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Correction of the DNA repair defect in xeroderma pigmentosum group E by injection of a DNA damage-binding protein

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ABSTRACT **Cells from a subset of patients with the DNA-repair-defective disease xeroderma pigmentosum complementation group E (XP-E) are known to lack a DNA damage-binding (DDB) activity. Purified human DDB protein was injected into XP-E cells to test whether the DNA-repair defect in these cells is caused by a defect in DDB activity. Injected DDB protein stimulated DNA repair to normal levels in those strains that lack the DDB activity but did not stimulate repair in cells from other xeroderma pigmentosum groups or in XP-E cells that contain the activity. These results provide direct evidence that defective DDB activity causes the repair defect in a subset of XP-E patients, which in turn establishes a role for this activity in nucleotide-excision repair *in vivo*.**

The cancer-prone genetic disease xeroderma pigmentosum (XP) is characterized by clinical and cellular hypersensitivity to UV radiation, correlated with a defect in nucleotide-excision repair of damaged DNA (for review, see ref. 1). On the basis of cell-fusion studies, XP patients have been divided into seven excision-repair-defective complementation groups (A–G) (as well as the excision-repair-proficient variant group V). Elucidating the molecular bases of the repair defects in these groups is an important step in understanding the mechanisms by which human cells defend against potentially toxic or mutagenic DNA damage.

Chu and Chang (2) reported that cells from two consanguineous XP-E patients lack a DNA damage-binding (DDB) activity that recognizes UV-irradiated DNA. The DDB protein has recently been purified to apparent homogeneity and characterized from human placenta and from HeLa cells (3–5); it is apparently identical to an activity first described from human placenta (6). DDB activity is associated with an ≈124-kDa polypeptide (3–5), which is isolated complexed with a 41-kDa protein. This stable heterodimer can, in turn, form a higher order complex (5, 7). The role of the DDB protein in nucleotide-excision repair is not known; indeed, there is as yet only indirect evidence that it is involved in DNA repair at all (8–11).

An obvious hypothesis is that a defect in DDB activity causes the DNA-repair defect in XP-E (2). However, when cells from other, unrelated XP-E patients were examined, only those from one additional XP-E individual were found to lack DDB activity, whereas cells from 12 other XP-E patients in 10 families showed normal levels of DDB activity that was indistinguishable from that of normal controls by several biochemical criteria (12–14). Although the correlation between XP-E and a Ddb^- cellular phenotype appears to be statistically significant when compared with the Ddb^+ phenotype of all non-XP-E cells analyzed to date (13, 14), the incomplete linkage raises doubts as to whether XP-E is caused by a defect in DDB activity. [We will use the terms Ddb^+ and Ddb^- to indicate the phenotypes of cell culture strains that contain or lack DDB activity, respectively. The designation Ddb^+ is not intended to imply that the DDB protein present in some XP-E cells is absolutely like wild type but to imply only that DDB activity can be detected in normal amounts in extracts from these strains.] Moreover, demonstration of a correlation between a defect in DDB activity and the XP-E disease neither proves a causal relationship nor confirms a role for the DDB protein in DNA repair. To directly address these issues, we examined the ability of purified DDB protein to correct the DNA-repair defect in XP-E cells.

MATERIALS AND METHODS

Cell Strains and Culture Media. XP-E fibroblast strains XP43TO, XP82TO, and XP93TO (15) were provided by Seiji Kondo, Tokyo Medical and Dental University. Fibroblast strains XP25RO (XP-A), XPCS2BA (XP-B), XP1TE (XP-C), XP1BR (XP-D), XP2RO (XP-E), XP126LO (XP-F), XP3BR (XP-G), TTD1BR (trichothiodystrophy, refs. 16–18), and C5RO (normal) were from the collection of D.B. and J.H.J.H. Normal fibroblast strain F65 was from the Naval Biomedical Research Laboratory, Oakland, CA. Cells were cultured in Dulbecco's modified Eagle's medium/10% fetal bovine serum/penicillin at 50 international units/ml/streptomycin at 50 μg/ml or in Eagle's minimal essential medium (BioWhittaker)/20% fetal bovine serum/penicillin at 50 international units/ml/streptomycin at 50 μg/ml containing double the concentrations of amino acids and vitamins recommended by the manufacturer.

Complementation analysis of XP93TO was confirmed by cell-fusion tests with XP2RO and XP25RO, performed according to standard protocols (19). UV-induced unscheduled DNA synthesis (UDS) in homo- or heterodikaryons [mean ± SEM, expressed as percentage of the mean level in normal control (C5RO) fibroblasts] was as follows: XP2RO homodikaryons, 63 ± 7; XP93TO homodikaryons, 55 ± 3; XP2RO × XP93TO heterodikaryons, 67 ± 3; XP25RO homodikaryons, 2.9 ± 0.3; XP93TO × XP25RO heterodikaryons, 82 ± 3.

Microinjection and UDS Measurement. DDB protein was purified from HeLa cells as described (5) and then concentrated and desalted in a Centricon 10 microconcentrator (Amicon). Injected samples were in phosphate-buffered saline (PBS) containing 0.005% (vol/vol) Triton X-100 and bovine serum albumin at 2 mg/ml. DDB activity was assayed by nitrocellulose filter binding, as described (5). One unit of

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Abbreviations: DDB, DNA damage-binding; XP, xeroderma pigmentosum; XP-E, XP complementation group E; UDS, UV-induced unscheduled DNA synthesis.

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DDB activity traps one fmol of UV-induced lesions in this assay. A DDB concentration of 3×10^6 units/ml yields approximately one cell equivalent of DDB activity per injection (see below).

Homopolykaryons prepared by fusion of diploid fibroblasts were used for injection experiments to help ensure that the nuclei of injected cells were not in S phase (because the high level of replicative synthesis of S-phase cells would obscure the much lower levels of incorporation from DNA-repair synthesis) and to provide multiple nuclei to score for DNA repair from each injection (20, 21). Homopolykaryons show UDS characteristics indistinguishable from those of monokaryons (22). Cell fusions were prepared by using heat-inactivated Sendai virus (23) or a 3-min treatment with 50% (wt/vol) polyethylene glycol in culture medium lacking fetal bovine serum. Fused cells were seeded onto glass slides marked with a grid and incubated ≥3 days before injection without changing the medium.

Injection via glass microneedles was as described (20–24). After injection, cells were incubated up to 2 hr at 37°C. UDS was then assayed essentially as described (23, 24). Briefly, cells were rinsed once with PBS, drained, then UV-irradiated with a total dose of 16 J/m^2 from a germicidal lamp (peak emission at 254 nm). The cells were placed immediately in labeling medium (culture medium containing dialyzed fetal bovine serum and [^{3}H-*methyl*] thymidine (≈50 mCi/mmol, 10 μCi/ml; 1 Ci = 37 GBq) and incubated at 37°C for 2 hr. Labeled cells were fixed, dried, and processed for *in situ* autoradiography. To facilitate comparison between experiments with different autoradiography exposure times, the results are normalized to the mean UV-induced UDS of an uninjected normal strain (C5RO or F65) assayed in parallel. Means for the control strains ranged from 90 to 140 grains per nucleus.

RESULTS

Heterodimeric DDB protein was purified from HeLa cells and injected via glass microneedles into the cytoplasm of Ddb$^-$ fibroblasts from the XP-E patient XP2RO. This injection stimulated DNA repair, measured as UDS, from 43% to 90% of the level found in normal cells (Table 1 and Fig. 1), shifting the distribution of autoradiographic grains per nucleus from the XP-E range (centered at 47 grains per nucleus in this experiment) to the range seen in a normal control strain (centered at 108 grains per nucleus) (Fig. 2). Although there was some variability from experiment to experiment in the absolute amount of UDS in XP2RO cells in comparison with normal controls (see, for example, Tables 1 and 2), correction upon injection of DDB protein was highly reproducible and represented an average stimulation of UDS of 2.1-fold over levels in uninjected cells (average for eight experiments). This is the level of stimulation expected for a biologically significant response because of the high residual DNA-repair capacity of XP-E cells (1). The increase in repair DNA synthesis depended on DDB protein because injection of buffer alone did not significantly alter UDS in this strain (Table 1 and Fig. 2*B*).

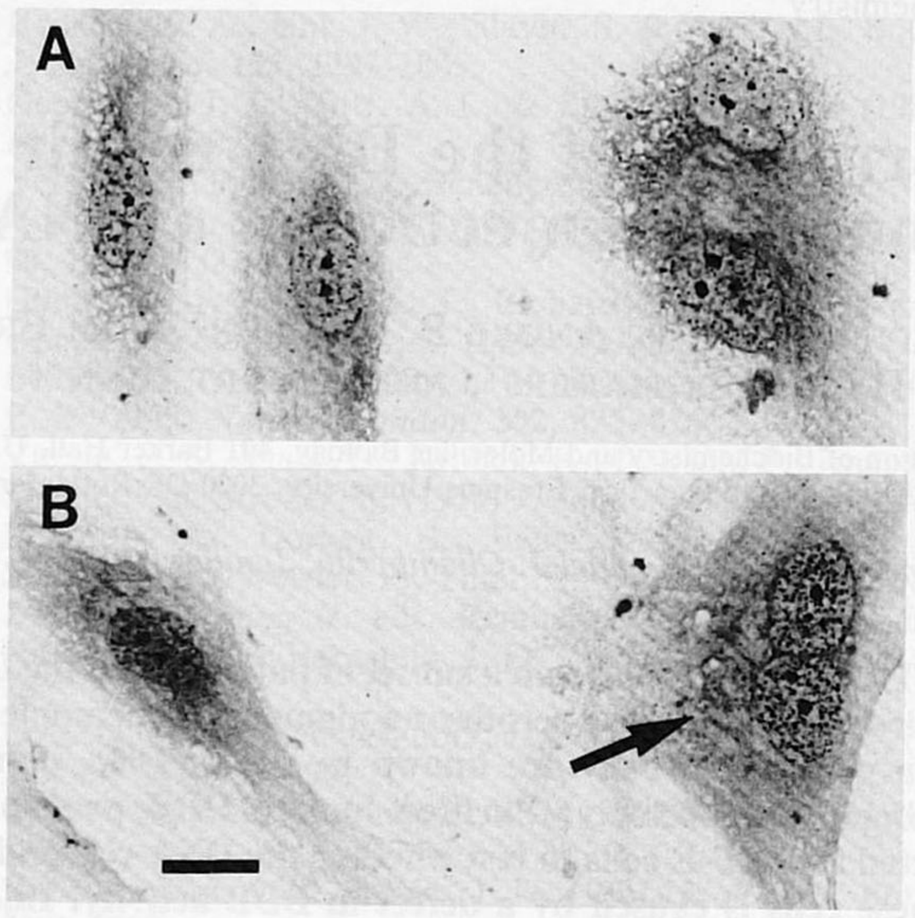

FIG. 1. Correction of the DNA-repair defect in XP-E cells. (*A*) Uninjected XP2RO dikaryon and monokaryons showing the residual UDS level of this strain. (*B*) XP2RO dikaryon (arrow) injected with DDB protein (6.0×10^6 units/ml) and uninjected monokaryons. The two dark bodies in the nuclei are the nucleoli. (Bar = 20 μm.)

DDB protein also corrected the DNA-repair defect in Ddb$^-$ cells derived from another XP-E patient, XP82TO (Table 1). As with XP2RO cells, enhancement of UDS in this strain was ≈two-fold, and the distribution of autoradiographic grain-count frequencies overlapped with the normal range (data not shown). (Fibroblasts from patient XP3RO, the third known Ddb$^-$ XP-E patient and a second cousin of patient XP2RO (25), were not available for testing.) These results provide direct evidence that a defect in DDB activity causes the repair defect in cells from a subset of XP-E patients, which in turn establishes that the DDB protein is involved in nucleotide-excision repair *in vivo*. Furthermore, these results rule out the possibility that the correlation between XP-E and defective DDB activity is due to a fortuitous genetic linkage between the DDB-encoding gene and the gene that, when defective, causes XP-E (13).

Correction of the DNA-repair defect is specific for XP-E. Within the variation inherent to UDS measurements, we never observed a significant stimulation of UDS upon injection of DDB protein into cells from each of the other excision-repair-defective XP groups or into cells from a patient with an excision-repair-defective form of trichothiodystrophy (16–18) (Table 2). In addition, the high level of UDS seen in DDB-injected XP-E cells depended on DNA damage because only a slight stimulation occurred in the very low levels seen with unirradiated XP-E cells [2 ± 0.2 (uninjected) vs. 4 ± 0.4 (DDB-injected) grains per nucleus for unirradiated XP2RO cells, compared with a typical UV-dependent response of 47 ± 2 (uninjected) vs. 97 ± 4 (DDB-injected) grains per nucleus].

Table 1. Microinjection of DDB protein into XP-E cells

Strain	Injection*	UDS,† % of normal control
	Experiment 1	
XP2RO	None	43 ± 2
	Buffer only	48 ± 2
	DDB	90 ± 4
	Experiment 2	
XP82TO	None	50 ± 3
	DDB	102 ± 6
	Experiment 3	
XP2RO	None	73 ± 5
	DDB	124 ± 8
XP82TO	None	32 ± 2
	DDB	66 ± 4
XP43TO	None	39 ± 3
	DDB	36 ± 2
XP93TO	None	28 ± 2
	DDB	30 ± 2

*DDB activities were 6.0×10^6 units per ml for Exp. 1 and 2.8×10^6 units per ml for Exp. 2 and 3.

†Data are the mean ± SEM of grain counts for 30–50 nuclei, expressed as percent of the mean UDS of an uninjected control strain [C5RO (Exp. 1) or F65 (Exp. 2 and 3)] assayed in parallel.

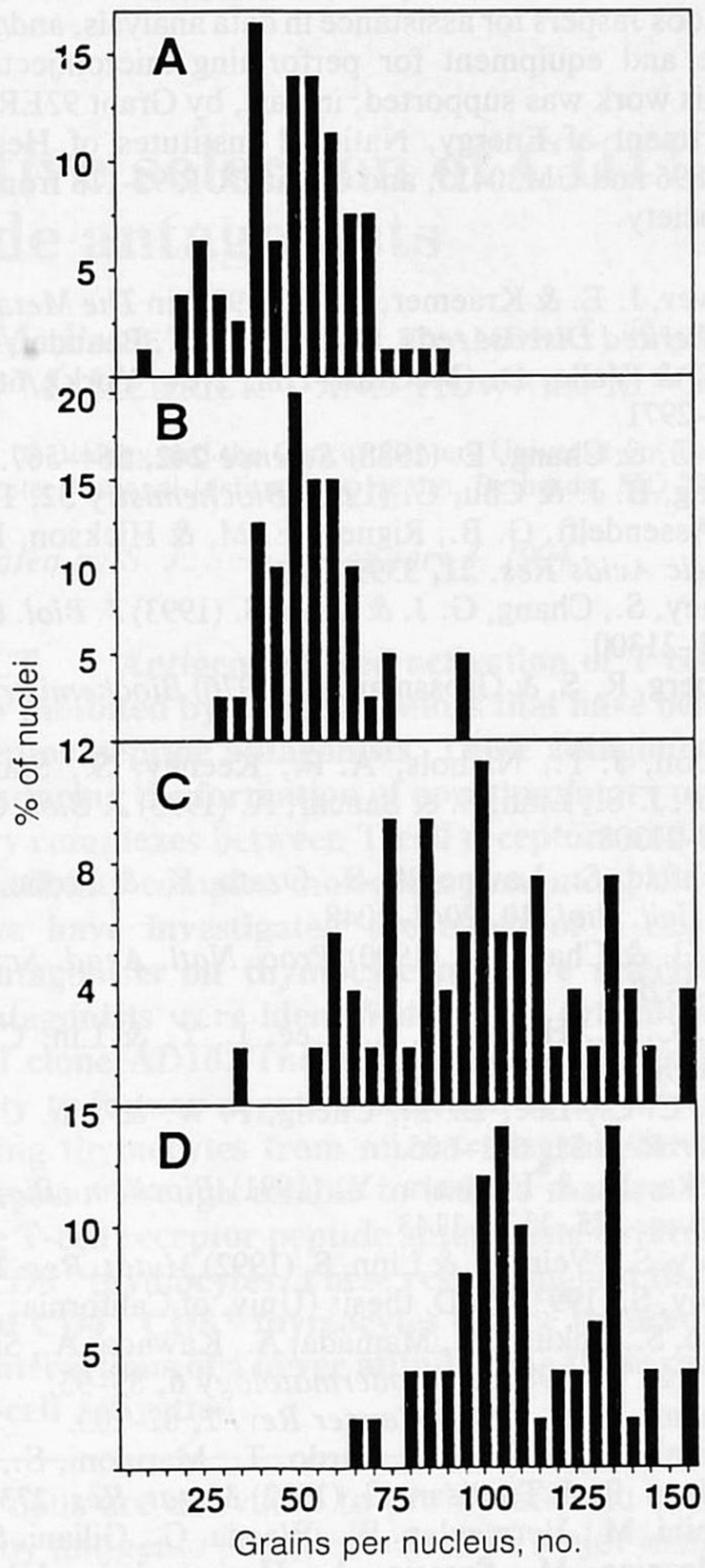

FIG. 2. Grain-count frequencies for XP2RO fibroblasts injected with DDB protein. (*A*) Uninjected XP2RO cells (47 ± 2 grains per nucleus, mean ± SEM). (*B*) XP2RO cells injected with PBS/0.005% Triton X-100/bovine serum albumin at 2 mg/ml (52 ± 2). (*C*) XP2RO cells injected with DDB protein, as described in the Fig. 1 legend (97 ± 4). (*D*) Uninjected C5RO (normal) cells (108 ± 3).

The amount of DDB protein injected relative to the levels found in normal cells can be estimated by assuming an injected volume of ≈50 fl (20) and a normal DDB level of 150 units per 10^6 cells (5, 13). In the experiments of Figs. 1 and 2, correction was achieved with the injection of ≈two cell equivalents (3×10^{-4} units) of DDB protein. However, injection of a 100-fold lower concentration of DDB protein (≈0.02 cell equivalent) gave ≈70% increase in UDS, and injection of a 30-fold lower concentration (≈0.07 cell equivalent) fully corrected the DNA-repair defect in XP2RO cells (Table 3), indicating that <10% of the normal amount of DDB activity was sufficient for wild-type levels of DNA repair in this assay. Injection of increased amounts of DDB protein beyond the minimum necessary for full correction gave no further stimulation of repair, as expected for true correction of the XP-E defect.

Table 2. Correction of the DNA-repair defect is specific for XP-E

Strain	XP group	UDS,* % of normal control: No injection	UDS,* % of normal control: DDB-injected†
Exp. 1			
XP25RO	A	4 ± 0.4	3 ± 0.2
XP1BR	D	27 ± 2	19 ± 1
XP126LO	F	11 ± 0.5	13 ± 0.9
XP2RO	E	70 ± 2	152 ± 8
Exp. 2			
XPCS2BA	B	21 ± 1	20 ± 1
XP1TE	C	32 ± 2	22 ± 3
XP2RO	E	49 ± 3	93 ± 6
Exp. 3			
XP3BR	G	5 ± 0.5	3 ± 0.3
TTD1BR	TTD	7 ± 0.6	6 ± 1
XP2RO	E	75 ± 4	118 ± 10

TTD, excision-repair-defective form of trichothiodystrophy.
*Mean ± SEM for 40–50 nuclei, normalized to the mean UDS of uninjected normal strain C5RO.
†Injection contained 6.0×10^6 units per ml.

Table 3. Determination of the minimum DDB concentration required for full correction

DDB,* units/ml	Cell equivalents per injection†	UDS,‡ % of normal control
No injection		42 ± 2
2.0×10^4	0.007	59 ± 4
6.0×10^4	0.02	73 ± 4
2.0×10^5	0.07	105 ± 7
6.0×10^6	2	90 ± 6

*Purified HeLa DDB protein was diluted to the indicated concentrations in PBS/bovine serum albumin at 0.2 mg/ml and then injected into XP2RO homopolykaryons.
†Cell equivalents per injection were estimated as described in text.
‡Data represent the means ± SEMs for 40–50 nuclei each, normalized to the mean UDS of normal strain C5RO.

We also examined the ability of normal DDB protein to correct the DNA-repair defect in the Ddb^+ class of XP-E cells. No effect on UDS was seen when DDB protein was injected into XP43TO or XP93TO fibroblasts under conditions that yielded correction of the DNA-repair defect in Ddb^- cells (Table 1). The ability of DDB protein to stimulate DNA repair thus appears to be limited to the Ddb^- class of XP-E cells in this assay.

DISCUSSION

Previous studies demonstrated that cells from some XP-E patients lack a DNA-binding activity specific for damaged DNA (2, 12, 13), but these studies could not establish a causal relationship between the defect in DDB activity and the XP-E DNA-repair defect. To address this question, we tested whether microinjection of DDB protein purified from repair-proficient cells could correct the DNA-repair defect in XP-E cells. Microinjection of purified DDB protein stimulated UV-induced DNA repair specifically in the Ddb^- class of XP-E cells. This stimulation appears to be a *bona fide* correction of the XP-E defect based on the following observations. (*i*) Repair DNA synthesis was stimulated to wild-type levels, and injection of DDB protein levels above the amount required for full correction gave no further stimulation. (*ii*) Stimulation of repair was specific for cells from XP-E. These results thus provide direct evidence that the DNA-repair defect in the XP2RO and XP82TO strains is caused by the defect in DDB activity.

The original studies that identified the DDB activity were intended to define damage-recognition activities that function in DNA repair (2, 6). However, there was only indirect evidence for a role of the DDB protein in DNA repair (8–11). Moreover, a high-mobility-group 1 protein recognizes damaged DNA and binds cisplatin adducts (26, 27). It appears to potentiate the lethal effects of platinum-DNA adducts, but not to be involved in the repair of these lesions (27–29). The results presented here provide direct evidence that the DDB protein functions in nucleotide-excision repair *in vivo*.

The role that the DDB protein plays in DNA repair remains in question. The mildness of the DNA-repair defect in XP-E (1) might suggest that the DDB protein functions in one of several parallel damage-processing pathways with overlapping specificities. Blocking one repair pathway by eliminating the DDB activity might only partially affect the sum total of DNA repair. Alternatively, the DDB protein might play an ancillary or stimulatory, rather than a central, role in DNA-damage recognition in nucleotide-excision repair. Binding by DDB protein might alter chromatin structure to facilitate access to DNA damage by other components of the DNA-repair machinery, or DDB protein might help to recruit processing enzymes to the sites of DNA damage in a manner such as that for the photolyases of *Escherichia coli* and *Saccharomyces cerevisiae*, which stimulate DNA repair in the absence of photoreactivating light by interacting with the nucleotide-excision repair system (30, 31). Such stimulatory or ancillary roles might be necessary to achieve maximally efficient or maximally rapid DNA repair but would not be absolutely required for repair to proceed.

One striking feature of the DDB protein is its abundance—nearly 10^5 copies per cell (5). This high number is surprising because the complete absence of DDB activity gives only a 50% reduction in DNA-repair capacity and, even more telling, introduction of <10% of the normal cellular amount results in normal DNA-repair levels in XP2RO cells. One interpretation of this abundance is that the DDB protein has another function in the cell aside from its role in DNA repair. Its relatively high affinity for undamaged DNA (7) raises the possibility that it may be a normal chromosomal constituent, although an unusual one because sequence analysis reveals no identity with known proteins (14, 32).

The molecular basis of the DNA-repair defect in the Ddb^+ of XP-E cells remains a puzzle. Two models can account for the Ddb^+ phenotype seen in most XP-E strains [assuming that the individuals studied here are truly members of complementation group E, as was independently confirmed for XP93TO (see *Materials and Methods*)]. (*i*) Different XP-E patients may have defects at distinct genetic loci that fail to complement one another in cell-fusion experiments (13). Such nonallelic noncomplementation could be the result of mutant and wild-type products of separate loci associating to form nonfunctional protein complexes, for example. The high-molecular-weight DDB protein complex and the heterodimeric structure of the core DDB protein (5, 14) provide obvious candidates for interacting gene products that, when mutated, might fail to cross-complement.

(*ii*) The protein present in the majority of XP-E strains could be altered at a domain other than its DNA-binding region (12, 13). Because the protein has no observed enzymatic activity (3, 5, 6), it presumably interacts with other proteins to mediate excision repair of target lesion(s). Mutations in domains involved in protein–protein interactions might allow DNA binding but not subsequent damage-processing events. This model could account for the inability of the DDB protein to correct the repair defect in Ddb^+ XP-E cells. For example, if the active form of the DDB protein *in vivo* is a higher order complex of the core heterodimer with other polypeptides, the accessory polypeptides in Ddb^+ XP-E cells might be sequestered in nonfunctional complexes with a defective endogenous heterodimer, unable to associate with the injected normal protein. Resolution of this issue must await molecular analysis of genes for the DDB subunits expressed in XP-E cells.

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