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# Identification and Characterization of XPC-Binding Domain of hHR23B

CHIKAHIDE MASUTANI,[1] MARITO ARAKI,[1] KAORU SUGASAWA,[2,3] PETER J. VAN DER SPEK,[3] AYUMI YAMADA,[1] AKIO UCHIDA,[1,2] TAKAFUMI MAEKAWA,[1] DIRK BOOTSMA,[3] JAN H. J. HOEIJMAKERS,[3] AND FUMIO HANAOKA[1,2]*

*Institute for Molecular and Cellular Biology, Osaka University, Suita, Osaka 565,[1] and Cellular Physiology Laboratory, The Institute of Physical and Chemical Research (RIKEN), Wako, Saitama 351-01,[2] Japan, and Department of Cell Biology and Genetics, Medical Genetic Centre, Erasmus University, 3000 DR Rotterdam, The Netherlands[3]*

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**hHR23B was originally isolated as a component of a protein complex that specifically complements nucleotide excision repair (NER) defects of xeroderma pigmentosum group C cell extracts in vitro and was identified as one of two human homologs of the *Saccharomyces cerevisiae* NER gene product Rad23. Recombinant hHR23B has previously been shown to significantly stimulate the NER activity of recombinant human XPC protein (rhXPC). In this study we identify and functionally characterize the XPC-binding domain of hHR23B protein. We prepared various internal as well as terminal deletion products of hHR23B protein in a His-tagged form and examined their binding with rhXPC by using nickel-chelating Sepharose. We demonstrate that a domain covering 56 amino acids of hHR23B is required for binding to rhXPC as well as for stimulation of in vitro NER reactions. Interestingly, a small polypeptide corresponding to the XPC-binding domain is sufficient to exert stimulation of XPC NER activity. Comparison with known crystal structures and analysis with secondary structure programs provided strong indications that the binding domain has a predominantly amphipathic α-helical character, consistent with evidence that the affinity with XPC is based on hydrophobic interactions. Our work shows that binding to XPC alone is required and sufficient for the role of hHR23B in in vitro NER but does not rule out the possibility that the protein has additional functions in vivo.**

Nucleotide excision repair (NER) is one of the most versatile DNA repair systems, because the process eliminates a remarkably wide range of structurally unrelated DNA lesions, such as UV-induced injury and numerous chemical adducts (2, 36). At least two distinct subpathways are discerned. One of the subpathways, transcription-coupled repair, rapidly eliminates lesions on DNA strands transcribed by RNA polymerases (13, 18, 19). This process is particularly important for the removal of types of damage for which the global genome repair subpathway, covering the entire genome, is too slow. Although most of the known complementation groups of the NER syndrome xeroderma pigmentosum (XP) are impaired in both of the subpathways, XP group C (XP-C) is particularly notable because its NER defect is limited to the global genome repair subpathway (32). By cDNA transfection, a partial *XPC* cDNA correcting the UV sensitivity of XP-C mutant cells was cloned (12). The level of *XPC* mRNA was greatly reduced in most XP-C cell lines tested, and nonsense and missense mutations have been identified in several cell lines (14). Using an in vitro NER system, on the other hand, we have previously reported cloning, purification, and characterization of a protein factor which biochemically corrects the defect of whole-cell extracts from XP-C mutant cells (17). This purified protein fraction contained XPC complexed with a 58-kDa protein, called hHR23B (human homolog of Rad23). Although the predicted amino acid sequence of XPC displays a limited homology to the *Saccharomyces cerevisiae RAD4* NER gene product, little functional information could be deduced from the primary sequence: only strong DNA binding has been demonstrated as a biochemical activity of this protein (17, 25). hHR23B shows overall amino acid sequence homology to the yeast NER factor Rad23. Intriguingly, we identified a second human Rad23 homolog, designated hHR23A (17). All of the *RAD23* gene products harbor a ubiquitin-like sequence at the N terminus, which appears to be important for the NER function, at least in yeast (35). Furthermore, these proteins contain two copies of the ubiquitin-associated domain, which has been suggested to be involved in ubiquitin metabolism (7, 31).

The role of Rad23 in yeast NER has not yet been clarified. *rad23* null mutants are only moderately sensitive to UV, like *rad7* and *rad16* mutants (21, 35). In contrast to *rad7* and *rad16*, whose NER defects are limited to global genome repair (23, 33), both NER subpathways seem to be impaired in *rad23* mutants (20). It has been recently reported that whole-cell extracts from yeast *rad23* mutant cells are totally inactive in NER reactions in vitro (5, 34). *rad4* and *rad23* mutant extracts failed to complement each other, suggesting that Rad4 and Rad23 proteins have a physical interaction in yeast cells (34), in analogy to the XPC-hHR23B complex in human cells. In agreement with this, Rad4-Rad23 protein complex could indeed be isolated from yeast cells (4). These findings suggest that Rad4 protein might not be stably expressed in *rad23* mutants and that the NER defect of the *rad23* extracts could be due to the absence of Rad4. Therefore, the requirement for Rad23 for in vitro NER has not yet been resolved.

We have previously expressed recombinant human XPC (rhXPC) and hHR23B (rhHR23B) proteins by using a baculovirus system and purified them to near homogeneity. In a reconstituted in vitro NER system, the rhXPC displayed some NER activity in the absence of rhHR23B, but the activity was significantly stimulated by coaddition of rhHR23B (28). On the other hand, Reardon et al. have recently reported that rhHR23B failed to stimulate rhXPC with a different NER

* Corresponding author. Mailing address: Institute for Molecular and Cellular Biology, Osaka University, 1-3 Yamada-oka, Suita, Osaka 565, Japan. Phone: (81) (6) 879-7975. Fax: (81) (6) 877-9382. E-mail: fhanaoka@imcb.osaka-u.ac.jp.

TABLE 1. Sequences of oligonucleotides used for site-directed mutagenesis

| Oligonucleotide | Sequence[a] |
|---|---|
| N1 | 5′-TCAGGGTGACCTCCATGGTGCCGCCGCGCAG-3′ |
| N2 | 5′-TCAGGGTGACCTGCATGGTATATCTCC-3′ |
| X1 | 5′-TCGAATTCGGATCCCTCGAGATCTTCATCAAAG-3′ |
| X2 | 5′-GTGGTGGTGCTCAAGATCTTCATCAAAG-3′ |
| 73M | 5′-TGGTCACCATGGCCACCACAAAG-3′ |
| 117M | 5′-AGTGGGGGCCATGGCAGGGACAG-3′ |
| 193M | 5′-CTCAGTTACCATGGTCTCGTAAG-3′ |
| 255M | 5′-GCTGCAGCCATGGCTGAAGACTG-3′ |
| 88X | 5′-AGCTGACTGCTCGAGTGTAGCTGG-3′ |
| 145X | 5′-AGGCTTCTCGAGTTTAGCTGCAC-3′ |
| 190X | 5′-CCATATTCTCGAGAGACTGACCCG-3′ |
| 222X | 5′-AAAGATACTCGAGTGCTCTGTCAG-3′ |
| 277X | 5′-CCGTAAAAACTCGAGGGGATGTCC-3′ |
| 304X | 5′-CCTATCTGCTCGAGTAACGCTGG-3′ |
| 333X | 5′-TGACCACCAGCCTCGAGAACTGGTTCATTTAAC-3′ |
| 383X | 5′-CGCTTGTATCTCGAGTCCTTCAGG-3′ |
| 276M | 5′-GATTCCGTAAAAATTCCATGGGATGTCCTCCAG-3′ |
| AX1 | 5′-GGCCTGGCTTCCTGCTCGAGCTCGTCATCAAAG-3′ |

[a] Introduced restriction sites recognized by *Nco*I (CCATGG) or *Xho*I (CTC GAG) are underlined.

assay reconstituted with purified proteins (22). To further examine the relationship between XPC-binding and -stimulating activities exerted by hHR23B, we identify here a domain which is responsible for both activities by generating a series of deletion mutants of rhHR23B.

## MATERIALS AND METHODS

**Construction of plasmids for protein expression in *Escherichia coli*.** Plasmid pUC-hHR23B (17), which carries *hHR23B* cDNA cloned into the *Eco*RI site of pUC19, was digested with *Eco*RI, and the resulting 2.9-kb cDNA fragment was inserted into the *Eco*RI site of an *E. coli* expression vector, pET-24d (Novagen), to generate pET-23BcDNA. The translation initiation site of hHR23B in pET-23BcDNA was converted to an *Nco*I site with an oligonucleotide, N1 (sequences of the oligonucleotides used for mutagenesis are shown in Table 1), and with a site-directed mutagenesis system, Mutan-K (Takara Shuzo), essentially according to the method of Kunkel et al. (9). All the mutagenesis experiments were done with this system. The resulting plasmid was digested with *Nco*I and then self-ligated to remove the 5′ untranslated sequence of hHR23B and to generate plasmid pET-23B(2Q-E), which encodes rhHR23B containing one amino acid change (of the second Q to E). This mutation was repaired by site-directed mutagenesis with oligonucleotide N2. The resulting plasmid, pET-23B, was used for nontagged rhHR23B expression. The stop codons in pET-23B(2Q-E) and pET-23B were converted to *Xho*I sites with oligonucleotide X1. The resulting products were digested with *Xho*I and self-ligated to remove the 3′ untranslated sequence and to fuse the C termini of the proteins in frame with eight amino acids (LEHHHHHH). The resulting plasmids were designated pET-23B(2Q-E)His and pET-23BHis, respectively.

The plasmids for expression of N-terminally truncated rhHR23B-His proteins were generated from pET-23B(2Q-E)His, which has an *Nco*I site at the translation start site. Another *Nco*I site was introduced with an oligonucleotide, and the product was digested with *Nco*I and then ligated. Oligonucleotides used were 73M for Δ1-72, 117M for Δ1-117, 193M for Δ1-192, and 255M for Δ1-255. The plasmids for expression of C-terminally truncated rhHR23B-His were generated from pET-23BHis, which has an *Xho*I site just before the histidine tag. Another *Xho*I site was introduced with an oligonucleotide, and the product was digested with *Xho*I and then ligated. Oligonucleotides used were 222X for Δ222-409, 277X for Δ278-409, 304X for Δ304-409, 333X for Δ333-409, and 383X for Δ383-409. To produce plasmids for expression of internally deleted rhHR23B-His, the *Xho*I site of pET-23BHis was converted to an undigestable sequence by *Xho*I with oligonucleotide X2. Two *Xho*I sites were introduced in the resulting plasmid at various places in the coding sequence by using different oligonucleotides, and the product was digested with *Xho*I and then ligated. Oligonucleotides used were 88X and 145X for Δ88-145, 145X and 190X for Δ145-190, 190X and 222X for Δ190-222, 222X and 277X for Δ222-275, 277X and 304X for Δ278-304, 304X and 333X for Δ304-333, and 333X and 383X for Δ333-383. For expression of rhHR23B(277-332)His, two mutations were introduced in pET-23B(2Q-E)His to generate an *Nco*I site and an *Xho*I site with oligonucleotides 276M and 333X, respectively. The resulting plasmid was digested with *Nco*I and then ligated, and the product was digested with *Xho*I and then ligated.

*hHR23A* cDNA was cloned into pET-11d as described previously (30). The plasmid was digested with *Xba*I and *Hin*dIII, and the resulting fragment was cloned into pET-24d digested with *Xba*I and *Hin*dIII. The resulting plasmid, pET-23A, was used for the expression of nontagged rhHR23A. The stop codon in pET-23A was converted to an *Xho*I site with oligonucleotide AX1, and the resulting product was digested with *Xho*I and ligated. The resulting plasmid, pET-23AHis, was used for the expression of rhHR23A-His. The introduced mutations were confirmed by DNA sequences with the ALFred DNA sequencer (Pharmacia).

**Expression of proteins in *E. coli* and their extraction.** Four milliliters of fresh full-grown culture of BL21(DE3) cells carrying expression constructs were inoculated into 200 ml of Luria-Bertani medium with 50 μg of kanamycin/ml and cultured at 37°C with vigorous shaking. The cells were grown to an optical density at 600 nm of 0.4 to 0.6, induced with 0.4 mM isopropylthioglucoside for 2 h, collected by centrifugation, washed once with ice-cold 50 mM Tris-HCl (pH 7.5)–10% sucrose, and suspended in 9 ml of lysis buffer (20 mM sodium phosphate [pH 7.8], 0.3 M NaCl, and protease inhibitors). The protease inhibitors contained 0.25 mM phenylmethylsulfonyl fluoride (PMSF), 50 μM EGTA, 0.2 μg of leupeptin/ml, and 0.1 μg of antipain/ml. The suspension was incubated on ice for 15 min with 1 mg of lysozyme/ml, frozen in liquid nitrogen, and thawed at 4°C. After three cycles of freezing and thawing, the sample was centrifuged in a Beckman 70.1 Ti rotor at 60,000 rpm for 30 min at 4°C. The supernatant of high-speed centrifugation was pooled as a lysate, and proteins were purified as described below.

**Purification of rhHR23 proteins.** During purification, the column behavior of rhHR23 proteins was monitored by sodium dodecyl sulfate-polyacrylamide gel electrophoresis (SDS-PAGE) and Coomassie brilliant blue staining. All purification steps described below were carried out at 4°C. To purify rhHR23B, the bacterial lysate was loaded on a phosphocellulose column (Whatman P11) equilibrated with buffer A (25 mM Tris-HCl [pH 7.5], 1 mM EDTA, 10% glycerol, 0.01% Triton X-100, 1 mM dithiothreitol, 0.1 mM PMSF) containing 50 mM NaCl. After the column was washed with the same buffer, flowthrough fractions were collected and brought to 35% saturation of ammonium sulfate (0.194 g of solid/ml). The precipitates were collected by centrifugation (20,000 × *g*, 30 min), redissolved in 10 ml of buffer B (25 mM Tris-HCl [pH 7.5], 1 mM EDTA, 1 mM dithiothreitol, 0.1 mM PMSF), and then dialyzed against buffer B containing 0.8 M ammonium sulfate. After the removal of insoluble materials by centrifugation, the dialysate was loaded on a butyl Sepharose 4FF column (Pharmacia) equilibrated with buffer B containing 0.8 M ammonium sulfate. The column was washed with the same buffer, and bound proteins were eluted with 100 ml of a decreasing (from 0.8 to 0 M) ammonium sulfate gradient. rhHR23B was eluted during the last part of the gradient. The fractions containing rhHR23B were pooled and directly loaded on a hydroxyapatite column (Bio-Gel HTP; Bio-Rad) equilibrated with buffer A containing 0.35 M KCl. After the column was washed with the same buffer, bound proteins were eluted with 45 ml of 0 to 0.25 M potassium phosphate (pH 7.5) gradient in buffer A containing 0.35 M KCl. rhHR23B was eluted at around 0.15 M potassium phosphate, but little further purification was achieved by this step. The peak fractions were dialyzed against buffer A containing 0.1 M NaCl and then loaded on a fast-performance liquid chromatography (FPLC) Mono Q HR5/5 column (Pharmacia) equilibrated with the same buffer. The proteins were eluted with 15 ml of 0.1 to 0.4 M NaCl gradient in buffer A. The peak fractions of rhHR23B were stored at −80°C.

For purification of rhHR23A, the crude extract was adjusted to 0.1 M NaCl and loaded on a Q-Sepharose fast flow column (Pharmacia) equilibrated with buffer A containing 0.1 M NaCl instead of phosphocellulose, because the phosphocellulose step of rhHR23B purification was not as effective. Bound proteins were eluted with 100 ml of a 0.1 to 0.5 M NaCl gradient in buffer A. Fractions containing rhHR23A (~0.3 M NaCl) were adjusted to 40% saturation of ammonium sulfate (0.226 g of solid/ml), and resulting precipitates were dissolved in 5 ml of buffer B. After dialysis against buffer B containing 0.8 M ammonium sulfate, rhHR23A was further purified by successive chromatographic steps with butyl Sepharose, hydroxyapatite, and FPLC Mono Q, as described above for rhHR23B purification.

**Purification of His-tagged proteins.** The lysate containing His-tagged proteins was adjusted to 20 mM imidazole and loaded onto nickel-chelating Sepharose columns equilibrated with buffer C (20 mM sodium phosphate [pH 7.8] and protease inhibitors) containing 0.3 M NaCl and 20 mM imidazole. The column was washed with the same buffer, and bound materials were eluted with buffer C containing 0.3 M NaCl and 60, 90, and 250 mM imidazole, sequentially. The His-tagged proteins eluted at 250 mM imidazole. In the case of rhHR23B(277-332)His, the protein was eluted from a nickel-chelating Sepharose column with a linear gradient of imidazole from 20 to 250 mM in buffer C containing 0.3 M NaCl. After the protein-rich fractions were pooled, glycerol was added to a concentration of 10% and the fractions were stored at −80°C until use. The concentrations of His-tagged proteins were 0.5 to 2.5 mg/ml and were diluted with buffer D (20 mM potassium phosphate [pH 7.5], 0.3 M KCl, 50% glycerol, 0.2 mg of bovine serum albumin/ml, and protease inhibitors) just before use.

**Preparation of other NER proteins.** The rhXPC was expressed in insect Sf9 cells infected with recombinant baculovirus expressing rhXPC and purified as described previously (28). HeLa XPC-hHR23B complex was purified as de-

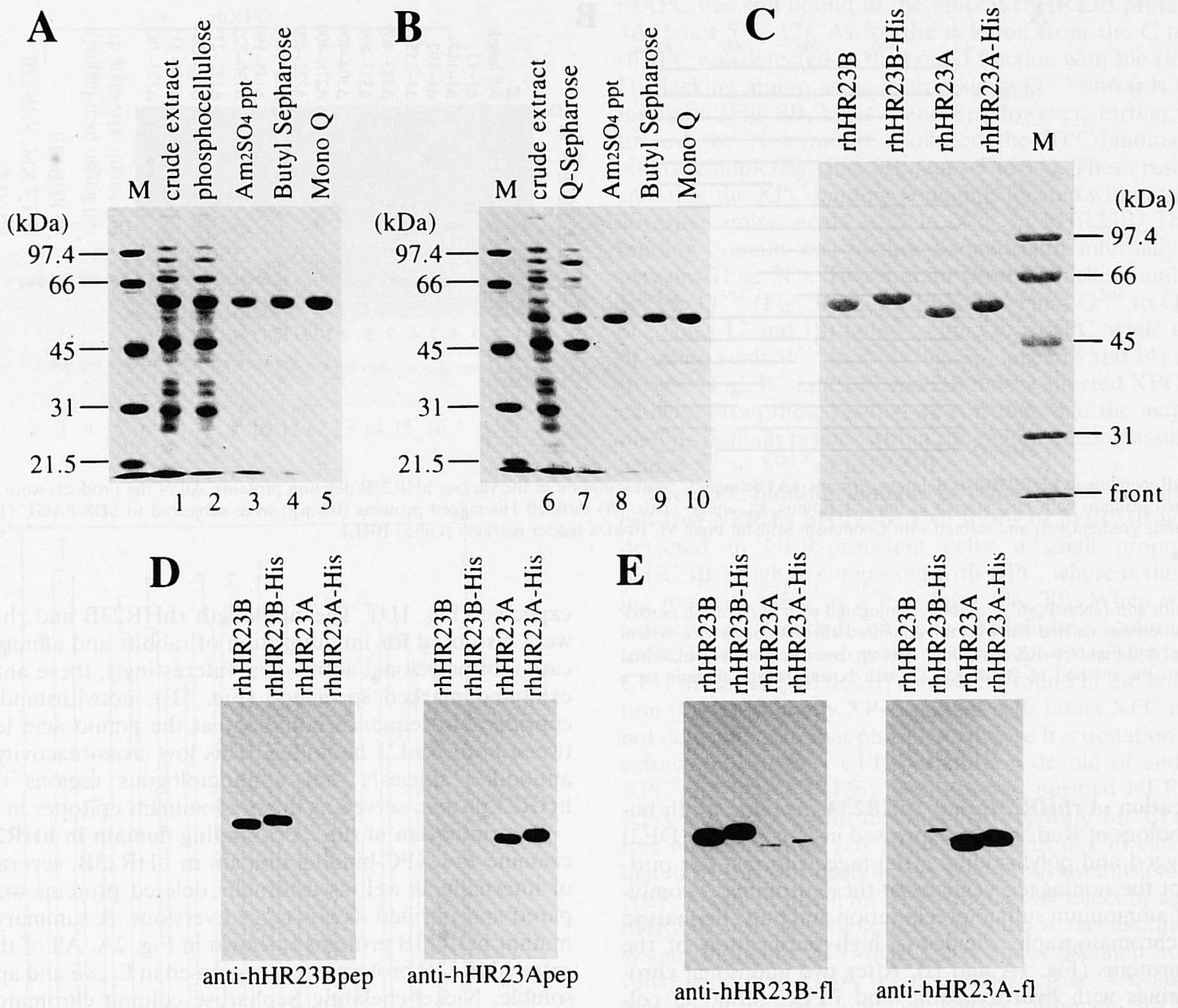



FIG. 1. Purified recombinant hHR23 proteins and specificities of antibodies. (A and B) Purification of rhHR23B (A) and rhHR23A (B). Protein samples from each purification step were subjected to SDS-PAGE (8% polyacrylamide gel) and stained with Coomassie brilliant blue. The amounts of proteins loaded were as follows: 10 μg (lanes 1 and 2), 2 μg (lane 3), 1 μg (lanes 4 and 5), 10 μg (lane 6), 5 μg (lane 7), 1.6 μg (lane 8), and 1 μg (lanes 9 and 10). M, low-molecular-mass marker (Bio-Rad). (C) Purified His-tagged and nontagged rhHR23 proteins (2 μg) were subjected to SDS-PAGE (9% polyacrylamide gel) and stained with Coomassie brilliant blue. (D and E) Purified proteins indicated (20 ng each) were subjected to immunoblot analysis with the antibodies shown below the panels. ppt, precipitate.

scribed previously (17). The replication protein A (RPA) (8) and proliferating cell nuclear antigen (PCNA) (11) were purified from 293 and HeLa cells as described previously.

**rhXPC-binding assay.** rhXPC (0.25 pmol) was mixed with His-tagged hHR23 proteins (2.5 pmol) in 60 μl of binding buffer (20 mM sodium phosphate buffer [pH 6.8], 0.3 M NaCl, 20 mM imidazole, 0.2 mg of bovine serum albumin/ml, 0.1% Triton X-100, and protease inhibitors). After incubation on ice for 1 h, 20 μl of a twofold suspension of nickel-chelating Sepharose with binding buffer was added and mixed at 4°C for 1 h. The mixture was centrifuged, and the supernatant was stored as unbound material. The resin was washed with binding buffer, and bound materials were eluted with binding buffer containing 250 mM imidazole. The unbound and bound materials were subjected to SDS-PAGE followed by immunoblot analysis.

**Fractionation of XP-C cell extracts.** Whole-cell extracts from XP4PASV cells for cell-free repair reactions were prepared and fractionated as described previously (28). The extract was loaded onto a phosphocellulose column equilibrated with buffer E (25 mM HEPES-KOH [pH 7.9], 1 mM EDTA, 10% glycerol, 0.01% Triton X-100, 1 mM dithiothreitol, and protease inhibitors) containing 0.2 M KCl. The column was washed with the same buffer, and the adsorbed proteins were eluted with buffer E containing 1 M KCl. The protein-rich fractions from flowthrough (column fraction I [CFI]) and the eluate (CFII) were concentrated by dialysis against buffer E containing 0.1 M KCl and 20% (wt/vol) sucrose and stored at −80°C.

**Cell-free DNA repair assay.** The standard reaction mixture (20 μl) contained 40 mM creatine phosphate-Tris (pH 7.7), 1 mM dithiothreitol, 5 mM $MgCl_2$, 2 mM ATP, 50 μM (each) dATP, dGTP, and dTTP, 10 μM dCTP, 0.5 μg of phosphocreatine kinase (Sigma type I), 6.4 μg of bovine serum albumin, 0.3 μg of nonirradiated pUC19 RFI DNA, UV-irradiated (200 J/$m^2$) simian virus 40 (SV40) minichromosomes (0.3 μg of DNA), 0.5 μg of RPA, CFII from XP4PASV cell extracts (20 μg of protein), and other proteins (XPC, hHR23B, and hHR23B derivatives). After incubation at 30°C for 90 min, PCNA (25 ng) and 37 kBq of [α-$^{32}$P]dCTP were added and the mixtures were further incubated at 30°C for 10 min. DNA was purified from the reaction mixtures, linearized by *Eco*RI digestion, and then electrophoresed in 1% agarose gels as described previously (16, 27). Autoradiography was performed at −80°C with Fuji New RX X-ray film. The incorporation of radioactive materials into SV40 DNA was quantified with the Fuji BAS2500 bioimaging analyzer.

**Antibodies.** The anti-XPC and anti-hHR23B-fl antibodies were obtained by immunization of rabbits with rhXPC and glutathione *S*-transferase–hHR23B, respectively, and purified by affinity chromatography as described previously (28). Anti-hHR23A-fl was obtained by immunization of rabbits with rhHR23A-His and purified with an affinity column. Anti-hHR23Bpep and anti-hHR23Apep were obtained by immunization of rabbits with synthesized polypeptides and were affinity purified. The amino acid sequences of the polypeptides used for immunization are DRESQAVVDPPQAASTGAPQ for anti-hHR23Bpep and REDKSPSEESAPTTSP for anti-hHR23Apep.

**Other methods.** SDS-PAGE was performed as described by Laemmli (10). Tricine–SDS-PAGE was performed as described by Schagger and von Jagow (24). For immunoblot analyses, proteins separated by electrophoresis were transferred onto polyvinylidene difluoride membranes (Immobilon-P; Millipore) at 8 V/cm for 12 h in ice-cold transfer buffer (50 mM Tris, 38.4 mM glycine, 0.01% SDS, 15% methanol). The membranes were successively incubated in blocking buffer (5% skim milk in 25 mM Tris-HCl [pH 7.5], 0.15 M NaCl, 0.1% Tween 20), with antibody (anti-XPC, anti-hHR23B, or anti-hHR23A) in blocking buffer,

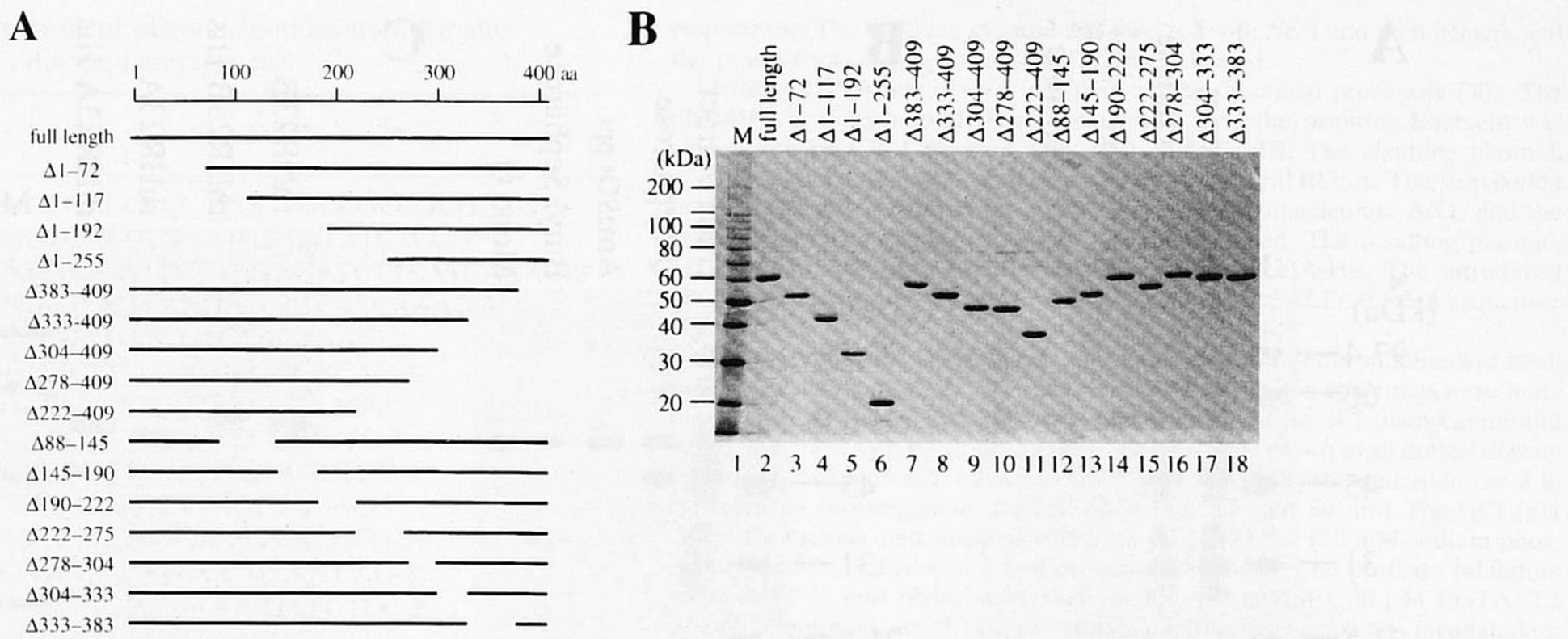



FIG. 2. Recombinant hHR23B-His deletion proteins. (A) Schematic representation of the various hHR23B deletion proteins. All of the products were expressed as His-tagged proteins with $His_6$ stretch at the C terminus. aa, amino acids. (B) Purified His-tagged proteins (0.5 μg) were subjected to SDS-PAGE (10 to 16% polyacrylamide gradient gel) and stained with Coomassie brilliant blue. M, 10-kDa ladder markers (Gibco BRL).

and then with anti-rabbit $F(ab')_2$ antibody conjugated with horseradish peroxidase. Detection was carried out with the enhanced chemiluminescence system (Amersham) and Fuji New RX X-ray film. Protein concentration was measured according to the method of Bradford (1), with bovine serum albumin as a standard.

## RESULTS

**Purification of rhHR23B and rhHR23A proteins.** Both human homologs of Rad23 were expressed in *E. coli* BL21(DE3) as nontagged and polyhistidine (His)-tagged forms. For purification of the nontagged versions of these proteins, a combination of ammonium sulfate precipitation and butyl Sepharose column chromatography yielded a high purification of the hHR23 proteins (Fig. 1A and B). After two additional chromatographies with hydroxyapatite and FPLC Mono Q columns, both hHR23 proteins were purified to near homogeneity. The His-tagged hHR23 proteins were purified by nickel-chelating Sepharose columns and migrated slightly more slowly than their nontagged counterparts on SDS-PAGE (Fig. 1C). These purified rhHR23 proteins were used in the following experiments as well as those described in the accompanying paper (29). Antibodies raised against unique synthetic polypeptides of the individual hHR23 proteins recognized each of the purified rhHR23 proteins in a specific manner, as expected (Fig. 1D). The full-length rhHR23B and rhHR23A were also used for immunization of rabbits and affinity purification of polyclonal antibodies. Interestingly, these antibodies exhibited marked specificity (Fig. 1E), notwithstanding the considerable sequence homology at the amino acid level between both Rad23 homologs. This low cross-reactivity of the antibodies suggests that nonhomologous regions of each hHR23 protein served as the predominant epitopes in rabbits.

**Determination of the XPC-binding domain in hHR23B.** To examine the XPC-binding domain in hHR23B, several series of internally as well as externally deleted proteins were prepared and purified as His-tagged versions. A summary of the mutant hHR23B proteins is shown in Fig. 2A. All of the truncated hHR23B proteins were expressed in *E. coli* and appeared soluble. Nickel-chelating Sepharose column chromatography was found to be an effective purification step for the truncated as well as the intact proteins, as shown in Fig. 2B. To test the XPC-binding activity of the mutant hHR23B, the His-tagged rhHR23B (full-length or truncated) proteins were incubated with rhXPC, which was purified from insect cells infected with a recombinant baculovirus (28). The His-tagged proteins were precipitated with nickel-chelating Sepharose beads, and the presence of rhXPC in supernatant (unbound) and precipitate (bound) fractions was assessed by immunoblotting. The His-

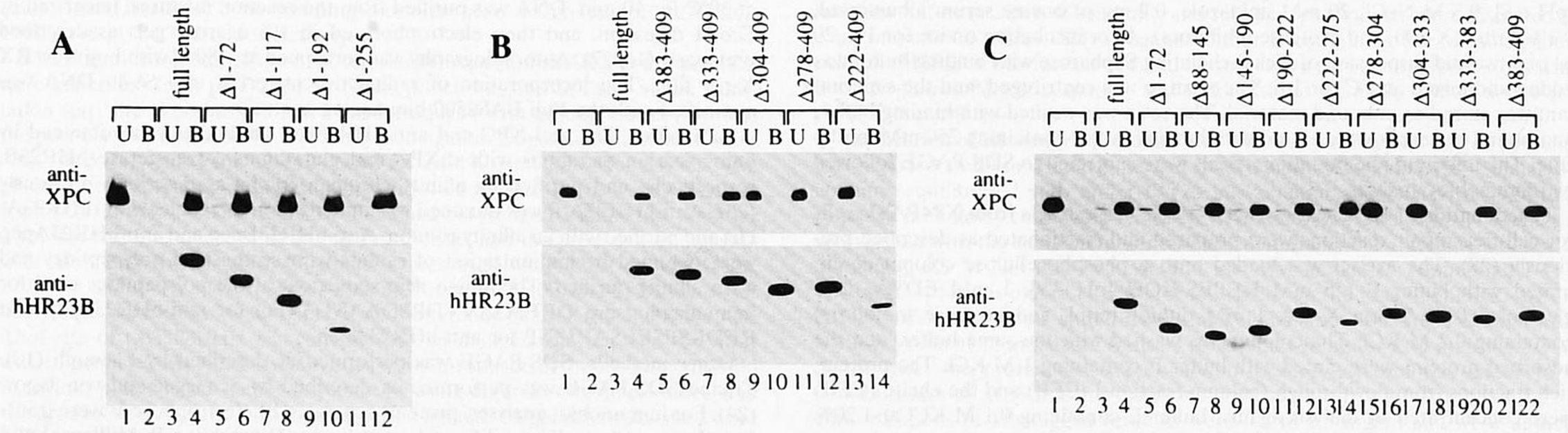
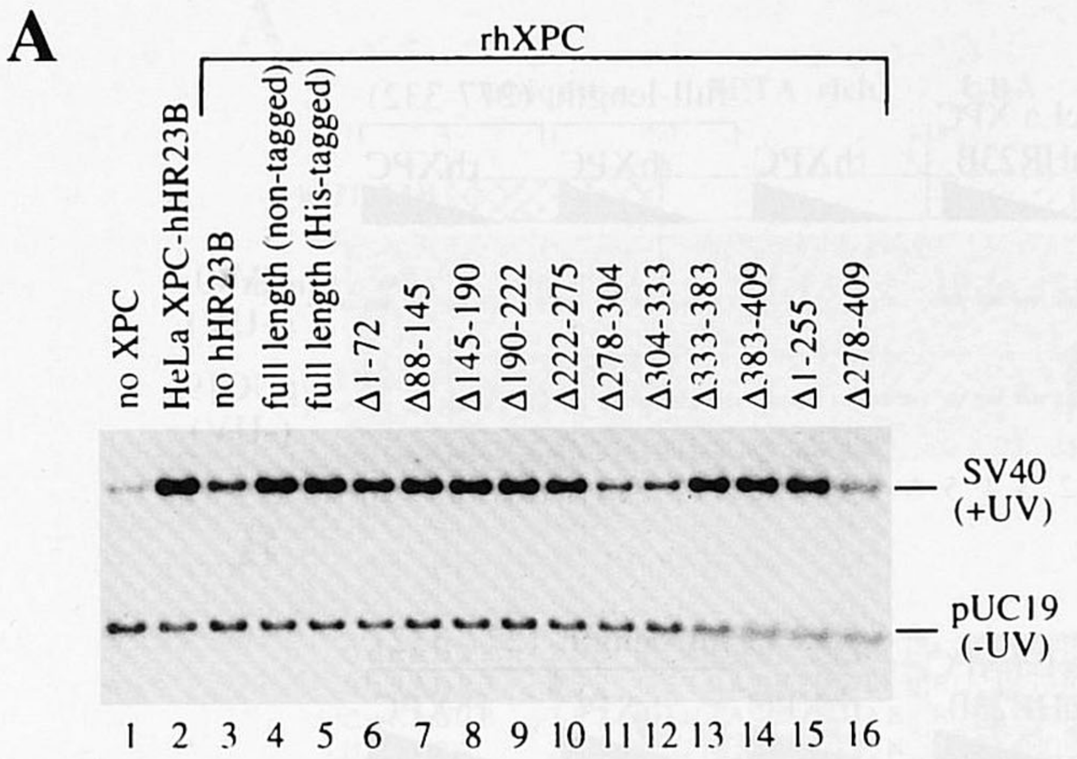



FIG. 3. rhXPC-binding activities of truncated rhHR23B-His proteins. rhXPC (0.25 pmol) and 2.5 pmol of His-tagged proteins were mixed, and materials unbound (U) and bound (B) to nickel-chelating Sepharose beads were recovered as described in Materials and Methods. A portion (20 μl) of each sample was subjected to SDS-PAGE (7.5 to 12.5% polyacrylamide gradient gels), and proteins were detected by immunoblotting with anti-XPC and anti-hHR23B-fl antibodies. (A) Deletions from N terminus. (B) Deletions from C terminus. (C) Internal deletions.



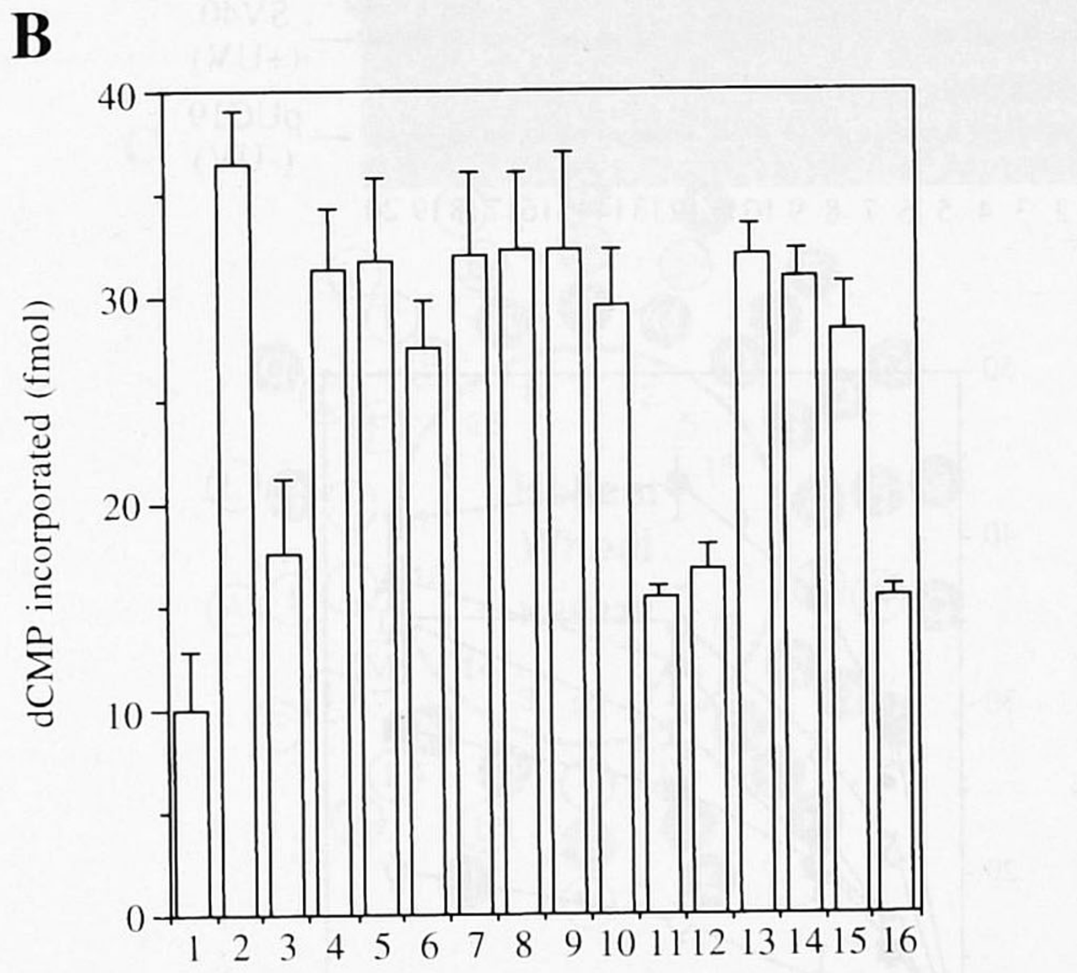
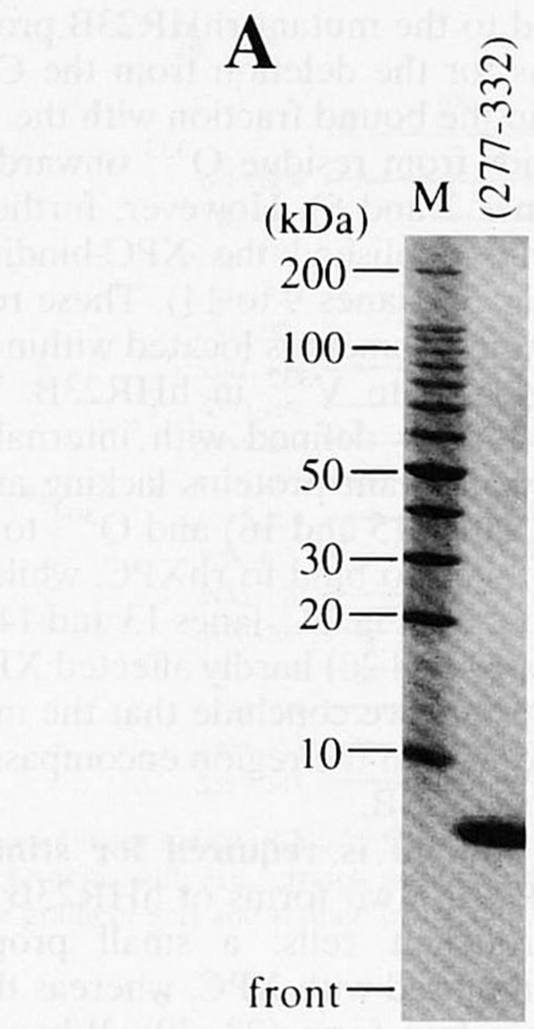



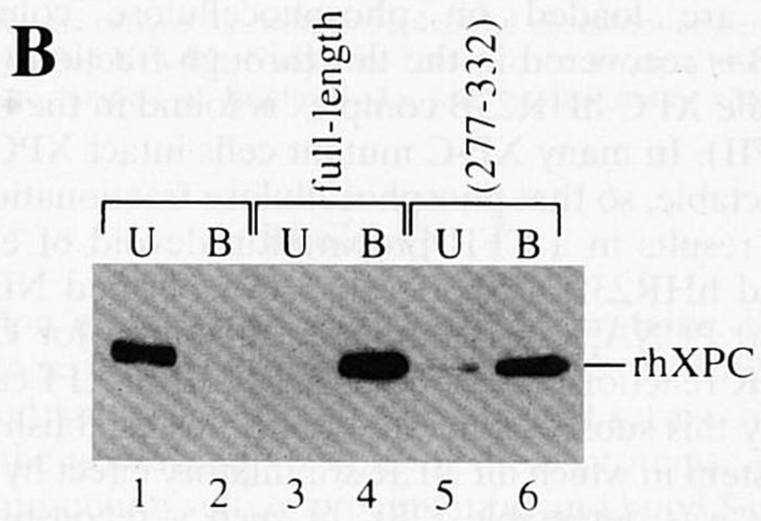

FIG. 4. Activities of the rhHR23B proteins containing various deletions in the reconstituted NER system. UV-irradiated SV40 minichromosomes were incubated with CFII from XP-C cells plus RPA with 20 fmol of XPC-hHR23B complex purified from HeLa cells (lane 2) or 20 fmol of rhXPC (lanes 3 to 16) in the presence (lanes 4 to 16) or absence (lane 3) of 100 fmol of rhHR23B (lane 4) or its derivatives (lanes 5 to 16) under standard assay conditions. After 90 min of incubation at 30°C, PCNA and [$\alpha$-$^{32}$P]dCTP were added and the mixture was incubated for another 10 min. DNA samples were purified, digested with *Eco*RI, and then subjected to agarose gel electrophoresis. (A) Autoradiogram of the gel. (B) Incorporation of dCMP into SV40 minichromosomes calculated and depicted as a graph. The average values and standard errors were calculated from three independent experiments. When unirradiated SV40 minichromosomes were used as a negative control, they reproducibly gave dCMP incorporations of 3.5 ± 1.5 fmol. The numbers of the bars correspond to those of the lanes in panel A.

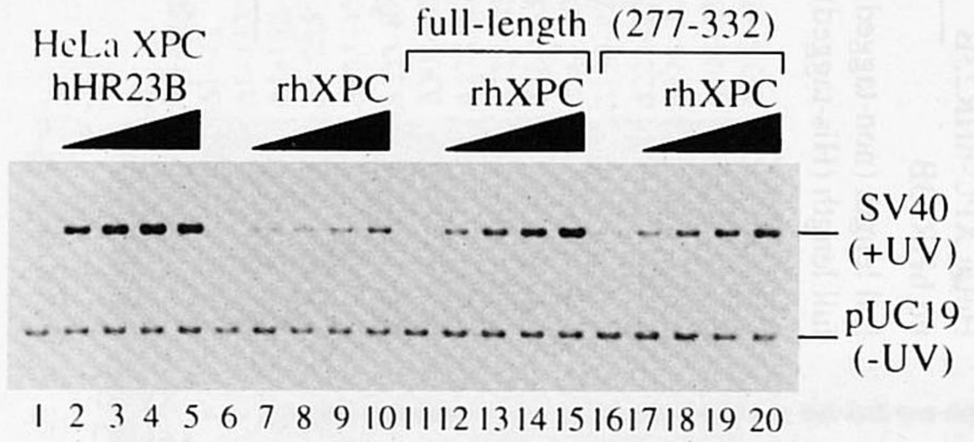
tagged rhHR23B proteins were also detected by immunoblotting with the antibody raised against the full-length protein. Some truncated proteins were recognized only weakly by the antibody, suggesting that the missing regions contained important antigenic determinants (see Discussion). As shown in Fig. 3, rhXPC itself did not bind to nickel-chelating Sepharose (Fig. 3, lanes 1 and 2). In the presence of full-length rhHR23B-His, however, a majority of the rhXPC was coprecipitated (Fig. 3, lanes 3 and 4), indicating that the two proteins formed a physical complex. The rhHR23B-His proteins with various deletions were also tested for XPC-binding activity. When no more than 255 amino acids were deleted from the N terminus, rhXPC was still bound to the mutant rhHR23B proteins (Fig. 3A, lanes 5 to 12). As for the deletion from the C terminus, rhXPC was detected in the bound fraction with the rhHR23B-His lacking amino acids from residue $Q^{333}$ onwards to the C terminus (Fig. 3B, lanes 7 and 8). However, further deletion toward the N terminus abolished the XPC-binding activity almost completely (Fig. 3B, lanes 9 to 14). These results indicate that the XPC-binding domain is located within the region covering amino acids $A^{256}$ to $V^{332}$ in hHR23B. The XPC-binding domain was further defined with internally deleted proteins (Fig. 3C). Two mutant proteins lacking amino acids $F^{278}$ to $Q^{304}$ (Fig. 3C, lanes 15 and 16) and $Q^{304}$ to $Q^{333}$ (Fig. 3C, lanes 17 and 18) failed to bind to rhXPC, while deletions of amino acids $V^{222}$ to $P^{275}$ (Fig. 3C, lanes 13 and 14) and $Q^{333}$ to $V^{383}$ (Fig. 3C, lanes 19 and 20) hardly affected XPC-binding activity. From these results, we conclude that the major XPC-binding domain resides within the region encompassing amino acids $F^{278}$ to $V^{332}$ of hHR23B.

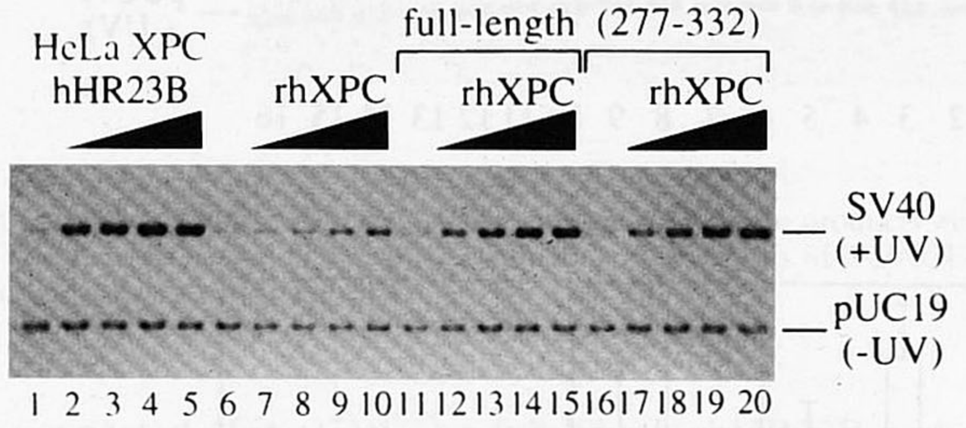
**The XPC-binding domain is required for stimulation of cell-free NER by hHR23B.** Two forms of hHR23B have been detected in NER-proficient cells: a small proportion of hHR23B is tightly complexed with XPC, whereas the remaining majority exists as a free form (28, 30). When whole-cell extracts are loaded on phosphocellulose columns, free hHR23B is recovered in the flowthrough fraction (designated CFI) while XPC-hHR23B complex is found in the bound fraction (CFII). In many XP-C mutant cells intact XPC protein is not detectable, so that phosphocellulose fractionation of XP-C extracts results in a CFII preparation devoid of endogenous XPC and hHR23B (28). Because two purified NER factors, RPA and PCNA, can functionally substitute for CFI in cell-free NER reactions (26, 28), all hHR23B in CFI can be eliminated by this substitution. We could thus establish an in vitro NER system in which an NER-stimulatory effect by exogenous hHR23B was detectable (28). In such a reconstituted NER system, authentic XPC-hHR23B complex (isolated from HeLa cells) supported DNA repair synthesis (Fig. 4A, lane 2). In agreement with our previous studies, although rhXPC alone displayed some NER activity in this system, the activity was significantly stimulated by coaddition of rhHR23B (Fig. 4A, lanes 3 and 4). rhHR23B-His showed a level of the XPC-stimulating activity similar to that of the nontagged protein (Fig. 4A, compare lanes 4 and 5), indicating that the His tag does not disturb the function of rhHR23B in the in vitro NER reaction. When various truncated rhHR23B-His proteins were assayed for stimulatory activity, three mutant proteins lacking the XPC-binding activity turned out to be inactive (Fig. 4A and B, lanes 11, 12, and 16 and corresponding bars). A mutant protein lacking the N-terminal ubiquitin-like sequence (i.e., Δ1-72) reproducibly showed slightly lower activity than the full-length protein (Fig. 4A, lane 6), but deletion of other regions hardly affected the stimulatory activity of rhHR23B. Thus, the XPC-binding domain is important for the stimulation of in vitro NER reactions by hHR23B and resides between amino acid residues 278 and 333.

**The XPC-binding domain of hHR23B can stimulate NER by itself.** The correlation between the XPC-binding and -stimulatory activities of the mutant rhHR23B proteins prompted us to investigate whether this domain alone is necessary and sufficient for XPC binding and stimulation. For this purpose, a small polypeptide which consists of amino acids $E^{277}$ to $V^{332}$ of hHR23B was expressed in *E. coli* in a His-tagged form and purified with a nickel-chelating Sepharose column (Fig. 5A). The purified protein, rhHR23B(277-332)His, was tested for XPC-binding and NER-stimulatory activities. As shown in Fig. 5B, rhXPC was coprecipitated with rhHR23B(277-332)His, in-



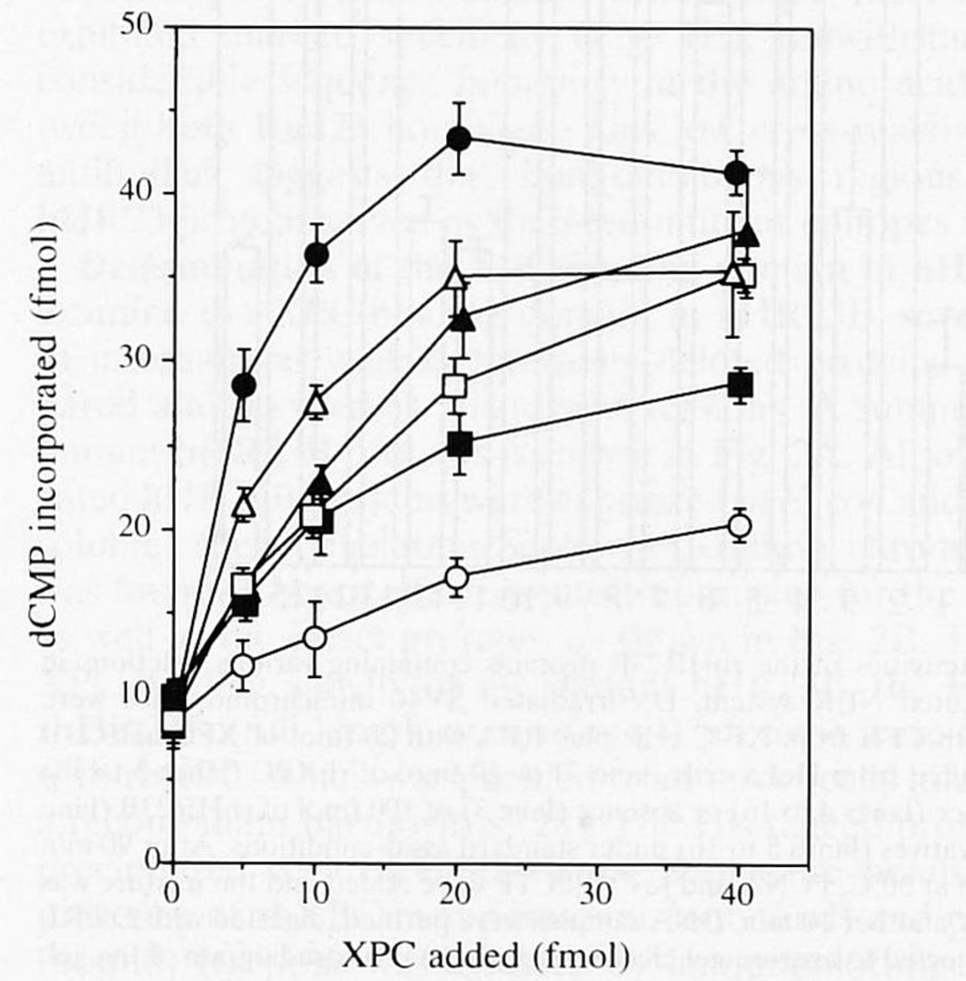
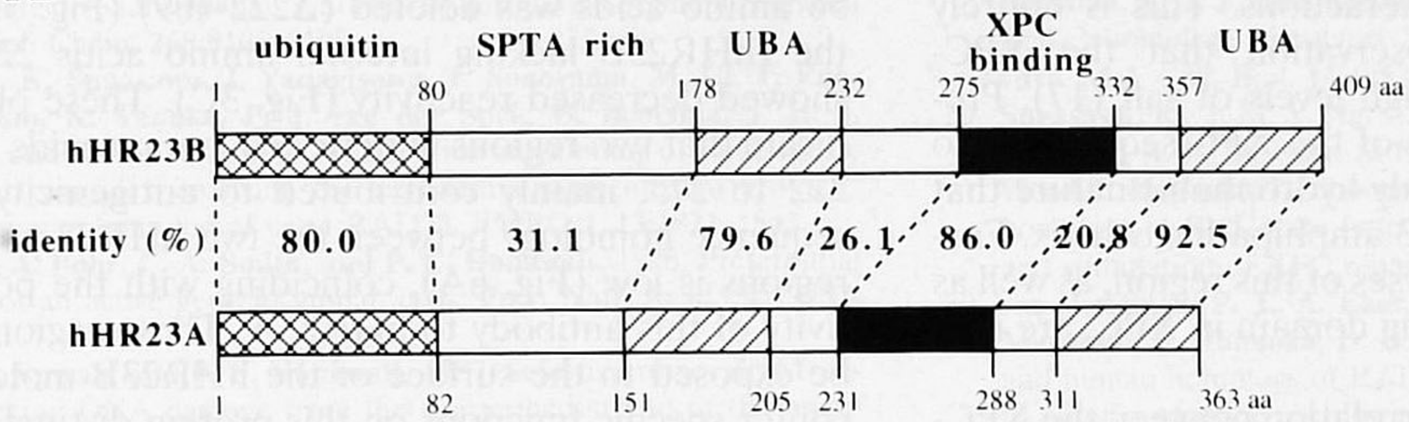
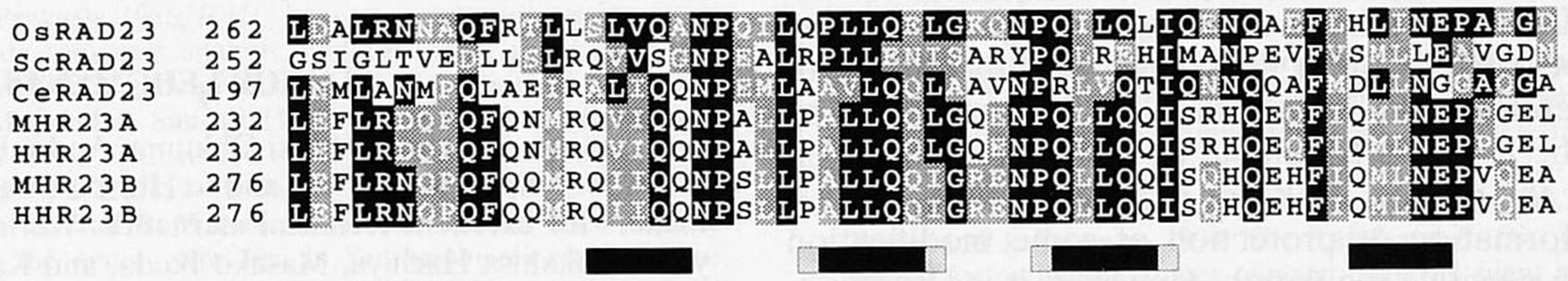




FIG. 5. Characterization of rhHR23B(277-332)His protein. (A) A portion (2 μg) of purified rhHR23B(277-332)His was subjected to Tricine–SDS-PAGE (16.5% polyacrylamide gel) and stained with Coomassie brilliant blue. M, 10-kDa ladder markers (Gibco BRL). (B) rhXPC-binding assay. rhXPC (0.25 pmol) was mixed with 2.5 pmol of rhHR23B-His (lanes 3 and 4) or rhHR23B(277-332)His (lanes 5 and 6), and materials unbound (U) and bound (B) to nickel-chelating Sepharose beads were subjected to SDS-PAGE followed by immunoblotting with anti-XPC antibodies. (C to E) NER assay in the reconstituted system. (C and D) UV-irradiated SV40 minichromosomes were incubated with CFII from XP-C cells plus RPA with increasing amounts of XPC-hHR23B complex purified from HeLa cells (lanes 1 to 5) or rhXPC (lanes 6 to 20) in the presence (lanes 11 to 20) or absence (lanes 6 to 10) of rhHR23B-His (lanes 11 to 15) or rhHR23B(277-332)His (lanes 16 to 20) under standard assay conditions. After 90 min of incubation at 30°C, PCNA and [α-$^{32}$P]dCTP were added and the mixture was incubated for another 10 min. DNA samples were purified, digested with *Eco*RI, and then subjected to agarose gel electrophoresis. Amounts of HeLa XPC-hHR23B or rhXPC were 5 fmol (lanes 2, 7, 12, and 17), 10 fmol (lanes 3, 8, 13, and 18), 20 fmol (lanes 4, 9, 14, and 19), and 40 fmol (lanes 5, 10, 15, and 20), respectively. Autoradiograms of the gels are shown. Fifty (C) or 200 (D) fmol of rhHR23B-His and rhHR23B(277-332)His were used. (E) Incorporation of dCMP into SV40 minichromosomes calculated and depicted as a graph. The average values and standard errors were calculated from two independent experiments. Symbols: filled circle, HeLa XPC-hHR23B; open circle, rhXPC alone; filled triangle, rhXPC with 50 fmol of rhHR23B-His; open triangle, rhXPC with 200 fmol of rhHR23B-His; filled square, rhXPC with 50 fmol of rhHR23B(277-332)His; open square, rhXPC with 200 fmol of rhHR23B(277-332)His.

dicating that this domain alone is enough for binding to XPC. Furthermore, these findings indicate that this part of the hHR23B protein, when overproduced in *E. coli*, adopts the correct conformation to permit interaction with its partner. At the same time, this argues against the possibility that the truncated hHR23B versions lacking XPC-binding activity were negative because of improper folding. When equimolar amounts of the full-length and mutant proteins were used, however, slightly more rhXPC remained in the unbound fraction with the severely truncated protein (Fig. 5B, compare lanes 3 and 5), suggesting that the affinity of rhHR23B(277-332)His for XPC may be somewhat lower than that of the



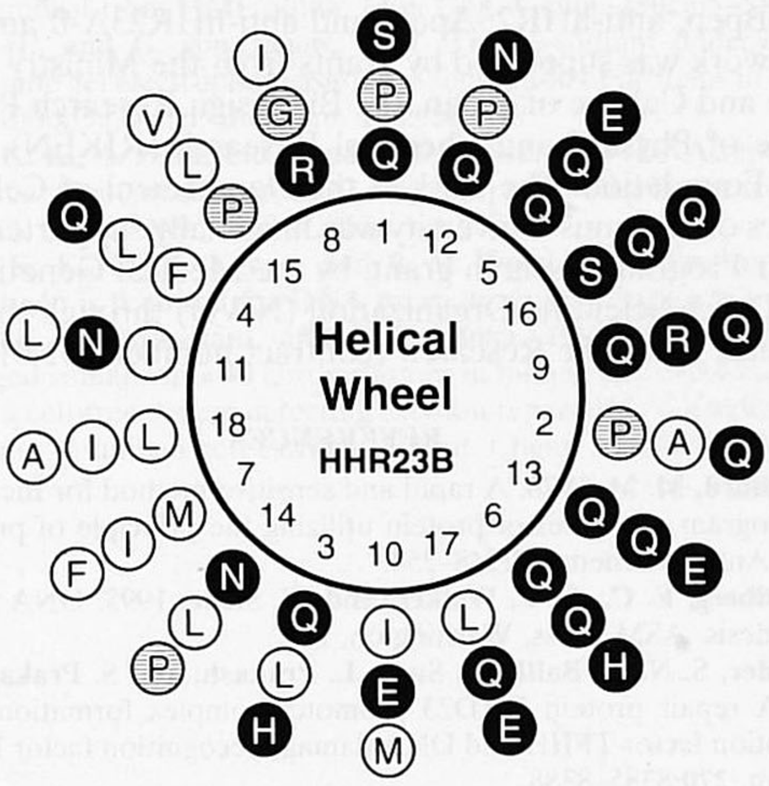




FIG. 6. Amino acid sequence and predicted structure of the XPC-binding domain. (A) Amino acid sequence homology between hHR23B and hHR23A proteins. Four highly homologous domains spaced by three nonidentical regions were found. The identity of each pair of domains between the hHR23B and hHR23A proteins is represented by a percentage. ubiquitin, ubiquitin-like region; SPTA rich, four kinds of amino acids (S, P, T, and A) are predominant in this region; UBA, ubiquitin-associated domain; aa, amino acids. (B) Comparison of amino acid sequences of the XPC-binding domains among Rad23 homologs. Identical and conservative amino acids are highlighted by solid and shaded boxes, respectively. Positions of the repetitive sequence, (P)QLLQQ(I), are shown below the sequence alignment. Os, *Oryza sativa*; Sc, *S. cerevisiae*; Ce, *Caenorhabditis elegans*. (C) Helical-wheel presentation of the amino acid sequence of the XPC-binding domain in hHR23B. Hydrophobic and hydrophilic amino acids are represented by solid and open circles, respectively.

full-length protein. The NER-stimulatory activities of the full-length and mutant rhHR23B were compared in the reconstituted NER system by titrating rhXPC in the presence of 50 fmol (Fig. 5C) or 200 fmol (Fig. 5D) of these proteins. When limited amounts (50 fmol) of the proteins were utilized, rhXPC activity was stimulated to a lesser extent by rhHR23B(277-332)His than by the full-length protein (see also Fig. 5E). However, 200 fmol of the mutant rhHR23B achieved the same level of XPC stimulation as that displayed by 50 fmol of the full-length protein, indicating that this domain (i.e., amino acids 277 to 332) is essentially sufficient for the stimulation of in vitro NER by hHR23B.

## DISCUSSION

**The XPC-binding domain in hHR23B.** In this study we identified the XPC-binding domain in hHR23B and showed that the domain plays an important role in the NER stimulation exerted by hHR23B. We have previously identified another human Rad23 homolog, designated hHR23A (17). The two human Rad23 homologs share a remarkable homology, especially in four regions, as depicted in Fig. 6A. From the N terminus, the first homologous region is the ubiquitin-like sequence whereas the second and fourth regions are so-called ubiquitin-associated domains (7, 31). The presence of these characteristic motifs strongly suggests that Rad23 and its mammalian homologs are involved in some aspect of ubiquitin metabolism, although the precise role of the ubiquitination pathway in NER remains to be elucidated. Intriguingly, the third homologous region corresponds precisely with the XPC-binding domain identified in these studies. Very recently, Li et al. have attempted to analyze the interaction between XPC and hHR23 proteins by using the yeast two-hybrid system (15). However, since only a C-terminal set of deletion mutants was made, the XPC-binding domain of hHR23B that we have identified here was missed.

The identification of the XPC-interacting domain responsible for the XPC NER activity in vitro prompted us to look more closely at this sequence. As shown in Fig. 6B, this region is highly conserved in all known Rad23 homologs and has a partly repetitive character containing various versions of the sequence (P)QLLQQ(I). Screening with a subselection of the Brookhaven database containing all known crystal structures at restricted stringency revealed matches with α-helical segments in catalase and myoglobin (Brookhaven PDB entries 1CAE and 2MM1, respectively), strongly suggesting that at least a part of this domain adopts an α-helical structure. In favor of this interpretation is our finding that the sequence discloses a highly amphipathic character when represented as a helical wheel (Fig. 6C). A striking bimodal distribution is apparent due to the clustering of the hydrophilic and hydrophobic residues on opposite sides of the helix. A similar picture emerges with all homologs of Rad23. Such a pronounced clustering is very unlikely to be coincidence. Although it cannot be ruled out that the XPC-binding domain partly adopts nonhelical structures, a strong prediction can be made that this domain

contains amphipathic helical features and that the affinity for XPC is based on hydrophobic interactions. This is entirely consistent with our previous observation that the XPC-hHR23B complex is resistant to high levels of salt (17). Preliminary computer-assisted analysis of the XPC sequence also revealed several regions with a highly hydrophobic nature that could mediate binding to the Rad23 amphipathic α-helix. Further mutational and structural analyses of this region, as well as determination of the hHR23-binding domain in XPC, are now ongoing.

We could observe a consistent correlation between the XPC-binding and NER-stimulatory activities with various mutant rhHR23B proteins. Interestingly, rhHR23B(277-332)His, which contains the XPC-binding domain but lacks most of the other regions, was still able to stimulate NER, although the activity appeared to be slightly lower than that of the full-length rhHR23B. Therefore, the stimulation of in vitro NER by hHR23B may be largely attributable to some direct effect on XPC caused by the binding itself, for instance, activation via a change in conformation or protection of some modification sites involved in negative regulation. However, it is also possible that this small polypeptide possesses still other functions, e.g., promotion of preincision complex assembly, as suggested for yeast Rad23 (3). Furthermore, although rhHR23B(277-332)His was essentially sufficient for binding to XPC, its affinity for XPC seemed to be lower than that of the full-length protein, suggesting that other regions outside the defined XPC-binding domain may be involved in stabilization of the complex.

**Roles of the ubiquitin-like sequence.** As does *S. cerevisiae* Rad23, hHR23B harbors a ubiquitin-like sequence at its N terminus. In yeast cells, this ubiquitin-like sequence was shown to be important for NER functions of Rad23 (35). However, a requirement for the ubiquitin-like sequence in hHR23B was unclear in our cell-free NER system, because the mutant rhHR23B lacking this sequence showed only slightly lower activity than the full-length protein. Very recently, Wang et al. (34) have examined interaction of Rad23 and Rad4 (the yeast XPC counterpart) proteins by using the yeast two-hybrid system. The Rad4-binding domain was mapped near the C terminus of Rad23, and the N-terminal ubiquitin-like sequence was not absolutely required for the interaction. Therefore, it is unlikely that the ubiquitin-like sequence plays a crucial role in complex assembly between Rad4/XPC and Rad23/hHR23B. One possible explanation is that the ubiquitin-like sequence may not be directly involved in the core part of the NER reaction but in some regulatory aspect in vivo which indirectly affects the repair capacity and UV sensitivity of cells. For instance, the ubiquitin-like sequence may control functions of Rad23 and its homologs by affecting turnover of these proteins in vivo, because one of the most extensively characterized functions of ubiquitin is triggering of protein degradation (6).

**Specificity of antibodies.** When we used full-length hHR23A and hHR23B proteins for immunization, the resulting polyclonal antibodies showed marked specificity for each protein despite high homology between the two hHR23 proteins (Fig. 1E). These findings suggest that the most antigenic epitopes reside in nonhomologous regions in each hHR23 protein. In agreement with this, the anti-hHR23B-fl antibody displayed different reactivities for rhHR23B-His proteins lacking various regions. Although the antibody still recognized rhHR23B(Δ1-117), further deletion to the 192nd amino acid markedly reduced the reactivity, which was then completely abolished by deletion of 255 amino acids from the N terminus (Fig. 3A). The antibody reacted with rhHR23B lacking C-terminal 132 amino acids (Δ278-409) to the same extent as the full-length protein, but the reactivity was reduced when a further region of 56 amino acids was deleted (Δ222-409) (Fig. 3B). Moreover, the rhHR23B lacking internal amino acids 222 to 275 also showed decreased reactivity (Fig. 3C). These observations indicate that two regions in hHR23B, amino acids 118 to 192 and 222 to 275, mainly contributed to antigenicity. Amino acid sequence homology between the two hHR23 proteins in these regions is low (Fig. 6A), coinciding with the poor cross-reactivity of this antibody to hHR23A. These regions are likely to be exposed to the surface of the hHR23B molecule and may confer specific functions on this protein distinct from those of hHR23A. Therefore, the antibodies may constitute valuable tools for further studies on the in vitro or in vivo functions of these proteins.

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