THE JOURNAL OF BIOLOGICAL CHEMISTRY
Vol. 273, No. 2, Issue of January 9, pp. 1092–1098, 1998
Printed in U.S.A.

Affinity Purification of Human DNA Repair/Transcription Factor TFIIH Using Epitope-tagged Xeroderma Pigmentosum B Protein*

(Received for publication, August 25, 1997, and in revised form, October 29, 1997)

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TFIIH is a high molecular weight complex with a remarkable dual function in nucleotide excision repair and initiation of RNA polymerase II transcription. Mutations in the largest subunits, the XPB and XPD helicases, are associated with three inherited disorders: xeroderma pigmentosum, Cockayne's syndrome, and trichothiodystrophy. To facilitate the purification and biochemical characterization of this intricate complex, we generated a cell line stably expressing tagged XPB, allowing the immunopurification of the XPB protein and associated factors. Addition of two tags, a N-terminal hexameric histidine stretch and a C-terminal hemagglutinin epitope, to this highly conserved protein did not interfere with its functioning in repair and transcription. The hemagglutinin epitope allowed efficient TFIIH immunopurification to homogeneity from a fractionated whole cell extract in essentially one step. We conclude that the predominant active form of TFIIH is composed of nine subunits and that there is one molecule of XPB per TFIIH complex. The affinity-purified complex exhibits all expected TFIIH activities: DNA-dependent ATPase, helicase, C-terminal domain kinase, and participation in *in vitro* and *in vivo* nucleotide excision repair and *in vitro* transcription. The affinity purification procedure described here is fast and simple, does not require extensive chromatographic procedures, and yields highly purified, active TFIIH.

Nucleotide excision repair (NER)[1] is a versatile DNA repair mechanism that removes a wide variety of lesions, such as UV-induced lesions and numerous chemical adducts (1, 2). The principal steps in the reaction mechanism of NER are recognition and demarcation of the lesion, probably involving chromatin remodelling and local helix opening, incision of the DNA on both sides of the lesion at some distance, removal of the damaged oligonucleotide, and, finally, repair DNA synthesis and ligation. In eukaryotes, this reaction requires about 30 polypeptides and has been reconstituted with purified components, including XPA, XPC/HHR23B, replication protein A, the structure-specific nucleases ERCC1/XPF and XPG, and the multisubunit basal transcription factor TFIIH (3–5). At least two subpathways can be discerned in the NER system. One of these, transcription-coupled repair, preferentially removes DNA damage from the transcribed strand of active genes, whereas lesions in the rest of the genome are repaired more slowly and less efficiently by the global genome repair pathway. TFIIH appears to be a core component of both excision subpathways.

Mutations in the two largest subunits of TFIIH, the XPB and XPD helicases, are associated with the rare genetically heterogeneous disorders xeroderma pigmentosum (XP), Cockayne's syndrome (CS), and trichothiodystrophy (TTD) (6, 7). Many complementation groups and considerable overlap have been established for these syndromes: seven complementation groups in XP (XP-A–XP-G), three of which include patients with combined XP and CS phenotypes (XP-B, XP-D and XP-G), two in the classical form of CS (CS-A and CS-B), and three in TTD (XP-B, XP-D, and TTD-A). The discovery of the dual function of XPB and XPD in both NER and transcription provides a rationale for the complex clinical features that are specifically associated with inherited defects in TFIIH subunits, such as seen in the combined XP/CS and the photosensitive form of TTD, that were difficult to explain solely on the basis of a NER defect. Thus, it was proposed that typical XP characteristics, such as UV-induced cutaneous abnormalities and predisposition to skin cancer, are due to inactivation of the NER function of TFIIH, whereas features typical for CS and/or TTD, such as neurodysmyelination, brittle hair, and growth defects, are due to a deficiency in the transcription function of TFIIH, possibly affecting only a subset of genes (8).

In addition to XPB and XPD, which exhibit DNA-dependent ATPase activities and are 3′-5′ and 5′-3′ DNA helicases, respectively (9, 10), seven more TFIIH subunits have been identified to date. CDK7 was identified as the catalytic subunit of the kinase activity of TFIIH that is able to phosphorylate the C-terminal domain (CTD) of the largest subunit of RNA polymerase II (11). Interestingly, CDK7 also constitutes a separate trimeric kinase complex that is possibly involved in cell cycle regulation together with the cyclin H and MAT1 subunits of TFIIH (12, 13). Furthermore, p44, the human homologue of yeast SSL1, and p34 contain zinc finger domains and possess putative DNA binding capacity (14). So far, no activity has been detected for the p62 and p52 subunits (15, 16). Whether these nine proteins constitute the TFIIH complex and whether the composition of TFIIH differs during NER and transcription initiation are yet unresolved issues.

The presence of two DNA helicases has implicated TFIIH in

* This work was supported in part by grants from the Netherlands Scientific Organization Section Medical Sciences (Project 901-01-151), the Dutch Cancer Society (Grant EUR-94–763), the Human Frontiers Science Program, INSERM, and CNRS. The research of G. W. was supported by a fellowship of the Royal Netherlands Academy of Arts and Sciences. The costs of publication of this article were defrayed in part by the payment of page charges. This article must therefore be hereby marked "*advertisement*" in accordance with 18 U.S.C. Section 1734 solely to indicate this fact.

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[1] The abbreviations used are: NER, nucleotide excision repair; XP, xeroderma pigmentosum; CS, Cockayne's syndrome; TTD, trichothiodystrophy; CTD, C-terminal domain; WCE, whole cell extract; HA, hemagglutinin; dtXPB, double-tagged XPB.

a helix-opening step during both transcription initiation and NER. It has been shown that such open-complex formation at the transcription start site depends on TFIIH and that the requirement for TFIIH is dependent on promoter topology and can be alleviated by premelted regions at the transcription start site (17–19). During NER, TFIIH is thought to convert a recognized damaged site into a substrate for the XPG and XPF/ERCC1 structure-specific nucleases by locally opening DNA around a lesion. The formation of an opened DNA conformation around a recognized lesion has been demonstrated; however, the direct involvement of TFIIH in this step has not been shown (20). Answers to these questions are hampered by the difficulty in obtaining large quantities of highly purified TFIIH. Therefore, we developed a procedure that facilitates the isolation of TFIIH using a human cell line expressing functional XPB provided with two tags. The affinity purification procedure described here is fast and simple, does not require extensive chromatographic procedures, and yields highly purified, active TFIIH.

EXPERIMENTAL PROCEDURES

General—Purification of nucleic acids, restriction enzyme digestion, gel electrophoresis of nucleic acids and proteins, immunoblotting, detection of proteins and nucleic acids were performed according to standard procedures (21).

Oligonucleotides and Plasmid DNA Construction—The coding sequence for the C-terminal HA epitope tag was added via PCR using oligonucleotide primer pairs p90 5′-CCCGGATCCTCAGCTAGCGTAATCTGGAACATCGTATGGGTATTTCCTAAAGCGCTTGAAG-3′ (3′ primer; underlined sequence encodes the HA epitope, and double underlined sequence indicates a *Bam*HI restriction site) and p33 5′-GGATCCACCATGGGCAAAAGAGACCG-3′ (5′ primer). Likewise, the hexahistidine tag was added using oligonucleotides p123 5′-CGCGCGGAATTCATGGGCAGCAGCCATCATCATCATCATCACAGCAGCGGCCTGGTGCCGCGCGGCAGCCATATGGGCAAAAGAGACCG-3′ (5′ primer; underlined sequence encodes a hexahistidine stretch and thrombin cleavage site, and double underlined sequence indicates an *Eco*RI site) and p41 5′-CGGGAAGTGGAGGGCCCACC-3′ (3′ primer). PCR fragments were cloned, sequenced, and confirmed to be free of PCR-introduced sequence errors. Full-length *XPB* and double-tagged *XPB* cDNA (*dtXPB* cDNA) were subcloned as *Eco*RI-*Bam*HI fragments in a modified pSG5 eukaryotic expression vector yielding plasmids pSHE3 and pSHE3HA, respectively. From pSHE3HA, the *dtXPB* cDNA was subcloned as a *Eco*RI-*Xho*I fragment in the eukaryotic expression vector pcDNA3 (Invitrogen) yielding plasmid pM300.

DNA Transfection and UV Survival Assay—Cells were cultured in a 1:1 mixture of Dulbecco's modified Eagle's medium and Ham's F-10 medium supplemented with 10% fetal calf serum and antibiotics. HeLa TK^- cells were transfected with vector pM300 by electroporation, and after selection with 1.0 mg/ml G418, individual clones were selected for expression of *dtXPB* by immunoblot analysis using anti-XPB antibodies.

Cell lines XP-wt and XP-t3 were generated by transfection of XPCS2BASV (XP-B) SV40-immortalized fibroblasts with *XPB* and *dtXPB* cDNA, respectively. Expression vector pSHE3 or pSHE3HA and a *neomycin*-selectable marker, were cotransfected using Lipofectin reagent essentially as described by the manufacturer (Life Technologies, Inc.). Stably expressing mass populations were obtained by selection with 300 μg/ml G418 and repeated UV irradiation at 1 day intervals (three doses of 4 J/m^2).

UV survival was assayed by [*methyl*-^{3}H]thymidine incorporation 3 days after irradiation as described (22). Proliferating cells were pulse-labeled with [*methyl*-^{3}H]thymidine (3 h) and then chased (1 h). Cell lysates were transferred to scintillation-counting vials, and survival was calculated as the average ratio of incorporated radiolabel in treated triplicates to that in four untreated control dishes.

Immunopurification of dtXPB Protein and Associated Factors—XP-t3 cells were cultured in suspension in RPMI 1640 medium supplemented with 10% fetal calf serum and antibiotics. Whole cell extracts (WCEs) (total, 530 mg) were prepared from frozen cell pellets (total, 50 ml packed cell volume) as described (23). Subsequently, WCEs were fractionated on heparin-Ultrogel (IBF, France) equilibrated in Buffer A (10 mM Tris-HCl, pH 7.8, 17% glycerol (v/v), 0.5 mM dithiothreitol, 5 mM $MgCl_2$) containing 0.1 M KCl and eluted with Buffer A containing 0.22, 0.4, and 1.0 M KCl as described (24). All of the dtXPB protein was present in the 0.4 M KCl fraction (designated Hep0.4; 175 mg of protein) as judged by immunoblot analysis similar to TFIIH. Typically, 10–12 ml (7.5–9.0 mg of protein) of the Hep0.4 fraction was successively incubated with 400 μg of purified 12CA5 anti-HA monoclonal antibody bound to 400 μl of protein G-Sepharose (Pharmacia Biotech Inc.) overnight at 4 °C. The resin was washed three times with 10 volumes of ice-cold buffer T (25 mM Tris-HCl, pH 7.9, 17% glycerol (v/v), 0.5 mM EDTA, 0.2 mM dithiothreitol, 5 mM $MgCl_2$) containing 0.4 M KCl and 0.1% Nonidet P-40 and twice with buffer T/0.1 M KCl containing 0.01% Nonidet P-40. Bound material was eluted for 1 h at 30 °C in 400 μl of buffer T/0.1 M KCl containing 0.01% Nonidet P-40, 0.2 mg/ml insulin, 2.0 mg/ml synthetic peptide corresponding to the HA epitope (sequence YPYDVPDYA), and 1.0 μg/ml aprotinin. This step was repeated once or twice. Routinely, nearly all dtXPB and associated factors were present in the first eluate as detected by immunoblotting. Purified proteins were stored at −80 °C.

The same procedure was used for immunoprecipitation of dtXPB directly from a WCE (15 mg) to analyze protein-protein interactions. However, in this case, bound material was washed five times with buffer containing 0.1 M KCl, 0.1% Nonidet P-40 and analyzed by immunoblotting after overnight elution at 4 °C with buffer containing 1.0 mg/ml peptide and 0.1 mg/ml bovine serum albumin (Sigma).

Microneedle Injection—Prior to microinjection, human primary fibroblasts were fused by inactivated Sendai virus as described earlier (8). Protein fractions were microinjected into the cytoplasm of XP polykaryons, and NER activity was measured by pulse labeling with [*methyl*-^{3}H]thymidine and *in situ* autoradiography as described (8). Repair activity was quantified by counting autoradiographic grains above at least 50 non-S phase nuclei. Primary cell lines used were XPCS1BA (XP-B), XP6BE (XP-D), XP126LO (XP-F), XP3BR (XP-G), TTD1BR (TTD-A), and C5RO (wild type).

In Vitro NER Assay—WCEs were prepared from repair-proficient HeLa and XP-t3 cells and repair-deficient SV40-immortalized XPCS2BASV (XP-B) cells (23). NER reactions (50 μl) contained 250 ng of plasmid DNA randomly damaged with *N*-acetoxy-2-acetylaminofluorene and as an internal control an equal amount of undamaged control plasmid of a different size, the indicated amount of extract, and purified proteins; the reactions were incubated for 3 h at 30 °C. DNA was purified, linearized with *Bam*HI, and analyzed on a 0.8% agarose gel (25, 26). Antibody depletion of extracts was performed as follows: for each reaction, 100 μg of WCE was incubated with 0.5 μl of anti-p62 ascites and 5 μl of protein G-agarose overnight at 4 °C. For the experiment shown in Fig. 6*A*, plasmid DNA was randomly damaged with *cis*-diaminedichloro-platinum(II) (16).

In Vitro Transcription Assay—Purified TFIIH was incubated with recombinant human TBP, TFIIB, and TFIIE and purified TFIIA, TFIIF, and RNA polymerase II as described earlier (24). After 15 min of preincubation at 25 °C with 70 ng of linearized template DNA containing the adenovirus 2 major late promoter, nucleotides were added, and transcription was allowed to proceed in a final reaction volume of 25 μl for 45 min at 25 °C. The 309-nucleotide runoff transcripts were analyzed by electrophoresis through a 5% acrylamide/50% urea gel.

Enzymatic Assays—ATPase reactions contained 20 mM Tris-HCl, pH 7.9, 4 mM $MgCl_2$, 1 mM dithiothreitol, 50 μg/ml bovine serum albumin, 150 ng of DNA and were performed essentially as described before (27). After 30 min of incubation at 37 °C, 25-μl reactions were stopped by adding 2 μl of 0.5 M EDTA and 25 μl of TE (10 mM Tris-HCl, pH 8.0, 0.1 mM EDTA). Of each reaction, 1 μl was analyzed by thin-layer chromatography using polyethylenimine-cellulose plates (Merck) run in 0.75 M KH_2PO_4. CTD kinase assays (20 μl) containing 20 mM Hepes-KOH, pH 7.9, 20 mM Tris-HCl, pH 7.9, 7 mM $MgCl_2$, 0.5 μg/ml bovine serum albumin, and 30 mM KCl were performed with 10 μg of a synthetic tetrapeptide of YSPTSPS as a substrate as detailed before (27). DNA helicase probes were prepared as described (27), and reactions (25 μl) contained 20 mM Tris-HCl, pH 7.9, 4 mM $MgCl_2$, 1 mM dithiothreitol, 50 μg/ml bovine serum albumin, 2 mM ATP, and 1 ng of DNA substrate and were incubated at 37 °C for 45 min. Displacement of the 24-mer oligonucleotide from M13mp18 single-stranded DNA was analyzed by 10% nondenaturing polyacrylamide gel electrophoresis and autoradiography (27).

Antibodies—The monoclonal antibodies recognizing TFIIH subunits were all described before (10, 12, 14–16). Monoclonal antibodies recognizing the HA epitope (28) were purified from 12CA5 hybridoma tissue culture supernatant by affinity chromatography on protein G-agarose according to established protocols (29).

RESULTS

Generation of a Cell Line Expressing Tagged XPB—To analyze interactions of the XPB protein with other proteins, including TFIIH under physiological conditions, and to facilitate the purification of active TFIIH, we decided to generate a human cell line stably expressing a tagged version of *XPB* (*dtXPB*) cDNA. To permit isolation of full-length XPB protein and allow purification of XPB on the basis of different reversible affinity purification steps, we chose to add two different types of tags, one on each end of the protein. Thus, coding sequences for a N-terminal hexahistidine stretch followed by a thrombin cleavage site and a C-terminal HA epitope tag (28, 30) were added to *XPB* cDNA fragments, and a full-length double-tagged *XPB* cDNA was constructed (Fig. 1*A*) and subcloned in eukaryotic expression vectors. To obtain cell lines stably expressing *dtXPB*, the cDNA vectors were transfected to two human cell lines. First, *dtXPB* cDNA and a *neomycin*-selectable marker were transfected into HeLa cells. After selection with G418, individual HeLa clones were obtained and analyzed by immunoblotting for the level of the dtXPB protein using anti-XPB antibodies. As shown in Fig. 1*B, lanes 1–5*, the double-tagged XPB can be conveniently discerned from the endogenous wild type XPB protein because of its increased size (the predicted molecular mass increases from 89,279 Da to 92,690 Da). In the 48 clones analyzed, various levels of dtXPB protein were detected in WCEs, with many clones expressing no or hardly detectable *dtXPB*, despite the fact that the *dtXPB* cDNA was under the control of the strong cytomegalovirus promoter, and multiple copies are expected to be integrated in the genome. Interestingly, in neither case did we observe a large overexpression, and clones expressing the highest level of *dtXPB* clearly showed decreased levels of the wild type endogenous protein as compared with the p62 core subunit of TFIIH (*e.g.* Fig. 1*B*, compare *clone 1-19* with *clones 1-6, 1-2*, and *1-7*). These findings suggest that the cellular content of XPB is kept within narrow concentration ranges by degrading excess protein and that there is competition between the endogenous wild type and exogenous tagged protein. Secondly, XP-B UV-sensitive cells were transfected, and after selection with G418 and repeated UV irradiation, a stably expressing mass population was established, designated XP-t3. As with the HeLa transfectants, immunoblot analysis of XP-t3 cell extracts indicated that dtXPB protein levels were comparable with the endogenous (mutant) XPB levels (Fig. 1*B, lanes 6–8*). Because the relatively high dtXPB protein levels in HeLa clone 1-19 varied during culturing and appeared to be more stable in the transfected XP-B cells, the XP-t3 cell line was further characterized and used for all experiments described here.

To characterize the functionality of the dtXPB protein *in vivo*, an UV survival experiment was carried out. Fig. 1*C* shows that *dtXPB* is able to reverse the UV sensitivity of XP-B cells to the same extent as wild type *XPB* cDNA, although both not to the same level as the wild type MRC5 transformed cell line used as a repair-proficient control. The NER activity was further analyzed using an *in vitro* assay. WCEs were prepared and incubated in the presence of both damaged and undamaged plasmid DNA and labeled nucleotides. The resulting incorporation of labeled nucleotides in the damaged plasmid DNA is due to repair DNA synthesis and a measure for NER activity. Fig. 1*D* shows that extracts prepared from XP-t3 cells were able to repair *N*-acetoxy-2-acetylaminofluorene-damaged DNA *in vitro*, whereas extracts prepared from XP-B cells display a strongly reduced repair activity. The above findings indicate that the dtXPB protein is functional in NER, implying that it most likely is incorporated in the TFIIH complex. Finally, the fact that these cells grow normally strongly suggests that the dtXPB protein is not interfering with the basal transcription initiation function of the complex.

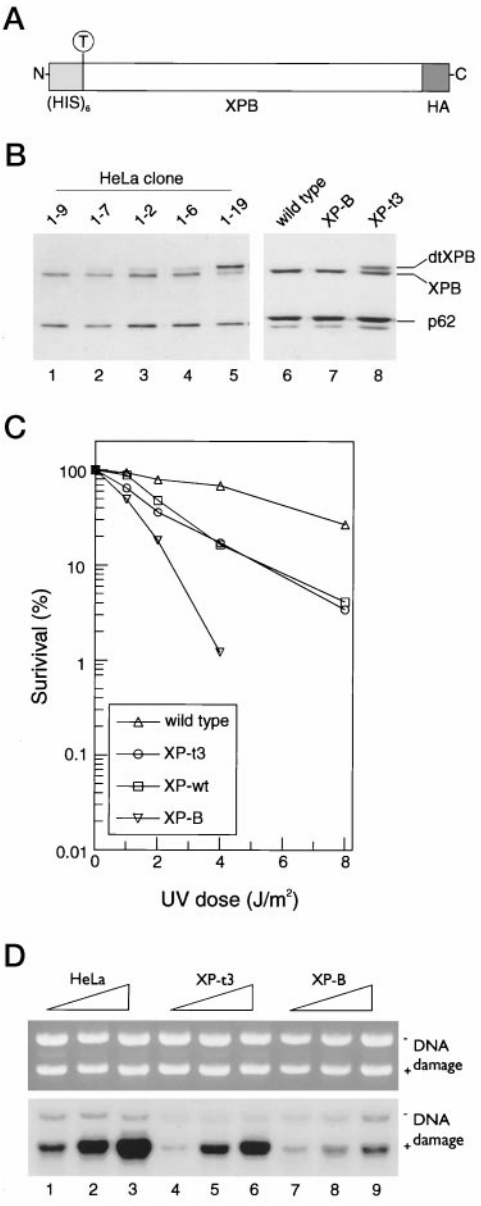

FIG. 1. **Analysis of the XP-t3 cell line expressing *dtXPB*.** *A,* schematic representation of the dtXPB protein. Indicated are the hexahistidine stretch ($(HIS)_6$), the thrombin cleavage site (*T*), and the HA epitope tag (*HA*). *B,* immunoblot analysis of cloned HeLa transfectants and the XP-t3 cell line containing varying levels of dtXPB protein. Due to the increased size of the double-tagged XPB, the protein migrates at a higher molecular mass. *Lanes 1–5*, HeLa clones containing varying levels of dtXPB protein; *lane 6*, wild type immortalized fibroblast (VH10); *lane 7*, XP-B immortalized fibroblast (XPCS2BASV); *lane 8*, XP-t3 transfected XP-B (XPCS2BASV) fibroblast. Indicated are the endogenous and tagged XPB proteins and the p62 subunit, used as internal reference for the amount of TFIIH in each lane. The p62 subunit appears in *lanes 6–8* as a doublet due to posttranslational modifications (T. Seroz, J. Auriol, and J.-M. Egly, unpublished results). *C,* dtXPB is functional in DNA repair. UV survival of XPCS2BASV (XP-B) fibroblasts and transfectants expressing tagged and wild type *XPB* cDNA (*XP-t3* and *XP-wt*, respectively). As a wild type control, MRC5 immortalized fibroblasts were used. *D, in vitro* NER activity measured in WCEs from HeLa (*lanes 1–3*), XP-t3 (*lanes 4–6*), and XP-B (*lanes 7–9*) cells using *N*-acetoxy-2-acetylaminofluorene-damaged plasmid DNA as substrate. Of each WCE, 50, 100, and 150 μg was used, respectively. *Upper panel*, ethidium bromide-stained gel of the linearized damaged and undamaged plasmids; *lower panel*, corresponding autoradiogram.

Analysis of TFIIH Factors Associated with dtXPB Pro-

tein—To identify proteins interacting with XPB, an immunoprecipitation experiment was carried out under physiological salt conditions (0.1 M KCl) using a repair- and transcription-competent XP-t3 WCE. As shown in Fig. 2, the dtXPB protein could be specifically and quantitatively immunoprecipitated using anti-HA monoclonal antibodies, and in addition to dtXPB, we could detect several TFIIH subunits in the bound fraction (XPD, p62, CDK7, and cyclin H). This confirms that dtXPB was incorporated in TFIIH. Furthermore, the fact that the relative intensities of XPB *versus* p62 are not significantly altered in the load, unbound, and the bound fractions indicates that the established XP-t3 cells harbor dtXPB in the majority of the TFIIH complexes. Interestingly, although all dtXPB protein was depleted from the WCE, none of the endogenous (mutant) XPB was detected in the bound fraction. This demonstrates that only one XPB subunit is present per complex: if the complex contained more than one XPB molecule, complexes with both the endogenous (mutant) subunit and the tagged protein would be expected to be present in the bound material.

Immunopurification of XPB and Associated Factors—Because a number of TFIIH subunits specifically co-immunoprecipitated with dtXPB protein using anti-HA antibodies, we set out to purify TFIIH on this basis and analyze the composition of the TFIIH complex (Fig. 3*A*). First, WCEs from XP-t3 cells were fractionated on heparin-Ultrogel as described (24). All dtXPB protein, as well as XPB and other known TFIIH subunits, were present in the 0.4 M KCl fraction, designated Hep0.4. Portions of this fraction were directly incubated without further fractionation with anti-HA resin to purify dtXPB and associated proteins. After incubation, the anti-HA resin was extensively washed, and bound material was eluted by competition with excess HA peptide. Subsequently, the composition of the eluate was analyzed by SDS-polyacrylamide gel electrophoresis and staining with silver nitrate. In addition to dtXPB, we identified only eight polypeptides ranging in molecular mass from 80 to 34 kDa that specifically and consistently immunoprecipitated with dtXPB and are thus XPB-associated factors (Fig. 3*B*). These associated proteins were all identified as known TFIIH subunits by two criteria: (i) reactivity with monoclonal antibodies specifically recognizing TFIIH subunits; and (ii) exact co-migration with known TFIIH subunits in SDS-polyacrylamide gel electrophoresis (data not shown). Finally, the staining intensity of the dtXPB subunit, compared with the other subunits, suggests that the dtXPB protein was predominantly present in stoichiometric amounts and not in a free form.

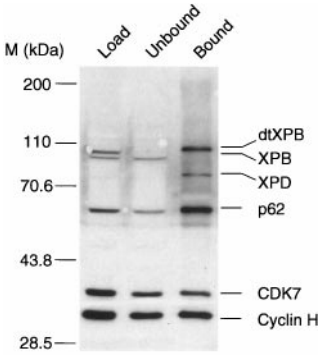

FIG. 2. **Immunoblot analysis of TFIIH factors associated with dtXPB in XP-t3 WCE using HA-antibody immunoprecipitation; dtXPB is incorporated in the TFIIH complex.** Indicated are the *load* (WCE), *unbound* (supernatant), and *bound* material (eluted with excess HA peptide). Because the amount of TFIIH was smaller in the lanes containing the load and unbound material, XPD appears as a very weak band in these two lanes as compared with the lane containing the bound fraction. The positions of the molecular weight markers used and the proteins detected by immunoblotting are indicated.

Presence of Additional NER and Transcription Factors in the Affinity-purified TFIIH Fraction—A number of NER and basal transcription factors (TFIIE, CSA, CSB, and XPG, among others) have been described to interact with the TFIIH complex, either as part of a RNA polymerase II holoenzyme (31, 32) or using isolated proteins (33, 34). Therefore, it was unexpected to find only nine polypeptides stained by silver nitrate in the immunopurified fraction. The silver-stained protein profile of affinity-purified TFIIH (Fig. 3*B*) does not exclude the possibility that substoichiometric amounts of other NER or transcription factors are present. Furthermore, the heparin fractionation might have disrupted salt-sensitive interactions. Therefore, the TFIIH-containing fraction that was immunoprecipitated directly from a WCE using low salt conditions was tested by immunoblot analysis for the presence of additional NER and transcription factors (Fig. 4). However, we were not able to detect the presence of any of the NER factors ERCC1, XPC, HHR23B, XPG, and CSB, RNA polymerase II, or any significant levels of the basal transcription factors tested. The absence of a stable interaction with CSB was confirmed by the reverse experiment, in which tagged CSB was immunoprecipitated and analyzed for the presence of TFIIH subunits (46). As a positive control, we detected the presence of the human homologue of yeast SUG1, a protein that we recently identified to interact with the XPB subunit of TFIIH (35). Notably, human SUG1 was below immunodetection level in the immunopurified fraction shown in Fig. 3*B*, indicating that this interaction was salt-sensitive under the conditions used. Similar results were obtained using fractionation of the WCE on a Ni-NTA column that has a high affinity for the hexahistidine stretch or by using a nuclear extract preparation (data not shown). The above results indicate that the interactions of TFIIH with the various NER and transcription factors are, at least under the various conditions we used, not stable.

Functional Characterization of Affinity-purified TFIIH—To determine whether the nine polypeptides identified in the silver-stained gel represent the active form of TFIIH, we tested whether the enzymatic DNA-dependent ATPase, DNA helicase, and CTD kinase activities that are associated with TFIIH were present in our purified preparations (27, 36). As shown in

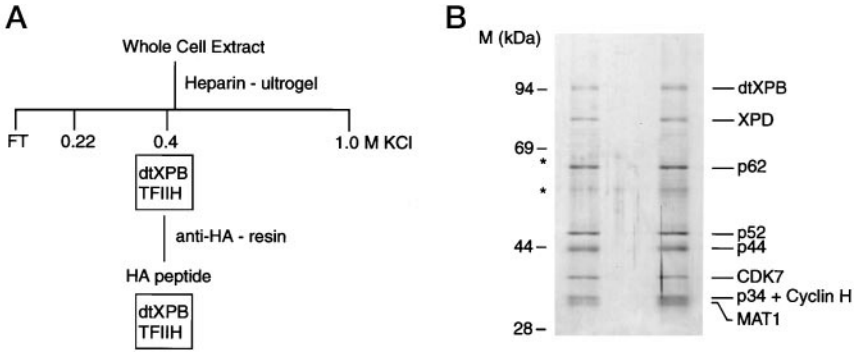

FIG. 3. **Immunopurification of dtXPB protein and associated factors.** *A,* schematic representation of the purification strategy. *B,* analysis of two independent anti-HA eluate preparations (10 μl) by 11% SDS-polyacrylamide gel electrophoresis stained with silver nitrate. The bands marked with asterisks (*) are also present in the empty lane and represent staining artifacts.

Fig. 5*A*, the ATPase activity detected was dependent on the presence of DNA and strongly stimulated by either circular M13 single-stranded or double-stranded supercoiled plasmid DNA. In addition, the DNA helicase and CTD kinase activities were readily detected (Fig. 5, *B* and *C*). In contrast, we were not able to detect any DNA nicking or exonuclease activity in the anti-HA fraction (data not shown).

Next, we tested the transcriptional activity of the anti-HA eluate (Fig. 5*D*). Addition of the purified complex was absolutely required in a fully defined reconstituted RNA polymerase II transcriptional reaction using the adenovirus 2 major late promoter. The anti-HA eluate contained only detectable TFIIH activity because omitting either TBP, TFIIB, TFIIE, or TFIIF abolished transcription completely, whereas omitting TFIIA resulted in a strongly decreased signal, in agreement with a stimulatory role for TFIIA in defined transcription reactions (Fig. 5*D*). These findings indicate the absence of detectable contaminating transcriptional activities in the TFIIH preparation. A quantitation of the enzymatic and transcriptional assays is presented in Fig. 5, *E* and *F*.

Finally, we characterized the activity of the affinity-purified TFIIH in *in vitro* NER assays. As shown in Fig. 6*A*, the affinity-purified complex was able to complement the NER defect in XP-B extracts. Quantitation of the incorporated labeled nucleotides in the damaged DNA allowed us to estimate the yield of active TFIIH, which was calculated to be ~12% (Table I), indicating that the anti-HA elution is rather efficient. Theoretically, the *in vitro* complementation could also be due to exchange of XPB with other TFIIH subunits. Therefore, we performed an antibody depletion experiment. Using anti-p62 antibodies, a HeLa WCE was depleted of TFIIH, which resulted in a co-depletion of NER activity (Fig. 6*B*, compare *lane 1* with *lanes 6* and *7*). As shown (Fig. 6*B, lanes 2–5*), the anti-HA eluate restored NER activity in the depleted HeLa WCE up to the level of the nondepleted extract, indicating that TFIIH complex formation involving XPB and p62 is essential for NER activity (25).

To analyze in more detail the NER activities of the purified TFIIH *in vivo*, a microneedle injection experiment was carried out. As anticipated, a strong stimulation of unscheduled DNA synthesis was readily observed directly after microinjection in XP-B and XP-D polykaryons, which was specific because it was not seen in XP-F and XP-G cells (Fig. 7, *A* and *C*). Interestingly, correction was also observed in TTD-A cells (Fig. 7, *B* and *C*) that contain a mutation in an as yet unidentified NER factor (37), in agreement with our earlier findings using highly purified TFIIH from HeLa cells by classical purification (8). These experiments indicate that TTDA either is an intrinsic component of TFIIH or is required for a modification of TFIIH, enabling it to function in repair (see "Discussion").

In conclusion, these experiments show that the immunopurification of dtXPB and associated proteins result in the rapid and efficient purification of TFIIH, which is active in NER and transcription.

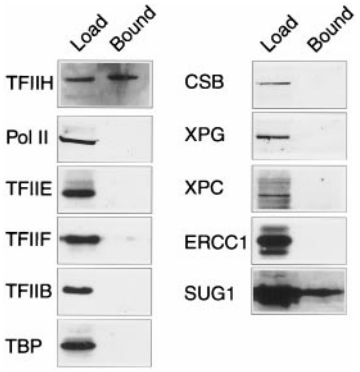

FIG. 4. **Association of TFIIH with NER and transcription factors.** None of the tested NER and/or basal transcription factors showed detectable association with TFIIH immunoprecipitated using HA-antibodies. As a positive control, human SUG1 was identified in the bound fraction. Antibodies used to identify multisubunit complexes recognized the p62 subunit of TFIIH, the RPB1 subunit of RNA polymerase II, the 34-kDa β-subunit of TFIIE, and the RAP74 subunit of TFIIF. Indicated are the *load* (WCE) and the *bound* fraction (eluted with excess HA peptide).

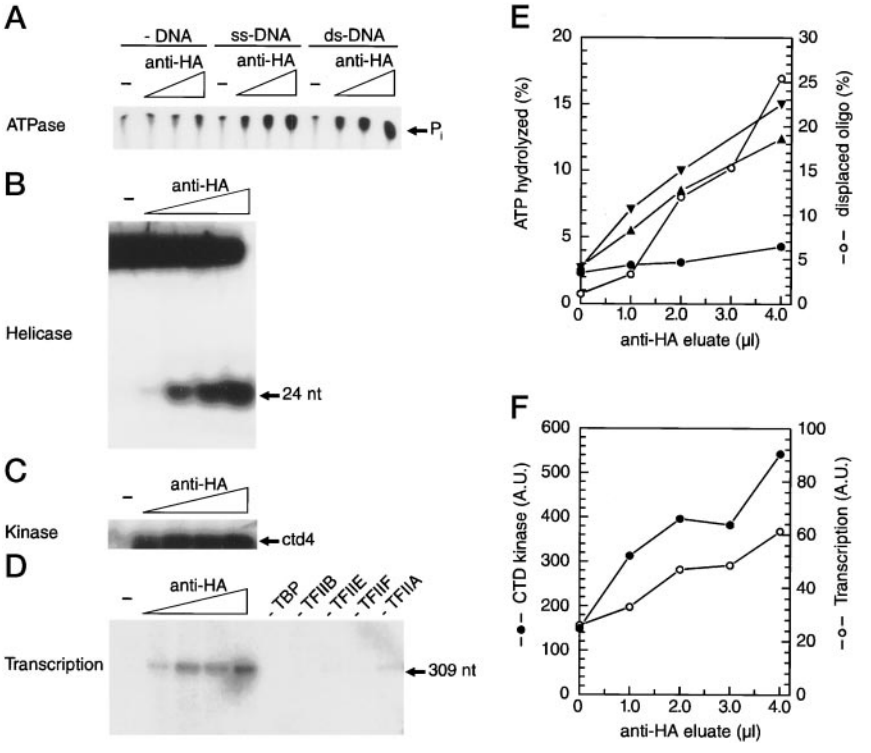

FIG. 5. **Analysis of TFIIH enzymatic and transcriptional activities in anti-HA eluates.** *A,* ATPase activity is strongly stimulated by single-stranded DNA (*ss-DNA*, M13mp18, 150 ng) or double-stranded DNA (*ds-DNA*, plasmid DNA, 150 ng). Increasing amounts of anti-HA affinity-purified TFIIH were used to measure ATPase activity (1, 2, and 4 μl). *B,* DNA helicase activity detected by displacement of a 24-mer oligonucleotide from M13mp18 single-stranded DNA. Indicated are increasing amounts of anti-HA eluate (1, 2, 3, and 4 μl). *C,* CTD kinase activity by increasing quantities of anti-HA eluate (1, 2, 3, and 4 μl). *D,* TFIIH transcriptional activity is present in anti-HA eluate. Indicated are increasing amounts of anti-HA eluate (0, 1, 2, 3, and 4 μl) added to a complete reaction or individual transcription factors omitted from the reaction containing anti-HA eluate (4 μl). *E,* quantitation of the ATPase and DNA helicase activities. The ATPase activity is represented by the percentage of ATP hydrolyzed in the absence of DNA (●), and in the presence of single-stranded DNA (▼) and double-stranded DNA (▲). The helicase activity is depicted by the percentage oligonucleotide displaced from the single-stranded DNA (○). *F,* quantitation of the CTD kinase (●) and transcriptional activities (○). Both the CTD kinase and transcription are represented in arbitrary units (*A.U.*).

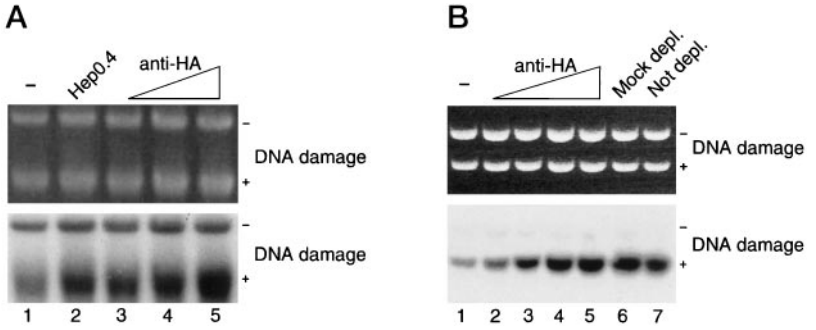

FIG. 6. **NER activity of affinity-purified TFIIH *in vitro*.** *A, in vitro* complementation of XP-B NER-deficient WCE (150 μg) by fractions from the XPB purification (*lane 1*, XP-B extract alone; *lane 2*, XP-B extract with 2 μl of Hep0.4 fraction; *lanes 3–5*, XP-B extract containing 2, 5, and 10 μl, respectively, of anti-HA fraction). *Upper panel*, ethidium bromide-stained gel; *lower panel,* autoradiogram. *B,* depletion of NER activity from HeLa WCE (100 μg/reaction) with anti-p62 monoclonal antibodies and restoration of NER activity with anti-HA eluate. *Lane 1*, depleted extract; *lanes 2–5*, depleted extracts containing 2, 4, 6, and 8 μl of anti-HA eluate, respectively; *lane 6*, mock-depleted extract; *lane 7*, nondepleted extract. *Upper panel,* ethidium bromide-stained gel; *lower panel,* autoradiogram.

TABLE I
Purification of the TFIIH complex using the dtXPB protein

Fraction	Protein	Activity[a]	Yield
	mg	*units*	*%*
WCE	54.4	—[b]	
Hep0.4	17.5	1731	100
Anti-HA	—[c]	208	12

[a] One unit of activity was defined as the amount of protein used to increase the incorporation 2-fold relative to the receiving XP-B extract (45).
[b] Activity of the WCE could not be determined.
[c] Protein concentrations were determined using the BCA protein assay (Pierce) and could not be reliably determined for the anti-HA fraction.

DISCUSSION

TFIIH was originally purified as a basal transcription factor from rat, yeast, and human (24, 38–40) and was first shown by Schaeffer *et al.* (9) to be involved in NER; this involvement was subsequently demonstrated by others as well (25, 33, 41). By immunopurifying the XPB protein using a cell line expressing functional tagged XPB, we describe an improved and facilitated purification for TFIIH free of contaminating NER and transcriptional activities that is an efficient, essentially one-step, procedure utilizing physiological elution conditions.

Utilizing this protocol, which avoids the high salt and hydrophobic chromatography conditions of the classical purification procedure, we identify TFIIH as a nine-subunit complex. In addition, we show that each complex contains only one molecule of the XPB helicase. The intensity of protein staining of the XPD subunit in the purified complex compared with XPB is consistent with the idea that the XPD helicase, also, is present on a molar basis in the complex. The occurrence of two helicase molecules per TFIIH complex is in agreement with the concept that the functional forms of a number of helicases are oligomers, generally dimeric or hexameric (42). As reported previously, CDK7 and cyclin H, together with MAT1, are part of both TFIIH and a separate trimeric complex in the cell (13). This is consistent with our findings in the immunoprecipitation experiment that CDK7 and cyclin H are relatively more abundant in the WCE and unbound fraction as compared with the XPB-associated fraction (see Fig. 2).

During the past years, all eight XPB-associated factors were identified and cloned as TFIIH subunits purified from both HeLa cells and from the budding yeast *Saccharomyces cerevisiae* (16, 43), suggesting that in solution in repair- and transcription-competent WCEs, at least the major form of TFIIH is composed of nine subunits and is active in NER, as well as transcription. However, the possibility cannot be excluded that substoichiometric and/or poorly stained subunits are essential for, for example, NER functioning, and therefore, definite proof that both the NER and transcriptional activity of TFIIH resides with the nine identified and cloned subunits awaits re-

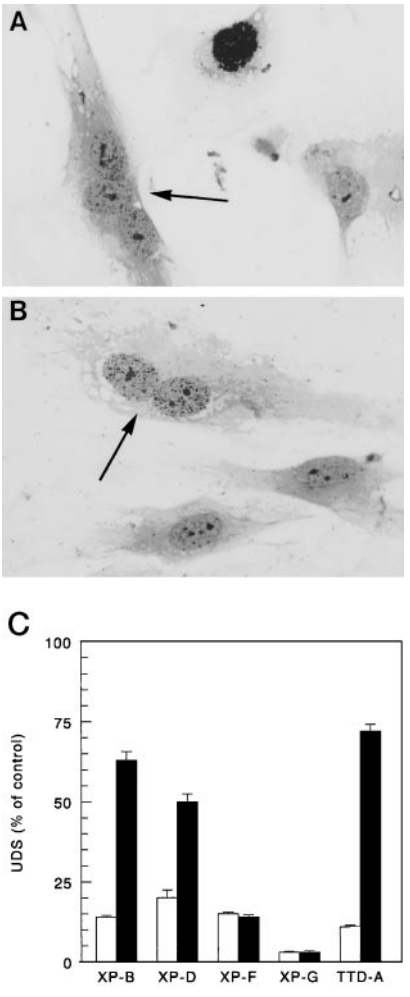

FIG. 7. **Correction of NER defect by microinjected affinity-purified TFIIH in XP-B, XP-D, and TTD-A cells *in vivo*.** *A* and *B,* micrographs showing the effect on NER activity of injection of the anti-HA eluate in XP-B (*A*) and TTD-A (*B*) cells. The injected fibroblasts (polynuclear, obtained by cell fusion prior to injection) are indicated by *arrows*. The heavily labeled cell in *A* is a noninjected S phase cell. *C,* quantitation of NER activity of injected XP and TTD-A polykaryons. The *bars* represent the average unscheduled DNA synthesis (*UDS*) level (obtained by counting grains above 50 nuclei), and S.E. values are indicated. As a control, unscheduled DNA synthesis of parallel-treated uninjected wild type fibroblasts (C5RO) were counted and arbitrarily set at 100%. *Open bars*, noninjected cells; *closed bars*, injected cells.

constitution of TFIIH from recombinant proteins.

One of the TFIIH factors that is not yet assigned to a subunit is TTDA. We are presently investigating whether any of the known TFIIH genes are mutated in TTD-A cells. However, it is theoretically also possible that TTDA is not a subunit of the TFIIH complex itself but is implicated in TFIIH modification as part of its function. Recently, we have identified human SUG1 as a protein interacting with the XPB subunit of TFIIH (35). Little is known about posttranslational regulation of TFIIH function and the role of factors like SUG1 that are thought to unfold or refold proteins in the context of several processes, including regulated proteolysis. Like SUG1, TTDA could play a role in TFIIH modification without being part of the complex. The inability to generate high levels of dtXPB protein, even when the cDNA was expressed under control of strong promoters, suggests an autoregulatory mechanism of XPB protein levels. For example, a similar observation was made in the case of overexpression of the NER protein ERCC1, which forms a complex with XPF, and TBP, which is part of the basal transcription factor TFIID (22, 44).

Using NER- and transcription-competent WCEs, physiological washing conditions, and nonoverexpressed functional dtXPB protein, we failed to observe, within our limits of detection, interactions with any NER and/or transcription factor tested, although some of them were reported previously by others (31–34). Several explanations can be put forward for this apparent discrepancy. Many methods for identification of protein-protein interactions use overexpressed, *in vitro* synthesized or purified proteins often involving heterologous expression systems. When the protein normally resides in a complex and has multiple interaction domains, it may exhibit promiscuous association behavior when studied in isolation because of the lack of its natural partners, improper folding, or lack of posttranslational modification. Alternatively or in addition, the interactions observed were not stable during our extract preparations and/or were transient or induced upon DNA binding.

The procedure described here greatly facilitates the isolation of active TFIIH. It is simple, fast, and reproducible and does not require extensive chromatographic procedures. In combination with specific procedures for extract preparation, it may be exploited further for the purification of holo-complexes involved in NER and/or transcription. Furthermore, this procedure may allow the isolation of TFIIH with mutated XPB subunits for biochemical analyses to obtain more insight into the requirements for the XPB helicase-mediated function in NER and transcription initiation. Because the reconstitution of TFIIH from recombinant source is lacking at this moment, it would also be of interest to add (epitope) tags to other TFIIH subunits for functional analysis of TFIIH with mutations in, for example, the second helicase subunit, XPD. These experiments are in progress.

Acknowledgments—We thank D. Bootsma, P. Chambon, and other members of our laboratories for continuous support and discussion. M. Chipoulet, T. Seroz, P. Vichi, and M. Rossignol are acknowledged for valuable help with experiments. M. Kuit is recognized for expert help with photography. The contributions to earlier experiments of M. Siep and J. van Kampen are acknowledged. We also thank Y. Lutz for antibody production and the IGBMC cell culture team.

REFERENCES

1. Friedberg, E. C., Walker, G. C., and Siede, W. (1995) *DNA Repair and Mutagenesis*, American Society for Microbiology Press, Washington, DC
2. Wood, R. D. (1996) *Annu. Rev. Biochem.* **65,** 135–167
3. Aboussekhra, A., Biggerstaff, M., Shivji, M. K. K., Vilpo, J. A., Moncollin, V., Podust, V. N., Protic, M., Hübscher, U., Egly, J.-M., and Wood, R. D. (1995) *Cell* **80,** 859–868
4. Mu, D., Park, C.-H., Matsunaga, T., Hsu, D. S., Reardon, J. T., and Sancar, A. (1995) *J. Biol. Chem.* **270,** 2415–2418
5. Guzder, S. N., Habraken, Y., Sung, P., Prakash, L., and Prakash, S. (1995) *J. Biol. Chem.* **270,** 12973–12976
6. Bootsma, D., Cleaver, J. E., Kraemer, K. H., and Hoeijmakers, J. H. J. (1998) in *The Metabolic and Molecular Bases of Inherited Disease* (Scriver, C. R., Beaudet, A. L., Sly, W. S., and Valle, D., eds) McGraw-Hill Books, New York, in press
7. Hoeijmakers, J. H. J. (1994) *Eur. J. Cancer* **30A,** 1912–1921
8. 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
9. Schaeffer, L., Roy, R., Humbert, S., Moncollin, V., Vermeulen, W., Hoeijmakers, J. H. J., Chambon, P., and Egly, J.-M. (1993) *Science* **260,** 58–63
10. 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
11. 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
12. Adamczewski, J. P., Rossignol, M., Tassan, J. P., Nigg, E. A., Moncollin, V., and Egly, J. M. (1996) *EMBO J.* **15,** 1877–1884
13. Rossignol, M., Kolb-Cheynel, I., and Egly, J. M. (1997) *EMBO J.* **16,** 1628–1637
14. Humbert, S., van Vuuren, A. J., Lutz, Y., Hoeijmakers, J. H. J., Egly, J.-M., and Moncollin, V. (1994) *EMBO J.* **13,** 2393–2398
15. Fischer, L., Gerard, M., Chalut, C., Lutz, Y., Humbert, S., Kanno, M., Chambon, P., and Egly, J.-M. (1992) *Science* **257,** 1392–1395
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. Parvin, J. D., and Sharp, P. A. (1993) *Cell* **73,** 533–540
18. Holstege, F. C. P., Tantin, D., Carey, M., van der Vliet, P. C., and Timmers, H. T. M. (1995) *EMBO J.* **14,** 810–819
19. Holstege, F. C. P., Van der Vliet, P. C., and Timmers, H. T. M. (1996) *EMBO J.* **15,** 1666–1677
20. Evans, E., Fellows, J., Coffer, A., and Wood, R. D. (1997) *EMBO J.* **16,** 625–638
21. Ausubel, F. M., Brent, R., Kingston, R. E., Moore, D. D., Seidman, J. G., Smith, J. A., and Struhl, K. (1994) *Current Protocols in Molecular Biology*. Greene Publishing Associates, New York
22. 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
23. Manley, J. L., Fire, A., Samuels, M., and Sharp, P. A. (1983) *Methods Enzymol.* **101,** 568–582
24. Gerard, M., Fischer, L., Moncollin, V., Chipoulet, J.-M., Chambon, P., and Egly, J.-M. (1991) *J. Biol. Chem.* **266,** 20940–20945
25. 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
26. Wood, R. D., Robins, P., and Lindahl, T. (1988) *Cell* **53,** 97–106
27. Roy, R., Schaeffer, L., Humbert, S., Vermeulen, W., Weeda, G., and Egly, J.-M. (1994) *J. Biol. Chem.* **269,** 9826–9832
28. Field, J., Nikawa, J. I., Broek, D., MacDonald, B., Rodgers, L., Wilson, I. A., Lerner, R. A., and Wigler, M. (1988) *Mol. Cell. Biol.* **8,** 2159–2165
29. Harlow, E., and Lane, D. (1988) *Antibodies. A Laboratory Manual*. Cold Spring Harbor Laboratory Press, Cold Spring Harbor, New York
30. Hoffmann, A., and Roeder, R. G. (1991) *Nucleic Acids Res.* **19,** 6337–6338
31. Ossipow, V., Tassan, J.-P., Nigg, E. A., and Schibler, U. (1995) *Cell* **83,** 137–146
32. Maldonado, E., Shiekhattar, R., Sheldon, M., Cho, H., Drapkin, R., Rickert, P., Lees, E., Anderson, C. W., Linn, S., and Reinberg, D. (1996) *Nature* **381,** 86–89
33. Drapkin, R., Reardon, J. T., Ansari, A., Huang, J. C., Zawel, L., Ahn, K., Sancar, A., and Reinberg, D. (1994) *Nature* **368,** 769–772
34. Iyer, N., Reagan, M. S., Wu, K.-J., Canagarajah, B., and Friedberg, E. C. (1996) *Biochemistry* **35,** 2157–2167
35. Weeda, G., Rossignol, M., Fraser, R. A., Winkler, G. S., Vermeulen, W., van 't Veer, L. J., Ma, L., Hoeijmakers, J. H. J., and Egly, J.-M. (1997) *Nucleic Acids Res.* **25,** 2274–2283
36. Serizawa, H., Conaway, R. C., and Conaway, J. W. (1993) *J. Biol. Chem.* **268,** 17300–17308
37. Stefanini, M., Vermeulen, W., Weeda, G., Giliani, S., Nardo, T., Mezzina, M., Sarasin, A., Harper, J. L., Arlett, C. F., Hoeijmakers, J. H. J., and Lehmann, A. R. (1993) *Am. J. Hum. Genet.* **53,** 817–821
38. Conaway, R. C., and Conaway, J. W. (1989) *Proc. Natl. Acad. Sci. U. S. A.* **86,** 7356–7360
39. Feaver, W. J., Gileadi, O., and Kornberg, R. D. (1991) *J. Biol. Chem.* **266,** 19000–19005
40. Flores, O., Lu, H., and Reinberg, D. (1992) *J. Biol. Chem.* **267,** 2786–2793
41. Wang, Z., Svejstrup, J. Q., Feaver, W. J., Wu, X., Kornberg, R. D., and Friedberg, E. C. (1994) *Nature* **368,** 74–76
42. Lohman, T. M., and Bjornson, K. P. (1996) *Annu. Rev. Biochem.* **65,** 169–214
43. Svejstrup, J. Q., Vichi, P., and Egly, J. M. (1996) *Trends Biochem. Sci.* **21,** 346–350
44. Zhou, Q., Lieberman, P. M., Boyer, T. G., and Berk, A. J. (1992) *Genes Dev.* **6,** 1964–1974
45. Robins, P., Jones, C. J., Biggerstaff, M., Lindahl, T., and Wood, R. D. (1991) *EMBO J.* **10,** 3913–3921
46. van Gool, A. J., Citterio, E., Rademakers, S., van Os, R., Vermeulen, W., Constantinou, A., Egly, J. M., Bootsma, D., and Hoeijmakers, J. H. J. (1997) *EMBO J.* **16,** 5955–5965