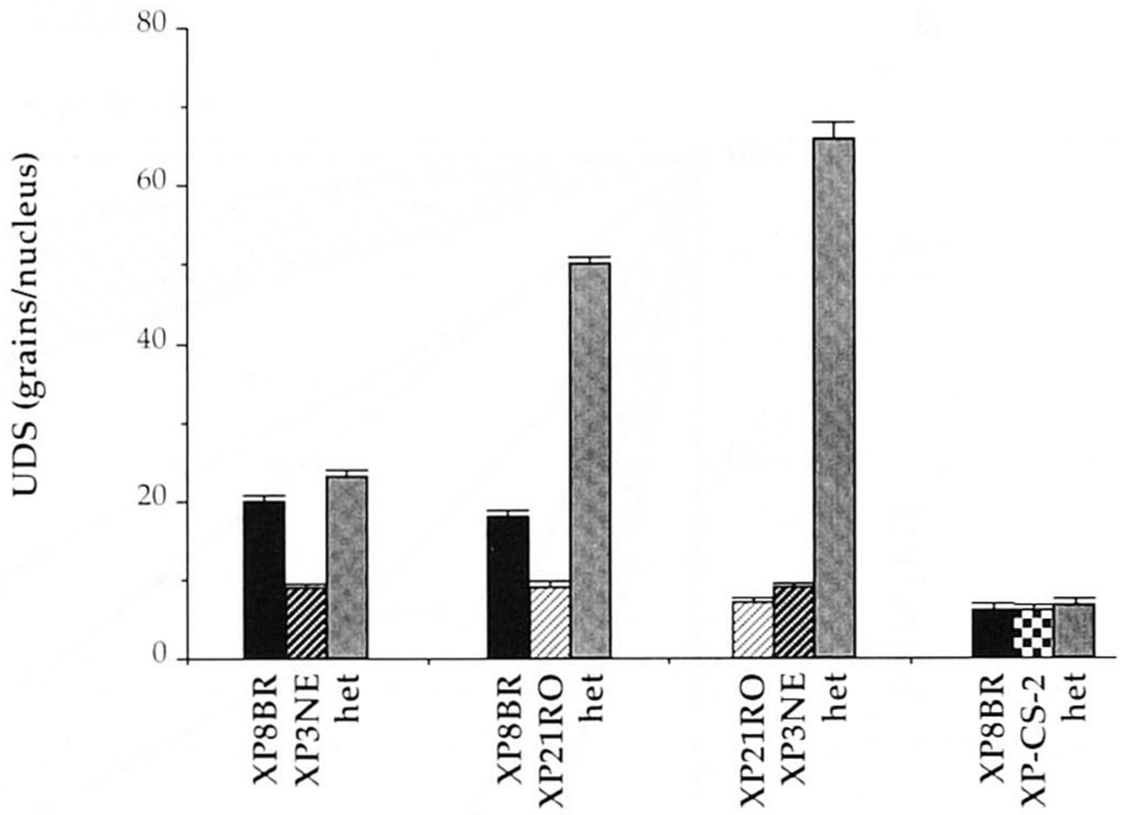


Figure 3 Complementation tests. Pairs of cell strains were fused and UV irradiated, and DNA repair synthesis was measured by autoradiography in homokaryons from the two donor cells and in heterokaryons. The UDS level in normal cells was 89 ± 3 grains/nucleus.

is one of the most sensitive cell strains that we have studied in our laboratory. Interestingly, we found that the other XP/CS patient in the XP-D group, XP-CS-2 (GM3248), was equally sensitive (fig. 1*a*). We have also analyzed the response of RNA synthesis to UV-C irradiation (fig. 1*b*), which we use as a rapid diagnostic test for CS and XP (Lehmann et al. 1993). With normal donors, 24 h after UV irradiation, RNA synthesis recovers to the level in unirradiated cells, even after a dose of 15 Jm^{-2} (e.g., see 1BR in fig. 1*b*). The defective responses in CS cell strains are generally manifest at doses of ⩾10 Jm^{-2}, but the response at 5 Jm^{-2} is close to normal (e.g., see CS3BR in fig. 1*b*). In contrast, even at 5 Jm^{-2}, XP8BR (like strain XPJCLO from group D) shows practically no recovery of RNA synthesis.

Measurement of nucleotide excision repair, by using a simple test for unscheduled DNA synthesis (UDS) (Lehmann and Stevens 1980), suggested an overall level of excision repair that was ~30%–40% of that in normal cells (data not shown). This deficiency was confirmed using the more rigorous procedure of repair replication (fig. 2).

Complementation Analysis

In order to assign the DNA repair defect to an XP complementation group, XP8BR cells were fused to XP3NE (group D) and XP21RO (group C) cells. After fusion, the cells were irradiated and UDS was compared in homo- and heterokaryons (fig. 3). Whereas fusion of XP8BR to XP21RO cells (group C) resulted in restoration of UDS to close to normal levels, as did fusion of XP21RO (group C) to XP3NE (group D), no such increase was observed when XP8BR was fused to XP3NE. These data permit us to assign XP8BR to complementation group D. In confirmation, we also found no complementation either between XP8BR and XP17PV from the XP-D group (not shown) or between XP8BR and XP-CS-2 (fig. 3).

Sensitivity of Circulating Lymphocytes

As with fibroblasts, the circulating lymphocytes of XP8BR were exquisitely sensitive to UV-C irradiation delivered to noncycling cells (fig. 4).

Molecular Analysis of the XPD Gene

It has recently been demonstrated that the human *ERCC2* DNA repair gene, cloned by its ability to correct the UV sensitivity of a hamster mutant from rodent complementation group 2 (Weber et al. 1990), was able to correct the UV sensitivity of XP-D cell lines (C. A. Weber, cited in Lehmann et al. 1992; also see Fletjer et al. 1992). We have analyzed this gene and found a variety of mutations in several TTD patients belonging to the XP-D

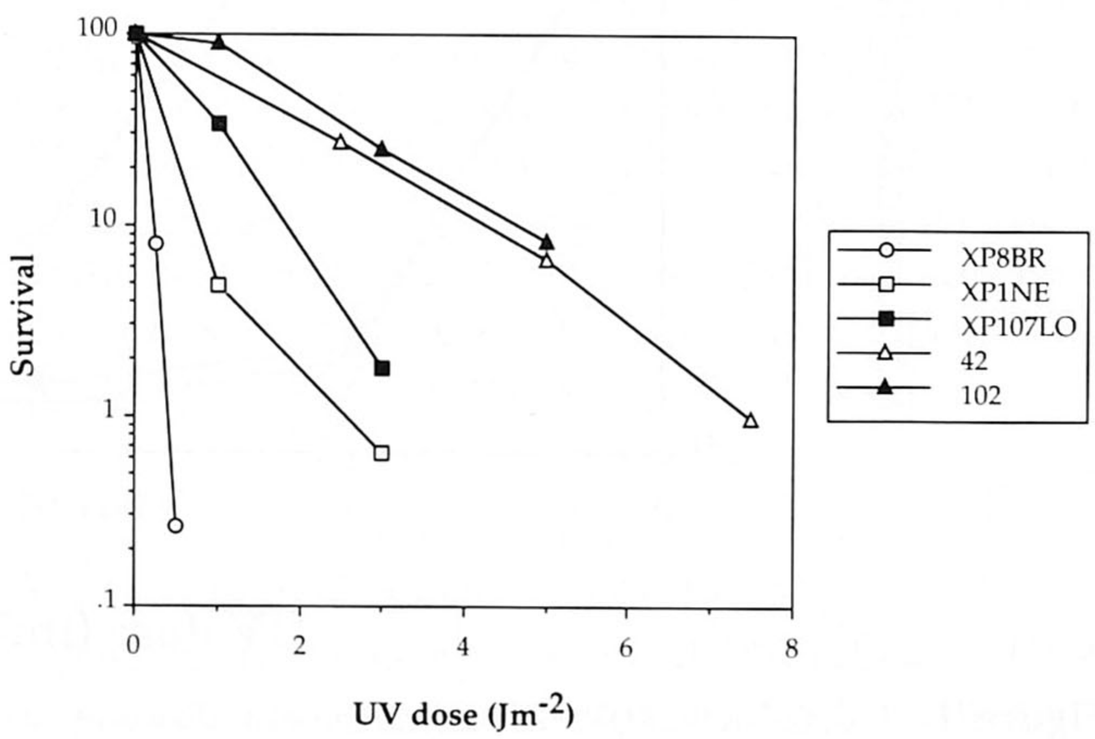


Figure 4 Survival of UV-irradiated Go lymphocytes. Lymphocytes from indicated donors were UV irradiated prior to stimulation with phytohemagglutinin and measurement of surviving cells by plating in Terasaki dishes.

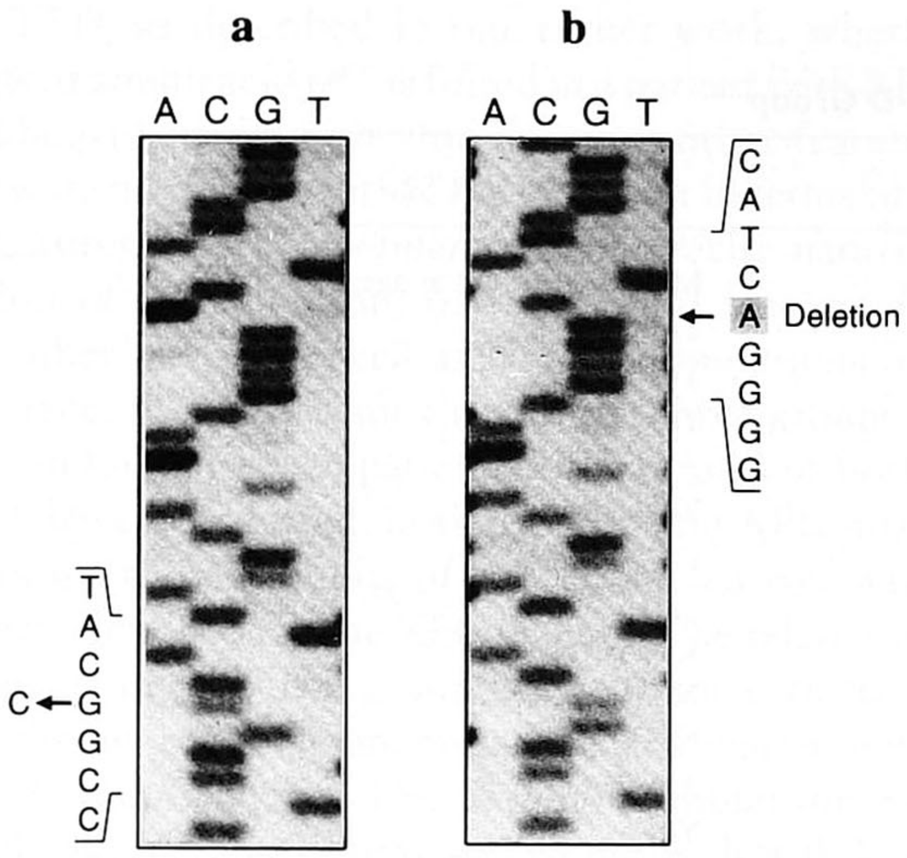


Figure 5 Sequencing of mutations in XP8BR. Autoradiographs of the sequencing gels showing G→C transversion at 2101 (*a*) and -A deletion at 2083 (*b*).

group, confirming that the *ERCC2* and *XPD* genes are one and the same (Broughton et al. 1994). Using reverse transcription–PCR, we have amplified the *XPD* gene from XP8BR in four fragments and sequenced these fragments by using a cycle sequencing procedure. Direct sequencing showed changes in the region around base numbers 2075–2105. The amplified 3′ fragments from bases 1401–2397 were cloned into a T vector, and several clones were sequenced. Two classes of clones were identified. In the first type there was a G→C transversion at base 2101, resulting in a gly675→arg change (fig. 5*a*). In the second type there was a deletion of adenine at base 2083 (fig. 5*b*). This puts the reading frame of the C terminus out of frame from amino acid 669, such that a stop codon is encountered at amino acid 708 and a truncated product results. In order to study the inheritance of the mutations, PCR was carried out on genomic DNA from XP8BR and his parents, by using primers BB28 and BB29, which amplify a 590-bp fragment, between bases 1925 and 2267, that includes two small introns. Direct sequencing showed that the patient's mother was heterozygous for the G→C transversion at 2101, whereas his father was heterozygous for the -A deletion at 2083 (results not shown). XP8BR is therefore a compound heterozygote with gly675→arg in the maternal allele and a frameshift at amino acid 669 in the paternal allele. Direct sequencing of the rest of the gene did not reveal any further alteration in the sequence of the *XPD* gene in XP8BR.

Discussion

The XP-D group is heterogeneous with regard to both the clinical features of the affected individuals and the extent of the DNA repair defect and cellular hypersensitivity to UV light (Johnson and Squires 1992). The group is represented by XP patients, who often have neurological abnormalities in addition to the skin lesions. In addition, photosensitive patients with TTD are deficient in excision repair of UV damage, and in most cases this deficiency has been assigned to the XP-D group (Stefanini et al. 1993*a*). One individual in this group, who has the features of both CS and XP, has been described elsewhere (Lafforet and Dupuy 1978; Moshell et al. 1983; Johnson et al. 1989; Vermeulen et al. 1991), and we have now identified a second such patient, XP8BR, who has both the freckling and pigmentation abnormalities typical of the exposed skin of XP individuals, together with cachectic dwarfism, failure to thrive, developmental delay, and pigmentary retinopathy and cataracts characteristic of severe forms of CS.

The clinical and cellular features of the two patients are remarkably similar (see table 2). Both had severe clinical features of CS from birth. The fibroblasts of both patients were exquisitely sensitive to the killing action of UV, despite a residual excision-repair level of ~30%–40%, which is similar to that in other XP-D cell strains. Repair-synthesis experiments measure the rate of repair immediately after UV irradiation, and it has been demonstrated that most of this early repair can be attributed to the removal of 6-4 photoproducts, which are excised much more rapidly than cyclobutane dimers (Mitchell et al. 1985). Galloway et al. (1994) have recently shown that in an XP-D cell strain there was very little removal of cyclobutane dimers from the bulk of the DNA, whereas there was a significant removal of 6-4 photoproducts. We propose that the similar levels of repair synthesis in XP8BR and other XP-Ds can be attributed to an ability to remove 6-4 photoproducts from the bulk DNA at a reduced rate. In XP-D cells, cyclobutane dimers are also not removed from active genes (L. Mullenders, personal communication). In order to account for the difference in survival between XP8BR (and XP-CS-2) and most other cell strains in the XP-D group, we postulate that XP-D cells are able to excise 6-4 photoproducts from active genes, whereas in XP8BR this activity is totally abolished. This would not be detected in measurements of repair synthesis. It may account for the clinical features of CS that are associated with the patient, since defective repair of active genes is a cardinal feature of CS cells (Venema et al. 1990).

The *XPD* gene is the homologue of *RAD3* of *Saccharomyces cerevisiae* and *rad15* of *Schizosaccharomyces pombe,* with three-way sequence identity of 50%–55% at the amino-acid level (Weber et al. 1990; Murray et al. 1992). All three genes contain seven conserved domains characteristic of DNA helicases, and DNA-DNA and DNA-RNA helicase activities have been characterized for the Rad3 protein (Sung et al. 1987; Naegeli et al. 1992). Recently the XPD protein has also been shown to have helicase activity (Sung et al. 1993). In addition to its involvement in excision repair, the *RAD3* gene has an essen-

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Table 2

Clinical and Cellular Features of XP/CS Patients in the XP-D Group

	XP8BR	XPCS2
Cause of death	Pneumonia at age $2^1/_2$ years	Multiple tumors at age 13 years
Developmental delay	Yes	Yes
Mental retardation	Yes	Yes
Spasticity	Yes	Yes
Failure to thrive	Yes	
Sun sensitivity	Photosensitive dermatitis, rash at age 2 mo	Yes
Height, weight, head circumference at birth	<3d percentile	<3d percentile
Cataracts	Yes	No
Deafness	No	No
Facies	Wizened	Abnormal
Freckling	Yes	Yes
Pigmentation	Yes	Yes
Skin tumors	No	Yes
Thin hair	Yes	
Nystagmus	Yes	
Pigmentary retinopathy	Yes	Yes
Calcification of basal ganglia	Yes	No
Myelination	Delayed	Neuropathy
Cell survival (D_{10})	.5 Jm^{-2}	.4 Jm^{-2}
Repair synthesis (% of normal)	30%	30%

tial function, as has also been inferred for the *XPD* gene. The nature of this function has recently been identified. The product of the human *XPD* gene, like that of the *XPB* gene (Schaeffer et al. 1993), is a subunit of the basal transcription factor TFIIH (BTF2) (Drapkin et al. 1994; Schaeffer et al. 1994; Vermeulen et al., in press), which is involved in loading RNA polymerase II molecules onto promoter sites. Similarly, the Rad3 protein is a constituent of the corresponding yeast transcription factor b (Feaver et al. 1993). In Rad3, it has been shown that the helicase activity is required for DNA repair but is not required for its essential function (Sung et al. 1988; Song et al. 1990). Mutations in the *XPD* gene have the potential, therefore, to affect excision repair, transcription, or both.

We have identified different mutations in the two alleles of the *XPD* gene in XP8BR, confirming the assignment to the XP-D group by complementation analysis. A frameshift at amino acid 669 in the paternal allele puts the C-terminal 92 amino acids out of frame and produces a truncated protein. Although the C-terminal 30 amino acids of the 760-amino-acid protein are the only part of the gene that is poorly conserved, 28 of the amino acids between 669 and 730 are identical in man, *S. cerevisiae,* and *S. pombe.* It is likely, therefore, that this mutation will have a severe effect on the function of the XPD protein. The second mutation changes gly 675 to arg. Gly 675 is conserved in both yeasts and lies in a very highly conserved region close to helicase domain VI, and, like previous mutations that we have identified in this region in a TTD patient (at amino acids 616 and 722) (Broughton et al. 1994), this mutation is sufficient to explain the severe UV sensitivity of XP8BR cells. Recent data (unpublished) of C. A. Weber and coworkers have shown that the single expressed allele in XP-CS-2 contains a gly602→asp change.

The effect of the mutation on transcription is much more difficult to ascertain, as is the relationship to the clinical features. We have previously postulated that deficiency in DNA repair alone will result in the clinical features of XP, whereas subtle defects in transcription could account for the much more complex and pleiotropic features of TTD and CS (Bootsma and Hoeijmakers 1993; Vermeulen et al., in press). Several of the mutations that we previously identified in the *XPD* gene in TTD patients were in the C-terminal 20% of the gene (Broughton et al. 1994). We would thus propose that this part of the gene is important for the transcriptional activity of the XPD protein to function fully. Several questions remain unanswered, however. First, in several of the TTD patients as well as in XP8BR, excision repair is severely reduced (by as much as 80%) and the cells are very sensitive to UV irradiation. Clearly the transcriptional function of the XPD protein cannot be reduced by 80%—such a defect would not be compatible with life. Thus, mutations affect repair and transcription differently. Second, if transcription is indeed altered in TTD and CS patients, why do mutations at amino acids 616 and 722 result in an individual

with TTD, as described in our earlier work, whereas a change at amino acid 675 is found in a patient with XP and CS? The patient did have thin hair, but other features of TTD were not found. Either the net result in terms of clinical features is very dependent on the precise nature and location of the mutation, or the clinical features result from other factors as well as from the mutations in the *XPD* gene. It is significant that, of the three groups (XP-B, -D, and -G) in which patients with features of both XP and CS have been found, both the XPB and XPD proteins are known to be subunits of TFIIH. Such a role has not yet been identified for the XPG protein. The relative rarity of patients in the XP-B group is consistent with the idea that cells can only tolerate mutations at a limited number of loci in the *XPB* gene. The majority of mutations would severely affect transcription and would be lethal. In contrast, within the XP population, the D group is rather common (15% of all patients examined, as reported in a 1987 survey by Kraemer et al. [1987]), and, together with TTD patients assigned to this group, it accounts for a very significant proportion of repair-deficient individuals. The implication is that cells can tolerate a much wider range of mutations in the *XPD* gene when compared with the *XPB* gene and that only a small part of the XPD protein is crucially involved in its transcriptional function. This is consistent with earlier findings with the *S. cerevisiae RAD3* homologue, in which many mutations were found that affected UV sensitivity (Song et al. 1990), whereas rather few affected its essential function.

Acknowledgments

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