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# HHR23B, a Human Rad23 Homolog, Stimulates XPC Protein in Nucleotide Excision Repair In Vitro

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**A protein complex which specifically complements defects of XP-C cell extracts in vitro was previously purified to near homogeneity from HeLa cells. The complex consists of two tightly associated proteins: the XPC gene product and HHR23B, one of two human homologs of the *Saccharomyces cerevisiae* repair gene product Rad23 (Masutani et al., EMBO J. 13:1831–1843, 1994). To elucidate the roles of these proteins in "genome-overall" repair, we expressed the XPC protein in a baculovirus system and purified it to near homogeneity. The recombinant human XPC (rhXPC) protein exhibited a high level of affinity for single-stranded DNA and corrected the repair defect in XP-C whole-cell extracts without extra addition of recombinant HHR23B (rHHR23B) protein. However, Western blot (immunoblot) experiments revealed that XP-C cell extracts contained excess endogenous HHR23B protein, which might be able to form a complex upon addition of the rhXPC protein. To investigate the role of HHR23B, we fractionated the XP-C cell extracts and constructed a reconstituted system in which neither endogenous XPC nor HHR23B proteins were present. In this assay system, rhXPC alone weakly corrected the repair defect, while significant enhancement of the correcting activity was observed upon coaddition of rHHR23B protein. Stimulation of XPC by HHR23B was found with simian virus 40 minichromosomes as well as with naked plasmid DNA and with UV- as well as *N*-acetoxy-2-acetylfluorene-induced DNA lesions, indicating a general role of HHR23B in XPC functioning in the genome-overall nucleotide excision repair subpathway.**

Various environmental agents such as radiation and chemicals cause DNA damage, which may lead to alterations in genetic information. DNA repair plays a crucial role in the prevention of such mutagenesis and consequent carcinogenesis and/or cell death. Nucleotide excision repair (NER) is one of the most important DNA repair pathways because it eliminates a wide variety of base lesions, including UV-induced cyclobutane pyrimidine dimers and [6-4]photoproducts, as well as certain chemical adducts (8). The molecular mechanism of NER in *Escherichia coli* is now understood in detail (9, 13, 21, 39). In this organism, only six proteins, UvrA, UvrB, UvrC, UvrD, DNA polymerase I, and DNA ligase, are sufficient to complete the NER reactions in vitro, whereas a much greater number of gene products are now known to be involved in eukaryotic NER reactions (14). The eukaryotic NER system consists of at least two distinct subpathways. One of these, transcription-coupled repair, preferentially eliminates DNA damage which hinders the advance of RNA polymerases on transcribed strands (4, 12, 25). Damage to the rest of the genome is repaired more slowly and less efficiently for some lesions by the "genome-overall" repair mechanism.

A number of NER gene products have been genetically identified. Xeroderma pigmentosum (XP) is a human autosomal recessive NER disease which is associated with extreme sensitivity to sunlight and a high incidence of skin abnormalities, including cancer. XP cells are hypersensitive to UV irradiation, and complementation analysis by cell fusion has identified at least seven complementation groups, XP-A to XP-G, all of which show defects in early steps of the NER reaction (37). Two genes implicated in XP, *XPA* and *XPC*, have been cloned by transfection of XP cells with mouse genomic DNA (36) or a human cDNA expression library (19). Another set of mammalian NER mutants has been established with cultured UV-sensitive rodent cell lines, in which at least 11 complementation groups have been identified (6). Several human genes which phenotypically correct the UV sensitivity of these mutants have been cloned. Among these, *ERCC* (excision repair cross-complementing rodent repair deficiency) genes *ERCC2*, *ERCC3*, and *ERCC5* appeared to be identical to genes *XPD*, *XPB*, and *XPG*, respectively (7, 28, 47). Additionally, the *ERCC6* gene has been shown to be responsible for complementation group B of another known NER disorder, Cockayne's syndrome (38). The XPG protein was found to be an endonuclease, implicated in the incision on the 3′ side of DNA adducts (27). Another cloned *ERCC* gene product, ERCC1, has recently been suggested to be complexed with protein factors which complement ERCC groups 4 and 11 and XP group F (3, 40) and to be possibly involved in incision on the 5′ side of DNA damage (2). More recently, the XPB and XPD proteins, both DNA helicases, were reported to be subunits of TFIIH, one of the basal transcription factors required for transcriptional initiation by eukaryotic RNA polymerase II (30, 31), revealing a dual functioning of these proteins in basal transcription and NER.

XP group C is unique in that its defect is limited to the genome-overall NER subpathway, transcription-coupled repair being normal (42). The converse is found in Cockayne's

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syndrome (24, 41). By transfection with a human cDNA expression library, Legerski and Peterson isolated a partial cDNA clone which corrected the UV sensitivity of XP-C cells (19). This gene, named *XPC*, shares limited homology with a known *Saccharomyces cerevisiae* NER protein, Rad4. The level of *XPC* mRNA was greatly reduced in most XP-C cell lines tested (19), and nonsense and missense mutations have been identified in several cell lines (20).

Development of a cell-free system which faithfully reproduces the in vivo reactions is a powerful strategy to investigate complicated biochemical processes, and several laboratories have reported the use of such cell-free systems for detection of NER (1, 33, 34, 45, 48). We have also developed a cell-free system which uses whole-cell extracts and UV-irradiated simian virus 40 (SV40) minichromosomes as templates (34). This system reproduced the NER defects of all XP complementation groups (22) and enabled us to identify biochemically a protein factor which corrects the defect in XP-C cell extracts (23). This factor, named XP-C correcting protein, was purified from HeLa cell nuclear extracts and was found to be a tight complex of two polypeptides with apparent molecular masses of 125 and 58 kDa. Cloning and sequence analysis of cDNAs encoding these proteins revealed that the 125-kDa polypeptide is an N-terminally extended version of the *XPC* gene product reported previously (23). The 58-kDa species was identified as one of two human homologs of the *S. cerevisiae* Rad23 NER protein, designated HHR23B (human homolog of Rad23) (23). Both the Rad23 and HHR23B proteins, as well as HHR23A, another human Rad23 cognate, contain ubiquitin-like sequences on their N termini. For *S. cerevisiae*, the importance of this domain in NER function has been suggested genetically (46). Unfortunately, for none of the HHR23 genes have corresponding human or rodent NER mutants been identified. It has been recently reported that yeast Rad23 protein may promote interaction with Rad14 and yTFIIH (10), but the biological significance of complex formation between the XPC and HHR23B proteins and the functions of HHR23B in NER, particularly in the genome-overall repair subpathway, have not yet been clarified. In the present study, recombinant human XPC (rhXPC) and recombinant HHR23B (rHHR23B) proteins were prepared to investigate the roles of these proteins in NER reactions in vitro.

## MATERIALS AND METHODS

**Cell culture and media.** Human 293, XP2OSSV (group A), XP4PASV, and XP3KA (group C) cells were grown at 37°C in Dulbecco's modified Eagle's medium (Nissui) supplemented with 10% fetal bovine serum. A lymphoblastoid cell line (GM2248B) from patient XP3BE (group C) was grown in suspension with RPMI 1640 medium supplemented with 15% fetal bovine serum. An insect cell line, *Spodoptera frugiperda* Sf9, was cultured at 27°C in TMN-FH medium supplemented with 10% heat-inactivated (56°C, 30 min) fetal bovine serum. TMN-FH medium was prepared from Grace's insect cell culture medium (Gibco-BRL), TC yeastolate, and TC lactalbumin hydrolysate (Difco) as described elsewhere (35).

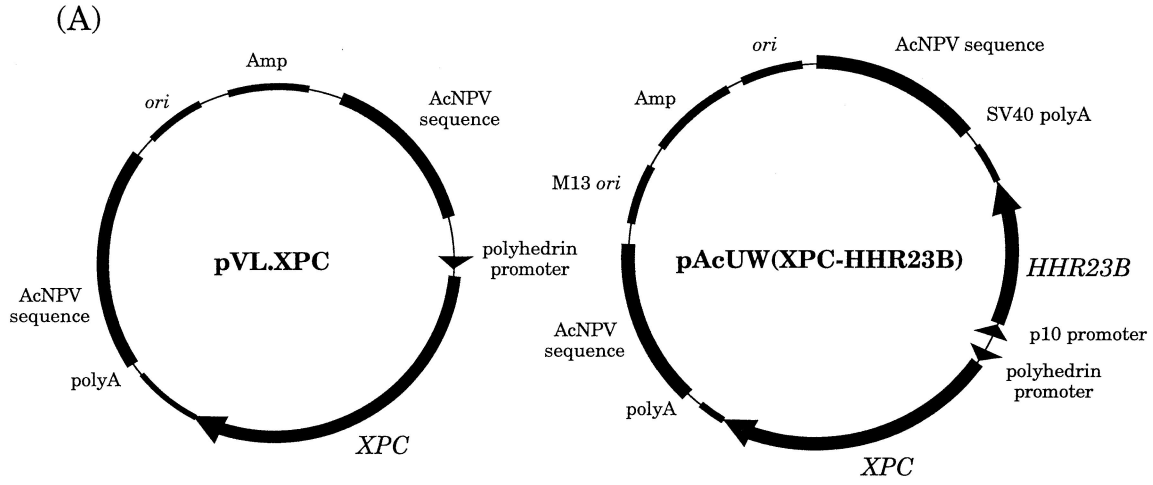
**Construction of recombinant baculoviruses.** Plasmid pBS.XPC, which carries human *XPC* cDNA cloned into the *Not*I site of pBluescript II KS+ (23), was digested with *Not*I, and the resulting 3.6-kb cDNA fragment was isolated. The fragment was inserted into the *Not*I site of a baculovirus transfer vector, pVL1393 (Invitrogen), to generate plasmid pVL.XPC. For simultaneous expression of the XPC and HHR23B proteins, the HHR23B cDNA cloned into pUC19 (23) was digested with *Ban*I and *Bln*I to obtain the 1.5-kb cDNA fragment. After treatment with Klenow fragment and subsequent addition of *Eco*RI linkers, the cDNA fragment was cloned into the *Eco*RI site of pAcUW31 (Clontech), generating plasmid pAcUW.HHR23B. The translational initiation site of XPC in plasmid pBS.XPC was converted to an *Nde*I site with an oligonucleotide, 5′-GACAAGCAACATATGGCTCGGAAAC-3′, and a site-directed mutagenesis system, Mutan-K (Takara Shuzo), essentially according to the method of Kunkel et al. (16). The resulting plasmid, pBS.XPC-*Nde*I, was digested with *Nde*I and *Bln*I to obtain the 3.3-kb XPC cDNA fragment. This cDNA fragment and *Bam*HI-digested pAcUW.HHR23B were blunt ended with Klenow fragment and then ligated to each other to generate plasmid pAcUW(XPC-HHR23B). Sf9 cells were cotransfected with either of the constructed plasmids and BaculoGold DNA (Pharmingen) to produce recombinant viruses, designated vVL.XPC and vAcUW(XPC-HHR23B), respectively. Both viruses expressed the human *XPC* gene under control of the polyhedrin promoter, while transcription of HHR23B in vAcUW(XPC-HHR23B) was driven by the p10 promoter (Fig. 1A).

**Baculovirus infection and extract preparation from the infected cells.** Monolayers of Sf9 cells were infected with the recombinant baculoviruses at room temperature for 1 h at a multiplicity of infection of 5 to 10. At 3 days postinfection, cells were collected by low-speed centrifugation and washed twice with ice-cold phosphate-buffered saline. To examine the total cellular proteins, the cell pellets were lysed in 10 volumes, relative to the packed cell volume (10× PCV), of 2× sodium dodecyl sulfate (SDS) sample buffer (1× concentration is 62.5 mM Tris-HCl [pH 6.8], 1% SDS, 5% glycerol, and 2% 2-mercaptoethanol) and heated to 95°C for 10 min. For fractionation, the pellets of the infected cells were suspended in 8× PCV of ice-cold NP lysis buffer [25 mM Tris-HCl (pH 8.0), 1 mM EDTA, 10% glycerol, 1% Nonidet P-40, 1 mM dithiothreitol, 0.25 mM phenylmethylsulfonyl fluoride, 50 μM ethylene-glycol-bis(β-aminoethyl ether)-*N*,*N*,*N*′,*N*′-tetraacetic acid (EGTA), 0.2 μg of aprotinin per ml, 0.2 μg of leupeptin per ml, 0.1 μg of antipain per ml]. All subsequent steps were carried out at 4°C. After incubation on ice for 30 min, the suspension was centrifuged at 800 × *g* for 10 min to obtain the supernatant fraction, designated as fraction S1. The remaining precipitate was resuspended by gentle pipetting in 8× PCV of NP lysis buffer containing 0.3 M NaCl and then was incubated on ice for 30 min with occasional agitation. The suspension was centrifuged at 12,000 × *g* for 15 min to be divided into the supernatant (S2) and precipitate (P) fractions. To examine the proteins remaining in fraction P, the pellets were homogenized in NP lysis buffer containing 0.3 M NaCl by sonication.

**Purification of recombinant proteins from insect cells.** For purification of recombinant proteins, 10 150-mm-diameter culture dishes of Sf9 cells were routinely infected. Behavior of the recombinant proteins was monitored by SDS-polyacrylamide gel electrophoresis (PAGE) and Western blot (immunoblot) analysis with antibodies raised against each protein (described below). For purification of the rhXPC-HHR23B protein complex, fraction S2 prepared from vAcUW(XPC-HHR23B)-infected Sf9 cells was loaded onto a phosphocellulose column (Whatman P11 [6 ml]) equilibrated with buffer A (25 mM Tris-HCl [pH 7.5], 1 mM EDTA, 10% glycerol, 0.01% Triton X-100, 1 mM dithiothreitol, 0.25 mM phenylmethylsulfonyl fluoride) containing 0.3 M NaCl. The column was washed with the same buffer, and the adsorbed proteins were eluted with buffer A containing 1 M NaCl. The eluate was adjusted to 0.6 M NaCl by dilution with buffer A and loaded onto a single-stranded DNA (ssDNA)–cellulose column (Sigma [4.3 mg of DNA per g; 2 ml]) equilibrated with buffer A containing 0.6 M NaCl. The column was washed with the same buffer, and then the proteins were eluted with buffer A containing 1.5 M NaCl. The eluate was dialyzed against buffer A containing 0.3 M NaCl and stored at −80°C. The rhXPC protein, which was free of HHR23B protein, was purified from Sf9 cells infected with vVL.XPC by the same procedures.

For purification of rHHR23B, fraction S1 from vAcUW(XPC-HHR23B)-infected Sf9 cells was used as a starting material. The conductivity of fraction S1 was adjusted to that of buffer A containing 0.05 M NaCl and loaded onto a phosphocellulose column equilibrated with the same buffer. The following two chromatography steps were performed with a fast-performance liquid chromatography system (Pharmacia). Flowthrough fractions from the phosphocellulose column were collected and then loaded at 0.1 ml/min onto a HiTrap-Q column (Pharmacia [5 ml]) equilibrated with buffer A containing 0.05 M NaCl. After the column was washed with 15 ml of the same buffer, proteins were eluted with 50 ml of a linear gradient of 0.05 to 0.4 M NaCl in buffer A. The rHHR23B protein was eluted at around 0.25 M NaCl. The peak fractions were collected and then further loaded at 0.5 ml/min onto a Bio-scale CHT2-I hydroxyapatite column (Bio-Rad [2 ml]) equilibrated with buffer A containing 0.2 M KCl. The column was washed with 10 ml of the same buffer, and elution was carried out with 24 ml of a linear gradient of 0 to 0.5 M potassium phosphate (pH 7.5) in buffer A containing 0.2 M KCl. The rHHR23B protein was eluted around 0.06 M potassium phosphate. The peak fractions were adjusted to 35% saturation of ammonium sulfate, kept on ice for 30 min, and then centrifuged at 12,000 × *g* for 15 min. The precipitates were suspended in buffer A and dialyzed against buffer A containing 0.3 M NaCl. Insoluble materials were removed by centrifugation, and the supernatant fraction was stored at −80°C.

**Preparation and fractionation of whole-cell extracts.** Whole-cell extracts for cell-free repair reactions were prepared as described previously. For fractionation of whole-cell extracts from HeLa or XP4PASV cells, the conductivity of the extracts was adjusted to buffer B (25 mM *N*-2-hydroxyethylpiperazine-*N*′-2-ethanesulfonic acid [HEPES]-KOH [pH 7.9], 1 mM EDTA, 10% glycerol, 0.01% Triton X-100, 1 mM dithiothreitol, 0.25 mM phenylmethylsulfonyl fluoride) containing 0.2 M KCl by the addition of 3 M KCl. Approximately 50 mg of protein from the extract was loaded onto a phosphocellulose column (5 ml) equilibrated with buffer B containing 0.2 M KCl. The column was washed with the same buffer, and the adsorbed proteins were eluted with buffer B containing 1 M KCl. The peak fractions from the flowthrough (CFI) and the eluate (CFII) were concentrated by dialysis against buffer B containing 0.1 M KCl and 20% sucrose and stored at −80°C. For reconstituted repair reactions with naked DNA substrates, CFII was prepared from XP3BE whole-cell extracts in the same way, except that the extracts were loaded on phosphocellulose at 0.1 M KCl.



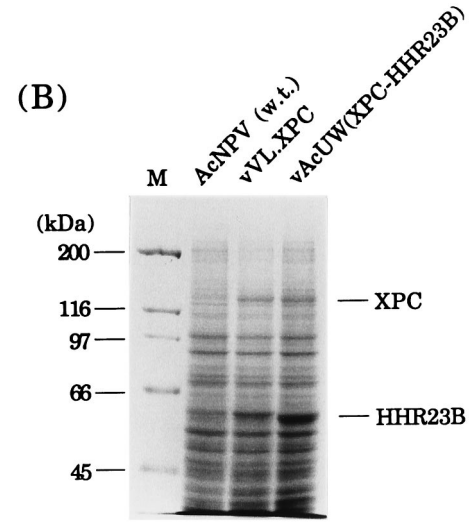




FIG. 1. Construction of recombinant baculoviruses expressing rhXPC with or without rHHR23B. (A) Baculovirus transfer plasmids used for generation of recombinant viruses. (B) Monolayers of Sf9 cells were infected with the indicated viruses. After incubation at 27°C for 3 days, the cells were lysed in SDS sample buffer and analyzed by SDS-PAGE (8% polyacrylamide) and subsequent Coomassie blue staining. Twenty micrograms of protein from each cell lysate was loaded in each lane. AcNPV, *Autographa californica* nuclear polyhedrosis virus; w.t., wild type.

**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 [$\alpha$-$^{32}$P]dCTP (37 to 74 kBq); 0.5 μg of phosphocreatine kinase (Sigma [type I]), 6.4 μg of bovine serum albumin, whole-cell extracts (80 μg of protein), and 0.3 μg of unirradiated pUC19 RFI DNA, in addition to UV-irradiated (400 J/m$^2$) or unirradiated SV40 minichromosomes (0.3 μg of DNA). Where indicated, fractionated extracts (CFI and/or CFII) and purified proteins (replication protein A [RPA], proliferating cell nuclear antigen [PCNA], XPC, and HHR23B) were substituted for whole-cell extracts. The reaction mixtures were incubated at 30°C for 3 h. DNA was purified from the reaction mixtures, linearized by *Eco*RI digestion, and then electrophoresed in 1% agarose gels as described previously (34). Autoradiography was performed at −80°C with Fuji New RX X-ray film and Kodak intensifying screens. The incorporation of radioactive materials into viral DNA was quantified with a Fujix BAS2000 Bio-Imaging analyzer. Data are presented as mean values with standard errors of the mean calculated from two or three independent experiments.

Repair reactions with naked plasmid DNA were carried out as described by van Vuuren et al. (40), except that XP3BE CFII and some purified proteins were used as indicated instead of whole-cell extracts and radioactive dATP was omitted from the first-stage reactions. After the incubation at 30°C for 90 min, PCNA and 74 kBq of [$\alpha$-$^{32}$P]dATP were added and the mixture was further incubated at 30°C for 15 min. DNAs were purified as described above, linearized by *Bam*HI, and fractionated through 1% agarose gel.

**Purification of RPA and PCNA.** The RPA (15) and PCNA (18) were purified from HeLa and 293 cells as described previously.

**Antibodies.** For immunization, rhXPC was partially purified from fraction P prepared from vVL.XPC-infected Sf9 cells. Fraction P was homogenized by sonication in buffer A containing 0.5 M NaCl. After the mixture was centrifuged at 12,000 × *g* for 15 min, the resultant supernatant was mixed with ssDNA-cellulose equilibrated with the same buffer and rotated gently at 4°C overnight. The resin was collected by low-speed centrifugation, washed with buffer A containing 0.5 M NaCl, and then packed into a column. After the column was further washed with the same buffer, bound proteins were eluted with buffer A containing 2 M NaCl and 50% ethylene glycol. The peak fractions determined by SDS-PAGE were dialyzed against buffer A containing 0.5 M NaCl. rhXPC was purified by excision from preparative SDS-PAGE and used for immunization of rabbits. Anti-XPC antibodies were affinity purified with the partially purified rhXPC fraction.

To obtain anti-HHR23B antibodies, HHR23B was expressed in *E. coli* as a fusion protein with either glutathione *S*-transferase (GST) or maltose-binding protein (MBP), with plasmid pGEX-2T (Pharmacia) or pMAL-c2 (New England Biolabs), respectively. The GST-HHR23B fusion protein was purified with glutathione-Sepharose (Pharmacia) under standard conditions and used for immunization of rabbits. Anti-HHR23B antibodies were affinity purified with the MBP-HHR23B fusion protein. Because the MBP-HHR23B protein was not bound to amylose resins, it was partially purified by conventional column chromatography with HiTrap-Q and Mono Q columns.

**Other methods.** SDS-PAGE was performed as described by Laemmli (17). For Western blot analyses, electrophoresed proteins 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), first with antibody (anti-XPC or anti-HHR23B) in blocking buffer, and then with anti-rabbit F(ab′)$_2$ antibodies conjugated with horseradish peroxidase (Amersham). 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 (5) with bovine serum albumin as a standard and with reagents purchased from Bio-Rad Laboratories.

## RESULTS

**Expression and extractability of rhXPC and rsHHR23B proteins.** To obtain large amounts of human XPC and HHR23B proteins, two types of recombinant baculovirus were constructed (Fig. 1A). One of the recombinant viruses, vVL.XPC, overexpressed XPC protein in insect cells, while the other, vAcUW(XPC-HHR23B), expressed both XPC and HHR23B proteins simultaneously. As shown in Fig. 1B, the rhXPC protein was easily detected by Coomassie blue staining in total protein extracts from Sf9 cells infected with either of the recombinant viruses. Expression of rHHR23B protein was also detected in the lysate from vAcUW(XPC-HHR23B)-infected cells.

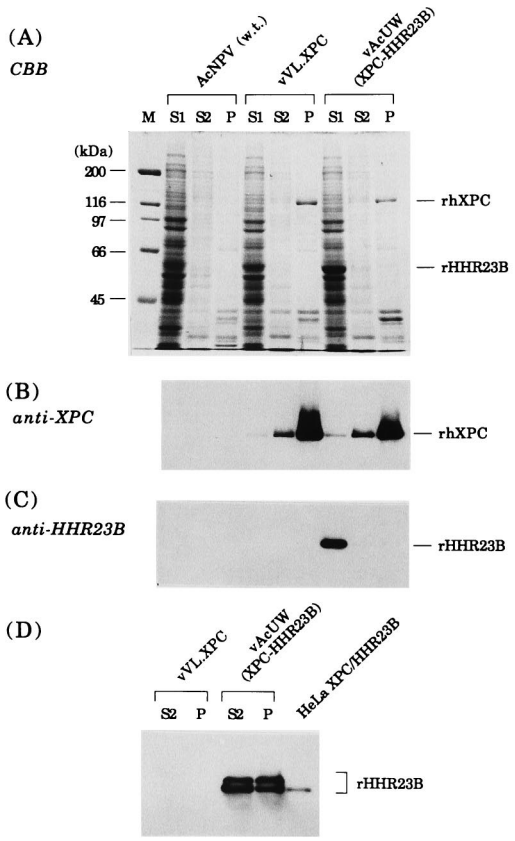



FIG. 2. Extraction properties of rhXPC and rHHR23B expressed in insect cells. (A) Monolayers of Sf9 cells in 60-mm-diameter dishes were infected with the indicated viruses and incubated at 27°C for 3 days. The cells were collected and fractionated as described in Materials and Methods. The volume of each of the resulting fractions, S1, S2, and P, was adjusted to 500 μl, and 3.3 μl of each fraction was analyzed by SDS-PAGE (8% polyacrylamide) and subsequent Coomassie blue staining. (B and C) Same samples as those shown in panel A were transferred onto a polyvinylidene difluoride membrane and subjected to Western blot analysis with anti-XPC (B) or anti-HHR23B (C) polyclonal antibodies. In this experiment, 0.33 μl of each fraction was loaded per lane. (D) rHHR23B in 5-μl aliquots of the fractions S2 and P from cells infected with the indicated viruses was visualized by Western blot analysis. AcNPV (w.t.), wild-type *Autographa californica* nuclear polyhedrosis virus.

To examine the extractability of these expressed proteins, the infected Sf9 cells were divided into three fractions. The infected cells were extracted with hypotonic buffer containing 1% Nonidet P-40 (NP lysis buffer [see Materials and Methods]) to obtain the supernatant (S1) fraction. The precipitate fraction was further extracted with buffer containing 0.3 M NaCl to obtain the supernatant (S2) and precipitate (P) fractions. As shown in Fig. 2A, the vast majority of rhXPC remained unextracted. These extraction properties of rhXPC were essentially unaffected by coexpression of rHHR23B. In marked contrast, most of the rHHR23B expressed in vAcUW(XPC-HHR23B)-infected cells was recovered in fraction S1, suggesting that most of rhXPC and rHHR23B were not complexed with each other when coexpressed in the insect cells.

The rhXPC remaining in fraction P was solubilized by sonication and then partially purified by ssDNA–cellulose column chromatography. With this protein fraction used as an antigen, anti-XPC polyclonal antibodies were obtained. These antibodies cross-reacted with a 125-kDa protein species in the XPC protein complex purified from HeLa cells as well as in total lysates from repair-proficient human 293 cells and an XP-A cell line, XP2OSSV, but not in lysates from two XP-C cell lines, XP4PASV and XP3KA (Fig. 3A), confirming that the band corresponded to the XPC protein. Polyclonal antibodies were raised against the GST-HHR23B fusion protein produced in *E. coli* and were affinity purified with the MBP-HHR23B fusion protein. The antibodies reacted specifically with the 58-kDa protein in the HeLa-XPC complex and also in the 293 and XP-A cell lysates (Fig. 3B). In contrast to XPC, the 58-kDa bands were also present in the XP-C cell lysates, indicating that HHR23B protein was normally expressed in the XP-C cells. Furthermore, the intensity of the XPC protein band in 20 μg of the 293 cell extract was much lower than the signal with 5 ng of the purified XPC-HHR23B complex (Fig. 3A), while the signal of HHR23B in the same amount of the extract was comparable to that with 30 ng of the purified complex (Fig. 3B). Thus, HHR23B protein is present in a large excess over XPC protein even in repair-proficient cells, suggesting that most of the HHR23B protein is not complexed with XPC protein.

With these antibodies, the expression and extractability of rhXPC and rHHR23B were reexamined. Although most of the rhXPC remained in fraction P, Western blot analyses revealed that a small but significant portion of the protein was extracted in fraction S2 from Sf9 cells infected with either vVL.XPC or vAcUW(XPC-HHR23B) (Fig. 2B). Similarly, a portion of rHHR23B was found to be resistant to the first hypotonic extraction and was present in the fractions S2 and P (Fig. 2C and D). Thus, fractions S2 and P from coexpressing cells contained both rhXPC and rHHR23B. Although rHHR23B in fraction S1 was detected as a single band, two HHR23B bands with apparent molecular masses of 61 and 58 kDa were detected in fractions S2 and P when larger amounts of the fractions were subjected to Western blot analyses (Fig. 2D). Since the lower band of the doublet comigrated with HHR23B in human cell extracts, a subpopulation of rHHR23B expressed in insect cells may undergo some posttranslational modification, such as phosphorylation.

**Purification of rhXPC and rHHR23B proteins.** We used the fraction S2 from coexpressing cells as a starting material for purification of the rhXPC-rHHR23B complex, because the results in Fig. 2A showed that, in other fractions, S1 and P, the molar ratios of the two recombinant proteins were quite different. Purification was carried out by procedures similar to those published previously for the purification of XPC protein complex from HeLa cell nuclear extracts (23). Two steps of

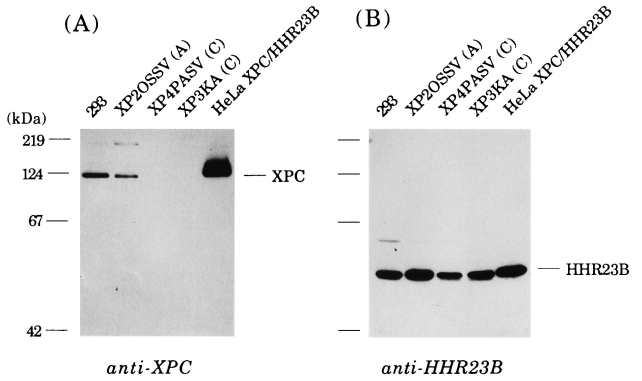



FIG. 3. Specificity of anti-XPC and HHR23B antibodies. Total cell lysates (each containing 20 μg of protein) from 293, XP2OSSV (group A), XP4PASV, and XP3KA (group C) cells were analyzed by Western blotting with anti-XPC (A) or anti-HHR23B (B) polyclonal antibodies. Five (A) or 30 (B) ng of XPC-HHR23B complex purified from HeLa cells was loaded in parallel.

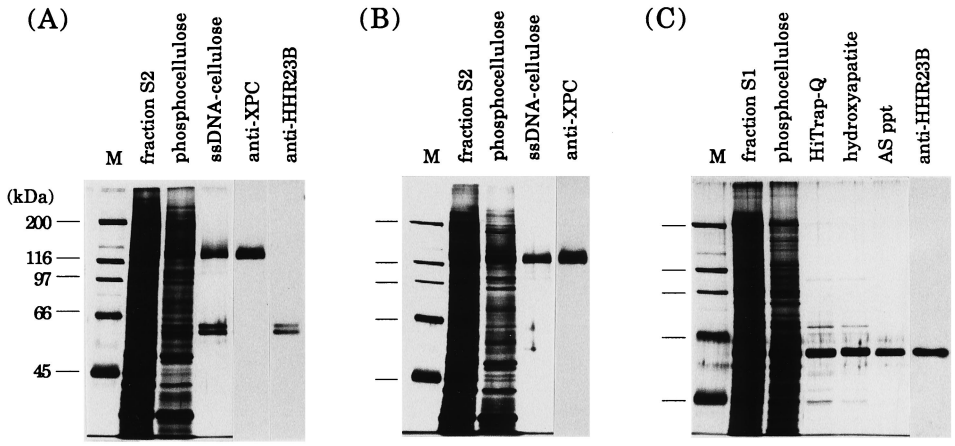



FIG. 4. Purification of recombinant proteins. (A) Purification of the rhXPC-rHHR23B complex. Aliquots of samples at each purification step indicated above the lanes were subjected to SDS-PAGE (8% polyacrylamide). Protein bands were visualized by silver staining. The final purified sample was subjected to Western blot analysis with anti-XPC or anti-HHR23B antibodies as indicated. (B) Purification of free rhXPC. (C) Purification of free rHHR23B. M, molecular mass markers; AS ppt, 35% ammonium sulfate precipitation.

column chromatography with phosphocellulose and ssDNA–cellulose gave a protein fraction consisting of three bands with apparent molecular masses of 125, 61, and 58 kDa on SDS-PAGE (Fig. 4A). The largest protein reacted with anti-XPC antibodies, and the smaller two reacted with anti-HHR23B antibodies as expected (Fig. 4A). Moreover, when the same purification procedures were applied to fraction S2 from Sf9 cells expressing rhXPC alone, the 61- and 58-kDa bands were not copurified with rhXPC (Fig. 4B), confirming that the two bands corresponded to subforms of rHHR23B which were complexed with rhXPC. The HHR23B-free rhXPC bound to ssDNA-cellulose in the presence of 0.6 M NaCl, as observed for the XPC-HHR23B complex. HHR23B itself did not bind to ssDNA-cellulose (data not shown), suggesting that the DNA binding activity can be attributed to XPC protein and not to HHR23B.

We also purified XPC-free rHHR23B from fraction S1 prepared from insect cells infected with the coexpression virus. Purification was performed with three steps of column chromatography and ammonium sulfate precipitation, which gave a protein fraction containing a 58-kDa polypeptide cross-reactive with anti-HHR23B antibody (Fig. 4C).

**rhXPC alone can complement the repair defect of XP-C cell extracts.** Each recombinant protein fraction obtained as described above was assayed for XP-C correcting activity in our cell-free DNA repair system with UV-irradiated SV40 minichromosomes. As shown in Fig. 5, the rhXPC-rHHR23B complex stimulated the cell-free NER reactions in whole-cell extracts from the XP-C cell line XP4PASV, although the correcting activity of the recombinant protein complex in all experiments was lower than that of similar amounts of the authentic XPC-HHR23B complex purified from HeLa cells. This

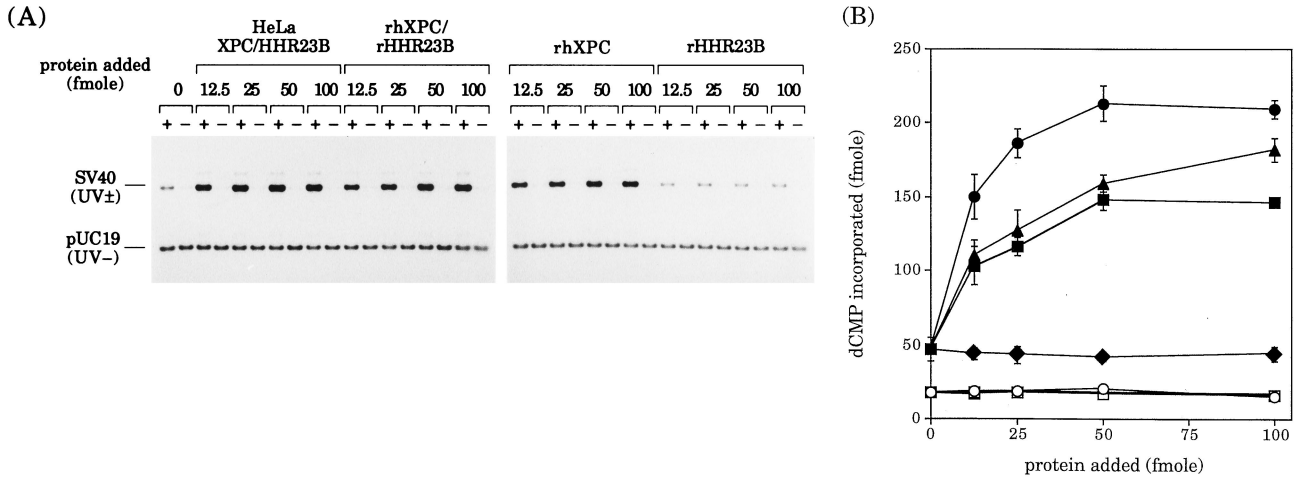



FIG. 5. XP-C correcting activity of the purified recombinant proteins in the cell-free NER system with XP4PASV whole-cell extracts. (A) UV-irradiated (UV+) or unirradiated (UV−) SV40 minichromosomes were incubated at 30°C for 3 h in standard reaction mixtures containing whole-cell extracts from XP4PASV cells and various amounts of purified proteins as indicated. DNA was purified, linearized by *Eco*RI, and then analyzed by 1% agarose gel electrophoresis and subsequent autoradiography. (B) Incorporation of $^{32}P$ radioactivity into each viral DNA band shown in panel A was quantified. Solid symbols show incorporation into UV-irradiated viral chromosomal DNA, and open symbols show incorporation into unirradiated viral DNA. Averages and experimental errors were calculated from three experiments. ● and ○, XPC-HHR23B complex purified from HeLa cells; ▲ and △, rhXPC-rHHR23B complex; ■ and □, free rhXPC; ◆ and ◇, free rHHR23B. In the present work, the amount of XPC-HHR23B complex was calculated on the basis of the observation that the two proteins form a 1:1 complex. Therefore, the reaction mixtures containing 100 fmol of XPC-HHR23B contained 100 fmol each of the two proteins.

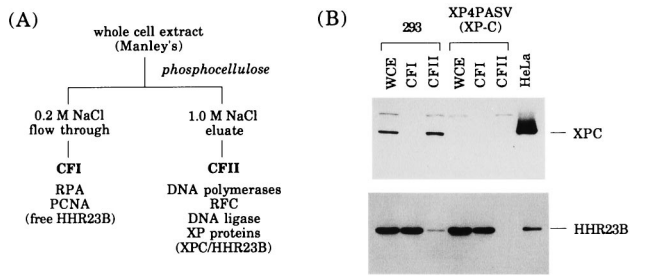



FIG. 6. Fractionation of whole-cell extracts (WCE) for the cell-free NER system. (A) Scheme of fractionation of whole-cell extracts. (B) Whole-cell extracts from 293 or XP4PASV cells were fractionated as shown in panel A, and the presence of XPC (upper panel) or HHR23B (lower panel) protein in CFI and CFII was visualized by Western blotting. Forty micrograms of protein from whole-cell extracts, 15 μg of protein of CFI, 10 μg of protein of CFII, or 10 ng of XPC-HHR23B complex purified from HeLa cells was loaded per lane. RFC, replication factor C.

could be due to a difference in protein modification between XPC from human cells and that from insect cells. The rhXPC, free of HHR23B, also complemented the repair defect in XP4PASV whole-cell extracts. This observation was not surprising, because Western blot analyses (Fig. 3) showed HHR23B protein to be normally expressed in XP4PASV cells. It is likely that exogenous rhXPC binds to endogenous HHR23B protein present in the XP4PASV cell extracts, resulting in reconstitution of functional rhXPC-HHR23B complex. We also tested the activity of rhXPC purified from fraction P: the bulk rhXPC hardly exhibited the XP-C correcting activity (data not shown). Therefore, only a small portion of rhXPC expressed in insect cells possessed the XP-C correcting activity, which was preferentially extracted into fraction S2. As expected, rHHR23B alone could not stimulate the cell-free NER reactions by XP4PASV cell extract.

**Fractionation and reconstitution of the cell-free DNA repair system with XP-C cell extract.** Because the whole-cell extract from XP-C cells contained endogenous HHR23B protein, it remained unclear whether the HHR23B protein was necessary for the cell-free NER reactions. To answer this question, we depleted HHR23B protein from the cell-free NER reactions by fractionation and reconstitution of whole-cell extracts. Phosphocellulose column chromatography of repair-proficient cell extracts yielded two protein fractions, a flowthrough fraction (CFI) and a bound fraction (CFII). Both CFI and CFII are required for the cell-free NER reactions, but CFI can be replaced by two purified proteins, RPA and PCNA (Fig. 6A [see reference 32]). As shown in Fig. 7, the NER reactions with SV40 minichromosomes were completely dependent on both CFI and CFII derived from 293 whole-cell extracts. Purified RPA and PCNA could substitute for CFI, and omission of either of the two proteins resulted in reduction in the repair synthesis level. Western blot analysis revealed that most of the HHR23B protein in 293 cell extracts was recovered in CFI by this fractionation procedure, whereas practically all of the XPC protein was present in CFII (Fig. 6B). Figure 6B also shows that a small portion of HHR23B protein was detected in the CFII from 293 cells. It is very likely that this subpopulation of HHR23B protein is complexed with XPC protein, because CFII prepared from XP4PASV cells under the same conditions contained no detectable level of HHR23B protein (Fig. 6B). Therefore, the replacement of CFI with RPA and PCNA resulted in omission of endogenous free HHR23B protein from the cell-free NER reactions.

**Stimulation of XP-C correcting activity by HHR23B protein in the reconstituted system.** The recombinant proteins were assayed for XP-C correcting activity in the reconstituted system with RPA, PCNA, and CFII from XP4PASV cells. As shown in Fig. 8, the HeLa XPC-HHR23B complex stimulated repair synthesis in the reconstituted system to achieve about threefold stimulation over background synthesis. Interestingly, free rhXPC alone showed only very weak stimulatory activity in the reconstituted system, in marked contrast to the results with whole-cell extracts (Fig. 5). On the other hand, the rhXPC-rHHR23B complex consistently and reproducibly stimulated repair synthesis in this system, but the maximum level of stimulation was approximately 40 to 50% of that of the HeLa XPC-HHR23B complex.

To examine the roles of HHR23B protein, rHHR23B was added to the reconstituted repair system in combination with rhXPC. In the presence of free rhXPC, rHHR23B stimulated repair synthesis in a dose-dependent manner up to a level nearly equivalent to that achieved by the rhXPC-rHHR23B complex (Fig. 9A). In contrast, rHHR23B showed little stim-

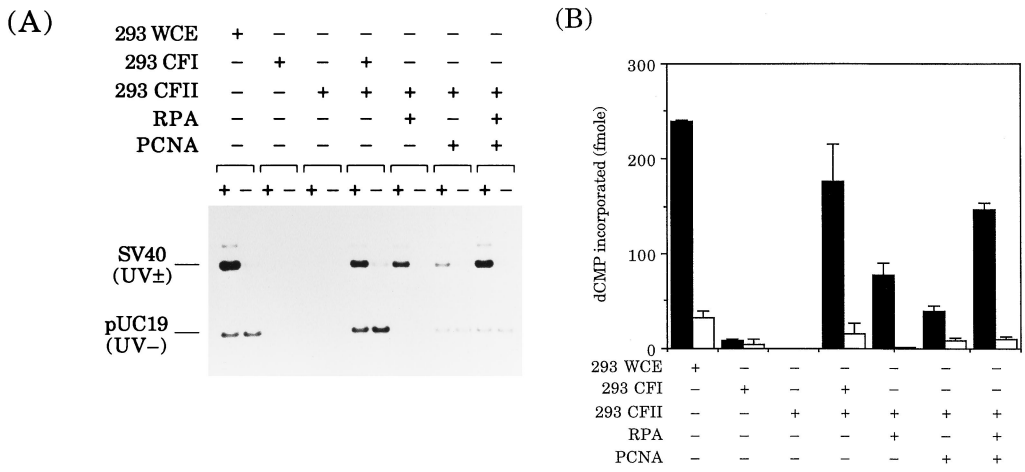



FIG. 7. Reconstitution of the cell-free NER reactions. (A) Cell-free NER reactions were carried out in which 293 whole-cell extract (WCE) was replaced by the indicated (+) components. The amounts of the components used were 80 μg for whole-cell extracts, 30 μg for CFI, 20 μg for CFII, 0.5 μg for RPA, and 24 ng for PCNA. (B) Incorporation of $^{32}P$ radioactivity into each viral band shown in panel A was measured. The solid bars indicate incorporation into UV-irradiated viral DNA, and the open bars indicate incorporation into unirradiated viral DNA.

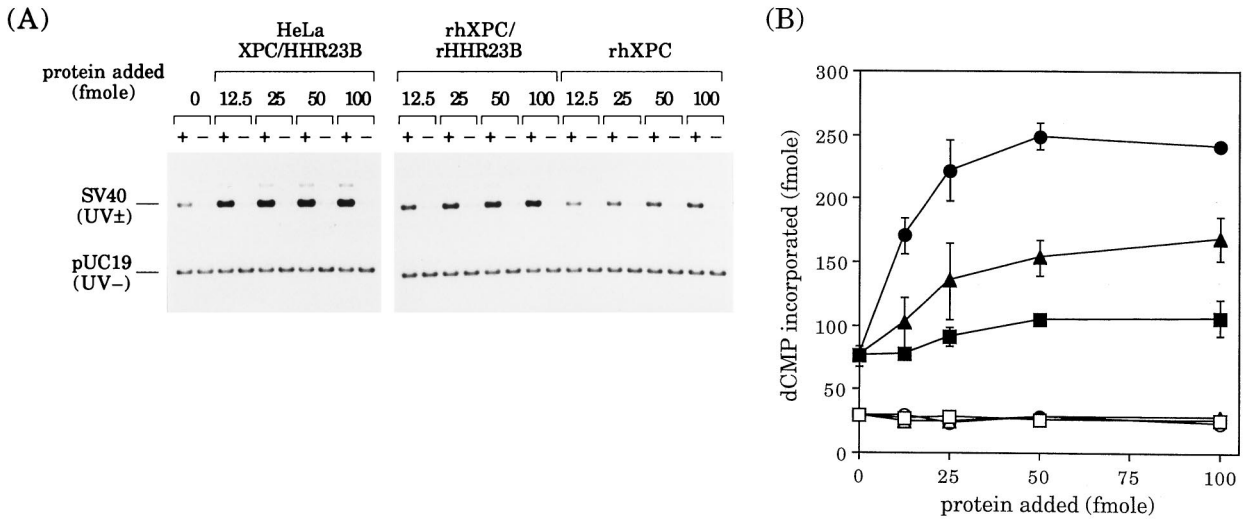



FIG. 8. Inability of rhXPC to stimulate repair synthesis in the reconstituted NER system with CFII from XP4PASV cells. (A) Cell-free NER reactions were performed with 20 μg of XP4PASV CFII, 0.5 μg of RPA, 24 ng of PCNA, and various amounts of proteins purified from HeLa or insect cells as indicated. (B) Incorporation of radioactivity in each viral band shown in panel A was measured and plotted. The incorporation into UV-irradiated viral DNA is shown by solid symbols, and incorporation into unirradiated viral DNA is shown by open symbols. Averages and experimental errors were taken from three experiments. ● and ○, HeLa XPC-HHR23B complex; ▲ and △, rhXPC-rHHR23B complex; ■ and □, free rhXPC.

ulatory activity by itself or in the presence of XPC protein prebound to HHR23B protein. Also when the amount of rhXPC was varied in the presence or absence of a fixed amount of rHHR23B, rhXPC again exhibited very weak stimulatory activity in the absence of rHHR23B, but the coaddition of rHHR23B significantly enhanced the activity of rhXPC (Fig. 9B).

To examine whether HHR23B stimulated the XPC NER function with naked DNA substrates instead of minichromosomes and to find out whether other lesions were repaired, the activities of the rhXPC and rHHR23B proteins were tested with plasmid DNA treated with *N*-acetoxy-2-acetylfluorene (AAF). A mixture of two different-sized plasmid DNAs, one undamaged and the other AAF-damaged, was incubated with RPA and CFII from XP-C cells, and then repair synthesis was allowed by the addition of PCNA and radioactive nucleotides. As shown in Fig. 10, 0.3 pmol of the authentic XPC protein complex stimulated the repair synthesis up to ninefold over the background synthesis, while the free rhXPC caused only twofold stimulation. In the presence of rHHR23B, however, the same amount of rhXPC gave approximately fivefold stimulation over the background level of synthesis, clearly indicating that the stimulatory effect of rHHR23B on the activity of rhXPC was even more pronounced than the effect of UV-damaged SV40 minichromosomes.

## DISCUSSION

In the present study, rhXPC and rHHR23B proteins were produced in insect cells with the baculovirus expression system. In *E. coli*, HHR23B protein was well expressed, but no detectable level of XPC protein expression was observed. This might be due to a number of AGA/AGG triplets encoding arginine in the *XPC* gene, whose tRNAs are very rare in *E. coli*. Although high levels of XPC protein were obtained in insect cells, most of the rhXPC was not active in our in vitro NER system and also was not solubilized under the rather mild extraction conditions adopted in the present study. Sedimentation analyses with glycerol density gradients showed that the bulk rhXPC was severely aggregated, while the soluble rhXPC was sedimented as a single monomer peak around the expected molecular weight (data not shown). It is likely that the bulk rhXPC is incorrectly folded and/or modified posttranslationally in insect cells.

We constructed a recombinant baculovirus expressing human XPC and HHR23B proteins simultaneously on the basis of anticipation that the coexpressed proteins may form specific complexes in insect cells. Although a small portion of each protein extracted in fraction S2 was purified in a complex form, the results shown in Fig. 2 indicate that the majority of the coexpressed proteins were not complexed. This may be explained by the finding that most of the rhXPC expressed in insect cells was not synthesized in a functional form as mentioned above. Interestingly, Western blot analyses indicated that HHR23B protein exists in large excess over XPC protein in human cells (Fig. 3 and 6). Moreover, HHR23B protein is expressed normally in XP-C cell lines, suggesting that this protein plays an additional role or roles other than complex formation with XPC. Further investigations are necessary to elucidate the putative multiple functions of HHR23B protein.

With a cell-free NER system, we purified the XPC-HHR23B protein complex, which complemented the repair defect in XP-C cell extracts. The rhXPC protein, free of HHR23B, could stimulate the repair synthesis of XP-C cell extracts (Fig. 5). These results support the idea that the disease XP group C is due to inactivation of the *XPC* gene and is not due to an indirect effect of a mutated XPC function on the HHR23B protein. However, our results also indicate that HHR23B protein plays an important role in the mammalian NER reaction (Fig. 8, 9, and 10). Disruption of the *S. cerevisiae RAD23* gene causes only a moderate degree of UV sensitivity, similar to that caused by null alleles of *RAD7* and *RAD16* (29). Recently, *RAD7* and *RAD16* have been shown to be essential for NER of nontranscribed DNA strands as well as of the silent mating type loci, but not for NER of transcribed strands (43). This phenotype thus closely resembles the selective global genome repair defect of XP-C cells. However, yeast *rad23* mutants may display a phenotype different from *rad7* and *rad16* mutants for

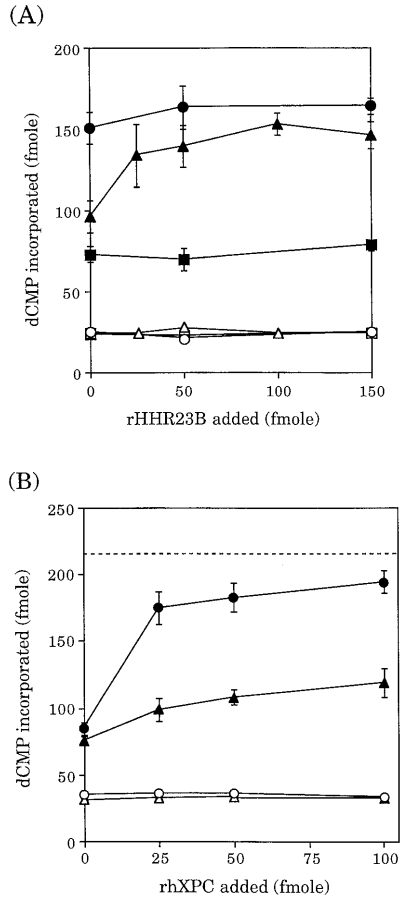



FIG. 9. Stimulation of XP-C correcting activity by HHR23B protein. (A) Various amounts of rHHR23B were added to the reconstituted cell-free NER reaction mixtures with CFII from XP4PASV cells in the presence or absence of 50 fmol of each indicated protein. ● and ○, rhXPC-rHHR23B; ▲ and △, free rhXPC; ■ and □, no protein added. Solid symbols represent incorporation into UV-irradiated viral DNA, and open symbols represent incorporation into unirradiated viral DNA. HeLa XPC-HHR23B complex (50 fmol) caused 260 ± 15 fmol of dCMP incorporation. (B) Repair synthesis in reaction mixtures containing various amounts of free rhXPC in the presence (● and ○) or absence (▲ and △) of 100 fmol of rHHR23B was plotted. Solid symbols represent incorporation into UV-irradiated viral DNA, and open symbols represent incorporation into unirradiated viral DNA. The dashed line shows the level of repair synthesis achieved by 100 fmol of rhXPC-rHHR23B complex. In both panels A and B, averages and experimental errors were calculated from the results of three experiments.

two reasons. First, Rad23 protein was recently shown to be complexed with Rad4 protein (11), which is the putative XPC counterpart, but—in contrast to XPC—Rad4 is involved in both NER subpathways (43). Second, in contrast to *rad7* and *rad16* mutants, *rad23* mutants were very recently found to be defective in removal of cyclobutane pyrimidine dimers from both transcribed and nontranscribed strands and were also found to be totally deficient in [6-4]photolesion repair, at least during the first 4 h post-UV irradiation (44). These findings point to a direct role of *RAD23* in both NER pathways in yeast cells. Our findings of free excess HHR23B (and another Rad23 homolog, HHR23A [unpublished observations]) also suggest multiple functions of this set of proteins in humans.

In all mammalian in vitro NER reactions described thus far, XP-C cell extracts appear to be almost totally inactive. Since XP-C cells are still proficient in transcription-coupled repair, this means that the in vitro repair reactions are driven, almost exclusively, by the mechanism of genome-overall repair. Although it is thus difficult to determine from our in vitro studies whether HHR23B protein plays any roles in transcription-coupled repair, the present data indicate that XPC protein is required for genome-overall repair of UV- and AAF-induced lesions and that HHR23B stimulates the function of XPC. Interestingly, Mu et al. (26) have very recently shown that with a cholesterol-damaged DNA substrate, dual incision occurs even in the absence of the XPC-HHR23B complex. Because of its high affinity to single-stranded DNA, it may be assumed, as discussed by Mu et al. (26), that the XPC protein complex promotes formation of a preincision complex and thus stabilize the locally unwound structure of damaged DNA. Some kinds of DNA lesions, which by themselves distort and destabilize the duplex DNA structure to a great extent, might not need such XPC function. On the other hand, Guzder et al. have claimed (10) that yeast Rad23 protein forms a higher-order complex with yTFIIH and Rad14 protein (the yeast XPA counterpart). Although it remains to be established whether such an interaction exists in the mammalian system, HHR23B, in an XPC-bound form, may mediate interactions with other repair proteins, such as XPA and TFIIH, to consequently promote the assembly of a preincision complex.

In the cell-free NER reactions with XP4PASV whole-cell extracts, the maximum level of repair stimulation achieved by the rhXPC-rHHR23B complex was 80 to 90% of that by the authentic XPC-HHR23B complex purified from HeLa cells (Fig. 5). On the other hand, in the reconstituted system, repair synthesis stimulated by the rhXPC-rHHR23B complex, as well as a combination of separately purified rhXPC and rHHR23B, consistently reached only 30 to 50% of that by the HeLa protein complex (Fig. 8, 9, and 10). Especially when we used UV-damaged SV40 minichromosomes as a substrate, the stimulation of rhXPC by rHHR23B was only twofold, compared with the background level of synthesis (Fig. 8 and 9). Two explanations for the modest stimulation are conceivable. First, the rhXPC and/or rHHR23B proteins are only partially active compared with the authentic XPC-HHR23B complex purified from HeLa cells, probably because of the lack of posttranslational modifications occurring in insect cells (the XPC protein and likely also the HHR23B protein are phosphorylated in mammalian cells) and/or because of improper folding of part of the recombinant protein molecules (as described in the first part of Discussion). In the reactions in which these proteins were added to whole-cell extracts (results shown in Fig. 5), the recombinant proteins may be modified to regain (nearly) full activity, but when added to the fractionated extracts, the modifying enzymes are likely to be absent. Protein kinases and/or phosphatases may be considered candidate activators of the recombinant proteins. Second, the UV-damaged SV40 minichromosome system shows a relatively high level of background DNA synthesis, resulting in reduced signal-to-noise ratios. With naked damaged DNA as a substrate and a slightly modified protocol, we could obtain a better signal-to-noise ratio (Fig. 10) in which the stimulation was fivefold. Obviously, the minichromosomes are a more natural substrate, and this is the reason why we work with this method, despite the fact that it is much more time-consuming. Although the calculated fold of stimulation in this system is quite modest in Fig. 8 and 9, the stimulation itself is very reproducible and is statistically significant, as shown by the error bars.

We think it is unlikely that HHR23B acts in an XPC-independent manner for three reasons. First, no stimulation was observed by the addition of HHR23B alone (Fig. 5, 9, and 10). Second, we showed that rhXPC and rHHR23B already form a complex by themselves within the insect cells (Fig. 2) and the

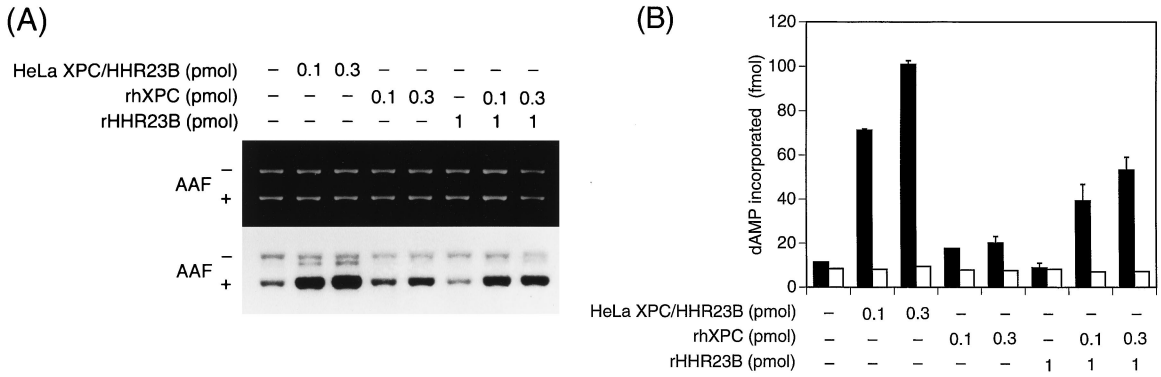



FIG. 10. Stimulatory effect of HHR23B in the naked DNA repair system. (A) Undamaged and AAF-damaged plasmids were incubated at 30°C for 90 min with 100 μg of CFII from XP3BE lymphoblastoid cells, 125 ng of RPA, and the proteins indicated above each lane. The rHHR23B protein used was produced in *E. coli* cells (expression and purification of this rHHR23B protein will be described elsewhere). After the incubation, 25 ng of PCNA and 74 kBq of [α-$^{32}$P]dATP were added, and incubation was continued for a further 15 min. DNA samples were purified, linearized by *Bam*HI, and fractionated by agarose gel electrophoresis. Ethidium bromide stain (upper panel) and an autoradiogram (lower panel) of the gel are shown. Radioactive bands observed between undamaged and AAF-damaged DNAs represent a trace of nicked circular, AAF-damaged plasmid DNA molecules which were not cut by *Bam*HI. (B) Quantitative analysis of the results shown in panel A. The dAMP incorporation into AAF-damaged (solid bars) and undamaged (open bars) plasmids is shown after normalization by the DNA recovery of each sample. For the incorporation into damaged DNA, standard errors were calculated from two independent experiments.

recombinant protein complex exhibited a higher level of activity than free rhXPC, as shown in Fig. 8 and 9. Third, in collaboration with R. D. Wood, who has set up a completely defined NER system starting from purified NER components (1), the rHHR23B stimulation of XPC correction was again observed to the same extent as that reported here (unpublished observations).

In our partial reconstitution system with UV-irradiated SV40 minichromosomes, the combination of CFII and RPA was capable of inducing a quite strong UV-dependent incorporation, with no detectable signal in the pUC19 internal control and the unirradiated SV40 minichromosomes (Fig. 7), indicating that PCNA dependency is not so strong in our cell-free system. In preliminary experiments, the weak dependency on PCNA was found to be specific for the chromatin template. Clarification of this difference between a naked DNA substrate and the chromatin template might help in elucidating the function(s) of PCNA in the in vitro and in vivo NER reactions.

There is another human homolog of the yeast Rad23 gene product, HHR23A. Since the HHR23A and HHR23B proteins exhibit a high degree of overall homology (57% identity and 76% similarity), it will be interesting to determine whether these two proteins are functionally interchangeable. This possibility and the characterization of HHR23A protein in human cells are currently under investigation in our laboratory and will be the subject of a forthcoming paper.

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