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-photoproducts. Mutations in the DNA-dependent ATPase/helicase subunits of TFIIH, XPB and XPD, are associated with three inherited syndromes as follows: xerodermia pigmentosa 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.

Nucleotide excision repair (NER)1 is one of the most versatile DNA-damage repair mechanisms in eukaryotic cells. A wide 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 IIH (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' or 3' side, 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 xerodermia 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

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 GXXGK), 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’TACGGCAAGCGCCTATGGTTCGTGATCTCGAAGCATA-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 pSVL vector (Amersham Pharmacia Biotech), yielding plasmids pM52 and pM124, and pcDNA3 (Invitrogen), yielding plasmids pM264 and pM265. The C-termina- l terminal epo tag was added by PCR using primers p117 5’-GCGGACAAC- CAGTGCAGGCCTCG-3’ (5’ primer) and p240 5’-AGGAAATTCTCAGCTA- GCGTAGATCCTGAACGCTCTCAGACTGCTCTC-3’ (3’ primer). PCR products were cloned into SacI-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-Scal and the Scal-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 UV436.12 expressing wild type and mutant HA-tagged XPD subunit. Following preincubation for 10 min at 30 °C, nucleotides were added (60 μM ATP, 10 μM CTP, 10 μM UTP, and 120 μM 3’-OMeGTP) and included for 10 min at 30 °C. Two μl of a freshly prepared 160 μM KmO4 was added for 20 s after which reactions were stopped by addition of 2 μl of 14.4 μM β-mercaptoethanol. DNA was cleaved at modified residues by piperidine and analyzed on 7% polyacrylamide by sequencing (31). KmO4 Sensitivity Assay of Transcription Intermediates—For the KmO4 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 μM ATP, 10 μM CTP, 10 μM UTP, and 120 μM 3’-OMeGTP) were added and incubated for 10 min at 30 °C. Two μl of a freshly prepared 160 μM KmO4 was added for 20 s after which reactions were stopped by addition of 2 μl of 14.4 μM β-mercaptoethanol. DNA was cleaved at modified residues by piperidine and analyzed on 7% polyacrylamide (acylamide:bisacrylamide = 19:1), 8.3 μm urea gel electrophoresis, after inactivation of RNA polymerase II by heating for 5 min at 65 °C and alkaline phosphatase treatment(31,941),(999,996).
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-induction of Unscheduled DNA Synthesis, 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 XPD 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 XPD containing TFIIH. This protocol consists of chromatography on heparin-Ultragel and anti-HA immunoprecipitation 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 Cdk9 components (CDK7, Cyclin H, and MAT-1) were more prominent as compared 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 the wild type level. The residual activity is most likely derived only from the second DNA-dependent ATPase subunit of TFIIH, XBP. 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 XBP 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-
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, irrespec-
tive 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 re-
sidue 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.

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 μl), 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 μl) 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 μl) using a peptide containing four repeats of the conserved repeat YPERTPS 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 (○) and K48R mutant (■) 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 (○) and K48R
mutant (■) XPD. E, quantitation of the CTD kinase activity in arbitrary units (A.U.) in the presence of TFIIH containing wild type (○) and K48R
mutant (■) XPD.

Requirement for XPD Helicase in DNA Repair and Transcription—To characterize further the possible effect of the K48R mu-
tation 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 mul-
tiple 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 presen-
tce of other basal transcription factors, RNA polymerase II,
and the appropriate nucleotides, and we analyzed the forma-
tion of tri- and dinucleotides with AdML + 3G template, Fig. 3C, lanes 1–8) and 15-nucleotide products (AdML + 15G template, Fig.
3c, lanes 9–16). As is evident from Fig. 3C, 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 agree-
mement 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 signif-
ificant difference is observed in the formation of tri- and dinucle-
otides 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-

A
wild type
K48R
ATPase

B
- wild type - K48R
Helicase

C
wild type K48R
Kinase

D

E

CTD kinase (A.U.)
regions with permanganate. Basal transcription factors, wild type or K48R mutant TFIIH, 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). 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 TFIIH 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 TFIIH were used (data not shown). Together, these comparative analyses between wild type and mutant TFIIH suggest that the XPD helicase activity is dispensable for transcription initiation by RNA polymerase II in vitro.

TFIIH 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 TFIIH 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 TFIIH 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 TFIIH are able to stimulate DNA repair synthesis. Surprisingly, different preparations of TFIIH 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 TFIIH. 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 TFIIH with K48R Mutant XPD—By using a DNA substrate containing a single 1,3-(GpTpG) intranstrand cisplatin cross-link, the formation of dual incisions around a

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**Fig. 3.** TFIIH containing inactive XPD DNA helicase allows transcription initiation by RNA polymerase II similar to wild type TFIIH. A, reconstituted transcription reactions. Reactions contained purified recombinant basal transcription factors TBP, TFIIA, TFIIH, TFIIF, and purified RNA polymerase II (lanes 1–7), wild type (1, 3, and 5 µl, lanes 2–4, respectively), or K48R XPD complex (1, 3, and 5 µl, 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 TFIIH activity. Transcription reactions contained TFIIH (3 µl; wild type, lanes 1–6, or K48R, lanes 6–13) 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 µl of TFIIH; 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).

**Fig. 4.** KMnO₄ 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 TFIIH (5 µl) 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) TFIIH 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.
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 XP-D 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 XP-D-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 XP-D 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 XP-D 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 efficiency of both incisions around a lesion depend on ATP hydrolysis and the DNA helicase activity of the XP-D subunit of TFIIH.

K48R XP-D 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 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 XP6B8 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 XP-D-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 lysates 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
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 compared 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 TFI IH 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) TFI IH 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 TFI IH 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 TFI IH (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 TFI IH 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 TFI IH 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-TFI IH can still support...
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 XPD 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 $\mu$m (51–53), and the apparent $K_m$ value for ATP in the oligo-displacement reaction is $\sim$100-fold higher (150–200 $\mu$m (18)). 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 XPD helicase is absolutely required for transcription in vivo (33, 54), it is possible that the limited opening in transcription is predominantly due to XPD 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 XPD and XPB 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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