Damage-specific DNA binding (DDB) activity purifies from HeLa cells as a heterodimer (p127 and p48) and is absent from cells of a subset (Ddb2) of xeroderma pigmentosum Group E (XPE) patients. Each subunit was overexpressed in insect cells and purified. Both must be present for the damaged DNA band shift characteristic of the HeLa heterodimer. However, overexpressed p48 peptides containing the mutations found in three Ddb2 XPE strains are inactive, and wild type p48 restores DDB activity to extracts from a fourth XPE Ddb2 strain, GM01389, in which compound heterozygous mutations in DDB2 (p48) lead to a L350P change from one allele and an Asn-349 deletion from the other. Although these results indicate that these mutations are each responsible for the loss of DDB activity, they do not affect nuclear localization of p48. In normal fibroblasts, a 4-fold increase in p48 mRNA amount was observed 38 h after UV irradiation, preceding a similar elevation in p48 protein and DDB activity at 48 h, implying that p48 limits DDB activity in vivo. Because DNA repair is virtually complete before 48 h, a role for DDB other than DNA repair is suggested.

The rare human hereditary disease, xeroderma pigmentosum (XP), is characterized biochemically by defective nucleotide excision repair (NER), which manifests clinically as sensitivity to ultraviolet light and a high incidence of skin cancer. Based on fusion studies of cells from XP patients, seven NER-defective complementation groups (A through G) and a post-replication repair-deficient variant group (XPV) have been identified (1, 2). Cell strains from a subset (Ddb2) of individuals carrying XP complementation group E (XPE) lack a damage-specific DNA binding (DDB) activity (3–5). Because DDB was reported to recognize many types of DNA lesions (6–11) and is inducible by treatment with DNA-damaging agents (7, 12, 13), DDB was originally expected to play a role in damage recognition prior to nucleotide excision repair. However, recent NER reconstitution studies have reported that DDB is not required in vitro (14–16). Nonetheless, microinjection of purified HeLa DDB heterodimer (p127, p48) into XPE cells restores in vitro DNA repair synthesis to normal levels in XPE Ddb2 strains but not in XPE Ddb2 strains or in cells from other XP groups (17). Sequencing of the cDNAs that encode the DDB heterodimer have identified single base mutations only in DDB2 (p48) of XPE Ddb2 cells. In the Ddb2 strains, XP2RO and XP3RO, a G → A transition at nucleotide +818 causes an R273H change in p48, whereas an A → G transition at nucleotide +730 causes a K244E change in XP82TO. Overexpression of wild type p48, but not of p127, in insect cells greatly increases DDB activity in extracts prepared from these cells, indicating that p48 is required for damage-specific DNA binding (18).

In the present study, we have reconstituted human DDB activity in an electrophoretic mobility shift assay by combining insect extracts containing individually overexpressed wild type p127 and p48. Both extracts were required for damage-specific DNA binding activity, but extracts containing mutant XP2RO- and XP82TO-p48 polypeptides were unable to complement p127, demonstrating that the mutations inactivate DDB activity in these XPE Ddb2 strains. The wild type human p127 and p48, which were overexpressed in insect cells, were purified by monitoring the reconstitution of DDB, and purified wild type p48 was able to restore DDB activity to an extract prepared from a fourth XPE Ddb2 cell strain, GM01389. Mutations were identified in the DDB2 gene in this strain. Because there are three potential nuclear localization signals in the p48 sequence, one of which is abolished by the mutation in XP82TO, fluorescence microscopy was used to determine the cellular localization of wild type and mutant p48. Finally, the relationships between DDB activity and p48 mRNA and protein levels in normal fibroblasts in response to UV damage were analyzed. The accompanying paper (19) investigates the cellular localization of p127 in both normal and XPE fibroblasts in response to UV damage.

**EXPERIMENTAL PROCEDURES**

**Cultures and Strains—**S19 insect cells, HeLa cells, and IMR-90 and XPE fibroblast strains were cultured as described previously (10, 18). The fibroblast XPE strain GM01389 was obtained from the Coriell Institute cell repository (Camden, NJ).

**Constructs for Overexpression of Mutant p48 in Insect Cells—**To construct the mutant XP2RO DDB2 transfection vector, a reverse transcription-polymerase chain reaction (PCR) product (18), containing
cDNA nucleotides +820 to +1724 of XP2RO DDB2, was digested with BsmI and ApoI. This restriction fragment (nucleotides +898 to +1690), containing the G → A mutation at +818, was ligated into pTB13 (wild type DDB2 cDNA in pBluescript SK (20)), which had been digested with BsmI and ApoI to remove the corresponding wild type cDNA region. The resulting vector was digested with StuI and BglII releasing the XP2RO cDNA sequence −62 to +1317, which was inserted into pBAcPAK8 (CLONTECH) at the StuI site to produce the transfection vector 2R0-p48/pBAcPAK8. To construct the mutant XP28TO DDB2 transfection vector, the A → G mutation at nucleotide +730 was introduced into the wild type DDB2 cDNA sequence by overlap extension PCR (21). Then the mutant DDB2 cDNA, containing nucleotides −11 to +1317, was inserted into pBAcPAK8 at the BsmIII and StuI sites to produce the transfection vector 82TO-p48/pBAcPAK8.

Transfection of SF9 cells, plaque selection, recombinant virus amplification, infection, and harvesting of cells was carried out according to the BacPAk Expression System protocol (CLONTECH). 7.5 × 10^6 Cells were infected with a multiplicity of infection of 5, and the surviving 4 × 10^4 cells were harvested 66 h postinfection, resuspended in 300 μl of 50 mM Tris-HCl, pH 8.0, 100 mM NaCl, 1 mM dithiothreitol (DTT), and lysed by sonication. The lysate was clarified by centrifugation for 15 min at 19,000 g prior to gel analysis and assay.

Overexpression and Purification of DDB p127—The purity of fractions was monitored by analysis on SDS-polyacrylamide gel electrophoresis, using a 10% acrylamide gel (Life Technologies, Inc.) for dodecyl maltoside (CM) samples. Activity was detected through reconstitution of DDB activity in an electrophoretic mobility shift assay (EMSA) by complementing p127 with 48. The p48 was overexpressed in SF9 cells, and 1 μg of the clarified extract was added to each reaction.

To construct the DDB1/p2Bac transfection vector, PCR was used to insert the insert Kozak sequence, GCCAC, immediately 5′ of the wild type ATG start codon of the DDB1 cDNA coding sequence. An XbaI and a SacI site were created by PCR at nucleotides −10 and +3425 (immediately 3′ of the wild type stop codon), respectively. This fragment, which contained the entire DDB1 cDNA coding sequence, was inserted into the p2Bac baculovirus vector (Invitrogen) at the XbaI and SacI restriction sites to yield DDB1/p2Bac.

To purify p127, 3 × 10^8 SF9 cells in 300 ml of EX-CELL 400 (JRH Biosciences, Lenexa, KS) with 2% fetal bovine serum and 1% Fungizone (Life Technologies, Inc.) were transfected with DDB1/p2Bac at a multiplicity of infection of 10. After 48 h in spinner flasks at 27 °C, the cells were harvested, resuspended in 15 ml of lysis buffer (57 mM potassium phosphate (pH 7.5), 1 mM DTT, 0.2 mM phenylmethylsulfonyl fluoride), lysing by sonication, and clarifying the lysate by centrifugation for 15 min at 19,000 g. The supernatant was loaded onto a 30-ml Whatman P11 phosphocellulose column pre-equilibrated with 100 mM potassium phosphate (pH 7.5), 1 mM MgCl_2, 60 mM KCl, 0.1 mg/ml bovine serum albumin, and 5 nM of poly(dI-dC) (nucleotide residues) (Midland Certified Reagent). After 15 min at room temperature, 8 fmol of 32P-labeled 77-base pair double stranded DNA, UV-irradiated with 6 kJ/m2, was added, and the reaction was incubated for an additional 15 min at 30–32 °C. Samples were electrophoresed on 0.5% polyacrylamide gels in Tris borate/EDTA buffer, and the dried gels were exposed to x-ray film or scanned using a Molecular Dynamics PhosphorImager. One unit of DDB activity is defined as that amount forming 1 fmol of DDB-DNA complex.

Sequence Analysis of DDB1 and DDB2 in GM01359—Mutation analysis of cDNA was performed as described previously (22). cDNA products were PCR amplified from the transfection vector 82TO-p48/pBacPAK8 (p127) and DDB2 (p48). Genomic DNA was amplified by PCR using the primers: I-580, 5′-GTGGTTTCTTCTTCTTCTTT-3′, and 1317, 5′-CTTCTCCCTGAGTTTCCAT-3′. Then the genomic DNA PCR products were cloned into a pGEM-T Easy Vector (Promega), and the clones were sequenced using the primer I-333, 5′-ATTAGTTGCGCTT-3′, to analyze for mutations in DDB2 at nucleotides +1045 to +1047 and nucleotide +1048.

Analysis of DDB Activity after UV Irradiation—1.0 × 10^6 IMR-90 fibroblasts were seeded onto 150-mm culture plates in 30 ml of Dulbecco’s modified essential medium (Life Technologies, Inc.) with 10% fetal bovine serum. After incubation for 4 days at 37 °C in 5% CO_2, the medium was aspirated off and replaced with 4 ml of cold phosphate-buffered saline (PBS) and the monolayer of subconfluent cells was irradiated with 12 J/m2 of 254-nm light. The PBS was then replaced with 30 ml of fresh culture medium, and the cells were returned to the incubator until harvest. Three to four plates were harvested at each time point: the cells were washed with PBS, layered with 4 ml of 0.05% trypsin/0.5 mM EDTA per plate, incubated at 37 °C for 20 min, and then 4 ml of fresh medium was added. The detached cells were pelleted, washed once with PBS, and resuspended in a total volume of 15 ml of PBS or which 12 ml was pelleted for cell-free extract and 3 ml was pelleted for RNA isolation (see below). Extract was prepared by resuspending the cells in 400 μl of lysis buffer (50 mM potassium phosphate (pH 7.5), 10% glycerol, 1 mM DTT), lysing by sonication, and clarifying the lysate by centrifugation for 15 min at 19,000 g. DDB activity was monitored by EMSA as described above using 1.5 or 5 μg of extract, rather than the in vivo DNA binding activity of 35-ml units.

Analysis of p48 mRNA Levels by Quantitative Competitive Reverse Transcription-PCR—To prepare competitive RNA, the DDB2 cDNA sequence from nucleotides +872 to +1196, with a deletion from +891 to +939, was inserted into a pT7-7 vector immediately 3′ of the T7 promoter. RNA was synthesized as per the Promega T7 RNA polymerase kit manual, using the medium for a 15 ml reaction in a total volume of 15 ml of PBS or which 12 ml was pelleted for cell-free extract and 3 ml was pelleted for RNA isolation (see below). Extract was prepared by resuspending the cells in 400 μl of lysis buffer (50 mM potassium phosphate (pH 7.5), 10% glycerol, 1 mM DTT), lysing by sonication, and clarifying the lysate by centrifugation for 15 min at 19,000 g. DDB activity was monitored by EMSA as described above using 1.5 or 5 μg of extract, rather than the in vivo DNA binding activity of 35-ml units.

Production of Polyclonal Antibodies against p48 and Immunoblotting—His<sub>t</sub>-tagged human p48 protein was overexpressed in Escherichia coli...
coli from the His$_6$-tagged vector, pET29b (Novagen). Polynucleotide antibodies were raised in rabbits against the affinity-purified recombinant His$_6$-p48 protein, and IgG was purified from serum by ammonium sulfate precipitation and protein A column purification (HiTrap protein A; Amersham Pharmacia Biotech).

**Analysis of DDB p48 Protein after UV Irradiation**—0.5 × 10$^9$ IMR-90 cells were seeded onto 100-mm culture plates in 10 ml of Dulbecco's modified essential medium (Life Technologies, Inc.) supplemented with 15% fetal bovine serum and incubated at 37 °C in 5% CO$_2$. When the cells reached early- to mid-log phase, the medium was aspirated off and replaced with cold PBS, and the monolayer of subconfluent cells was irradiated with 12 mJ/cm$^2$-nm light. The PBS was then replaced with fresh culture medium, and the cells were returned to the incubator until harvest. Eight plates were harvested at each time point. Whole cell extracts were prepared as follows. The cell pellet was resuspended in 200 µl of hypotonic buffer (10 mM Tris-HCl (pH 8.0), 5 mM MgCl$_2$, and protease inhibitors (Complete; Roche Molecular Biochemicals). After 15 min, the cells were lysed by 50 strokes in a Dounce homogenizer. Potassium glutamate buffer (250 mM Tris-HCl (pH 8.0), 10 mM MgCl$_2$, 0.3 M potassium glutamate (pH 7.8), 5 mM DTT, 50% glycerol, and protein inhibitors) was added, and the extract was incubated for 30 min with occasional gentle mixing. The extract was clarified by ultracentrifugation for 1 h at 50,000 rpm (106,000 × g) in a Beckman 100.3 rotor at 4 °C and then dialyzed against 50 mM Tris-HCl (pH 8.0), 1 mM DTT, and 10% glycerol. 50 µg of extract was resolved by 2D SDS-PAGE and transferred to nitrocellulose paper, and a 200-fold dilution of purified p48 antibody was used for immunoblotting.

**Fluorescence Microscopy of DDB p48 Subcellular Localization**—Full-length cDNAs for both wild type and mutant DDB p48 were amplified by PCR with native p75 DNA polymerase (Stratagene) using primers 5'-ACCGATCAGCTCCAGAAGAAC-3' and 5'-TAGGGATCCATTGGCTCCCAAGAAAC-3', corresponding to cDNA nucleotides 12 to +16 and +1268 to +1293, respectively. The PCR products were digested with BamHI and then cloned into the mammalian expression vector pEGFP-C1 (CLONTech). The pEGFP-p48, pEGFP-p48RO, and pEGFP-p48TO, correspond to GFP-p48 wild type, XP2RO, and XP82TO vector pEGFP-C1 (CLONTech). The pEGFP-p48, pEGFP-p48RO, and pEGFP-p48TO, correspond to GFP-p48 wild type, XP2RO, and XP82TO vector pEGFP-C1 (CLONTech).

**RESULTS**

**DDB2 Mutations Inactivate UV-specific Binding of DDB in Cell-Free Extracts**—DDB activity can be reconstituted in an EMSA by mixing insect cell extracts containing individually overexpressed wild type p127 and p48 subunits (Fig. 1). Both subunits are required to produce the DDB-DNA complex band formed by the DDB heterodimer purified directly from HeLa cells (10).

The mutant p48 proteins, XP82TO-p48 and XP2RO-p48, containing the amino acid substitutions K244E and R273H, respectively, were overexpressed in a baculovirus-insect cell system (Fig. 2). The migration of XP82TO-p48 with a Mr of 42,500, and wild type p48, migrated at a temperature (Fig. 2). The migration of XP2RO-p48 with a Mr of 41,000, and wild type p48, was absent, demonstrating that the single mutations in both XP2RO and XP82TO are sufficient to inactivate DDB activity (Fig. 1). This rules out an alternative possibility that the DDB2 mutations in these cell strains are not responsible for the Ddb2$^{-}$ phenotype and that a third (unidentified) subunit of DDB could be defective in all XPE strains.

**Overexpression of p48 and p127 DDB subunits in Sf9 cells**—Clariﬁed extracts containing the indicated overexpressed DDB subunits were electrophoresed on a 7–12% SDS-polyacrylamide gel, followed by Brilliant Blue-G colloidal staining (Sigma). M, molecular mass marker (Amersham Pharmacia Biotech) with molecular sizes indicated to the left in kilodaltons. Arrows indicate the bands due to overexpression at positions corresponding to wild type p48 and XP2RO-p48 at Mr of 41,000, XP82TO-p48 at Mr of 42,500, and wild type p127 at Mr of 127,000.
p48 overexpressed in insect cells. A, SDS-PAGE analysis of p48 fractions visualized by Brilliant Blue-G colloidal staining. Lane 1, 10 μg of cell-free extract (5210 units); lane 2, 9-μg phosphocellulose fraction (5440 units); lane 3, 1.3-μg DEAE fraction (1400 units); lane 4, 1.3-μg S300 fraction (1190 units). B, SDS-PAGE analysis of p48 fractions. Lane 1, cell-free extract from uninfected Sf9 cells; lane 2, Sf9 extract containing overexpressed p48; lane 3, p48 overexpressed in Sf9 cells and purified through phosphocellulose, DEAE-Sepharose, and Superose6 columns. p48 is the lower band of the doublet. M, molecular mass marker (Amersham Pharmacia Biotech) with molecular sizes indicated to the left in kilodaltons. CFE, clarified cell-free extract; PC, phosphocellulose fraction; DE, DEAE-Sepharose fraction; SC, sizing column fraction.

Although the p48 polypeptide was soluble in the cell-free extract, it represented only 3–4% of the total cellular protein when overexpressed (Figs. 2 and 3B). This material was purified by chromatography on phosphocellulose, DEAE-Sepharose, and Superose6 columns as described under “Experimental Procedures” and summarized in Table I. Because p48 eluted from the Superose6 column at a position corresponding to a Mr of 41,400, it would appear to be a monomer in solution. This is consistent with its SDS-PAGE migration at 41,000 and its predicted molecular weight of 47,966 based on the cDNA sequence. The final material represented approximately 20% of the total protein in the peak Superose6 fraction as analyzed by SDS-PAGE (Fig. 3B). (The p48 polypeptide is the lower band of a doublet that migrates at the anticipated Mr of 41,000.) We have not had evidence for p48 acting other than stoichiometrically. For example, an estimated 0.2 fmol of p48 from the peak Superose6 fraction and 165 fmol of DDB p127 formed approximately 0.2 fmol of the DDB-damaged DNA complex, indicating that p48 acted stoichiometrically in the binding reaction, rather than catalytically. As expected, increasing the amount of p48 increased the level of DDB binding proportionately (data not shown).

Wild Type p48 Restores DDB Activity to Extracts from XPE DDB-deficient GM01389 Cells—Clarified extract prepared from GM01389 diploid fibroblasts was deficient in DDB activity (Fig. 4), confirming the previously reported identification of this strain as Ddb− (24). Addition of overexpressed wild type p48 purified from insect cells restored repair activity, indicating that p48 is also defective in this XPE Ddb− strain. Sequence analysis of cDNA prepared from DDB1 (p127) and DDB2 (p48) mRNA of GM01389 cells identified compound heterozygous mutations in DDB2, which was confirmed by sequencing the genomic DNA in the affected region. In one allele, a T → C transition at nucleotide +1049 caused a L350P change, whereas in the other allele a deletion of nucleotides +1045 to +1047 resulted in the loss of Asn-349 (Table III). No mutations were observed in the DDB1 cDNA of the strain. Hence, as in the three previously identified Ddb− strains, there was no wild type p48 present.

Maximum Increases in p48 mRNA Levels, p48 Protein, and DDB Activity, Post-UV Irradiation, Are Observed after DNA Repair Is Completed—When early- to mid-log phase IMR-90 fibroblasts were irradiated with 12 J/m2 UV light and harvested at various times up to 72 h after treatment, cell survival reached a minimum of 40% after 38 h (Fig. 5A), whereas p48 mRNA levels reached a maximum at that same time (Fig. 5C). After an initial level of 17 copies per unirradiated normal cell extract to 32P-labeled DNA substrate was monitored by the electrophoretic mobility shift assay. Lane 1, 1.5-μg GM01389 cell extract; lane 2, mixture of 1.5 μg of the GM01389 cell extract and 4 fmol of the DDB p48 Superose6 fraction; lane 3, 1.5 μg of the XP95TO (Ddb+) cell extract.

![Image](http://www.jbc.org/content/272/32/21425/F3.large.jpg)

**FIG. 3.** Purification of DDB p127 and DDB p48 overexpressed in insect cells. A, SDS-PAGE analysis of DDB p127 fractions visualized by Brilliant Blue-G colloidal staining. Lane 1, 10 μg of cell-free extract (5210 units); lane 2, 9-μg phosphocellulose fraction (5440 units); lane 3, 1.3-μg DEAE fraction (1400 units); lane 4, 1.3-μg S300 fraction (1190 units). B, SDS-PAGE analysis of DDB p48 fractions. Lane 1, cell-free extract from uninfected Sf9 cells; lane 2, Sf9 extract containing overexpressed p48; lane 3, p48 overexpressed in Sf9 cells and purified through phosphocellulose, DEAE-Sepharose, and Superose6 columns. p48 is the lower band of the doublet. M, molecular mass marker (Amersham Pharmacia Biotech) with molecular sizes indicated to the left in kilodaltons. CFE, clarified cell-free extract; PC, phosphocellulose fraction; DE, DEAE-Sepharose fraction; SC, sizing column fraction.

![Image](http://www.jbc.org/content/272/32/21425/F4.large.jpg)

**FIG. 4.** Wild type p48 restores DDB activity to a DDB-deficient GM01389 cell-free extract. Binding of DDB in a clarified GM01389 fibroblast extract to 32P-labeled DNA substrate was monitored by the electrophoretic mobility shift assay. Lane 1, 1.5-μg GM01389 cell extract; lane 2, mixture of 1.5 μg of the GM01389 cell extract and 4 fmol of the DDB p48 Superose6 fraction; lane 3, 1.5 μg of the XP95TO (Ddb+) cell extract.
to that observed in the IMR-90 normal fibroblasts (data not shown). In cell-free extracts from late-log phase IMR-90 fibroblasts, the induced response of DDB activity to UV irradiation is much slower than in early- to mid-log phase cells, with only a 2-fold increase observed at 72 h (data not shown).

**DDB2 Mutations in XPE Ddb** XP2RO and XP82TO Do Not Affect Nuclear Localization of p48—By fluorescence microscopy, recombinant GFP-p48 was observed exclusively in the nucleus for wild type and mutant p48 (Fig. 7). The control GFP protein alone showed whole cell distribution. This is in agreement with its small molecular size (26 kDa) and its lack of nuclear localization signals. Wild type DDB p48 and XP2RO mutant p48 were strictly localized in the nucleus. This is not surprising, because the three nuclear localization signals at amino acid positions 2–5 (PKKR), 3–6 (KKRP), and 240–243 (HKKK) are intact in these polypeptides. A nuclear localization prediction using the PSORT program (web version 6.4 (27)) indicated that there was a 70% certainty that p48 would be localized to the nucleus. The single amino acid change, K244E, in XP82TO p48 effectively abolished the third predicted nuclear localization signal within this mutant p48 but failed to change its nuclear localization. These results are in agreement with previously reported studies with T7-tagged p48 immuno-

**TABLE III**

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<tr>
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<td>T → C</td>
<td>L350P</td>
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<td>L350P</td>
<td>6/11</td>
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<td>AAC deletion</td>
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<td>AAC deletion</td>
<td>Loss of Asn-349</td>
<td>5/11</td>
</tr>
</tbody>
</table>

* Ratio of number of clones with mutations to the number of clones sequenced. These values are consistent with GM01389 being a complex heterozygote of DDB2.

**FIG. 5.** Response of p48 mRNA levels, p48 protein, and DDB activity to UV irradiation. IMR-90 early- to mid-log phase fibroblasts were irradiated with 12 J/m² UV light, incubated in fresh medium, and harvested at the times indicated. A, the number of viable cells was determined by trypan blue staining and are expressed relative to the cell number at the time of irradiation. B, p48 mRNA levels were monitored by QC-RT-PCR as described under “Experimental Procedures.” PCR products were resolved on a 5% native polyacrylamide gel and quantitated using a PhosphorImager (Molecular Dynamics) and ImageQuant™ 1.2. C, analysis of the immunoblot in Fig. 6 by ImageQuant. D, 5 µg of cell-free extract and 2 fmol of 32P-labeled UV-irradiated double stranded DNA were used per reaction. DDB activity was monitored by the electrophoretic mobility shift assay and quantitated using a PhosphorImager (Molecular Dynamics) and ImageQuant.

**FIG. 6.** DDB p48 is induced by UV irradiation. IMR-90 early- to mid-log phase fibroblasts were irradiated with 12 J/m² UV light, incubated in fresh medium, and harvested at the times indicated. 50 µg of cell-free extract was resolved by 10% SDS-PAGE, transferred to a nitrocellulose filter, and probed with polyclonal antibodies raised against His6-p48 polypeptide, and the filter was analyzed by autoradiography.

**FIG. 7.** Nuclear localization of wild type and mutant p48. Human normal diploid fibroblast IMR-90 cells were transfected with plasmid DNA coding for GFP alone or GFP-p48 fusions. Cells were fixed as described under “Experimental Procedures” and stained with DAPI 24 h after transfection. Fluorescence from DAPI and GFP was observed under a Zeiss Axioshot fluorescence microscope.
fluorescence microscopy (28). However, to rule out the possibility that a requisite factor for p48 nuclear localization is absent in XPE Ddb− cells, GFP-p48 constructs were transfected into XP2RO and XP82TO cells. Mutant and wild type p48 proteins were still observed in the nucleus (data not shown).

**DISCUSSION**

Although the proteins correlated with XPA, XPB, XPC, XPD, XPF, XPG, and XPV have been identified and studied, the XPE protein has remained elusive. One contributing factor is that XPE patients exhibit the mildest clinical signs of UV sensitivity and skin cancer of all the xeroderma pigmentosum groups. This is reflected biochemically in that cells from group E individuals have a relatively high level of nucleotide excision repair as determined by an unscheduled DNA synthesis level of 40–60%. Consequently, classification of XPE patients has been difficult, and functional complementation of XPE has not isolated the XPE gene.

In 1988 Chu and Chang (3) reported that a damage-specific DNA-binding protein was absent in cells from two XPE patients, XP2RO and XP3RO. However, further studies (4, 5) discovered only one additional XPE strain, XP82TO, that lacked this binding activity, whereas cells from 13 other patients classified as XPE had normal levels of DDB activity. Three additional XPE Ddb− strains have since been reported (24). Because the DDB activity is absent in only a subset of XPE patients, the relationship between XPE and the DDB protein is unclear. Is the DDB deficiency directly related to nucleotide excision repair? The fact that microinjection of the DDB heterodimer (p127, p48) purified from HeLa cells restored unscheduled DNA synthesis to Ddb− but not Ddb+ cells (17) suggested that DDB was indeed an “XPE factor.” In addition, the high affinity of DDB for many forms of DNA lesions implied that DDB was a damage recognition factor for NER.

Cells from the XP2RO and XP3RO patients, who were second cousins, have a single base transition that gives rise to a R273H substitution in p48. A single base transition in XP82TO, that lacked this binding activity, whereas cells from 13 other patients classified as XPE had normal levels of DDB activity. Three additional XPE Ddb− strains have since been reported (24). Because the DDB activity is absent in only a subset of XPE patients, the relationship between XPE and the DDB protein is unclear. Is the DDB deficiency directly related to nucleotide excision repair? The fact that microinjection of the DDB heterodimer (p127, p48) purified from HeLa cells restored unscheduled DNA synthesis to Ddb− but not Ddb+ cells (17) suggested that DDB was indeed an “XPE factor.” In addition, the high affinity of DDB for many forms of DNA lesions implied that DDB was a damage recognition factor for NER.

Cells from the XP2RO and XP3RO patients, who were second cousins, have a single base transition that gives rise to a R273H substitution in p48. A single base transition in XP82TO results in a R273H substitution. No DDB2 mutations were observed in the XPE Ddb− strains, nor were any mutations found in the DDB1 (p127) gene in any of the XPE strains investigated (18). However, it remained possible that these DDB2 mutations were not responsible for the Ddb− phenotype. For example, a third DDB subunit could be defective in all XPE strains.

In this report we have used functional assays to demonstrate that the DDB2 mutations are directly responsible for the loss of DDB activity in the XP2RO, XP3RO, and XP82TO Ddb− strains. In addition, using human p48 overexpressed in and purified from insect cells we were able to restore DDB activity to extract prepared from a fourth XPE Ddb− strain, GM01389, which was recently reclassified by Otrin and coworkers (24). Moreover, mutational analysis of cDNA and genomic sequences of the DDB2 gene in this strain identified compound heterozygous mutations: One allele has a L350P change, and the other allele has a deletion of Asn-349. No mutations were found in the DDB1 gene. Identification of DDB2 mutations in each of these four XPE Ddb− strains and the functional assays support the recommendation of Cleaver and colleagues (29) that XPE should be defined through molecular terms because of difficulties in assignment by complementation studies. By this definition, XPE would contain only those cell strains that have a mutation in DDB2.

The question still remains as to the function(s) of DDB in the cell. The original proposal that DDB is a damage recognition factor in nucleotide excision repair has yet to be substantiated. DDB is not required in in vitro reconstitution DNA repair assays and can even inhibit excision of damage (16). Possibly, the DDB heterodimer inhibits repair in vivo so as to modulate the rate of excision or allow damage bypass by a DNA polymerase. Because p48, but not p127, is induced by DNA-damaging agents (26), we analyzed the time response of p48 mRNA levels and DDB activity after UV irradiation of normal diploid fibroblasts. p48 mRNA levels peaked at 38 h, followed by a similar, almost 4-fold increase in DDB activity 48 h after UV irradiation. However, these peaks occur after a majority of DNA repair has been completed (25), suggesting an alternative role for DDB. (It is feasible, though unlikely, that this late induction could represent a replenishment of DDB once repair is complete and cell division resumes.)

DDB p127 interacts with several viral transactivating proteins: protein X of hepatitis B virus (30, 31), and the V proteins of paramyxovirus SV5, mumps virus, human para-influenza virus 2, and measles virus (32). DDB can associate with the transcription factor, E2F1, and function as a transcriptional partner of E2F1, whereas XP2RO and XP82TO p48 mutant proteins are severely impaired in stimulating E2F1-activated transcription (28). Watanabe and colleagues (33) reported that DDB p127 also binds to the cytoplasmic domain of the transmembrane protein, Alzheimer’s amyloid precursor protein (APP). They proposed that p127 may move between the cytosol and nucleus as part of a signal transduction process that regulates gene transcription in response to DNA damage. Because p48 transports p127 to the nucleus (28), a feasible model is that excess p127 is anchored in the cytoplasm by binding to APP. In response to damage, p48 mRNA levels increase, resulting in increased production of p48 protein. This de novo p48 may compete with APP for binding to p127 and transport it to the nucleus, as reflected by the observed increase in DDB activity after UV irradiation. XP2RO and XP82TO mutant p48 are defective in nuclear import of the large DDB subunit (28).

Hwang et al. (34) reported that the presence of p48 was not required for subsequent binding of p127 to DNA damage and that only a small fraction of DDB was a heterodimer either in solution or bound to DNA. Yet the DDB heterodimer is stable during purification (10), and in this report we show elution characteristics during chromatography of human p127 overexpressed alone in insect cells that are very different from the heterodimer. Also DDB activity can be reconstituted only by mixing purified p127 and p48, and p48 acts stoichiometrically in binding to damaged DNA in vitro and apparently in vivo. However, although the heterodimer produces a distinct DNase I footprint on DNA substrates containing unique UV photoproducts (8), the footprint for p127 alone indicates that both subunits need not be bound to damaged DNA. Hence, it is possible that, if bound to p48, and only after such binding, p127 can bind to DNA and remain bound even if p48 dissociates. However, the correlation of p48 and DDB induction by UV irradiation suggests that such a “catalytic” role for p48 may not normally exist in vivo. We have shown that the single base substitutions found in DDB2 of the XPE Ddb− strains XP2RO and XP82TO do not affect the nuclear localization of DDB p48. This observation, combined with the inability of these mutant p48 proteins to complement p127, suggests that the XPE Ddb− mutations affect the direct interaction of the two subunits that is required for both nuclear localization of the heterodimer and its subsequent binding to damaged DNA.

The abundance of DDB (∼105 copies per cell; Ref 10 and Fig. 5D) implies that DDB plays an important role in the cell. Moreover, its specificity and high affinity for damaged DNA suggests it has a role in DNA damage responses, but its late induction after UV irradiation proposes alternative roles. The interacting subunits of DDB may couple transcription to DNA damage by sensing such damage, whereas the individual DDB...
subunits perform different functions in the cell. We expect that the purified individual DDB subunits will allow us to study the function(s) of both the heterodimer and the individual subunits in the multiple potential roles of DDB in transcription, DNA damage responses, and DNA repair.

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Human Damage-specific DNA-binding Protein p48: CHARACTERIZATION OF XPE MUTATIONS AND REGULATION FOLLOWING UV IRRADIATION
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