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TFIIH with Inactive XPD Helicase Functions in Transcription Initiation but Is Defective in DNA Repair*

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TFIIH is a multisubunit protein complex involved in RNA polymerase II transcription and nucleotide excision repair, which removes a wide variety of DNA lesions including UV-induced photoproducts. Mutations in the DNA-dependent ATPase/helicase subunits of TFIIH, XPB and XPD, are associated with three inherited syndromes as follows: xeroderma pigmentosum with or without Cockayne syndrome and trichothiodystrophy. By using epitope-tagged XPD we purified mammalian TFIIH carrying a wild type or an active-site mutant XPD subunit. Contrary to XPB, XPD helicase activity was dispensable for *in vitro* transcription, catalytic formation of trinucleotide transcripts, and promoter opening. Moreover, in contrast to XPB, microinjection of mutant XPD cDNA did not interfere with *in vivo* transcription. These data show directly that XPD activity is not required for transcription. However, during DNA repair, neither 5' nor 3' incisions in defined positions around a DNA adduct were detected in the presence of TFIIH containing inactive XPD, although substantial damage-dependent DNA synthesis was induced by the presence of mutant XPD both in cells and cell extracts. The aberrant damage-dependent DNA synthesis caused by the mutant XPD does not lead to effective repair, consistent with the discrepancy between repair synthesis and survival in cells from a number of XP-D patients.

variety of DNA lesions, including UV-induced photoproducts and bulky chemical adducts, can be removed by this multienzyme system. After recognition of a damaged site, a region around the lesion is locally unwound, and two incisions flanking the lesion are made on the damaged strand. The subsequent removal of the damaged oligonucleotide is succeeded by gap-filling DNA synthesis and ligation to restore the original DNA sequence (1–3). Six protein factors are required for the early incision stage (4, 5). The XPC-hHR23B complex, which binds with high affinity to DNA damage and represents the primary damage recognition factor (6), XPA, RPA, and transcription factor IIIH (TFIIH), are involved in verification of the lesion, ATP-dependent local unwinding of DNA around the damage, and organization of the remainder of the NER complex (7, 8). This provides a substrate for the structure-specific endonucleases ERCC1-XPF and XPG, which incise the damaged strand asymmetrically on the 5' and 3' sides, respectively.

In addition to its role in NER, TFIIH is one of five basal transcription factors required for accurate initiation of transcription of protein coding genes by RNA polymerase II (Refs. 9 and 10 and see Ref. 11 for review). In the initiation reaction, TFIIH is required for the ATP-dependent formation of melted regions around the transcription start site (12). This requirement is alleviated by a pre-melted promoter region (12, 13) or negative supercoiling that can induce transient DNA melting (14, 15). TFIIH consists of nine subunits for which all genes have been cloned (16, 17). Several enzymatic activities have been identified for TFIIH and attributed to individual subunits; both of the largest two components, XPB and XPD, are DNA-dependent ATPases and DNA helicases (18–20), whereas CDK7 is a protein kinase that is capable of phosphorylating the C-terminal domain of the largest subunit of RNA polymerase II (21). Non-lethal defects in the two helicase subunits, XPB and XPD, give rise to the inherited disorders xeroderma pigmentosum (XP), Cockayne's syndrome (CS) combined with XP, or trichothiodystrophy (TTD) (22). Some of the clinical features of these syndromes, such as UV sensitivity and predisposition to skin cancer (characteristic for XP), may be due to defective functioning of TFIIH in NER, whereas other symptoms, such as severe growth retardation, neurodysmyelination (XP/CS and TTD), and brittle hair (TTD), may be caused by a subtle defect in the transcriptional activity of TFIIH (see Refs. 23 and 24 for further explanation). Mutations in the XPD gene frequently result in an unaccounted for property in which XP-D cell lines have relatively high levels of damage-dependent DNA

Nucleotide excision repair (NER)¹ is one of the most versatile DNA-damage repair mechanisms in eukaryotic cells. A wide

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¹ The abbreviations used are: NER, nucleotide excision repair; TF, transcription factor; XP, xeroderma pigmentosum; CS, Cockayne's syndrome; TTD, trichothiodystrophy; PCR, polymerase chain reaction; HA, hemagglutinin; CHO, Chinese hamster ovary; WCEs, whole cell ex-

tracts; DTT, dithiothreitol; BSA, bovine serum albumin; UDS, unscheduled DNA synthesis.

synthesis which does not generally correlate with residual repair activity and UV sensitivity (25, 26).

To obtain insight into the biochemical defects associated with mutations in TFIIH, we examined the contribution of the XPD-associated enzymatic activities to TFIIH function. An XPD mutant was used with a defined single amino acid change in the Walker type A nucleotide-binding motif (consensus GXGKT), lysine to arginine at position 48 (K48R), which preserves the positive charge. Biochemical analysis of the corresponding mutation in the *Saccharomyces cerevisiae* homolog of XPD, Rad3p, has demonstrated that this mutation dramatically impairs ATP hydrolysis, although the protein retains the ability to bind ATP (27). Yeast cells carrying the same mutation are viable but UV-sensitive (27, 28).

In this report we describe the effect of an active-site XPD mutant on mammalian TFIIH function. We provide direct evidence that XPD helicase activity is dispensable for basal transcription. In contrast, we show that XPD helicase activity is required for the formation of both the 5' and 3' incision around a site of DNA damage.

MATERIALS AND METHODS

Oligonucleotides, Site-directed Mutagenesis, and Plasmid DNA Construction—The mutation in the nucleotide binding domain encoded by the XPD cDNA was generated by site-directed mutagenesis using oligonucleotide p142 5'-TCAGGCACCGGGCGAACAGTATCCCTG-3'. The desired mutation was verified by sequence analysis, and XPD wild type and mutant cDNAs were cloned as *EcoRI* fragments into eukaryotic expression vector pSVL, a modified pSVM vector (Amersham Pharmacia Biotech), yielding plasmids pM52 and pM124, and pcDNA3 (Invitrogen), yielding plasmids pM264 and pM265. The C-terminal HA epitope tag was added by PCR using primers p117 5'-GCCGAAAC-CAGTGTGCCCC-3' (5' primer) and p240 5'-AGGAATCTCAGTAC-GCGTAATCTGGAACATCGTATGGGTATCCGAGCTGCTGAGCAAT-CTGCTC-3' (3' primer). PCR products were cloned into *SacII-EcoRI* sites of pBluescript (Stratagene) and sequenced to ensure the absence of any PCR-generated errors. Eukaryotic expression vectors containing cDNA encoding wild type and K48R mutant HA-tagged XPD were generated by ligation of the *EcoRI-ScaI* and the *ScaI-EcoRI* cDNA fragments into the *EcoRI* site of pcDNA3, yielding plasmids pM435 and pM436.

Isolation of TFIIH Containing Wild Type and K48R Mutant XPD—Cell lines UV435.12 and UV436.1 expressing wild type and K48R mutant HA-tagged XPD cDNA, respectively, were generated by transfection of CHO UV5 cells with plasmids pM435 and pM436 using Lipofectin reagent (Life Technologies, Inc.) essentially as suggested by the manufacturer. After selection with G418 (0.75 mg/ml), independent clones were isolated and tested for expression by immunoblotting of cell lysates using anti-HA 12CA5 monoclonal antibodies. Whole cell extracts (WCEs) were prepared from 3 liters of suspension culture of either cell line (29) and loaded onto a heparin-Ultrogel column (IBF, France) equilibrated in buffer A (25 mM HEPES-KOH (pH 7.8), 1 mM EDTA, 1 mM DTT, 10% (v/v) glycerol) containing 0.1 M KCl and step-eluted in buffer A containing 0.1, 0.22, 0.4, and 1.0 M KCl. The 0.4 M KCl fraction containing all of the TFIIH was incubated for 16 h with 100 μ l of protein G-Sepharose beads (Amersham Pharmacia Biotech) and 250 μ g of monoclonal antibody 12CA5, essentially as described before (30). The resin was recovered by centrifugation (3 min, 1000 rpm) and washed 3 times in buffer T (50 mM Tris-HCl (pH 7.9), 0.2 mM EDTA, 0.5 mM DTT, 17% (v/v) glycerol) containing 0.4 M KCl and 0.1% Nonidet P-40 and twice in buffer T containing 0.1 M KCl and 0.01% Nonidet P-40. Bound TFIIH was eluted from the resin in two washes with the latter buffer supplemented with 2.0 mg/ml HA peptide (YPYDVPDYA) for 1 h at 30 °C. TFIIH was dialyzed to remove the peptide in buffer T containing 0.05 M KCl and 0.01% Nonidet P-40 for enzymatic and NER analysis or buffer A (20 mM HEPES-KOH (pH 7.9), 17% glycerol (v/v), 1 mM EDTA, 1 mM DTT, and 0.1 M KCl; BSA was added to a final concentration of 0.2 mg/ml) for transcriptional analysis and stored in aliquots at -80 °C. During purification, all procedures were carried out at 4 °C, except where noted otherwise.

Analysis of TFIIH-associated Enzymatic Activities—Purified TFIIH was analyzed for ATPase, CTD kinase, and DNA helicase activities as described before (30).

Analysis of *In Vitro* Transcription Activity—Analysis of transcription

activity was carried out with highly purified or homogeneous preparations of recombinant human TBP, TFIIB, TFIIE, TFIIF, and affinity-purified RNA polymerase II from calf thymus as detailed before (12, 15). Reactions (20 μ l volume) contained 12 ng of TBP, 50 ng of TFIIB, 80 ng of TFIIF, 30 ng of RNA polymerase II, 75 ng of TFIIE, and TFIIH as indicated and contained 12 mM HEPES-KOH (pH 7.9), 60 mM KCl, 5 mM MgCl₂, 3 mM (NH₄)₂SO₄, 0.6 mM EDTA, 0.7 mM DTT, and 120 μ g/ml BSA.

Standard transcription reactions contained 100 ng of linearized p Δ ML (200) template (14) containing the adenovirus major late promoter, 60 μ M ATP, 60 μ M UTP, 10 μ M [α -³²P]CTP (80 Ci/mmol), and 120 μ M 3'-OMeGTP, as described (15).

For analysis of tri- and 15-nucleotide RNA product formation, 5 ng of AdML + 3G or AdML + 15G template DNA (372-base pair *PvuII* fragment), respectively, were used (31). Partial non-template DNA sequences (underlined are the natural transcription start site and the first G residue) are as follows: AdML + 3G, 5'-CCTCACGCTCTTCTCTAGAGTC-3', and AdML + 15G, 5'-CCTCACTCTTCTCTCTAGAGTC-3'.

After preincubation in the above described reaction mixture for 45 min at 30 °C, nucleotides were added (60 μ M ATP, 3 μ M [α -³²P]CTP (267 Ci/mmol), 10 μ M UTP, 120 μ M 3'-OMeGTP, and reactions were incubated for the indicated time. Reactions were analyzed as described by 20% polyacrylamide (acrylamide:bisacrylamide = 19:1), 8.3 M urea gel electrophoresis, after inactivation of RNA polymerase II by heating for 3 min at 68 °C and alkaline phosphatase treatment (31).

KMnO₄ Sensitivity Assay of Transcription Intermediates—For the KMnO₄ analysis of open complex formation, radiolabeled probe (0.2–0.4 ng of *EcoRI-HindIII* AdML + 15G fragment, labeled at the non-template strand (12)) was incubated in the above reaction mixture for 45 min at 30 °C. After this preincubation, nucleotides (60 μ M ATP, 10 μ M CTP, 10 μ M UTP, and 120 μ M 3'-OMeGTP) were added and incubated for 10 min at 30 °C. Two μ l of a freshly prepared 160 mM KMnO₄ was added for 20 s after which reactions were stopped by addition of 2 μ l of 14.4 M β -mercaptoethanol. DNA was cleaved at modified residues by piperidine and analyzed on 7% polyacrylamide (acrylamide:bisacrylamide = 19:1), 8.3 M urea gel electrophoresis as described (12, 31).

NER *In Vitro*, DNA Synthesis Assay—WCE were prepared from repair-proficient cell lines CHO AA8, and repair-deficient CHO NER mutant UV5. Reactions contained plasmid randomly damaged with *N*-acetyl-AAF and an equal amount of undamaged control plasmid with a different size, the indicated amounts of extracts and purified protein, and labeled nucleotides as described before (32, 33). After incubation for 3 h at 30 °C, DNA was isolated, linearized, and analyzed by agarose gel electrophoresis.

Nucleotide Excision Repair *In Vitro*, Detection of Incision Products—Covalently closed circular DNA containing a single 1,3-intrastrand d(GpTpG)-cisplatin cross-link (Pt-GTG) was produced as described (5). Repair was carried out in 8.5- μ l reaction mixtures for the fully defined reconstituted reactions or in 10- μ l reaction mixtures for the cell extract complementation. Repair reactions were carried out in buffer containing 45 mM HEPES-KOH (pH 7.8), 70 mM KCl, 5 mM MgCl₂, 1 mM DTT, 0.3 mM EDTA, 10% glycerol, 2.5 μ g BSA, and 2 mM ATP. Complementation reactions were supplemented with 40 mM phosphocreatine (di-Tris salt) and 0.5 μ g of creatine kinase. Each reconstituted reaction contained 50 ng of RPA, 45 ng of XPA, 10 ng of XPC-hHR23B complex, 50 ng of XPG, 20 ng of ERCC1-XPF complex, and either 1.5 μ l of HeLa TFIIH Heparin fraction IV (34) or 1.5 and 3 μ l TFIIH containing wild type or K48R mutant XPD subunit. Following preincubation for 10 min at 30 °C, 50 ng of Pt-GTG was added, and reactions were continued for 90 min at 30 °C (5 and 30 min, respectively, for complementation reactions). Reactions were stopped by rapid freezing. Six ng of an oligonucleotide was added, complementary to the excised DNA fragment and with 4 extra G residues at the 5' end. The excision products were radiolabeled with 0.1 units of Sequenase version 2.0 polymerase and 1 μ Ci of [α -³²P]dCTP, separated on a 14% polyacrylamide denaturing gel, and visualized by autoradiography as described (35).

For the detection of 3' and 5' incisions, the (Pt-GTG) plasmid was digested with *AvaII* at 140 base pairs 3' from the lesion, radiolabeled at the 3' end using DNA polymerase I (Klenow fragment) and [α -³²P]dTTP, and purified over a G-50 Sephadex column. Repair reactions were performed as in the dual incision assay, and DNA products were analyzed by denaturing 6% polyacrylamide gel electrophoresis as described previously (7).

Microneedle Injection of cDNA Expression Vectors and Assay for RNA Synthesis and UV-induced Unscheduled DNA Synthesis (UDS)—Microinjection of cDNA expression plasmids in nuclei of XP-D primary fibroblasts (XP1BR and XP6BE) as well as control cells (C5RO) was per-

formed as described previously (36). Briefly, at least 3 days prior to injection cells were fused with inactivated Sendai virus and seeded onto coverslips. After nuclear injection of cDNA constructs, cells were incubated in normal culture medium for 24 h to allow expression of the injected DNA molecules. Subsequently, UDS was determined after UV-C irradiation (15 J/m²), incubation for 2 h in [³H]thymidine-containing culture medium, fixation, and *in situ* autoradiography. Grains above nuclei were counted. RNA synthesis was determined by counting autoradiographic grains above the nuclei of injected cells, after labeling with [³H]uridine during a pulse-labeling period of 1 h in normal culture medium.

UV Survival and Analysis of Unscheduled DNA Synthesis of Transfected Cells—Transfections of CHO UV5 (37) and human HD2 (38) cells with plasmids pM265 and pM266 were performed using Lipofectin reagent (Life Technologies, Inc.) essentially as described by the manufacturer. After selection with G418 (0.75 mg/ml for CHO cells; 0.3 mg/ml for HD2 cells), single clones were isolated and selected for expression of wild type and mutant XPD by immunoblot analysis. UV survival was assayed using [³H]thymidine incorporation (39). Briefly, 3 days after irradiation with UV, proliferating cells were pulse-labeled with [³H]thymidine (4 h), followed by a chase (2 h). Cells were washed and lysed in 0.05 N NaOH. Quantification of the [³H]thymidine incorporation by liquid scintillation counting provides a measure of cell viability. For analysis of unscheduled DNA synthesis, cells were plated onto glass coverslips. After 24 h, cells were irradiated, pulse-labeled in [³H]thymidine-containing culture medium for 2 h, fixed, and subjected to *in situ* autoradiography (36).

RESULTS

Isolation of TFIIH Complexes Containing Wild Type and K48R Mutant XPD Subunit—To investigate the role of ATP hydrolysis by the XPD helicase subunit of TFIIH during NER and transcription initiation, we isolated TFIIH complexes containing wild type and mutant XPD protein. A cDNA encoding wild type XPD containing a C-terminal HA epitope tag and a cDNA encoding a similarly HA-tagged K48R mutant protein were generated. In the mutant, the highly conserved lysine residue in the Walker type A nucleotide binding motif was replaced by the similarly charged arginine (GKT → GRT, see Fig. 1A). UV-sensitive, NER-deficient Chinese hamster ovary (CHO) cells from complementation group 2 (UV5), carrying a mutation in the XPD gene, were transfected with cDNA encoding wild type and K48R protein, respectively, and independent clones stably expressing HA-tagged XPD were isolated. By using anti-HA monoclonal antibodies, immunoblot analysis of cell lysates from two selected clones expressing wild type and mutant HA-tagged XPD, respectively, shows a specific band at the expected molecular mass of XPD (Fig. 1B). The protein level of the wild type and K48R mutant appeared comparable.

The ability of HA-tagged XPD to correct the defective repair of UV5 cells was assessed by determining the UV survival of the transfected cells. The results presented in Fig. 1C show that a cDNA encoding HA-tagged XPD is able to confer UV resistance to UV5 cells close to the level of untagged XPD cDNA and in the range of the parental repair-proficient cell line AA8. This indicates that addition of the HA epitope tag does not negatively influence the function of the XPD protein in NER.

To obtain highly purified wild type and K48R XPD complexes from whole-cell extracts (WCE), a fast and efficient two-step purification protocol was employed, previously developed for the purification of HA-tagged XPB containing TFIIH. This protocol consists of chromatography on heparin-Ultrogel and anti-HA immunosorption followed by peptide elution (30). Isolated complexes were subjected to SDS-polyacrylamide gel electrophoresis and stained with silver nitrate (Fig. 1D). In addition to XPD, proteins with molecular masses ranging from 32 to 90 kDa were identified corresponding to all nine known TFIIH subunits as is indicated in the figure (Fig. 1D). As expected, the bands corresponding to XPD and the three CAK components (CDK7, Cyclin H, and MAT-1) were more prominent as com-

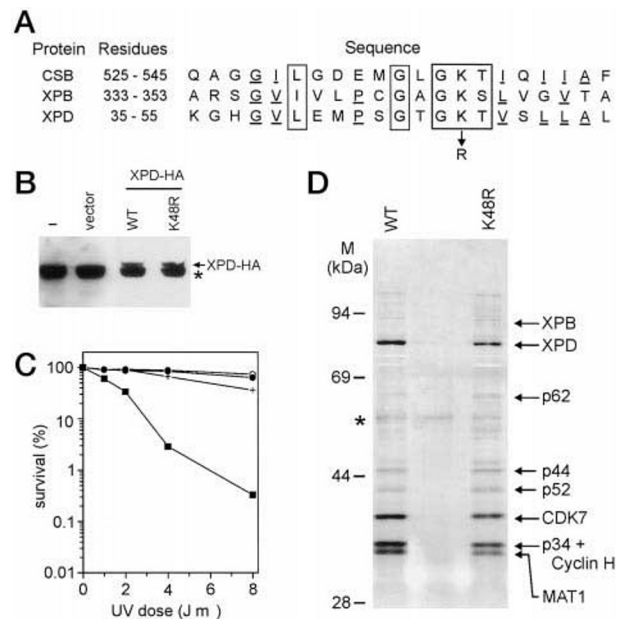


FIG. 1. Purification of wild type and K48R XPD-containing TFIIH complex from mammalian cells. A, homology of the conserved Walker type A nucleotide-binding motif of XPD with the sequences of the XPB and CSB proteins. Identical or similar residues are *underlined*; residues of the consensus nucleotide-binding motif are *boxed*. Indicated is the introduced point mutation at position 48. B, immunoblot analysis of CHO UV5 transfectants using anti-HA 12CA5 monoclonal antibodies. Lysates were obtained from untransfected (–), G418-resistant cells transfected with empty vector (vector), and cells transfected with human cDNA encoding XPD-HA wild type (WT) and XPD-HA K48R protein. The position of HA-tagged XPD is indicated with an *arrow*, and cross-reactive material that cannot be precipitated by 12CA5 antibodies is marked with an *asterisk*. C, UV survival analysis of CHO UV5 mutant cells (■), transfected with cDNA encoding (untagged) human XPD (●), and HA-tagged XPD (+) protein, and the parental wild type cell line AA8 (○). D, purified wild type (WT) and K48R XPD-TFIIH complexes were subjected to 11% SDS-polyacrylamide gel electrophoresis, and proteins were visualized by staining with silver nitrate. Indicated are the positions of the molecular mass markers and the TFIIH subunits identified on the basis of apparent molecular mass. The identities of p44 and cyclin H were confirmed by immunoblotting using antibodies that cross-react with Chinese hamster TFIIH factors (not shown). The band corresponding to the p52 subunit could not be assigned conclusively because antibodies raised against the human protein did not cross-react. Keratin artifact bands are marked with an *asterisk*.

pared with the other TFIIH subunits. This is in agreement with the finding that XPD resides in two complexes, TFIIH and XPD-CAK (40). We were not able to separate efficiently XPD-CAK and TFIIH by subsequent chromatography as their purification properties are very similar. However, since the staining patterns of the wild type and K48R mutant fractions were almost identical, the activities of the HA fractions could be directly compared.

Comparison of TFIIH-associated Enzymatic Activities of Wild Type and K48R Mutant XPD Complexes—Several enzymatic activities are associated with TFIIH (41, 42). The data in Fig. 2, A and D, and data from similar experiments showed that the ATPase activity of the K48R complex is reduced to about half of the wild type level. The residual activity is most likely derived only from the second DNA-dependent ATPase subunit of TFIIH, XPB. The reduction we observe is similar to that seen using recombinant TFIIH lacking the XPD subunit (47). These results suggest that both the XPD and XPB subunits contribute significantly to the ATPase activity of TFIIH.

The DNA helicase activities were compared using a radiolabeled 24-mer oligonucleotide annealed to single-stranded M13 DNA. This substrate design allows measurement of DNA heli-

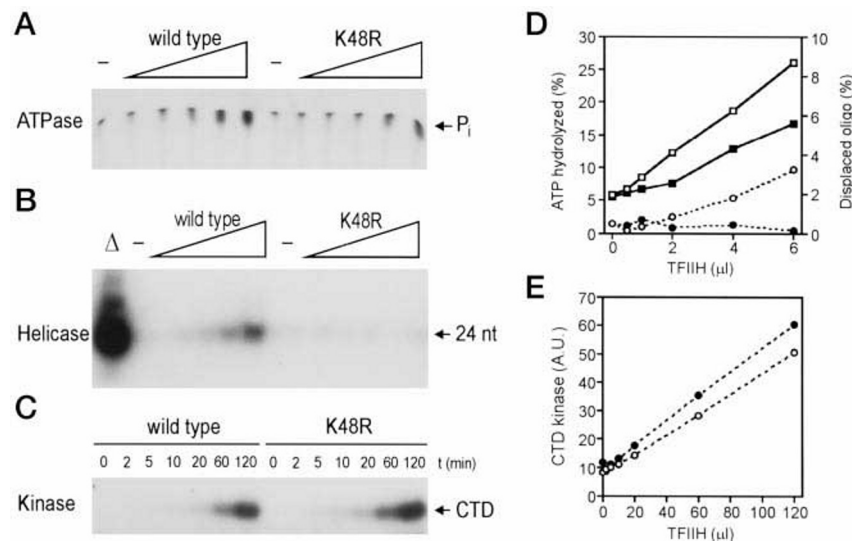


FIG. 2. Comparison of TFIIH-associated enzymatic activities of wild type and K48R XPD complex. *A*, autoradiogram showing reduction of DNA-dependent ATPase activity of K48R XPD complex. Radiolabeled ATP was incubated in the presence of 150 ng of M13 single-stranded DNA with increasing amounts of XPD complex (0, 0.5, 1, 2, 4, and 6 μ l), and the released phosphate was separated by thin layer chromatography. *B*, DNA helicase activity is absent in K48R XPD complex. Helicase activity was measured in the presence of increasing quantities XPD complex (0, 0.5, 1, 2, 4, and 6 μ l) as detected by the release of a radiolabeled 24-mer oligonucleotide from single-stranded M13 DNA. Δ indicates the heat-denatured control. *C*, CTD kinase activity is not affected by the K48R mutation in XPD. Activity was measured in the presence of wild type or K48R XPD complex (2 μ l) using a peptide containing four repeats of the conserved repeat YSPSTSPS of the large subunit of RNA polymerase II and incubated with radiolabeled ATP. *D*, quantitation of the ATPase and DNA helicase activities. The ATPase activity is represented as the percentage ATP hydrolyzed in the presence of TFIIH containing wild type (\square) and K48R mutant (\blacksquare) XPD. The DNA helicase activity is depicted as the percentage oligonucleotide displaced from the single-stranded DNA in the presence of TFIIH containing wild type (\circ) and K48R mutant (\bullet) XPD. *E*, quantitation of the CTD kinase activity in arbitrary units (A.U.) in the presence of TFIIH containing wild type (\circ) and K48R mutant (\bullet) XPD.

case activity independent of helicase polarity. As shown in Fig. 2, *B* and *D*, wild type XPD complex is able to release annealed oligonucleotide from the M13 DNA. However, within the limits of detection, the mutant K48R XPD complex added in the same quantity appears to be completely deficient in the ability to release the labeled oligonucleotide. This result indicates that the XPD DNA helicase is absolutely required for the DNA-unwinding activity of TFIIH measured in this assay, irrespective of DNA polarity. The fact that both the TFIIH-associated ATPase and DNA helicase activities are severely affected is consistent with the notion that the introduced point mutation in the nucleotide binding motif of XPD abolishes its associated enzymatic activities.

Finally, we characterized the kinase activity that is able to phosphorylate the C-terminal domain of the largest subunit of RNA polymerase II, RPB1. Equal amounts of both wild type and K48R XPD complexes were incubated with a synthetic tetra-repeat heptapeptide corresponding to the conserved repeat of RPB1 in the presence of radiolabeled ATP. The CTD peptide was phosphorylated in a time-dependent manner with similar efficiency by both wild type and K48R XPD complexes (Fig. 2, *C* and *E*).

TFIIH Containing Inactive XPD ATPase Subunit Supports Transcription Initiation—To investigate whether the K48R effect on ATPase and helicase activity has consequences for the function of TFIIH in basal transcription, we tested both wild type and mutant XPD fractions in RNA polymerase II-mediated *in vitro* transcription assays. Wild type and K48R XPD complexes were incubated in the presence of homogenous recombinant TBP, TFIIB, TFIIE, TFIIF, and highly purified RNA polymerase II, linearized DNA template containing the adenovirus major late promoter, and ribonucleotides. Interestingly, correctly initiated transcripts were produced in the presence of either wild type and K48R XPD complex (Fig. 3*A*). This was specifically due to TFIIH activity in both wild type and K48R mutant preparations since omission of any basal factor from

the reaction mixture did not yield appreciable transcript levels (Fig. 3*B*). The level of transcription supported by K48R mutant TFIIH varied between different preparations within a 2-fold range as compared with wild type and precluded detection of a statistically significant difference in transcription activity between wild type and K48R preparations.

To characterize further the possible effect of the K48R mutation in the XPD subunit of TFIIH on transcription, we looked at early stages of transcription initiation. RNA products shorter than 11 nucleotides accumulate with time, due to multiple rounds of abortive synthesis and initiation by the same RNA polymerase II complex (31). If subtle differences, such as the dynamic transition from closed to open conformation, exist in the presence of mutant TFIIH, they may become apparent from analysis of small RNA products. Therefore, we incubated mutant and wild type TFIIH with template DNA in the presence of other basal transcription factors, RNA polymerase II, and the appropriate nucleotides, and we analyzed the formation of trinucleotides (with AdML + 3G template, Fig. 3*C*, lanes 1–8) and 15-nucleotide products (AdML + 15G template, Fig. 3*C*, lanes 9–16). As is evident from Fig. 3*C*, the products from the AdML + 3G template, tri- and mainly dinucleotides (31), increase with time due to multiple rounds of abortive synthesis and initiation. This is not observed with the AdML + 15G template, which does not allow abortive transcription in agreement with previous results (31). Note that much more tri- and dinucleotide products are formed, which incorporated only one (labeled) CTP, as compared with the 15-nucleotide product, which incorporated six CTPs. Importantly, however, no significant difference is observed in the formation of tri- and dinucleotides and 15-nucleotide transcripts in the presence of either wild type or mutant TFIIH.

Open Complex Formation in Transcription Is Unaffected with Inactive XPD Helicase—Since TFIIH is implicated in melting of DNA during the transcription initiation event, we analyzed the promoter-template region for single-stranded re-

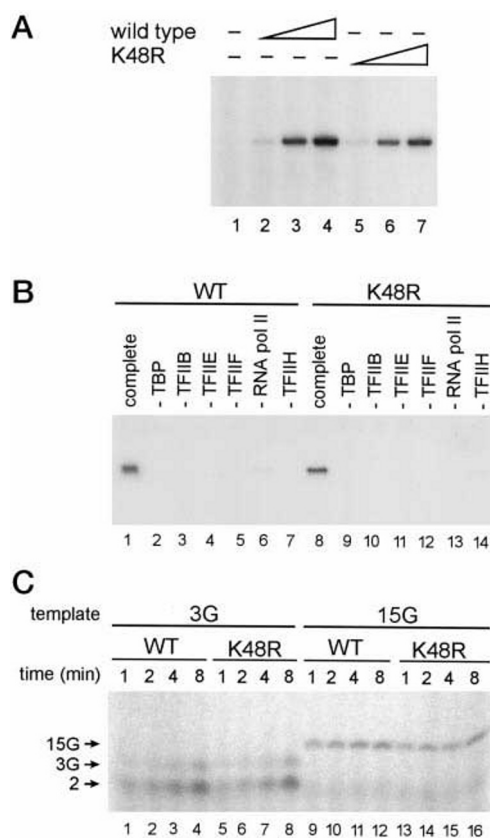


FIG. 3. TFIIF containing inactive XPD DNA helicase allows transcription initiation by RNA polymerase II similar to wild type TFIIF. *A*, reconstituted transcription reactions. Reactions contained purified recombinant basal transcription factors TBP, TFIIB, TFIIE, TFIIF, and purified RNA polymerase II (lanes 1–7), wild type (1, 3, and 5 μ l, lanes 2–4, respectively), or K48R XPD complex (1, 3, and 5 μ l, lanes 5–7, respectively) and linearized template DNA containing the AdML promoter and a G-less cassette of 200 base pair. *B*, stimulation of RNA polymerase II transcription by wild type (WT) and K48R complex is specifically due to TFIIF activity. Transcription reactions contained TFIIF (3 μ l; wild type, lanes 1–6, or K48R, lanes 8–13) and all basal transcription factors and RNA polymerase II (lane 1 and 8), or a single factor omitted as indicated above the lanes (lanes 2–7 and 9–14). *C*, time course analysis of the formation of short RNA products. Reactions contained basal transcription factors and RNA polymerase II (3 μ l of TFIIF; wild type, lanes 1–4 and 9–12, or K48R, lanes 5–8 and 13–16), and template DNA (AdML + 3G, lanes 1–8; AdML + 15G, lanes 9–16).

regions with permanganate. Basal transcription factors, wild type or K48R mutant TFIIF, and RNA polymerase II were incubated with template DNA to allow the formation of preinitiation complexes. Subsequently, ATP was added to allow transient initial opening of the promoter region (12, 31). The specific activity of the TFIIF preparation was not sufficient to allow detection of the unstable initial opening of the promoter region (Fig. 4, lanes 1–4). However, upon addition of ATP, UTP, CTP, and 3'-OMeGTP that allows the formation of a specific 15-nucleotide transcript, permanganate sensitivity of thymidine residues at position +5 to +13 was clearly enhanced (Fig. 4, compare lanes 1 and 3 and 4 and 6). Importantly, the increased sensitivity of this region, which indicates the formation of an open region downstream the initiation site, was found in the presence of both wild type and mutant TFIIF containing an inactive XPD helicase subunit.

Earlier stages of open complex formation, in which a region upstream of the start site is still open, were analyzed using template DNA that allows stalling of the polymerase after the formation of a 4-nucleotide transcript (31). Again, no difference could be discerned when wild type or mutant TFIIF were used (data not shown). Together, these comparative analyses be-

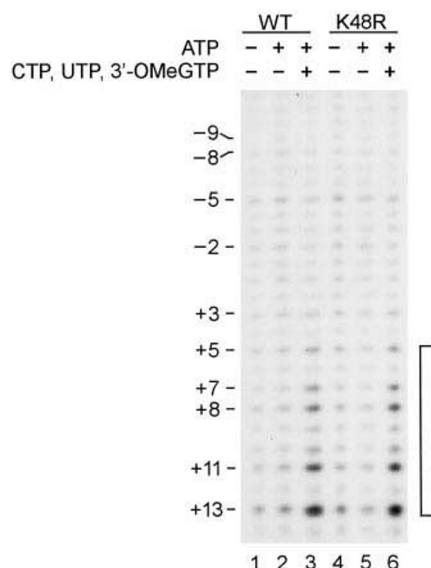


FIG. 4. KMnO_4 sensitivity of the AdML promoter region around the start site. Open regions from +5 to +13 are efficiently detected in the presence of both wild type (WT) and mutant TFIIF (5 μ l) after production of a 15-nucleotide transcript. The DNA fragment (labeled at the non-template strand) containing the AdML promoter was incubated with wild type (lanes 1–3) or mutant (lanes 4–6) TFIIF and other basal factors. The sensitivity to potassium permanganate modification was subsequently determined in the absence of nucleotides (lanes 1 and 4) or in the presence of ATP (lanes 2 and 5), or ATP, CTP, UTP, and 3'-OMeGTP (lanes 3 and 6). Indicated are the positions of thymidine residues with respect to the transcriptional start site as determined with a G + A ladder (not shown). The bar indicates the open region of the non-template strand.

tween wild type and mutant TFIIF suggest that the XPD helicase activity is dispensable for transcription initiation by RNA polymerase II *in vitro*.

TFIIF Containing Inactive XPD ATPase Subunit Is Able to Support Damage-dependent DNA Synthesis—To analyze the requirement for ATP hydrolysis by the XPD subunit of TFIIF during NER, we carried out *in vitro* repair synthesis reactions. A WCE was prepared from CHO UV5 repair-deficient cells and incubated in the absence or presence of wild type and K48R mutant TFIIF complexes, AAF-damaged DNA and undamaged control plasmid, and radiolabeled dATP and nucleotides. After incubation, plasmid DNA was isolated, linearized, and subjected to agarose gel electrophoresis. Ethidium bromide staining indicated that equal amounts of DNA were recovered from each reaction (Fig. 5, upper panel). The corresponding autoradiogram (Fig. 5, lower panel) shows that some radiolabel is incorporated in the damaged plasmid incubated with UV5 WCE alone compared with the non-damaged control, whereas increasing amounts of wild type TFIIF are able to stimulate DNA repair synthesis. Surprisingly, different preparations of TFIIF containing K48R XPD were also able to stimulate DNA synthesis specifically in the plasmid containing AAF damage, although to a lower level as compared with wild type samples. From the experiment shown in Fig. 5, and other similar experiments, we estimate that the mutant complex stimulates incorporation of nucleotides to about 25% of the wild type level. These findings suggested that the K48R XPD mutation might not completely impair the NER function of TFIIF. Therefore, we analyzed in more detail the formation of NER reaction intermediates.

The Formation of Incisions around DNA Damage Is Defective in the Presence of TFIIF with K48R Mutant XPD—By using a DNA substrate containing a single 1,3-d(GpTpG) intrastrand cisplatin cross-link, the formation of dual incisions around a

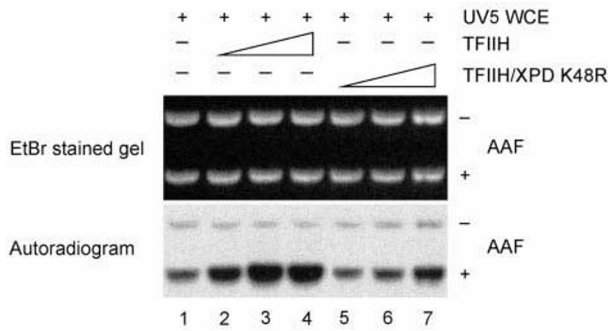


FIG. 5. **K48R XPD complex stimulates damage-dependent DNA synthesis *in vitro*.** Wild type (1, 2, and 4 μ l, lanes 2–4, respectively) and K48R XPD complex (1, 2, and 4 μ l, lanes 5–7, respectively) were assayed for complementing activity of CHO UV5 (XP-D) extracts (125 μ g, lanes 1–7). The positions of the undamaged (–) and AAF-damaged (+) plasmids are indicated. *Upper panel*, ethidium bromide-stained agarose gel. *Lower panel*, autoradiogram of the same gel.

DNA lesion was studied in detail using a specific and sensitive method that detects the 24–32-nucleotide platinated oligonucleotides released by the NER system. We assessed the activity of the mutant TFIIH in NER using a fully reconstituted system (Fig. 6A) and by complementation of two TFIIH-defective cell lines (Fig. 6B). For the latter purpose WCE were prepared from two human repair-deficient cell lines, XP-B (GM2252) and XP-D (GM2485). In the presence of the purified NER factors, wild type immunopurified TFIIH allows the formation of incisions on both sides of the lesion as does TFIIH purified by traditional chromatography from HeLa cells (Fig. 6A). However, reaction mixtures containing TFIIH with mutant K48R XPD subunit showed no detectable release of damaged oligonucleotide (Fig. 6A). We estimate from other experiments that the sensitivity of the assay would detect dual incision activity as low as 5% of the wild type level. Mutant K48R XPD-TFIIH complex was, however, able to complement weakly an extract derived from XP-B cells (Fig. 6B). Correction of the incision defect by the mutant complex is lower than correction by wild type TFIIH, presumably because exchange of TFIIH subunits is needed for complementation of extract containing mutated XPB with mutant XPD TFIIH complex. Consistent with this, when wild type or mutant complex was added to extracts from XP-D cells, only the wild type was able to correct the incision defect (Fig. 6B).

To establish whether mutant TFIIH allows the formation of uncoupled 3' or 5' incisions, the single lesion DNA substrate was labeled at the 3' end with respect to the lesion and was incubated with purified components. This assay detects all 3' incisions, arising during the dual incision reaction or as uncoupled 3' or 5' incisions (see diagrams at the right of Fig. 7). In the presence of wild type TFIIH, 3' incisions were readily formed (during the dual incision reaction, Fig. 7, lanes 6 and 7). However, neither 3' nor 5' incisions were detected when the reactions were performed with TFIIH containing mutant XPD subunit (Fig. 7, lanes 8 and 9). As a positive control, uncoupled 5' incisions were efficiently placed in the presence of E791A mutant XPG protein (Fig. 7, lanes 4 and 5) (43). Taken together, these data indicate that the specific placement and efficient formation of both incisions around a lesion depend on ATP hydrolysis and the DNA helicase activity of the XPD subunit of TFIIH.

K48R XPD cDNA Stimulates Unscheduled DNA Synthesis but Does Not Confer UV Resistance to XP-D Cells—The physiological relevance of the above findings for NER was investigated. Therefore, the cDNAs encoding the wild type and K48R protein were injected into nuclei of living primary human XP-D fibroblasts. Upon injection of wild type cDNA, unscheduled

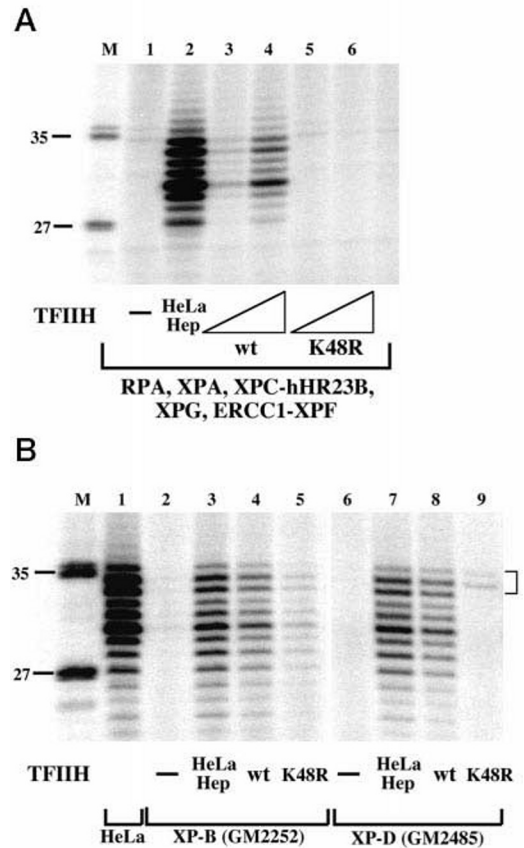


FIG. 6. **Dual incision activity of wild type and K48R mutant XPD *in vitro*.** *A*, reconstitution of nucleotide excision repair *in vitro* with purified components; reactions included either HeLa TFIIH (1.5 μ l of heparin (Hep) fraction IV) or wild type (wt) or K48R mutant TFIIH (1.5 and 3 μ l) as indicated; lane M, size markers (pBR322-*Msp*I digest). *B*, complementation of XP-B and XP-D defective cell extracts with wild type or K48R mutant TFIIH; each reaction contained 20 μ g of whole cell extract protein and was complemented with 1 μ l of HeLa TFIIH or 2 μ l of wild type XPD or K48R mutant complex as indicated; lane M, size markers; the weak bracketed bands (lane 9) occasionally arise from nonspecific end labeling of the complementary oligonucleotide used in the detection method.

DNA synthesis (UDS) was stimulated up to the level of wild type cells (Table I). Interestingly, injection of mutant cDNA encoding the K48R protein stimulated the residual UDS of the XP6BE XP-D fibroblast to about 50% of wild type levels. The *in vivo* observed increase in UDS roughly correlates with the increase in DNA synthesis measured in the *in vitro* assay (see Fig. 5). Similar results were obtained using a different XP-D cell line (XP1BR, data not shown). We failed to detect any dominant effect exerted by the mutant protein on either RNA synthesis or UDS after injection of wild type fibroblasts (data not shown).

To investigate whether the observed increased UDS levels represent a low level of DNA repair, the cDNA encoding wild type and K48R protein were transfected into XPD-deficient CHO and human cells, and UV survival was analyzed. Fig. 8A shows an immunoblot of CHO UV5 cell lysates obtained from selected clones expressing wild type and mutant cDNA. By using a monoclonal antibody raised against human XPD, a specific band with equal intensity corresponding to the expected molecular mass of XPD is observed in lanes containing lysates from UV5 cells transfected with wild type or mutant human cDNA. This band is not observed in lanes containing lysate from UV5 cells or empty vector-transfected cells, indicating that UV5 cells express the human cDNA to the same level and that the monoclonal antibody does not cross-react

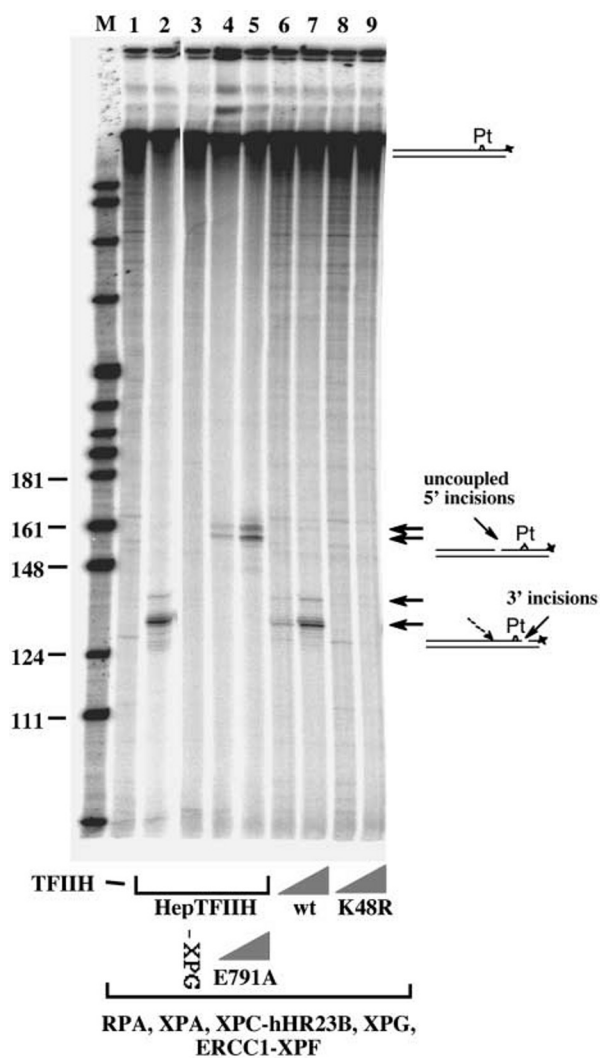


FIG. 7. Activity of TFIIH with wild type or K48R mutant XPD around a single Pt-GTG lesion. Reconstitution of nucleotide excision repair *in vitro* using an assay that reveals specific sites of enzymatic incision on the 3' or 5' sides of the platinum lesion. The (Pt-GTG) duplex substrate is radiolabeled on the damaged strand 140 nucleotides 3' of the adduct. Lane M, size markers (pBR322-*Msp*I digest). Reactions contained purified components except as indicated: lane 1 contains no TFIIH; lanes 2–5 contain HeLa TFIIH (1.5 μ l of heparin (Hep) fraction IV); lane 3 contains no XPG protein; lanes 4 and 5 contain 0.75 and 1.5 ng, respectively, of E791A mutant XPG (43); lanes 6–9 contain the indicated TFIIH complexes containing wild type (wt) or K48R mutant XPD subunit; uncoupled 5' incision and 3' incision products are indicated.

TABLE I
Microinjection of cDNA encoding XPD(K48R) protein stimulates unscheduled DNA synthesis in living XP-D fibroblasts

Cell type	cDNA injected	UDS grains/nucleus ^a	Percentage
C5RO (wild type)	Non-injected	65.8 \pm 1.8	100 \pm 2.7
	XP6BE(XP-D)	11.5 \pm 0.6	17.5 \pm 5.0
XP6BE(XP-D)	XP6BE(XP-D)	66.8 \pm 2.4	101.5 \pm 3.5
	XP6BE(XP-D)	31.0 \pm 1.7	47.2 \pm 5.4

^a Autoradiographic grains above the nuclei of at least 50 cells were counted.

with the mutant Chinese hamster XPD protein. The UV survival experiment (Fig. 8B) shows that the wild type human cDNA confers UV resistance to UV5 cells, up to the level of the parental cell line AA8. However, the mutant cDNA encoding K48R XPD failed to confer significant UV resistance as com-

pared with empty vector-transfected UV5 cells as noted before (44).

To analyze further whether the observed increase of UDS represents NER events and to exclude that the inability of the human mutant cDNA to confer UV resistance to CHO XPD-deficient cells is due to a subtle cross-species complementation defect, a similar experiment was performed using human XP-D cells. Expression of the cDNA encoding wild type and K48R mutant proteins was analyzed by immunoblotting (Fig. 8C). A specific band was only observed in lysates containing the wild type and K48R proteins in approximately equal amounts when compared with a lysate derived from immortalized wild type cells. Interestingly, the antibody did not recognize the endogenous mutant protein present in the XP-D cells. As in the case of transfection of CHO cells, the cDNA encoding K48R XPD failed to confer UV resistance to human cells, whereas the cDNA encoding the wild type protein did, almost to the level of wild type control cells (Fig. 8D). However, transfection of cDNA encoding K48R XPD resulted in a statistically significant increase of UDS in human XP-D cells (Fig. 8E, *p* value 0.05), up to approximately 50% of wild type levels, which was not observed with empty vector-transfected cells. These results are in agreement with the microinjection experiments and indicate that cellular UV resistance and efficient NER requires XPD helicase activity. However, significant damage-dependent DNA synthesis is supported by TFIIH containing an enzymatically inactive XPD subunit.

DISCUSSION

Role of XPD in Transcription—In this paper, five criteria are presented that indicate directly that mammalian XPD activity is not required for RNA polymerase II-mediated basal transcription as follows: (i) TFIIH containing an inactive XPD helicase is able to support basal transcription in a reconstituted system with highly purified components; (ii) neither enzymatic formation of tri(di)-nucleotide transcripts nor productive formation of 15-nucleotide transcripts are significantly affected; (iii) open complex formation after synthesis of a 15-nucleotide transcript is nearly identical in the presence of either wild type or K48R mutant XPD; and (iv) microinjection of XPD cDNA encoding K48R protein does not interfere with RNA synthesis in living fibroblasts and therefore does not seem to have dominant-negative effects. This is in striking contrast to the corresponding mutation in the XPB helicase subunit, which was found to paralyze transcription and DNA repair in a dominant fashion (33). (v) Stable transformants carrying the K48R mutant protein in TFIIH are viable, suggesting that the mutation does not impair transcription. These findings are in agreement with and further extend observations made in *S. cerevisiae* (45, 46) and studies using recombinant TFIIH (47).

In contrast to the requirement for the enzymatic activity of XPD, it is evident that the physical presence of the protein is required for transcription and viability (48, 49). Mutations in XPD that interfere with transcription should therefore compromise other functions of the protein, perhaps in addition to a defect in the ATPase activity, such as interactions with other transcription proteins or stability of TFIIH *in vivo* (24).

Role of XPD in NER—The findings in this paper show that if a mutation inactivates only the enzymatic activity without significantly altering the conformation of the XPD protein, then this results in considerable damage-dependent DNA synthesis. However, the observed synthesis does not reflect effective DNA repair, because a cDNA encoding the mutant protein is unable to confer significant UV resistance to XP-D cells, and TFIIH containing mutant XPD is unable to place efficiently either 5' or 3' incisions at defined positions around a DNA lesion. It is possible that the K48R XPD-TFIIH can still support

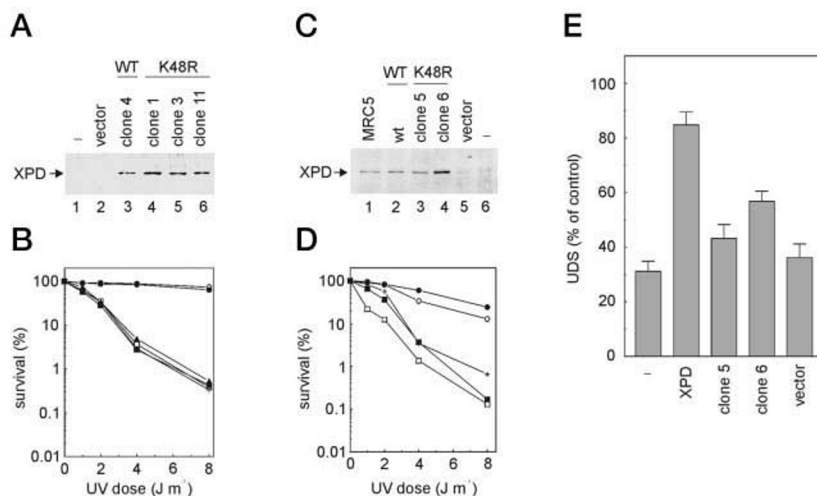


FIG. 8. cDNA encoding K48R XPD protein does not confer UV resistance to CHO or human cells but stimulates unscheduled DNA synthesis in human cells. **A**, immunoblot analysis using anti-XPD monoclonal antibodies 2F6. Lysates were obtained from CHO UV5 cells (lane 1), or CHO cells transfected with empty vector (lane 2), cDNA encoding wild type (WT) XPD (clone 4, lane 3), or cDNA encoding K48R XPD (clone 1, 3, and 11, lanes 4–6, respectively). **B**, UV survival analysis of UV5 CHO cells transfected with empty vector (+), cDNA encoding wild type XPD (○), or cDNA encoding K48R XPD (□, clone 1; ■, clone 3; ▲, clone 11), and the parental cell line AA8 (●). **C**, immunoblot analysis of lysates of human MRC5 immortalized wild type fibroblasts (lane 1), HD2 cells transfected with cDNA encoding wild type XPD (lane 2), or cDNA encoding K48R XPD (clone 5 and 6, lanes 3 and 4), empty vector transfected (lane 5) and untransfected HD2 cells (lane 6). **D**, UV survival analysis of HD2 human cells transfected with empty vector (+), cDNA encoding wild type XPD (○), or cDNA encoding K48R XPD (■, clone 5; □, clone 6), and a wild type cell line MRC5 (●). **E**, analysis of UDS of HD2 transfected cells. Levels of UDS are represented as values relative to those of wild type MRC5 cells. Error bars indicate the standard error of the mean.

NER at 5% or less of the normal level, below our limit of detectability. However, this is not sufficient to account for the 25–50% residual UDS seen in many XP-D cells (25, 26) nor for the ability of K48R XPD-TFIIH to increase UDS in cells (Table I and Fig. 8) and increase damage-dependent synthesis in extracts (Fig. 5). The origin of this substantial damage-dependent DNA synthesis is still unknown. It appears not to arise from normally placed NER incisions (Figs. 6 and 7), and it does not result in lesion repair that increases cellular survival (Fig. 8 and see Refs. 25 and 26). The data suggest that mutant XPD-TFIIH stimulates some type of damage-dependent nicking that is not localized at normal NER sites and does not lead to lesion removal. Further study will be necessary to understand this long standing enigma. For example, is the excessive UDS in XP-D cells dependent on other NER gene products such as XPA or XPG? Nevertheless, a new insight is obtained from the present data, which is that XPD lacking catalytic activity can directly promote such UDS.

Mechanisms of Unwinding by TFIIH—In the oligonucleotide displacement assay, no activity could be observed with TFIIH containing K48R XPD subunit. This is in agreement with the observation that the contribution of the XPB helicase is minor compared with that of XPD in this type of assay (47, 50). However, opening of a promoter region in transcription initiation is supported by the XPD mutant form of TFIIH within the limits of the assay in a fashion indistinguishable from the wild type complex. Obviously, in these two assays different activities associated with TFIIH are measured. This is also reflected in the apparent K_m for ATP in the two reactions as follows: in transcription, the K_m value for (d)ATP has been determined to be 0.25–2 μM (51–53), and the apparent K_m value for ATP in the oligo-displacement reaction is ≈ 100 -fold higher (150–200 μM) (18).² A reason for these large differences may be that in transcription TFIIH is differently positioned in the presence of other factors and opening of only 6 base pairs may already be sufficient (12). Since the XPB helicase is absolutely required for transcription *in vivo* (33, 54), it is possible that the limited

opening in transcription is predominantly due to XPB activity. The differential requirement for XPD activity in transcription and NER may originate from the fact that in the NER reaction the initial unwinding by TFIIH must be more extended, demanding both XPB and XPD activities, before other factors can participate in opening and stabilize the unwound structure.

Implications for Human Disease—Mutations in XPD can result in XP, XP/CS, and TTD syndromes, whereas mutations in XPB, which are much more infrequent, are associated with XP/CS and TTD. It has been proposed that the additional clinical manifestations seen in XP/CS and TTD are due to a defect in the transcription function of TFIIH. The fact that only very few XPB mutations are identified as compared with XPD correlates with the notion that XPB plays a more important role in transcription initiation. Likewise, the observation that some XPD mutations give rise only to XP correlates with the data presented here that the function of XPD in NER and transcription can be separated. If the enzymatic activity of XPD is indeed dispensable for transcription, then this would predict that an allele encoding the K48R mutation would result in an XP-like phenotype. No such mutation has been identified yet, although a mutation in the Walker type A domain has been described (G47R) causing an XP phenotype (55) without clear symptoms associated with a transcriptional defect. To test further the above hypothesis and to establish the significance of the XPD helicase activity for transcription *in vivo*, it would be of interest to generate a mouse model by introducing the K48R point mutation in the murine XPD gene by homologous recombination.

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REFERENCES

- Friedberg, E. C., Walker, G. C., and Siede, W. (1995) *DNA Repair and Mutagenesis*, American Society for Microbiology Press, Washington, D. C.
- Wood, R. D. (1997) *J. Biol. Chem.* **272**, 23465–23468
- de Laat, W. L., Jaspers, N. G. J., and Hoeijmakers, J. H. J. (1999) *Genes Dev.* **13**, 768–785
- Mu, D., Park, C.-H., Matsunaga, T., Hsu, D. S., Reardon, J. T., and Sancar, A. (1995) *J. Biol. Chem.* **270**, 2415–2418

² F. C. P. Holstege and H. Th. M. Timmers, unpublished results.

5. Moggs, J. G., Yarema, K. J., Essigmann, J. M., and Wood, R. D. (1996) *J. Biol. Chem.* **271**, 7177–7186
6. Sugasawa, K., Ng, J. M. Y., Masutani, C., Iwai, S., van der Spek, P. J., Eker, A. P. M., Hanaoka, F., Bootsma, D., and Hoeijmakers, J. H. J. (1998) *Mol. Cell* **2**, 223–232
7. Evans, E., Fellows, J., Coffey, A., and Wood, R. D. (1997) *EMBO J.* **16**, 625–638
8. Evans, E., Moggs, J. G., Hwang, J. R., Egly, J.-M., and Wood, R. D. (1997) *EMBO J.* **16**, 6559–6573
9. Gerard, M., Fischer, L., Moncollin, V., Chipoulet, J.-M., Chambon, P., and Egly, J.-M. (1991) *J. Biol. Chem.* **266**, 20940–20945
10. Flores, O., Lu, H., and Reinberg, D. (1992) *J. Biol. Chem.* **267**, 2786–2793
11. Orphanides, G., Lagrange, T., and Reinberg, D. (1996) *Genes Dev.* **10**, 2657–2683
12. Holstege, F. C. P., Van der Vliet, P. C., and Timmers, H. T. M. (1996) *EMBO J.* **15**, 1666–1677
13. Tantin, D., and Carey, M. (1994) *J. Biol. Chem.* **269**, 17397–17400
14. Parvin, J. D., and Sharp, P. A. (1993) *Cell* **73**, 533–540
15. Timmers, H. T. M. (1994) *EMBO J.* **13**, 391–399
16. Marinoni, J. C., Roy, R., Vermeulen, W., Miniou, P., Lutz, Y., Weeda, G., Seroz, T., Molina Gomez, D., Hoeijmakers, J. H. J., and Egly, J. M. (1997) *EMBO J.* **16**, 1093–1102
17. Svejstrup, J. Q., Vichi, P., and Egly, J. M. (1996) *Trends Biochem. Sci.* **21**, 346–350
18. Schaeffer, L., Roy, R., Humbert, S., Moncollin, V., Vermeulen, W., Hoeijmakers, J. H. J., Chambon, P., and Egly, J. (1993) *Science* **260**, 58–63
19. Schaeffer, L., Moncollin, V., Roy, R., Staub, A., Mezzina, M., Sarasin, A., Weeda, G., Hoeijmakers, J. H. J., and Egly, J. M. (1994) *EMBO J.* **13**, 2388–2392
20. Drapkin, R., Reardon, J. T., Ansari, A., Huang, J. C., Zawel, L., Ahn, K., Sancar, A., and Reinberg, D. (1994) *Nature* **368**, 769–772
21. Roy, R., Adamczewski, J. P., Seroz, T., Vermeulen, W., Tassan, J.-P., Schaeffer, L., Nigg, E. A., Hoeijmakers, J. H. J., and Egly, J.-M. (1994) *Cell* **79**, 1093–1101
22. Hoeijmakers, J. H. J. (1994) *Eur. J. Cancer* **30**, 1912–1921
23. Vermeulen, W., van Vuuren, A. J., Chipoulet, M., Schaeffer, L., Appeldoorn, E., Weeda, G., Jaspers, N. G. J., Priestley, A., Arlett, C. F., Lehmann, A. R., Stefanini, M., Mezzina, M., Sarasin, A., Bootsma, D., Egly, J.-M., and Hoeijmakers, J. H. J. (1994) *Cold Spring Harbor Symp. Quant. Biol.* **59**, 317–329
24. de Boer, J., de Wit, J., van Steeg, H., Berg, R. J. W., Morreau, H., Visser, P., Lehmann, A. R., Duran, M., Hoeijmakers, J. H. J., and Weeda, G. (1998) *Mol. Cell* **1**, 981–990
25. Johnson, R. T., and Squires, S. (1992) *Mutat. Res.* **273**, 97–118
26. Paterson, M. C., Middlestadt, M. V., MacFarlane, S. J., Gentner, N. E., Weinfeld, M., and Eker, A. P. M. (1987) *J. Cell Sci.* **6**, (suppl.) 161–176
27. Sung, P., Higgins, D., Prakash, L., and Prakash, S. (1988) *EMBO J.* **7**, 3263–3269
28. Sung, P., Guzder, S. N., Prakash, L., and Prakash, S. (1996) *J. Biol. Chem.* **271**, 10821–10826
29. Manley, J. L., Fire, A., Samuels, M., and Sharp, P. A. (1983) *Methods Enzymol.* **101**, 568–582
30. Winkler, G. S., Vermeulen, W., Coin, F., Egly, J.-M., Hoeijmakers, J. H. J., and Weeda, G. (1998) *J. Biol. Chem.* **273**, 1092–1098
31. Holstege, F. C. P., Fiedler, U., and Timmers, H. T. M. (1997) *EMBO J.* **24**, 7468–7480
32. Wood, R. D., Robins, P., and Lindahl, T. (1988) *Cell* **53**, 97–106
33. van Vuuren, A. J., Vermeulen, W., Ma, L., Weeda, G., Appeldoorn, E., Jaspers, N. G. J., van der Eb, A. J., Bootsma, D., Hoeijmakers, J. H. J., Humbert, S., Schaeffer, L., and Egly, J.-M. (1994) *EMBO J.* **13**, 1645–1653
34. Marinoni, J. C., Rossignol, M. E., and Egly, J. M. (1997) *Methods* **12**, 235–253
35. Shivji, M. K. K., Moggs, J. G., Kuraoka, I., and Wood, R. D. (1998) in *DNA Repair Protocols: Eukaryotic Systems* (Henderson, D. S., ed) Humana Press Inc., Totowa, NJ
36. Vermeulen, W., Scott, R. J., Potger, S., Muller, H. J., Cole, J., Arlett, C. F., Kleijer, W. J., Bootsma, D., Hoeijmakers, J. H. J., and Weeda, G. (1994) *Am. J. Hum. Genet.* **54**, 191–200
37. Weber, C. A., Kirchner, J. M., Salazar, E. P., and Takayama, K. (1994) *Mutat. Res.* **324**, 147–152
38. Johnson, R. T., Squires, S., Elliott, G. C., Koch, G. L. E., and Rainbow, A. J. (1985) *J. Cell Sci.* **76**, 115–133
39. Sijbers, A. M., van der Spek, P. J., Odijk, H., Van den Berg, J., Van Duin, M., Westerveld, A., Jaspers, N. G. J., Bootsma, D., and Hoeijmakers, J. H. J. (1996) *Nucleic Acids Res.* **24**, 3370–3380
40. Drapkin, R., Le Roy, G., Cho, H., Akoulitchev, S., and Reinberg, D. (1996) *Proc. Natl. Acad. Sci. U. S. A.* **93**, 6488–6493
41. Roy, R., Schaeffer, L., Humbert, S., Vermeulen, W., Weeda, G., and Egly, J.-M. (1994) *J. Biol. Chem.* **269**, 9826–9832
42. Serizawa, H., Conaway, R. C., and Conaway, J. W. (1993) *J. Biol. Chem.* **268**, 17300–17308
43. Constantinou, A., Gunz, D., Evans, E., Lalle, P., Bates, P. A., Wood, R. D., and Clarkson, S. G. (1999) *J. Biol. Chem.* **274**, 5637–5648
44. Kadkhodayan, S., Salazar, E. P., Ramsey, M. J., Takayama, K., Zdzienicka, M. Z., Tucker, J. D., and Weber, C. A. (1997) *Mutat. Res.* **385**, 47–57
45. Sung, P., Bailly, V., Weber, C., Thompson, L. H., Prakash, L., and Prakash, S. (1993) *Nature* **365**, 852–855
46. Feaver, W. J., Svejstrup, J. Q., Bardwell, L., Bardwell, A. J., Buratowski, S., Gulyas, K. D., Donahue, T. F., Friedberg, E. C., and Kornberg, R. D. (1993) *Cell* **75**, 1379–1387
47. Tirode, F., Busso, D., Coin, F., and Egly, J.-M. (1999) *Mol. Cell* **3**, 87–95
48. Guzder, S. N., Qiu, H., Sommers, C. H., Sung, P., Prakash, L., and Prakash, S. (1994) *Nature* **367**, 91–94
49. de Boer, J., Donker, I., de Wit, J., Hoeijmakers, J. H. J., and Weeda, G. (1998) *Cancer Res.* **58**, 89–94
50. Coin, F., Marinoni, J.-C., Rodolfo, C., Fribourg, S., Pedrini, M. A., and Egly, J.-M. (1998) *Nat. Genet.* **20**, 184–188
51. Conaway, R. C., and Conaway, J. W. (1988) *J. Biol. Chem.* **263**, 2962–2968
52. Dvir, A., Garrett, K. P., Chalut, C., Egly, J.-M., Conaway, J. W., and Conaway, R. C. (1996) *J. Biol. Chem.* **271**, 7245–7248
53. Holstege, F. C. P. (1996) *The Mechanism of RNA Polymerase II Promoter Opening*. Ph.D. thesis, Utrecht University, Utrecht, The Netherlands
54. Qiu, H., Park, E., Prakash, L., and Prakash, S. (1993) *Genes Dev.* **7**, 2161–2171
55. Taylor, E. M., Broughton, B. C., Botta, E., Stefanini, M., Sarasin, A., Jaspers, N. G. J., Fawcett, H., Harcourt, S. A., Arlett, C. F., and Lehmann, A. R. (1997) *Proc. Natl. Acad. Sci. U. S. A.* **94**, 8658–8663