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Overproduction, Purification, and Characterization of the XPC Subunit of the Human DNA Repair Excision Nuclease*

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Joyce T. Reardon, David Mu‡, and Aziz Sancar§

From the Department of Biochemistry and Biophysics, University of North Carolina School of Medicine, Chapel Hill, North Carolina 27599

Xeroderma pigmentosum complementation group C gene (XPC) encodes a protein of 125 kDa which is present in a tight complex with a 58-kDa protein encoded by the human homolog of the yeast RAD23 gene, HHR23B (Masutani, C., Sugasawa, K., Yanagisawa, J., Sonoyama, T., Ui, M., Enomoto, T., Takio, K., Tanaka, K., van der Spek, P. J., Bootsma, D., Hoeijmakers, J. H. J., and Hanaoka, F. (1994) *EMBO J.* 13, 1831–1843). The XPC-HHR23B complex is required for excision of thymine dimers from DNA in a human excision nuclease system reconstituted from purified proteins. In order to understand the role of the XPC-HHR23B complex in excision repair, we have overexpressed each subunit alone and the heterodimer in heterologous systems, purified them, and characterized their biochemical properties. We find that both XPC and the heterodimer bind DNA with high affinity and UV-damaged DNA with slightly higher preference. Surprisingly, we find that the XPC subunit alone is sufficient for reconstitution of the human excision nuclease and that the HHR23B subunit has no detectable effect on the excision activity of the reconstituted system.

Nucleotide excision repair is a general repair system which is particularly suited for removing bulky DNA lesions such as thymine dimers and cisplatin-d(GpG) diadducts (Friedberg *et al.*, 1995; Sancar, 1996). A defect in excision repair causes xeroderma pigmentosum (XP)[1] in humans. XP patients are hypersensitive to sunlight and develop skin cancers at a young age and at high frequency; some patients, in addition, exhibit neurological symptoms (Cleaver and Kraemer, 1989). Proteins encoded by seven XP genes, XPA through XPG, are required for the dual incision (excision) step of nucleotide excision repair. In addition to the proteins encoded by the XP genes, the ERCC1 protein, the replication protein RPA, and the multimeric transcription factor TFIIH (two of the subunits are XPB and XPD) are required for the dual incision (Mu *et al.*, 1995, 1996; Guzder *et al.*, 1995b). Furthermore, upon purification of XPC complementing activity, it was found that the 125-kDa protein encoded by XPC (Legerski and Peterson, 1992; Masutani *et al.*, 1994) was in a complex with a protein of 58 kDa which is highly homologous to the yeast excision repair protein encoded by the RAD23 gene (Watkins *et al.*, 1993; Masutani *et al.*, 1994). In humans there are two genes with sequence homology to RAD23, and these were named human homolog of RAD23 A and B (HHR23A and HHR23B), respectively; only the protein encoded by HHR23B is found in a complex with XPC protein (Masutani *et al.*, 1994).

Although XPC and HHR23B appear to be tightly bound and the final purification step for XPC yields these two proteins in 1:1 stoichiometry (Masutani *et al.*, 1994; Aboussekhra *et al.*, 1995; Mu *et al.*, 1996), there was no genetic or biochemical data from mammalian systems indicating that HHR23B is involved in excision repair. To clarify the role of HHR23B in repair, we expressed XPC and HHR23B, separately and in a complex using baculovirus/insect cells and plasmid/*Escherichia coli* expression systems, purified these proteins, and characterized them. We found that XPC and the XPC-HHR23B complex bind to DNA nonspecifically and with relatively high affinity and to UV-damaged DNA with slightly higher affinity. Both forms of XPC are capable of complementing cell-free extracts of XP-C mutants and are active in reconstituting excision nuclease activity in a completely defined system. We conclude that with naked DNA under our experimental conditions HHR23B does not play a direct role in excision repair.

MATERIALS AND METHODS

Plasmid Construction and Baculovirus Stock Establishment—Two constructs were used for protein expression in insect cells (Fig. 1*A*). For p2Bac.XPC-HHR23B, a 1.8-kb *Sma*I-*Dra*I fragment from pUC19.HHR23B (Masutani *et al.*, 1994) was subcloned into the p2Bac vector (Invitrogen) at the *Pvu*II site under the control of the PH promoter (p2Bac.HHR23B), followed by subcloning of the 3.6-kb *Not*I fragment from pBSIISK(+).XPC (Masutani *et al.*, 1994) at the *Not*I site under the control of the P10 promoter. For p2Bac.XPC, only the *Not*I fragment was subcloned into the p2Bac vector. Sf21 cells were transfected with either p2Bac.XPC-HHR23B or p2Bac.XPC using the BaculoGold Transfection Kit (Pharmingen). Recombinant virus stocks were established from single plaques and identified by polymerase chain reaction (PCR) amplification of viral DNA using a combination of p2Bac vector and gene-specific primers. Standard procedures (O'Reilly *et al.*, 1994) were used for cell culture and infection by recombinant baculoviruses as well as for amplification and titering of the virus stocks.

To express recombinant proteins in bacterial cells, two constructs were used (Fig. 1*B*). For pHis.HHR23B, a 1.2-kb PCR fragment was subcloned into pQE-30 (Qiagen). The PCR primers were designed to introduce a 5′-*Bam*HI site while simultaneously deleting the first two (Met-Gln) and substituting the 3rd and 4th amino acids (from Val-Thr to Gly-Ser) and to generate a 3′-*Sal*I site beyond the stop codon. For pMBP.XPC, a 2.5-kb PCR fragment was subcloned into pMAL-c2 (New England Biolabs). Primers were designed to generate a blunt end at the first ATG codon of pXPC-3 (Legerski and Peterson, 1992) and to introduce a 3′-*Sal*I site beyond the stop codon. The insert was in-frame with

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§ To whom correspondence should be addressed: Dept. of Biochemistry and Biophysics, CB# 7260, University of North Carolina at Chapel Hill, Chapel Hill, NC 27599-7260. Tel.: 919-962-0115; Fax: 919-966-2852.

[1] The abbreviations used are: XP, xeroderma pigmentosum; bp, base pair(s); CFE, cell-free extract; ds-DNA, double-stranded DNA; DTT, dithiothreitol; ERCC, excision repair cross-complementing; HHR, human homolog of RAD; his, 6× histidine tag; MBP, maltose-binding protein; nt, nucleotide(s); kb, kilobase pair(s); PCR, polymerase chain reaction; RPA, replication protein A; ss-DNA, single-stranded DNA; TFIIH, transcription factor IIH.

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TABLE I
Oligomers used for binding studies

Oligomer	Sequence
1	5′-GGGGCGAATTCGAGCTCGCCCGGGATCCTCTAGAGTCGACCTGCTGCAGCCCAAGCTTGGC
2	5′-GAGCGCCAAGCTTGGGCTGCAGCAGGTCGACTCTAGAGGATCCCGGGCGAGCTCGAATTCGCCC
3	5′-GCCAAGCTTGCGCTCGCTCGTAGCTTCTCAGGGTGGCCAGCTGGC
4	5′-GCCAGCTGGCCACCCTGAGAAGCTACGAGCGAGCGCAAGCTTGGC
5	5′-CATGGGACTACAAGGACGACGATGACAAGC
6	5′-CATGGCTTGTCATCGTCGTCCTTGTAGTCC
7	5′-GCTCGAGCTAAATTCGTCAG
8	5′-CTGACGAATTTAGCTCGAGC

malE sequences (data not shown), but only a truncated fusion protein was produced. Therefore, the pMBP.XPC DNA was digested with *Hin*dIII and *Sal*I; the 1.3-kb fragment was gel-purified, and ends were filled in with the Klenow enzyme, recut with *Sal*I, and subcloned into pMAL-c2 creating pMBP.XPC(445C), which encodes the *malE* gene fused in-frame to the 445 amino acids from the carboxyl terminus of XPC.

Cell-free Extracts and Protein Purification—Mammalian cell-free extracts (CFE) were prepared from an XPC fibroblast line (patient 21RO, GM00709A, NIGMS Human Mutant Cell Repository, Camden, NJ) and from a rodent homolog of XPG (cell line UV135, CRL1867, ATCC Respository, Rockville, MD). Extracts were prepared according to the method of Manley *et al.* (1980), dialyzed into buffer A (25 mM Hepes-KOH, pH 7.9, 0.1 M KCl, 12 mM $MgCl_2$, 0.5 mM EDTA, 2 mM DTT, 12.5% glycerol), and stored at −80 °C. For immunoblot analyses, extracts were also prepared from XPC-1BE, GM02246B; XPC-3BE, GM02248B; XPC-9BE, GM02498B; XPC-1MI, GM02634; XPV-PHBE, GM02449B; XPV-115LO, GM02359. Protein concentrations were measured by the Bradford method (Bradford, 1976) using reagents from Bio-Rad. The polypeptides required for *in vitro* reconstitution of nucleotide excision repair were purified, analyzed, and stored as described (Mu *et al.*, 1995, 1996).

To prepare recombinant XPC, insect cells (Sf21) were infected with baculoviral stocks at multiplicity of infection = 15. Following a 41–43-h incubation, extracts were prepared as described above and dialyzed into buffer B (20 mM potassium phosphate, pH 7.5, 0.01% Triton X-100, 1 mM EDTA, 1 mM DTT, 10% glycerol) containing 0.15 or 0.3 M KCl. Protein concentrations were determined as described, and expression of recombinant proteins as well as purification was monitored by immunoblot analyses as described below.

For purification of recombinant XPC, CFE (20 mg) was applied to a phosphocellulose P11 (Whatman) column (4 ml), washed with 40 ml of buffer B containing 0.15 M KCl, and bound proteins were eluted with buffer B containing 1.0 M KCl. The XPC fractions were pooled, adjusted to 0.6 M KCl by dilution with buffer B, and applied to a single-stranded DNA-cellulose (Sigma) column (5 ml). The column was washed with 15 ml of buffer B containing 0.6 M KCl, and bound proteins were eluted with buffer B containing 1.5 M KCl. The XPC fractions were pooled, dialyzed against buffer B containing 0.15 M KCl, and applied to a DEAE-agarose (Bio-Rad) column (1 ml). XPC does not bind to this column, and further purification is not achieved, but this chromatographic step removes contaminating DNA which interferes with subsequent analyses. The flow-through fractions containing XPC were pooled and stored at −80 °C.

For purification of the XPC-HHR23B complex, a similar scheme was used with the following modifications. The CFE was dialyzed into buffer B containing 0.3 M KCl, 24 mg were applied to a phosphocellulose column (4 ml) which was washed with 25 ml of the same buffer, and bound proteins were eluted as described above. The XPC-HHR23B-containing fractions were pooled, adjusted to 0.3 M KCl by dilution with buffer B, and applied to a single-stranded DNA cellulose (Sigma) column (5 ml); this column was washed with 5 ml of buffer B containing 0.3 M KCl, and bound proteins were eluted with a step gradient of 0.3–1.5 M KCl in buffer B, with highly purified XPC-HHR23B eluting at 0.9–1.2 M KCl. XPC-HHR23B fractions were pooled, dialyzed against buffer B containing 0.3 M KCl, and applied to a DEAE-agarose (Bio-Rad) column (3 ml) which was washed with buffer B containing 0.3 M KCl; fractions were pooled and stored at −80 °C.

For purification of histidine-tagged HHR23B, *E. coli* strain DH5αF′lacI^q was transformed with pHis.HHR23B, and cells were grown at 37 °C to A_{600} ~0.6 and induced for 4 h with isopropyl-1-thio-β-D-galactopyranoside at 0.5 mM. The bacterial cells were collected, washed with phosphate-buffered saline, treated with lysozyme at 1 mg/ml, and disrupted by sonication (8 × 15 s with a Branson Sonicator set at maximum power). Clarified extract was obtained by centrifugation at 35,000 rpm (Beckman Ti60 rotor), and His-HHR23B was purified by Ni-NTA chromatography (Qiagen) according to the manufacturer's directions. CFE (100 mg in 10 ml) was mixed with 10 ml of resin for 16 h at 4 °C, and then the CFE-resin mixture was poured into a column (15 cm × 1.5 cm). Following extensive washing with buffer C (50 mM sodium phosphate, pH 6, 0.3 M NaCl, 10% glycerol), bound proteins were eluted with a step gradient of 0.05 to 0.5 M imidazole in buffer C, with His-HHR23B eluting at 0.15–0.3 M imidazole. His-HHR23B-containing fractions were pooled, dialyzed into buffer A, and stored at −80 °C.

For purification of the MBP-XPC(445C) fusion protein, the initial processing was as described above for His-HHR23B except the 4-h induction was with 0.3 mM isopropyl-1-thio-β-D-galactopyranoside. Clarified extract (200 mg in 150 ml) was applied to an amylose column (5 ml, New England Biolabs), and the manufacturer's directions were followed for elution of fusion proteins with 10 mM maltose. MBP-XPC(445C)-containing fractions were pooled, dialyzed into buffer A, and stored at −80 °C.

Antiserum Production and Immunoblotting—Two antigens, MBP-XPC(445C) and His-HHR23B, were used for the generation of anti-XPC and anti-HHR23B antisera. The bacterially expressed proteins were mixed with adjuvant (RIBI Immunochem Research, Inc.) and used to immunize rabbits at 2–3-week intervals (0.5 mg of protein per injection) with serum samples being collected after the second injection. These antisera react with XPC and HHR23B, respectively, in immunoblot analyses. For immunoblotting, proteins were separated by SDS-polyacrylamide gel electrophoresis and transferred to nitrocellulose membranes (Schleicher and Schuell, Protran) using standard procedures for electroblotting. Antigen-antibody complexes were detected using the alkaline phosphatase method (Promega) or enhanced chemiluminescence (Amersham).

DNA Binding Assays—Several oligonucleotides were prepared for the DNA binding assays, but all substrates within a particular category were prepared in essentially the same manner. Approximately 50 pmol of each oligonucleotide were 5′-end-labeled with T4 polynucleotide kinase (New England Biolabs) and [γ-^{32}P]ATP (7000 Ci/mmol; ICN). Labeled oligonucleotides were either ethanol-precipitated alone (ss-DNA) or in the presence of unlabeled complementary oligomers (ds-DNA), and DNA was resuspended in annealing buffer (40 mM Tris, pH 7.4, 100 mM NaCl, 4 mM $MgCl_2$). To obtain ds-DNA substrates, the coprecipitated oligomers were heated at 90–95 °C for 5 min and slow cooled to <30 °C (about 3 h). Both ss- and ds-DNA were separated from unincorporated [γ-^{32}P]ATP or nonhybridized complementary strands by electrophoresis on 6–8% polyacrylamide gels, followed by electroelution, ethanol precipitation, and resuspension in annealing buffer. For preparing damaged DNA, the DNA was irradiated with 4–5 kJ/m^2 using a germicidal lamp (254 nm). DNA was stored at 4 °C in annealing buffer and diluted just prior to use in binding buffer. The substrates used (Table I) include (*a*) 64-nt labeled oligomer (ss-DNA), part of which was annealed to 61-mer (oligo 2) to generate a 60-bp duplex (ds-DNA) with a 1-base overhang at the 5′ end and 4-base overhang at the 3′ end, (*b*) 45-nt labeled oligomer (ss-DNA), an aliquot of which was annealed to 45-mer (oligo 4) to generate a 45-bp duplex (ds-DNA), (*c*) 30-nt labeled oligomer (ss-DNA), half of which was annealed to 30-mer (oligo 6) to generate a 26-bp duplex (ds-DNA), with 4-base overhangs at both the 5′ and 3′ ends, and (*d*) 20-nt labeled oligomer (ss-DNA), part of which was annealed to 20-mer (oligo 8) to generate a 20-bp duplex (ds-DNA).

For the binding assays, DNA (~0.3 nM) was mixed with the indicated amounts of protein in 15 μl of binding buffer (30 mM Hepes-KOH, pH 7.9, 4 mM potassium phosphate, pH 7.5, 100 mM KCl, 3.2 mM $MgCl_2$, 0.4 mM DTT, 0.3 mM EDTA, 0.002% Triton X-100, 2 mM ATP, 2% glycerol, 1 μg of bovine serum albumin). Following a 30-min incubation at 30 °C,

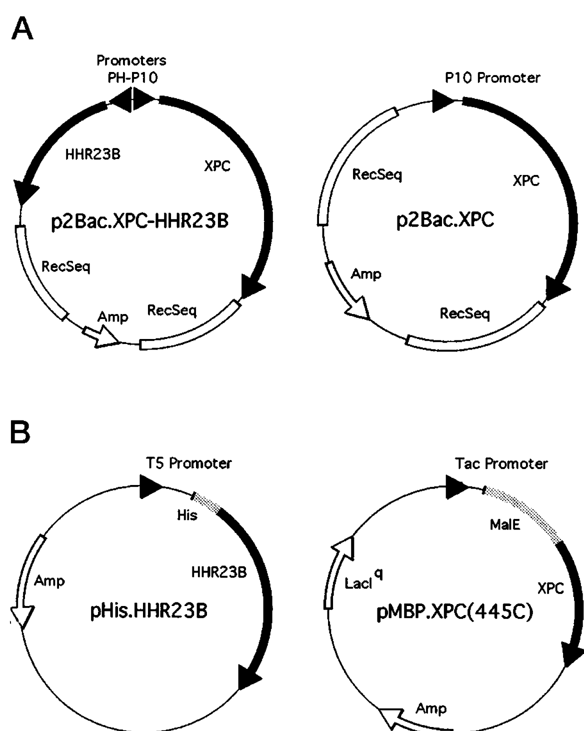

FIG. 1. **Plasmids for recombinant protein expression.** Four constructs were used in this study. *A*, p2Bac.XPC and p2Bac.XPC-HHR23B were used for the expression and purification of recombinant proteins used in DNA binding and repair assays. *B*, protein expressed with pHis.HHR23B was used for both of these assays as well as for antisera production. Recombinant protein expressed with pMBP.XPC(445C) was used only for the generation of antisera. RecSeq indicates recombination sequences for integration into the viral genome; *Amp* is the ampicillin resistance gene, β-lactamase.

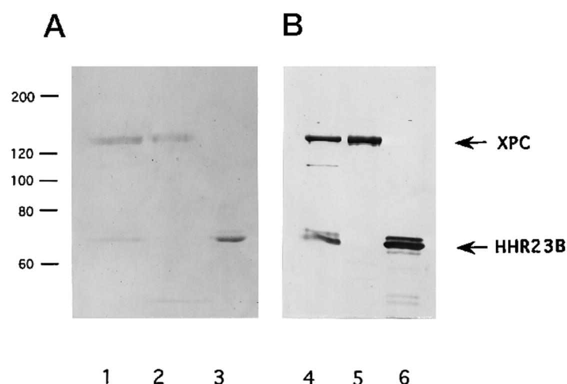

FIG. 2. **Analysis of purified proteins.** Polypeptides were separated by electrophoresis through a 10% SDS-polyacrylamide gel and detected by staining with Coomassie Brilliant Blue (*A*, *lanes 1–3*) or by immunoblot analysis with alkaline phosphatase reagents (*B*, *lanes 4–6*). *Lane 1*, rXPC-HHR23B, 1.2 μg, and *lane 4*, 0.5 μg of the same fraction; *lane 2*, 0.6 μg, and *lane 5*, 0.25 μg, of rXPC; *lanes 3* and *6*, 1.3 μg and 0.8 μg, respectively, of His-HHR23B. *Numbers* to the *left* show positions of size markers (Life Technologies, Inc. 10-kDa protein ladder).

glycerol was added to 7%, and the protein-DNA complexes were separated by electrophoresis at a constant current of 25 mA at room temperature using 5% polyacrylamide gels containing acrylamide/methylenebisacrylamide at 30:1 in 50 mM Tris borate and 1 mM EDTA, pH 8.3. The gels were autoradiographed for visual inspection and quantitatively analyzed with an Ambis systems scanner.

Repair Assays—The excision assay, utilizing internally labeled 140-mer cholesterol-A substrate (Matsunaga *et al.*, 1995; Mu *et al.*, 1996) or a thymine dimer (T<>T) was used to assay repair activity for complementation of XPC cell-free extracts by rXPC and rXPC-HHR23B or for reconstitution of excision repair by either rXPC-HHR23B or rXPC ±

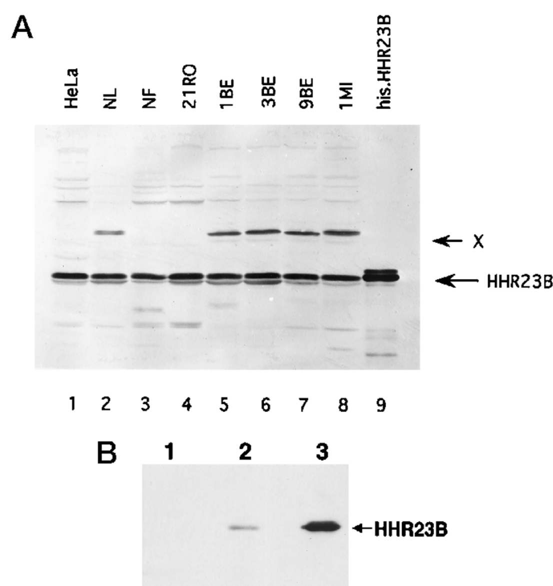

FIG. 3. **Western blot analyses of XPC cell-free extracts (CFEs) and purified protein fractions.** Polypeptides were separated by electrophoresis through a 10% SDS-polyacrylamide gel, transferred to nitrocellulose, and probed with antisera. *A*, immunoblot analysis (alkaline phosphatase reagents) of CFEs from HeLa (*lane 1*), normal lymphoblastoid (*NL*, *lane 2*), and normal fibroblast (*NF*, *lane 3*) cells compared with CFEs from various XPC strains (*lanes 4–8*). Approximately 50 μg of CFE were resolved in *lanes 1–8* and 0.5 μg of his.HHR23B in *lane 9*. The *large arrow* indicates HHR23B while the *smaller*, *upper arrow* indicates a slower-migrating, cross-reacting material (*X*) unique to lymphoblastoid cells. *B*, immunoblot analysis (enhanced chemiluminescence) of purified proteins used in the types of experiments illustrated in Fig. 6. *Lane 1* contained 15 ng of TFIIH, 20 ng of XPG, and 20 ng of XPF-ERCC1. *Lane 2* contained 5 ng of XPC-HHR23B purified from HeLa cells. *Lane 3* contained 20 ng of recombinant HHR23B purified from *E. coli*.

HHR23B. Briefly, for complementation, increasing amounts of CFE (60–100 μg) were mixed with a constant amount of either rXPC or rXPC-HHR23B and incubated for 60 min at 30 °C in 25 μl of excision buffer (35 mM Hepes-KOH, pH 7.9, 8 mM Tris-HCl, pH 7.5, 66 mM KCl, 32 mM NaCl, 5.6 mM $MgCl_2$, 0.8 mM DTT, 0.4 mM EDTA, 2 mM ATP, 0.0004% Triton X-100, 20 μM each of dATP, dCTP, dGTP, and dTTP, 0.2 mg/ml bovine serum albumin, and 2.9% glycerol). Then, the mixtures were incubated with Proteinase K for 15 min at 37 °C, and the DNA was purified by phenol/phenol chloroform/ether extractions followed by ethanol precipitation, and the products were analyzed on 10% polyacrylamide sequencing gels. Similar conditions were used for the reconstitution experiments, except highly purified proteins were used in place of the mutant CFE and rXPC was compared with natural XPC (native, heterodimer purified from HeLa CFE) under optimal reaction conditions and under suboptimal conditions to test for the effect(s) of HHR23B, and the repair reaction was for 2.5 h at 30 °C.

RESULTS

Purification of Recombinant XPC and XPC-HHR23B—We attempted to overproduce XPC and HHR23B in *E. coli*, but failed to express full-length XPC in *E. coli* either from its own initiation codon or in the form of a fusion protein with maltose-binding protein (MBP). Nevertheless, the MBP-XPC fusion protein containing the carboxyl-terminal 445 amino acids of XPC was expressed and used for generating XPC antibodies used in the present study.

To obtain functional recombinant XPC, we cloned the gene into the baculovirus expression vector p2Bac (Invitrogen) either alone or together with HHR23B. Additionally, HHR23B was cloned into an *E. coli* expression plasmid. Fig. 1 shows the plasmid constructs used in the current study. Full-length soluble proteins were expressed with these constructs. The XPC protein and XPC-HHR23B heterodimer were purified essentially as described by Masutani *et al.* (1994). The HHR23B

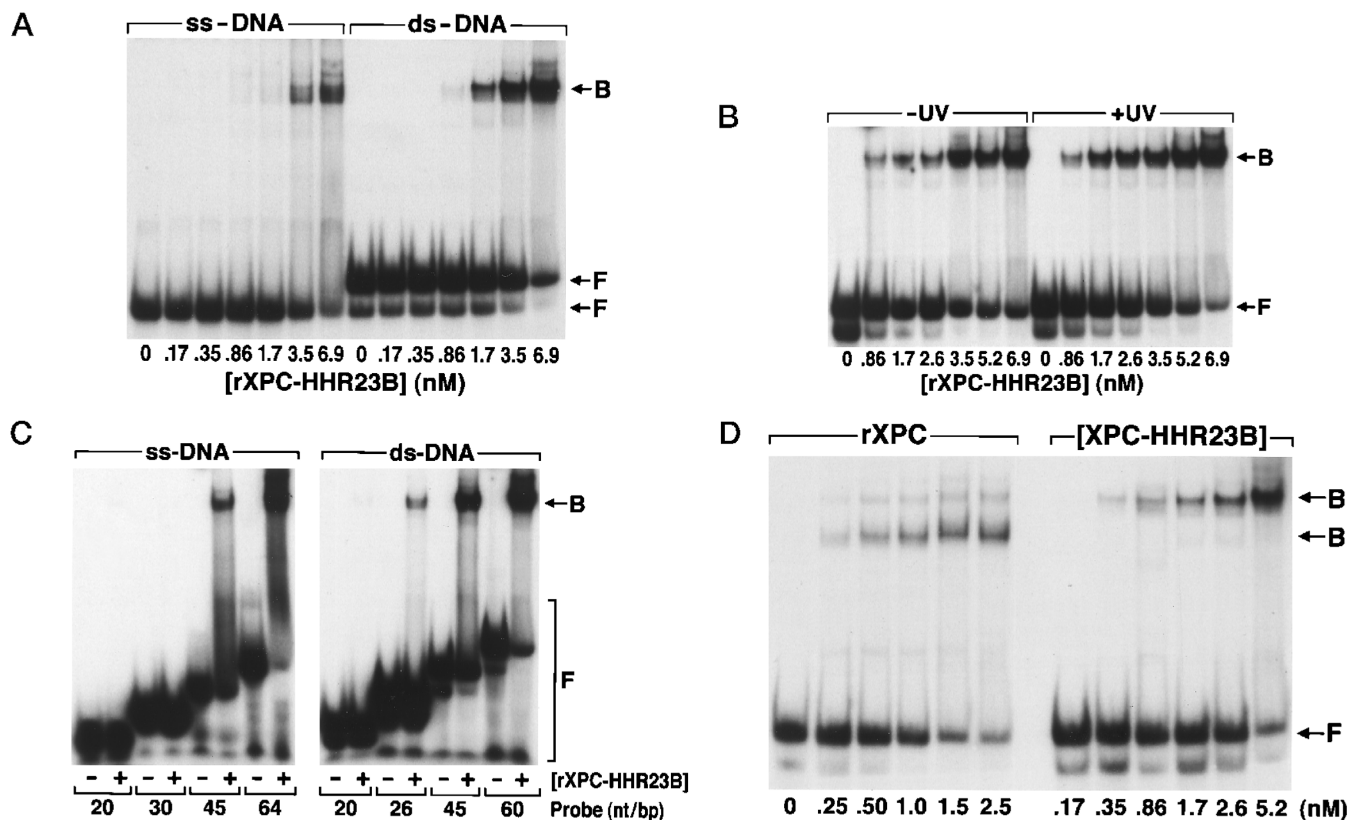

FIG. 4. **DNA binding by XPC and XPC-HHR23B.** Assay conditions were as described under "Materials and Methods"; for *A* and *B*, the DNA concentration was 0.3 nM and protein concentrations were as indicated. *A*, binding of rXPC-HHR23B to single-stranded 64-mer (ss-DNA) and double-stranded 60-bp duplex (ds-DNA). Quantitative analyses of the data revealed that 50% binding was achieved with 4.8 nM and 5.2 nM XPC with single- and double-stranded 60-mers, respectively. *B*, binding of rXPC-HHR23B to undamaged (−*UV*) and damaged (+*UV*) double-stranded 60-bp duplex. For UV-DNA, 4 nM gave 50% binding. *C*, binding of rXPC-HHR23B to single-stranded oligomers (20-mer, 30-mer, 45-mer, and 64-mer) and double-stranded duplexes (20 bp, 26 bp, 45 bp, and 60 bp). DNA concentration was 0.3 nM and rXPC-HHR23B was at 10.5 nM. At this protein concentration, only 40% binding was observed with ss- or ds-45-mer, and 50% binding was estimated to be 13 nM. Less than 5% of 26-bp duplex was bound, and no binding was detected for 30-mer or 20-mer (ss- and ds-DNA). *D*, binding of rXPC and XPC-HHR23B to 60-bp duplex. DNA concentration was 0.3 nM and protein concentrations were as indicated; heterodimer used in this experiment was from its natural source (HeLa cells). As with recombinant heterodimer, 50% binding was at 5.2 nM while rXPC bound 50% substrate at 2 nM. For all panels, the faster migrating free DNA species is single-stranded DNA.

protein was expressed in soluble form and in full-length in *E. coli* and was purified from *E. coli* by nickel-affinity chromatography. Fig. 2 shows the analyses of purified proteins by Coomassie Blue staining and Western blotting.

Expression of HHR23B in XPC Mutant Cell Lines—Since one of the goals of this study was the investigation of the properties of the XPC protein in the absence of HHR23B, we wished to know whether or not the XP-C mutant cell-free extracts contained HHR23B protein at normal or reduced levels. Cell-free extracts from a variety of cell lines were tested by Western blotting. Fig. 3*A* shows that XPC mutants have normal levels of HHR23B. In fact, during purification of XPC complementing activity from HeLa cells, we found that HHR23B is in vast molar excess over the XPC polypeptide and separates from the heterodimer readily by ion exchange chromatography.[2]

In addition, our purified repair factors were tested for the presence of HHR23B to ascertain that factors other than the XPC fraction were free of HHR23B, and, hence, complementation experiments done with recombinant XPC reflected the activity of this subunit alone. Fig. 3*B* reveals that the repair factors purified from HeLa cells contained no detectable HHR23B (<0.2 ng per excision assay) and hence any activity arising from the addition of XPC to the reconstituted system could safely be ascribed to XPC alone.

DNA Binding Properties—The natural as well as the recombinant XPC-HHR23B were purified through DNA-cellulose affinity chromatography and hence were known to be DNA-binding proteins. For quantitative analysis of DNA binding properties, we conducted electrophoretic mobility shift assays with various types of DNAs. The heterodimer (both recombinant and natural forms) binds to single-stranded and double-stranded DNA (60-bp duplex) with comparable affinities (Fig. 4*A*) and with a K_D of $\sim 5 \times 10^{-9}$ M (calculated from the 50% binding point); heavily irradiated 60-bp duplex (about 3 photolesions per molecule) was bound with slightly higher affinity ($K_D \sim 4 \times 10^{-9}$ M, Fig. 4*B*). The heterodimer bound to the 45-bp duplex with somewhat lower affinity and to a 26-bp duplex very weakly; no binding with 20-bp oligomer could be detected over the concentration range used (Fig. 4*C*); similar binding properties were observed when the probe was single-stranded DNA. The HHR23B subunit on its own has no affinity for DNA (data not shown). Interestingly, the XPC subunit binds the 60-bp duplex with approximately 2-fold higher affinity than the heterodimer (Fig. 4*D*), suggesting that in fact HHR23B reduces the intrinsic DNA binding affinity of XPC.

Reconstitution of Excision Nuclease with XPC-HHR23B and with the XPC Polypeptide—Although mutations in XPC confer sensitivity to DNA damage (Li *et al.*, 1993), there is no genetic evidence from mammalian systems implicating HHR23B in repair. We wished to find out whether HHR23B plays a role in repair by conducting complementation and reconstitution experiments using the XPC polypeptide and the XPC-HHR23B

[2] C. P. Selby and A. Sancar, unpublished results.

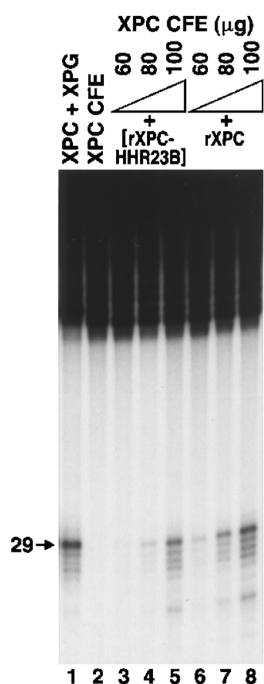

FIG. 5. **Complementation of XPC mutant cell-free extracts by rXPC and rXPC-HHR23B.** rXPC-HHR23B (5 ng, *lanes 3–5*) or rXPC (2. 5 ng, *lanes 6–8*) were added to the indicated amounts of XPC CFE and tested for complementation of excision nuclease activity. Control reactions are in *lane 1* (50 μg of XPC + 50 μg of XPG CFE) and *lane 2* (100 μg of XPC CFE). The prominent 29-mer excision product is indicated.

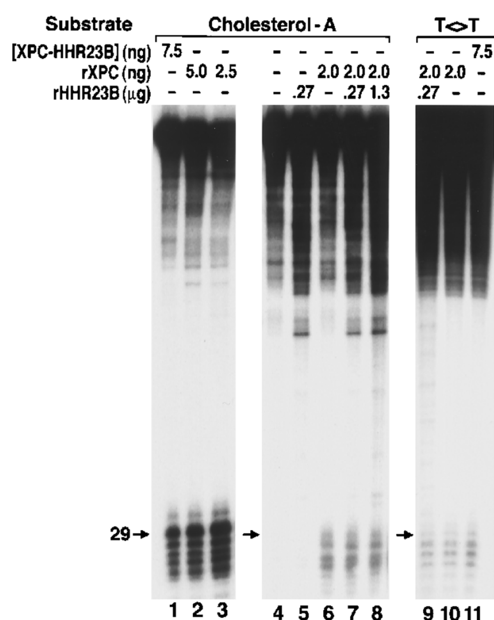

FIG. 6. **Reconstitution of excision nuclease with rXPC and the effect of rHHR23B.** Assay conditions were as described under “Materials and Methods.” The amounts of XPC-HHR23B, rXPC, and rHHR23B (his-tagged) are indicated, and other repair factors included in all reaction mixtures were as follows: XPA, 25 ng; TFIIH, 2 ng; XPG, 4 ng; XPF-ERCC1, 9 ng; and RPA, 250 ng. The 29-mer excision product is indicated.

heterodimer. We found that both forms are active in complementing XP-C cell-free extracts (Fig. 5). This experiment, which reveals that recombinant XPC is active in excision repair, indicates that the XPC polypeptide made in the absence of HHR23B folds properly. However, these experiments do not show whether XPC can function without HHR23B because XPC cells appear to have normal levels of HHR23B protein (Fig. 3*A*) which could reconstitute the XPC-HHR23B heterodimer upon addition of XPC to the cell-free extract. To address this particular question, we conducted experiments with a defined system of purified excision repair proteins not containing detectable levels of HHR23B (Fig. 3*B*). Fig. 6 shows that XPC alone is sufficient to reconstitute the excision nuclease to the same extent as the heterodimer purified from its natural source (HeLa cells). Supplementing HHR23B to the defined excision repair system reconstituted with XPC alone did not alter the rate of excision.

A previous study (Mu *et al.*, 1996) showed that a cholesterol substitutent attached with a shorter linker to the phosphodiester backbone (cholesterol-B) than the one used in the current work (cholesterol-A) was excised normally even in the absence of XPC-HHR23B. Therefore, it was conceivable that these excisions in the absence of HHR23B or even the XPC-HHR23B complex were peculiar to these unnatural substrates. Hence, we tested the classic substrate for human excision nuclease, cyclobutane thymine dimer, in our reconstituted system. As evident from Fig. 6, *lanes 9* and *10*, even the XPC polypeptide alone is sufficient for reconstitution and there is no detectable effect of HHR23B on excision under these experimental conditions.

Thus, we conclude that HHR23B does not play a direct role in excision repair but may modulate the XPC activity. Indeed, HHR23B may have a negative effect on both DNA binding and excision activity because we reproducibly observe better binding (Fig. 4*D*) and excision (Figs. 5 and 6) with rXPC alone compared to the heterodimer.

DISCUSSION

The yeast RAD23 gene belongs to the RAD3 epistasis group, whose members participate in excision repair. Null mutations of genes in this group, such as RAD1, completely abolish excision repair (Prakash *et al.*, 1993). In contrast, deletion of RAD23 increases the UV sensitivity of yeast cells only modestly (Perozzi and Prakash, 1986; Watkins *et al.*, 1993) indicating that, unlike RAD1 and other members of the RAD3 epistasis group, RAD23 is not essential for excision. Thus, extrapolating from yeast genetics, one would expect that the same would be true in humans. However, even though the yeast and human excision repair systems are highly homologous (Prakash *et al.*, 1993; Friedberg *et al.*, 1995; Sancar, 1996), certain significant differences have been found. In particular, it was found that the 3′ nick of the dual incision could occur in the absence of XPF-ERCC1 in humans (Mu *et al.*, 1996) but requires the entire complement of excision repair proteins in yeast (Guzder *et al.*, 1995b). Thus, *a priori*, it was not possible to predict whether XPC without HHR23B would be sufficient to reconstitute the human excision nuclease. Indeed, it was reported that it was not possible to separate XPC from HHR23B without losing the XPC correcting activity (Masutani *et al.*, 1994). The results presented in this report indicate that the HHR23B polypeptide is not required for the excision repair function of the XPC protein and that HHR23B modulates XPC activity, perhaps by interacting with and masking the DNA binding domain of XPC. It is possible that the stringent conditions used to separate XPC from HHR23B in the previous study inactivated the XPC protein.

In light of our results, it is reasonable to ask whether HHR23B plays any role in repair. To answer this question, in addition to the yeast *rad23* phenotype, one must take the following findings into account. First, in humans, two RAD23 homologs were found which exhibit 57% sequence identity to one another and 30–34% sequence identity to RAD23. Second, of the two polypeptides, only HHR23B was found in a complex with XPC (Masutani *et al.*, 1994). Third, RAD23 (Watkins *et*

al., 1993) and HHR23 belong to the ubiquitin-fusion family of proteins in that the NH_2-terminal 80 amino acids are highly homologous to ubiquitin (Masutani *et al.*, 1994). The precise role of ubiquitin in these proteins is not known but is thought to function as a chaperone in assisting proper folding and assembly and thermostability (Finley *et al.*, 1989; Garrett *et al.*, 1994; Aso *et al.*, 1995). Fourth, the Rad23 protein is not essential for, but promotes complex formation between TFIIH and Rad14 (XPA homolog) proteins (Guzder *et al.*, 1995a). In humans TFIIH seems to be directly bound to XPA without the aid of other proteins (Park *et al.*, 1995) although the possibility that HHR23B stimulates XPA-TFIIH interaction has not been ruled out. Fifth, the yeast homolog of XPC, the Rad4 protein, is required for global excision repair (Prakash *et al.*, 1993) but not for excision repair of the transcribed strand of rDNA genes which are transcribed by RNA polymerase I (Verhage *et al.*, 1996a). In contrast, in humans the template strand of the polymerase II-transcribed sequences is repaired in XP-C mutants at an essentially normal rate (Mellon *et al.*, 1987; Kantor *et al.*, 1990; Venema *et al.*, 1990, 1991; Evans *et al.*, 1993). In fact, it has been demonstrated *in vitro* that for certain lesions, even in the absence of transcription, excision repair occurs without XPC-HHR23B (Mu *et al.*, 1996). Thus, not only HHR23B but even XPC is dispensable for excision nuclease activity under certain conditions or with certain substrates.

The following model is consistent with most of these observations. The HHR23B subunit of the heterodimer may be involved in nucleosome disassembly or reorganization which makes DNA accessible to the XPC subunit and other factors of the excision nuclease system. Alternatively, HHR23B may modulate the activity of XPC and perhaps function as a chaperone molecule. Indeed, preliminary experiments suggest that HHR23B protects the XPC subunit from thermal inactivation as tested by the DNA binding assay.[3] The role of XPC may be to stabilize the local unwinding that is thought to occur in the preincision complex. Thus, with naked DNA (such as the 140-mer substrate), there is no need for HHR23B. Similarly, in transcribed DNA where local unwinding is provided by the RNA polymerase stalled at a lesion or with certain lesions which intrinsically cause local unwinding, XPC is not needed and hence excision occurs at a near-normal rate in the absence of the XPC-HHR23B heterodimer. This model is in agreement with the finding of Miller *et al.* (1982) that yeast *rad23* mutants removed pyrimidine dimers at about 60% of wild type levels. In contrast, McCready (1994) and Verhage *et al.* (1996b) using different methodologies did not find dimer removal from the genome overall or from transcriptionally active genes in *rad23* mutants. Further studies are needed to establish the function of XPC, to determine whether there are bona fide differences between the roles of HHR23B in humans and RAD23 in yeast, and to reconcile these seemingly contradictory reports regarding the requirement of RAD23 for excision repair in yeast.

[3] J. T. Reardon and A. Sancar, unpublished results.

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