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Characterization of XPC-hHR23B complex in nucleotide excision repair and investigation of another role of hHR23 proteins.

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ABBREVIATIONS

AP	: apurinic or apyrimidinic (site in DNA)
bp	: base pairs
BSA	: bovine serum albumin
CPD	: cyclobutane pyrimidine dimer
CS	: Cockayne syndrome
DMSO	: dimethylsulfoxide
DTT	: dithiothreitol
EDTA	: ethylene dinitrilo tetraacetic acid
ERCC	: excision repair cross complementing
GGR	: global genome repair
hHR23	: human homolog of RAD23
IPTG	: isopropyl-ß-D-thiogalactoside
J	: Joule
kbp	: kilobase pair
kDa	: kilodalton
MBP1	: multiubiquitin binding protein
Mcb1	: multiubiquitin-chain-binding protein
N-AAAF	: *N*-Acetoxy-2-acetyl-2-aminofluorene
NER	: nucleotide excision repair
nm	: nanometer
nt	: nucleotide
PAGE	: polyacrylamide gel electrophoresis
PBS	: phosphate-buffered saline
PCNA	: proliferating cell nuclear antigen
PCR	: polymerase chain reaction
RAD	: radiation sensitive
RF-C	: replication factor C
RPA	: replication factor A
SDS	: sodium dodecyl sulfate
SV40	: Simian virus 40
TCR	: transcription coupled repair
TFIIH	: transcription initiation factor II H
Tris	: tris (hydroxymethyl) aminomethane
TTD	: trichothiodystrophy
UBA	: ubiquitin associated
UBC	: ubiquitin conjugation
UDS	: unscheduled DNA synthesis
UV	: ultraviolet
UV-DDB	: uv-damaged DNA binding protein
uvr	: ultraviolet resistance
XP	: xeroderma pigmentosum
XP-A (to G)	: XP complementation group A (to G)
4-NQO	: 4-nitroquinoline-1-oxide
(6-4)PP	: pyrimidine (6-4) pyrimidone photoproduct

PREFACE

The aim of this thesis is to elucidate the role of XPC-hHR23B complex in nucleotide excision repair (NER) and to explore the unknown function of hHR23 proteins. To be able to discuss these subjects, two short general introductions are given in Chapter 1. Just before starting the Chapters 2 and 3, the abstract for each of them is presented. Chapters 2 and 3 are constructed from three different parts, INTRODUCTION, RESULTS, and DISCUSSION, respectively. The CONCLUSION for them is placed just after Chapter 3 followed by EXPERIMENTAL PROCEDURES and REFERENCES.

Chapter 1. GENERAL INTRODUCTIONS

I. General introduction to DNA repair

Studies on DNA repair of higher eukaryotes show the dramatic development in these days. It is no exaggeration to say that the field of DNA repair becomes one of the most notable subjects in the basic biology as well as the field of DNA replication, transcription and cell cycle. In this introduction, some basic affairs about NER are described in short paragraphs.

DNA can be subjected to numerous alterations, which can be caused by endogenous metabolic processes or by exogenous agents, like UV irradiation or chemical compounds (Fig. 1). The injury on DNA may cause catastrophic problems for all living things that adopt DNA as a carrier of the genetic information through the inhibition of, such as, DNA replication and transcription. These damages in DNA may lead to cancer, aging and cell death. Therefore, all organisms have to develop systems to recognize and remove DNA injury. So far, a number of distinct DNA repair pathways have been identified as follows: alkyltransferase, NER, base excision repair (BER), mismatch repair, recombination repair, post-replication repair, and photoreactivation. Among them, NER is one of the most

important pathways because this can recognize and remove various types of DNA damage including the major UV-induced DNA lesions, CPDs and (6-4)PPs, as well as intrastrand crosslinks and bulky DNA adducts caused by chemicals such as 4-NQO or *N*-AAAF.

(1) *NER pathway: E. coli*

The basic mechanism of NER has been studied in the *Escherichia coli* (Fig. 2). A complex consisting of UvrA and UvrB is thought to scan the DNA for local distortions caused by the damage. After scanning DNA lesion, the $UvrA_2B$ complex unwinds the DNA around the lesion. The two UvrA proteins leave from the complex, and remained UvrB protein induces the conformational change in the double helix around the damage. Then UvrC is introduced on UvrB-DNA complex to incise the damaged strand; UvrC at the eighth phosphodiester bond 5' of the lesion, and it is unknown whether UvrC, UvrB or both are responsible for cleaving at the fifth phosphodiester bond 3' of the lesion. After removing the damaged part by UvrD helicase activity, the gap of DNA is filled and closed by DNA polymerase I and ligase.

(2) *Identification of NER proteins from eukaryotes*

Many yeast mutants that are sensitive to UV-radiation or to X-rays, so-called *rad* mutants, have been isolated (Friedberg 1998; Prakash *et al.* 1993). Characterization of double mutants allowed classification of the *RAD* genes into three epistasis groups: the *RAD3*, the *RAD6*, and the *RAD51* groups, involving NER, post-replication repair, and recombination repair, respectively. Several types of experiments including the measurement of UV-sensitivity of mutants in the *RAD3* epistasis group have contributed to insight in the NER process in yeast. The results of these experiments identified a group of genes that encode

proteins which are believed to be essential for the damage recognition and dual incision of NER. Moreover, many of the Rad proteins involving NER have been purified and biochemically characterized.

On the other hand, many of the human genes involving NER were isolated by DNA transfection of normal genomic DNA or cDNA into both repair-deficient laboratory-induced mutant rodent cell lines and human cell lines derived from patients with autosomal recessive disease xeroderma pigmentosum (XP), which has been characterized by hypersensitivity to sunlight (UV), pigmentosum abnormalities and a highly elevated risk of developing cancer on sun-exposed areas of the skin (Boulikas 1996). The group of genes which were isolated from the experiments using laboratory-derived rodent cells are called as Excision Repair Cross Complementing (*ERCC*) genes (Boulikas 1996). Insight into the biochemistry of NER in eukaryotes has been obtained by these isolating proteins and analyzing NER in extracts from both yeast and mammalian cells (Wang *et al.* 1993; Wood *et al.* 1988). Finally, the highly defined reconstituted systems have been used to identify which proteins and protein complexes are responsible for damage-specific incisions in NER (Aboussekhra *et al.* 1995; Guzder *et al.* 1995b; Mu *et al.* 1995). Since the eukaryote NER proteins and process are remarkably conserved through yeast to man (Table 1), NER mechanism and machinery for eukaryote will be described below based upon information of mammalian NER system.

(3) *NER deficiency*

Several complementation groups of NER-deficient cell lines have been established, such as laboratory-derived rodent cells or human cell lines from patients with one of the several rare repair disorders, from the result of cell fusion experiments. Cell fusion experiments showed that the XP cell lines could be divided into at least eight complementation groups. Seven of the XP groups (designated XP-A through XP-G) have severe problems in the incision step

of NER pathway, whereas cells of the eighth group (the variants) have problems in post-replication repair pathway. As well as XP, there are two other autosomal recessive diseases which have deficiency in NER, non cancer-prone disorder Cockayne syndrome (CS) and the photosensitive subset of trichothiodystrophy (TTD) patients (Lehmann 1995). The phenotype of CS and TTD patients which shows NER defects include sun sensitivity, but less severe than in XP, and stunted growth, neural dysmyelination and disturbed sexual development (Table 2). Neither CS nor TTD patients show an elevated risk of skin tumor formation. Cell fusion and microinjection of cloned NER genes have also revealed genetic heterogeneity within these NER syndromes. It has been identified that CS has been divided at least to three different complementation groups (Tanaka *et al.* 1981; Lehmann 1982). Classical CS is caused by mutation in the *CSA* or *CSB* genes. Extremely rare CS patients have been registered with simultaneous symptoms of XP and CS. These patients are assigned to XP-B, -D, and -G (designated as XP/CS). On the other hand, most of TTD which has NER deficiency falls in the same group as XP-D (Kleijer *et al.* 1994). Moreover, one TTD family was reported which belongs to XP-B group and a third group, TTD-A, constitutes a distinct NER-deficient complementing group (Hoeijmakers 1994). Therefore, features of both the CS and the TTD are caused by particular mutations in at least five and three genes, respectively.

(4) *NER pathway: mammalian*

Many details of the NER reaction mechanism are being elucidated with both the purified proteins and the reconstituted systems. At a minimum, the dual incision step of the NER reaction requires XPA, RPA, XPC-hHR23B complex, TFIIH, XPG, and XPF-ERCC1 complex in mammalian or the homologous components of yeast (Sancar 1996; Wood 1996). Although models for the NER pathway in mammalian cells have been reported by some distinct laboratories, there is fairly general agreement as follows. In initial stage of NER,

damage recognition probably involves proteins such as XPA and RPA (Asahina *et al.* 1994; Robins *et al.* 1991; Burns *et al.* 1996; He *et al.* 1995), which associate with one another and are able to bind to the damaged DNA. XPC-hHR23B complex may also be involved in damage recognition (Reardon *et al.* 1996). TFIIH includes the XPB and XPD DNA helicase as an essential subunit (Svejstrup *et al.* 1996) and is involved in local opening of DNA around a site of damage. Recently, it is reported that XPC may also be necessary for opening (Evans *et al.* 1997). Both the XPG and the XPF-ERCC1 complex, which are structure-specific endonucleases, are responsible for cleaving on the 3'- and 5'-sides of a lesion, respectively (Mu *et al.* 1996; O'Donovan *et al.* 1994). The excised fragment is replaced by the temporal DNA synthesis called as unscheduled DNA synthesis (UDS) which requires DNA polymerase δ or ε, PCNA, RF-C, and RPA. The NER process is completed by sealing the repair patch of about 30 nt with DNA ligase.

Transcription-coupled repair (TCR) and global genome repair (GGR) were established by taking advantage of the observation that removal of CPDs from different part of genome displays different kinetics and efficiency. One of the immediate effects of DNA damage is the blockage of transcription. To allow rapid resumption of this vital process, TCR involves repair of DNA lesions on the transcribed strands of expressed genes with high efficiency. This is in contrast to lesions that occur on the non-transcribed strands of active genes, or in unexpressed genes. NER for these lesions is processed by GGR. The models of TCR and GGR are presented in Fig. 3. All XP, XP/CS and TTD, except for XP-C, complementation groups are deficient in TCR and GGR. A specific defect in TCR but not in GGR is encountered in CS. It appears that cells with mutations in one of the two CS genes, *CSA* or *CSB*, display a severe defect in efficient and fast repair of the transcribed strands of active genes. Interestingly, in contrast to CS, XP-C cell has the defect in GGR, but not in TCR.

(5) *XPC and hHR23 proteins*

Originally, the functional *XPC* gene was identified by DNA transfection of normal cDNA (however it was truncated in its N-terminal) into XP group C (Legerski and Peterson 1992). On the other hand, using *in vitro* system based on cell-free extracts capable of performing NER on damaged SV40 minichromosomes (Masutani *et al.* 1993; Sugasawa *et al.* 1993), Masutani *et al.* (1994) identified the XP-C complementing factor, independent to Shivji *et al.* (1994), as a heterodimer constituted by 125 kDa and 58 kDa proteins by the authentic protein purification procedures. Peptide sequence analyses of purified proteins revealed that 125 kDa protein was identical to the previously cloned XPC with the N-terminal extension and the partner protein was one of the human homologs of Rad23 (hHR23).

XPC binds DNA with a preference for single-stranded (Reardon *et al.* 1996; Sugasawa *et al.* 1996) or damaged (Reardon *et al.* 1996) DNA. As indicated in the previous section, XPC-hHR23B complex is specifically associated with the process of GGR. Computer-associated analysis revealed the partial homology between XPC and Rad4 (Henning *et al.* 1994; Legerski and Peterson 1992). The *rad4* mutant cells are completely deficient in NER (McCready 1994) while XP-C cells retain the TCR activity (Venema *et al.* 1990; Venema *et al.* 1991). Phenotypically, the yeast *rad7* and *rad16* mutants resemble human XP-C cells. Both mutants exhibit normal levels of CPD repair of the transcribed strand, whereas the transcriptionally inactive strands of the active genes are not repaired as are the repressed genes (see Hanawalt and Mellon 1993; Verhage *et al.* 1994) .

The yeast Rad23 was isolated by functional complementation of mutants which shows a moderate sensitivity to UV and psolaren crosslink. The *rad1*, *rad2*, *rad3* and *rad4* mutants are completely unable to repair either CPDs or (6-4)PPs and all exhibit a very high sensitivity to UV-irradiation. This is in contrast to the yeast *rad23* mutants. However, no repair of both UV induced damages is observed in the rad23 mutants after 50 J/m^2 UV-irradiation (McCready 1994; Verhage *et al.* 1996). Therefore this intermediate UV-sensitivity of *rad23* mutants is not due to similar defects in GGR as described for *rad7* and *rad16* mutants by Verhage *et al.* (1996). Intriguingly, the *Rad23* gene is found to encode a ubiquitin-like

fusion protein (Watkins *et al.* 1993). Mutation analysis has demonstrated that the ubiquitin-like portion could be replaced by the ubiquitin sequence without affecting Rad23 activity on NER (Watkins *et al.* 1993). However, removal of this conserved domain affected the UV-survival (Watkins *et al.* 1993). On the other hand, two distinct hHR23 proteins have been independently identified (Masutani *et al.* 1994). The hHR23B protein was purified with XPC as a heterodimer, and the *hHR23A* gene was obtained by the computer-assisted sequence analysis. In the respective reconstituted highly purified NER systems, the yeast Rad23 and the human hHR23B seem required for incision activity. From the sequence analyses, some intriguing features in the hHR23 proteins were revealed. The domain that is present in multiple enzyme classes of the ubiquitination pathway was found in the hHR23 proteins (van der Spek *et al.* 1996b). This domain called UBA shows similarity to the C-terminal portion of mammalian multiubiquitinating enzyme $E2_{25K}$. The comment about the linkage between the hHR23 proteins and the ubiquitin pathway is given in Chapter 1-II (4). The region that is responsible for binding to XPC in hHR23B was determined using various type of hHR23B mutants, and shows a high homology between hHR23A and hHR23B (Masutani *et al.* 1997) (Fig. 4). Using *in vitro* repair system, it has been demonstrated that the hHR23 proteins might be at least partially functionally redundant (Sugasawa *et al.* 1997). Sugasawa *et al.* (1997) identified that both hHR23A and hHR23B might be able to bind to XPC and stimulate the activity of XPC in *in vitro* NER system.

For more detailed discussions of DNA repair and mutagenesis, see Friedberg *et al.* (1995) and Vos (1995).

II. General introduction to ubiquitin-dependent proteolysis

Proteasome is an essential component of the ATP-dependent proteolytic pathway in eukaryotic cells. It has been known that most of the cellular proteins are degraded by this

pathway. It is very important for all living cells to regulate the content and composition of intracellular proteins. In these several years the dramatic advances in knowledge about the mechanisms for intracellular protein degradation have brought out. The proteasome has been identified as multi-polypeptide complex present in the cytoplasm as well as in the nucleus and is involved in both ubiquitin-dependent and -independent degradation. Although the form of the proteasome most often isolated is the 20S particle composed by fourteen distinct subunits (Table 3), the larger structure which degrades ubiquitinated proteins by ATP-dependent way is called as 26S (2000 kDa) proteasome. Besides the 20S particle, the 26S complex contains approximately twenty additional polypeptides (Table 4) called as "19S complex", or the "22S regulator". It has been identified that this additional complex regulates substrate specificity and provides multiple enzymatic function (reviewed by Coux *et al.* 1996; Rivett *et al.* 1997; Seeger *et al.* 1997; Tanaka and Tsurumi 1997).

So far, a number of informative reviews concerning the proteasome has appeared, and it is impossible to cover this field within the limits of this introduction. Therefore, the ubiquitin-dependent proteolysis will be mentioned in this introduction.

(1) *Ubiquitin conjugation pathway*

Ubiquitin constituted from only seventy-six amino acids is one of the most abundant polypeptides (~6x 10^7 molecules in each cell of our body) and one of the most conserved protein in evolution. Usually, the ubiquitin molecules are not found as a free form, but conjugated to other polypeptides. The ubiquitin conjugation pathway is divided into three steps (Fig. 5). At first, the ubiquitin-activated enzymes (E1) activate the C-terminal glycine residue of a ubiquitin molecule, then activated ubiquitin makes the complex with one of the ubiquitin-conjugating enzymes (UBC or E2). The E2-ubiquitin complex links the ubiquitin moiety via its C-terminal glycine residue to the target protein, with or without the help of one of the ubiquitin ligases (E3). This ubiquitination pathway and the proteins participating to it,

are also highly conserved almost as well as ubiquitin itself. The ubiquitination finally leads to ATP-dependent degradation of the targeted protein by the 26S proteasome (Fig. 6). (reviewed by Ciechanover 1994; Ciechanover and Schwartz 1994; Wilkinson 1995)

(2) *The "ubiquitin receptor," S5a*

Substrate selection by the 26S proteasome is presumably mediated by the interaction of specific components of the regulatory complex with the multiubiquitinated proteins. Although little is known about this step, a human regulatory complex subunit, S5a, was identified as a candidate which binds to multiubiquitin chain with high affinity (Ferrell *et al.* 1996). Sequence analysis revealed that homologs of this gene are present in a variety of other eukaryotes, including *Saccharomyces cerevisiae*, *Arabidopsis thaliana*, and *Drosophila melanogaster* (Fig. 7). It is reported that *A. thaliana* and *S. cerevisiae* homologs, designated as MBP1 (van Nocker *et al.* 1996a) and Mcb1/Sun1 (van Nocker *et al.* 1996b; Kominami *et al.* 1997), also bind to multiubiquitin chain with high affinity. Interestingly, recombinant MBP1 can inhibit the degradation of the ubiquitinated proteins by the purified 26S proteasome, without affecting the peptidase activities of the proteasome (Deveraux *et al.* 1995). Moreover, in *Xenopus* egg extracts, MBP1 inhibits ubiquitin-mediated degradation of cyclin B (Deveraux *et al.* 1995). These results strongly suggest that both MBP1 and Mcb1 (probably all S5a homologs) might be involved in ubiquitin recognition by the 26S proteasome. However, surprisingly, Mcb1/Sun1 is not essential for *S. cerevisiae* (van Nocker *et al.* 1996b; Kominami *et al.* 1997). Presumably, S5a is not the sole factor which recognizes the ubiquitinated protein, and may interact with only a subset of ubiquitinated substrates.

(3) *Ubiquitin-like proteins*

It was identified that all organisms harbor many functional copies of ubiquitin genes. Moreover, at least one of the genes was a poly-ubiquitin gene, harboring variable number of ubiquitin coding elements, and thus encoding a poly-ubiquitin precursor protein. On the other hand, the mono-ubiquitin genes are often fusion genes as they encode the ubiquitin moiety in frame with a C-terminal extension peptide (CEP). The CEPs were appeared to represent ribosomal polypeptides. In addition, the family of ubiquitin-like proteins is constantly growing (Table 5). This family has a ubiquitin-like sequence with C-terminal extensions or a C-terminal ubiquitin-like extension (designated as N-terminal extension peptide: NEPs). This family is found to represent at least two distinct groups. The first one is a functionally conserved (especially the C-terminal glycine residues) ubiquitin-like portions which have similar functions as the classical ubiquitin (e.g. UCRP, NEDD8, SF3a120). The second one is a class of ancient "normal ubiquitin"-fusion proteins which lost the cleavage site between ubiquitin and the C-terminal extension (e.g. BAT3, GdX, SIII p18, hHR23 proteins, Dsk2, SMT3).

(4) *DNA repair and ubiquitination*

The first link between hHR23 proteins and ubiquitin pathway is mentioned in the previous section. The second link is found in the sequence of hHR23 proteins as well as Rad23, that each contains two repeated and highly conserved about fifty amino acids UBA domains (Fig. 4), a structure of unknown function that is found in several ubiquitin hydrolases, E2 and E3 proteins, as well as in several protein kinases (van der Spek *et al.* 1996b). Moreover, it is intriguing that the majority of hHR23 proteins are free from XPC in cells (Sugasawa *et al.* 1996). However these ubiquitin related sequences of hHR23B is not necessary to bind and stimulate the activity of XPC in *in vitro* repair system (Masutani *et al.* 1997). Therefore, it has been strongly suggested that hHR23 proteins might be involved in the ubiquitin-

dependent degradation pathway.

Ubiquitination of RNA polymerase II large subunit has been observed after exposing cells to UV-radiation or cisplatin. Interestingly, ubiquitination of RNA polymerase II after UV irradiation is deficient in CS cells. Bregman *et al.* (1996) reported that UV-induced ubiquitination of RNA polymerase II can be restored by introducing cDNA encoding the CSA and CSB proteins into CS-A and CS-B cells, respectively.

S. cerevisiae Rad6 involved in DNA repair and DNA damage-induced mutagenesis is a member of the expanding family of E2 enzymes. The *rad6* mutants are extremely sensitive to DNA damage introduced by UV-radiation, X-rays, and alkylating agents. In humans, intriguingly, the *Rad6* homologous gene is duplicated, and the proteins encoded by the distinct two genes human homolog of Rad6 (hHR6) A and B can complement the DNA repair of *rad6* mutants. It has been suggested that both Rad6 and hHR6 proteins are required for post-replication repair.

For more detailed discussions of the mechanism of proteasome and the linkage between DNA repair and ubiquitination, see Oliver *et al.* (1996) and Koken (1996).

ABSTRACT

Abstract for Chapter 2

XPC-hHR23B complex was isolated as a complementing factor which restores nucleotide excision repair defects of XP-C cell extracts *in vitro*. Previously, we reported that human XPC and hHR23B proteins are consisted of 940 and 409 amino acids, respectively. However, both polypeptides have no indicative motifs for their functions. However, from a series of biochemical study, some features of XPC-hHR23B complex has been appeared. i) XPC-hHR23B complex binds to the damaged DNA efficiently. ii) XPC-hHR23B complex works on the transcriptionally inactive DNA and/or the opposite strand of transcriptionally active strand. iii) XPC requires hHR23 proteins to play a role as a complementing factor in NER.

Here I describe that XPC-hHR23B complex associates with TFIIH both *in vivo* and *in vitro*. Immunoprecipitation experiment with the antibody raised against a putative subunit of TFIIH implied that XPC-hHR23B complex may have an interaction with TFIIH in cell extract. This interaction was identified using purified proteins and recombinant proteins. From these results, I concluded that XPC has affinities with both XPB and p62 that are subunits of TFIIH. Recently, it was reported that XPC will participate in open complex formation in global genome repair by Evans *et. al.* (1997). Therefore, I examined whether XPC-TFIIH interaction has any biochemical meanings in NER. I performed the precipitation experiments using DNA-cellulose with the whole-cell extracts that are derived from NER-proficient and -deficient cell lines. In the precipitants from the NER-proficient cell extract, TFIIH was detected by immunoblotting despite its very low DNA-binding activity. Then, I supposed that TFIIH was retained on DNA through the interaction with DNA binding protein(s) such as XPA, XPC, and RPA which are believed as damage-recognition proteins in NER. From the results of NER-deficient cell extracts, I defined that the recovery of TFIIH from cell extract by DNA was dependent on XPC-hHR23B complex,

but not on others. Moreover, I found that, in cell-free extract, XPC-hHR23B complex is necessary for efficient recruitment of TFIIH onto the *N*-acetoxy-2-acetyl-2-aminofluorene (*N*-AAAF) treated DNA.

From these results, I concluded that XPC-hHR23B complex plays an important role in GGR to recruit TFIIH onto the site of damage, and to initiate NER.

Abstract for Chapter 3

We recently identified that both of hHR23A and hHR23B (designated as hHR23 proteins) are the human homologs of the *Saccharomyces cerevisiae* NER gene product Rad23, and they have a function for supporting XPC activity in NER reaction. However, it has been suspected that hHR23 proteins will have other roles except for NER because of its structural features. We reported previously that hHR23 proteins have an ubiquitin-like domain in its N-terminus and two UBA-like domains at the center and in its C-terminal region. Furthermore, most of the hHR23 proteins are free from XPC *in vivo*, suggesting that they have other roles beside NER.

In this study, I identified that hHR23 proteins bind directly with S5a, one of the subunits of 26S proteasome. Using the yeast two-hybrid system, both the full-length and the N-terminal truncated cDNA encoding human S5a (hS5a) were isolated as one of the possible partners of hHR23B. From co-precipitation experiments using the various types of deletion mutants, both of the ubiquitin-like domain of recombinant hHR23B (designated as rhHR23B (1-87)) and the C-terminal region of hS5a are necessary to interact with each other. Since it has been known that S5a binds ubiquitin moieties *in vitro*, it was supposed that the ubiquitin-like portion of hHR23B might be involved in the physical interaction with hS5a. Therefore, I performed the binding assays using intact rhHR23B and rhS5a with either ubiquitin monomer or rhHR23B (1-87) as a competitor. Intriguingly, rhHR23B (1-87) inhibited the interaction of rhHR23B and rhS5a while ubiquitin monomer did not show any effect. This suggests that S5a has higher affinity to the ubiquitin-like portion of

hHR23B than to the ubiquitin monomer. Moreover, in glycerol density gradient fractions of HeLa S100, hHR23 proteins were detected with hS5a in the same fractions which contain the proteasome activity. These results suggest that hHR23 proteins associate with 26S proteasome through the interaction with hS5a *in vivo*.

From these results, I supposed that a part of hHR23 proteins might be involved in the proteolytic pathway in cells.

Chapter 2. XPC-hHR23B complex plays an important role in recruitment of TFIIH onto damaged DNA.

INTRODUCTION

Nucleotide excision repair (NER) is the main pathway which is conserved in wide range species from prokaryote to eukaryote to remove the variety of damage from DNA (Friedberg *et al.* 1995). From studies on this pathway using *Escherichia coli,* it had been identified that the NER pathway is well controlled by the phased enzymatic reaction. So far, many proteins which are involved in this pathway have been purified and characterized in *E. coli.* Recently, the analyses of the eukaryotic NER reaction show the prompt progress in the identification and the characterization of NER proteins using yeast, rodent, and human cells which have deficiency in NER. It has been appeared that the eukaryotic NER proteins have more diversity than that of prokaryote in its number and its function. Both xeroderma pigmentosum (XP) and Cockayne's syndrome (CS) are the human autosomal recessive hereditary diseases which have deficiency in repair activity. It has been known that one or some of NER proteins are deficient in XP and CS patients. So far, the seven complementing groups (A to G) in XP and the two complementing groups (A and B) in CS have been identified by biochemical studies, and most of the NER genes have been cloned. Moreover, using cell-free repair assays, additional factors involved in NER have also been identified such as RPA and PCNA (Coverley *et al.* 1992; Shivji *et al.* 1992; Nichols and Sancar 1992). Based on these studies, including studies on the *in vitro* reconstitution of NER system using purified proteins (Aboussekhra *et al.* 1995; Mu *et al.* 1995), some models for the eukaryotic NER mechanism have been proposed. However, there are some controversy about which protein recognizes the DNA damage and how other NER proteins are introduced to the site of damage to repair.

Among proteins involved in NER, XPA, XPC, UV-DDB, and RPA are consider to be the damage recognition protein because they have an activity to bind to the UV-irradiated

or cisplatin treated DNA (Hirschfeld *et al.* 1990; Coverley *et al.* 1992; Jones and Wood 1993; Mu *et al.* 1996). Among them, in most of NER models, XPA is believed to be a major factor for damage recognition and NER complex formation because of several lines of evidences as follows: i) XPA is indispensable for both the transcription-coupled repair (TCR) and the global genome repair (GGR) (Tanaka *et al.* 1990). ii) XPA associates with other NER related factors such as ERCC1, RPA and TFIIH *in vitro* (Li *et al.* 1994; Park and Sancar 1994; Matsuda *et al.* 1995; Nocentini *et al.* 1997). iii) The affinity of XPA-RPA complex for damaged DNA is much increased than XPA alone (He *et al.* 1995). iv) XPA column eluates of HeLa extract are sufficient to show NER activity *in vitro* (He and Ingles 1997). v) Purified TFIIH was recovered with GST-XPA by glutathione-Sepharose in the presence of UV-irradiated and -nonirradiated DNA (Nocentini *et al.* 1997).

We previously cloned the full-length *XPC* gene and showed that it encodes a protein of 940 amino acids containing none of authentic motifs (Masutani *et al.* 1994). So far, we identified that XPC binds to UV-irradiated dsDNA preferentially depending on hHR23B (human homolog of RAD23) (our unpublished observation). Although the putative role of XPC-hHR23B complex in NER has been still unclear, it is well known that this complex works in GGR but not in TCR where the DNA structure is dramatically changed by the transcription machinery, especially by RNA polymerase II (RNAPII). At transcriptionally active site, double-stranded DNA is opened by the transcription machinery. When RNAP is stalled by lesions on DNA, it is supposed that, at this opened structure, RNAPII will be replaced efficiently by the NER machinery. On the other hand, in GGR, it is predicted that the structure of damaged DNA should be opened to introduce the NER machinery onto lesion. Recently, Evans *et al.* (1997) reported that XPC might be necessary for the opening reaction adjacent to the lesion, and the presence of both XPA and XPC will provide further opening that extend toward the lesion sites. On the other hand, it is reported that XPC was co-purified with TFIIH which is a putative NER factor (Drapkin *et al.* 1994) or with RNAPII containing TFIIH (Maldonado *et al.* 1996) from HeLa nuclear extract. From these evidences, we proposed that XPC might also be involved in the processing of DNA to allow

access for the repair enzymes to DNA to do GGR. It is particularly important to elucidate the function of XPC-hHR23B complex to establish the proper model of NER.

From the results of co-precipitation assay, we reported here that XPC-hHR23B complex and recombinant human XPC (rhXPC) alone but not recombinant hHR23B (rhHR23B) associated with purified TFIIH directly *in vitro*. Moreover, from the results of protein precipitation experiments using DNA-cellulose, we demonstrated that XPC-hHR23B complex is necessary to introduce TFIIH onto *N*-acetoxy-2-acetyl-2-aminofluorene (*N*-AAAF) treated dsDNA in cell extract. These results strongly suggest that XPC-hHR23B complex might be involved in the recruitment of TFIIH onto the damaged DNA in GGR.

RESULTS

Association of XPC-hHR23B complex with TFIIH in cell free extract.

Using an *in vitro* NER system, we have previously reported cloning, purification, and characterization of a protein factor complementing the NER defect of the whole-cell extract from the XP-C mutant cells. So far, there are some reports which suggest the association between XPC-hHR23B complex and TFIIH (Drapkin *et al.* 1994; Maldonado *et al.* 1996). Moreover, it was demonstrated that Rad23 promotes complex formation between TFIIH and Rad14, which is a *Saccharomyces cerevisiae* homolog of human XPA protein (Guzder *et al.* 1995a). Therefore, I demonstrated some experiments to make it clear whether some kind of physical interaction exists between XPC-hHR23B complex and TFIIH or not. To examine the interaction among them, co-immunoprecipitation experiments were performed with antibody raised against cyclin H which is one of the putative subunits of TFIIH. The anti-cyclin H antibody conjugated to Protein G-Sepharose beads was incubated with the whole-cell extracts derived from the NER proficient human cells. The presence of both XPC-hHR23B complex and TFIIH in precipitant fractions were assessed by immunoblotting with antibody raised against XPC and p62, which is one of the essential subunits of TFIIH, respectively. As shown in Fig. 8, both p62 and XPC were identified in the precipitated fraction incubated with anti-cyclin H antibody (Fig. 8, lane 3) but not in the pre-immune bound fraction (Fig. 8, lane 2). This result suggests, although it is very weak, that XPC-hHR23B complex can associate with TFIIH in cell extract.

XPC-hHR23B complex interacts with TFIIH directly *in vitro*.

To establish whether the interaction of XPC-hHR23B complex and TFIIH is mediated by other proteins present in cell extract, co-precipitation experiments were performed using recombinant human XPC (rhXPC), recombinant hHR23B (rhHR23B), and purified TFIIH from the HeLa nuclear extract. HeLa TFIIH were incubated with GST-tagged rhHR23B, which had previously been incubated with either the presence or the absence of rhXPC. The

GST-rhHR23B proteins were precipitated with GSH-Sepharose beads, and the presence of TFIIH in precipitated fractions was assessed by immunoblotting using anti-p62 antibody. As shown in Fig. 9A, the p62 protein was detected in the rhXPC-added fractions (Fig. 9A, lane 4). This indicated that either XPC-hHR23B complex could interact directly with HeLa TFIIH. To confirm this result, hexahistidine-tagged rhHR23B conjugated to Sepharose beads was used. The rhHR23B-6His conjugating Sepharose, which previously incubated in either the presence or the absence of rhXPC, were mixed with purified TFIIH, and precipitation of TFIIH was performed. The presence of TFIIH was assessed by immunoblotting with anti-p62 antibody. Western blot analysis revealed that HeLa TFIIH was also detected in the fraction which is previously incubated with rhXPC (Fig. 9B, lane 3). These results indicated that XPC-hHR23B complex binds with TFIIH through interaction of XPC and TFIIH constituents. Iyer *et al.* (1996) previously examined the protein-protein interactions among subunits of human TFIIH with the yeast two-hybrid system. They reported that both XPB and XPD proteins might be able to interact with other TFIIH subunits, and also demonstrated that XPG would associate with multiple subunits of human TFIIH (Iyer *et al.* 1996). To assess which subunit of TFIIH interacts with XPC-hHR23B complex, several experiments were performed using GST-tagged TFIIH subunits. Each of GST-TFIIH subunits was adsorbed to glutathione-Sepharose beads, previously. The presence of XPC-hHR23B complex was assessed by immunoblotting with anti-XPC antibody. When XPC-hHR23B complex purified from the HeLa nuclear extract was examined, XPC was detected in both GST-XPB and GST-p62 bound fractions by immunoblotting (Fig. 10A, lanes 3 and 5), while neither rRPA nor rPCNA were detected in any bound fractions (Fig. 10D and 10E). From the result of both rhXPC and rhHR23B (Fig. 10B and 10C), it is elucidated that rhXPC interacted with GST-XPB and GST-p62 (Fig. 10B, lanes 3 and 5) almost as well as HeLa XPC-hHR23B complex, whereas rhHR23B did not. Interestingly, the quite small amount of XPC were detected in the GST-p44 and GST-MO15 bound fractions (Fig. 10, lanes 6 and 9). These results indicate that XPC-hHR23B complex associates specifically with human TFIIH through at least either

XPC-XPB or XPC-p62 interaction in cell extract.

TFIIH is recruited efficiently onto dsDNA with the presence of XPC-hHR23B complex in cell extract.

The result of *in vitro* precipitation experiments strongly suggested that XPC-hHR23B complex might be able to associate with TFIIH. Then, to define the meaning of the interaction of XPC-hHR23B complex and TFIIH, several series of precipitation assay were performed. As previously reported, XPC-hHR23B complex shows high affinity to both the single-stranded (ss) and the double-stranded (ds) DNA (Masutani *et al.* 1994; Sugasawa *et al.* 1996; Reardon *et al.* 1996). Thus I proposed the precipitation of XPC-hHR23B complex using ssDNA- and dsDNA-cellulose from the whole-cell extract. As shown in Fig. 11, several NER factors including XPC, TFIIH (p62), XPA, and RPA (RPA32) were detected in the dsDNA-cellulose bound fraction, independently to either the presence or the absence of both ATP and Mg^{2+}, from the whole-cell extract prepared from the NER proficient 293 cells (Fig. 11, lanes 3 and 5). Although XPC, XPA and RPA have been known as a DNA binding protein, it is reported that TFIIH show scarcely low affinity to both the UV-irradiated and the non-irradiated dsDNA (Nocentini *et al.* 1997). Therefore, it was supposed that the efficient precipitation of TFIIH by dsDNA-cellulose was caused by the association of TFIIH with other DNA binding proteins. I supposed that TFIIH might be somehow introduced onto DNA through associations with the DNA binding proteins including XPA, RPA, and XPC-hHR23B, which are known as damage recognition proteins. To proof this idea, the precipitation experiments were performed using the whole-cell extracts prepared from NER-deficient cell lines. As in the case of 293, from XP-B, XP-D, XP-G, CS-A, and CS-B whole-cell extracts, p62 was detected as well as XPC, XPA, and RPA32 in both the ssDNA- and the dsDNA-cellulose bound fractions (Fig. 12A and 12B). Then, I performed same experiments against both XP-C and XP-A whole-cell extracts. From the XP-A whole-cell extract, all proteins were identified, except for XPA, in the DNA-cellulose bound fraction by immunoblotting (Fig. 13, lanes 11 and 12). In contrast to this, from the XP-C

whole-cell extract, p62 was not found in the DNA-cellulose bound fraction nevertheless both XPA and RPA32 were detected (Fig. 13, lanes 7 and 8) as much as the case of the 293 cells (Fig. 13, lanes 3 and 4). This implied that TFIIH is recruited onto DNA in XPC-dependent manner but independent to other NER factors. To proof this hypothesis, the precipitation assays using dsDNA-cellulose were performed with the whole-cell extract prepared from the XP-C cells. The XP-C whole-cell extract was incubated with either the presence or the absence of the HeLa XPC-hHR23B complex, previously. As shown in Fig. 14, in the dsDNA-cellulose bound fraction, the precipitated p62 from the XP-C whole-cell extract was detected depend on HeLa XPC-hHR23B complex by immunoblotting (Fig. 14, lane 9), as much as the case of the 293 cells (Fig. 14, lane 3). Therefore, these results strongly indicate that XPC-hHR23B complex is necessary to recruit TFIIH onto dsDNA in cell extract.

TFIIH was recruited efficiently onto *N*-AAAF treated DNA depend on XPC-hHR23B complex.

Furthermore, to elucidate the importance of XPC-hHR23B complex that would be necessary to recruit TFIIH onto DNA, I designed the experiments using DNA treated by *N*-acetoxy-2-acetyl-2-aminofluorene (*N*-AAAF) to introduce the bulky adducts. It has been known that *N*-AAAF predominantly reacts at the C^8-position of deoxynucleosine residues, causing dG-AF and dG-AAF adducts. To introduce AAF-damages which are repaired principally by the NER pathway, PCR products were treated with *N*-AAAF as described previously (van Vuuren *et al.* 1993). Therefore, I supposed that XPC-hHR23B complex-dependent recruitment of TFIIH onto damaged DNA will be observed. As shown in Fig. 15A, using PCR products treated by *N*-AAAF as indicated time, the precipitation assay was performed. The presence of precipitated proteins was assessed by immunoblotting. From the whole-cell extract of 293, XPC and TFIIH (p62) were recovered efficiently with the *N*-AAAF-treated DNA compared to adduct-free DNA (Fig. 15A, lanes 3 to 6). The amount of both proteins was increased depend on incubation time with *N*-AAAF. In contrast to this, the amount of precipitated RPA32 was relatively decreased when *N*-AAAF treated DNA was used (Fig.

15A, lanes 3 to 6). Furthermore, I could not detect any XPA in the adduct-free DNA bound fraction, and the very low amount of XPA was recruited with *N*-AAAF treated DNA (Fig. 15A, lanes 3 to 6). However, as I demonstrated, XPA will be precipitated by DNA-cellulose. The difference in the amount of XPA between DNA-cellulose and PCR products is supposed that because of the difference in the amount of DNA used in these experiments (see EXPERIMENTAL PROCEDURES). Therefore, I speculated that the precipitation of proteins with small amount of DNA was due to their high affinity to damaged DNA (Fig 15A, lanes 4 to 6). As I mentioned before, TFIIH is reported to show scarcely low affinity to both UV-irradiated and non-irradiated dsDNA (Nocentini *et al.* 1997). Therefore I supposed that TFIIH might be recruited onto damaged DNA in XPC-hHR23B dependent manner as shown in Fig. 14. To examine the role of XPC-hHR23B complex in cell extract, the precipitation experiment was performed using the *N*-AAAF treated (60 min) DNA with the whole-cell extract prepared from the XP-C cells. Western blot analyses revealed that TFIIH was not precipitated with the adduct-free DNA (Fig. 15B, lane 3) from XP-C cell extract. Interestingly, the damaged DNA, which can precipitate TFIIH efficiently from 293 cell extract, failed to collect TFIIH from XP-C cell extract (Fig. 15B, lane 4). However, TFIIH was detected in the damaged DNA-bound fraction in XPC-hHR23B complex dependent manner (Fig. 15B, lane 5). These results strongly indicate that the role of XPC-hHR23B complex in NER is the recruitment of TFIIH onto damaged DNA in cell extract.

DISCUSSION

XPC-hHR23B complex was purified from the HeLa cell nuclear extracts as a protein factor which is sufficient to complement the DNA repair deficiency of the XP-C whole cell extracts in the cell-free NER reaction (Masutani *et al.* 1994; Shivji *et al.* 1994). Although no enzymatic activity was detected in the purified complex, it has been appeared that this complex is required for the DNA repair reaction at least before the step of excision (Mu *et al.* 1996; Shivji *et al.* 1994). During the purification, XPC-hHR23B complex exhibited high affinity for single-stranded DNA (Masutani *et al.* 1994; Shivji *et al.* 1994). Moreover, using the recombinant protein of both human XPC and hHR23B (designated as rhXPC and rhHR23B), high affinity of XPC-hHR23B complex for both double-stranded and UV-irradiated DNA has been also clarified (Reardon *et al.* 1996). However, the functional roles of XPC-hHR23B complex in NER are still unknown.

In contrast to XPC-hHR23B complex, XPA is one of the proteins which would be involved in the damage recognition in NER reaction. It has been well known that XPA preferentially binds to several types of damages, which are known to be the substrates for NER, introduced by UV, cisplatin, and *N*-AAAF treatment (Jones and Wood 1993; Asahina *et al.* 1994; He *et al.* 1995). The affinity of XPA for damaged DNA has been augmented in the presence of RPA (He *et al.* 1995), probably through the interaction of XPA and RPA (Li *et al.* 1995). In addition, ERCC1 has also been known to enhance the affinity of XPA for the damaged DNA when ERCC1 interacts with XPA (Nagai *et al.* 1995). Moreover, it has been reported that XPA recruits TFIIH to the damaged DNA *in vitro* (Nocentini *et al.* 1997; Park *et al.* 1995). From these evidences, at present, XPA has been believed as the best candidate for the initiator of the NER event. Another damage-binding protein, UV-DDB, was isolated as a protein complex (124 and 41 kDa) which exhibit the high affinity for some lesions (Keeney *et al.* 1993). However, the experiment using the reconstituted NER system indicated that UV-DDB plays a regulatory but not an essential role in the NER process (Aboussekhra *et al.* 1995).

In this thesis, I presented the physical association between XPC-hHR23B complex and TFIIH. There are some reports which suggest interaction of TFIIH and several proteins including XPC in human, or Rad4, which is supposed to be a yeast XPC homolog (Drapkin *et al.* 1994; Svejstrup *et al.* 1995; Maldonado *et al.* 1996; He and Ingles 1997). However, no evidences have been obtained to prove the association of TFIIH with XPC or Rad4 (Guzder *et al.* 1996; van der Spek *et al.* 1996a). In yeast, interaction of TFIIH and Rad23 was identified (Guzder *et al.* 1995a). In contrast to this, I demonstrated that TFIIH associates with XPC-hHR23B complex at least through XPC-XPB and/or XPC-p62 interactions, while both the TFIIH and the components of TFIIH did not show any affinity to hHR23B. Moreover, hHR23B might be omitted from NER components in the reconstituted system (Reardon *et al.* 1996). However, I cannot exclude the possibility that hHR23B somehow affect the association of TFIIH with XPC since hHR23B as well as hHR23A are necessary to stimulate the NER activity of rhXPC protein in the cell-free NER reaction (Masutani *et al.* 1997; Sugasawa *et al.* 1997).

Furthermore, I demonstrated that XPC-hHR23B complex is necessary to recruit TFIIH efficiently onto damaged DNA in the whole cell extract. As shown in Fig. 15B, TFIIH was precipitated by DNA in XPC-hHR23B complex dependent manner. However, this result does not deny the scenario that XPA is necessary to initiate the NER reaction. Actually, the incision reaction has never been observed without XPA in the reconstituted excision reaction (Mu *et al.* 1997). From the property of XPA, I supposed that XPA would be necessary to assemble the repair factors (e.g. XPF-ERCC1, RPA) on the damaged site through protein-protein interactions, whereas XPC-hHR23B complex recruit TFIIH onto substrate in cell extract.

On the other hand, it is well known that XPC-hHR23B complex works in GGR but not in TCR, where the DNA structure is dramatically changed by the transcription machinery. It has been supposed that double-stranded DNA might be remained the opening structure when RNA polymerase II is stalled at a lesion during transcription on the damaged template. Consequently, one could reasonably assume that XPC would be necessary for the

excision repair on the base paired duplex substrate, but not on the substrate which has opened structure. Interestingly, it has been reported that XPC (with or without hHR23B) might be omitted from the NER components to excise the cyclobutane thymine dimer from the bubble substrate (Mu and Sancar 1997; Mu *et al.* 1997). Moreover, for repair of a particular cholesterol moiety built into a DNA backbone, the XPC subunit is not required even for repair (Mu *et al.* 1996). These findings strongly suggest that XPC is dispensable for certain DNA structures. It has been known that some kind of damage will change the DNA structure by distortion of the helical structure. Probably, these DNAs would be the suitable substrate for repair factors to assemble without XPC-hHR23B complex. This kind of difference in the structure of the NER substrate may be the one of reasons for the superiority of TCR to GGR.

Recently, we demonstrated the several experiments to identify the positioning of XPC-hHR23B complex in GGR, and concluded that XPC-hHR23B complex is a very first damage detector. These data suggest that XPC is necessary in the immediate early stage of repair reaction before the XPA protein recognizes the damage (our unpublished data). Based on my results in addition to other findings, I proposed the model for the complex formation of repair factors on damaged DNA (Fig. 16). On the base paired substrate, the damage would be detected by XPC-hHR23B. XPC-hHR23B complex may introduce a conformational change in the substrate. Although there is no evidence indicating such a conformational change in the substrate by XPC-hHR23B complex, XPA will assemble on the damaged site. Then TFIIH would be recruited onto the substrate. Probably, RPA is supposed to be already involved in this complex through interaction with XPA (Li *et al.* 1995). XPG might be able to associate with TFIIH (Iyer *et al.* 1996; our unpublished data) and RPA (He *et al.* 1995). On the other hand, it has been identified that RPA and XPA would interact with XPF and ERCC1 (Matsunaga *et al.* 1996), respectively. Therefore, XPG and XPF-ERCC1 complex would be introduced onto the substrate through such interaction.

Although the entire figure is still unknown at present, the future work with the repair

system using a chromatin DNA will provide us a more detailed model for GGR in cells.

Chapter 3. hHR23 proteins interact with 26S proteasome subunit S5a through the ubiquitin-like portion.

INTRODUCTION

Nucleotide excision repair (NER) in eukaryotic cells is a complex biochemical process supported by the multiple gene products. It has been revealed that NER is evolutionarily highly conserved among eukaryotes. The number and the function of the proteins participating in this repair system are common among eukaryotes from the numerous experiments using the UV-sensitive eukaryote cells derived from yeast mutants, the laboratory-induced rodent cell lines, and the cells from the human autosomal recessive disease patients. However mammalian equivalents are still missing for the several yeast NER genes. We previously reported the cloning and analyses of three human NER genes, *XPC*, and two distinct human homologs of Rad23 (*hHR23*) gene, which are involved in the complementation of the NER defect of xeroderma pigmentosum group C (Masutani *et al.* 1994). This finding filled one of the remaining gaps in the parallels between yeast and human. Interestingly, two distinct *hHR23* genes have been independently identified. It has been revealed that the *hHR23B* gene encodes the protein purified with XPC as a complex, while the *hHR23A* gene was obtained by the computer-assisted sequence analysis. The sequence analyses revealed some intriguing features of hHR23 proteins, as well as Rad23. All of three gene products harbor a ubiquitin-like sequence at the N-terminus, and contain two copies of the "ubiquitin associated (UBA) domain" (van der Spek *et al.* 1996b), which appears to be present in the enzyme classes involved in the ubiquitination pathway (Hofmann and Bucher 1996). However, the ubiquitin-like domain of Rad23 is required for its biological function in yeast (Watkins *et al.* 1993), Masutani *et al.* (1997) reported both the ubiquitin-like sequence and UBA domains of hHR23B might not be directly involved in the core part of the NER reaction *in vitro*, because the mutant hHR23B lacking these sequences showed only slightly lower activity than the full-length protein. One of the possible

explanations for this dispensability of the ubiquitin related sequences of hHR23 proteins is that there is a link between hHR23 proteins and the ubiquitin pathway. Since the ubiquitin-conjugating pathway is involved in the proteolytic degradation of proteins, these ubiquitin related sequences may control the functions of the hHR23 proteins by affecting turnover of these proteins *in vivo*. It has also been appeared that the ubiquitin-dependent proteolysis is involved in additional cellular process such as DNA repair, chromosome condensation and decondensation, and cell cycle control (Koken 1996).

Intriguingly, Western blot analyses indicate that the majority of hHR23 proteins are free from XPC in human cells, and exist even in the XP-C cell lines (Sugasawa *et al.* 1996; van der Spek *et al.* 1996a). These results suggest that the hHR23 proteins may have additional novel roles in cells other than complex formation with XPC.

I describe here the physical association of the hHR23 proteins with one of the regulatory subunits of 26S proteasome, S5a, which is identified as a multiubiquitin chain binding protein. This is the first report that indicates the association of the hHR23 proteins with the ubiquitin-dependent proteolysis.

RESULTS

hHR23B interacts with hS5a, a component of the human 26S proteasome regulatory subunit.

To isolate protein(s) which interacts with hHR23B, I performed the yeast two-hybrid screening. The hHR23B was expressed in the pGBT9 yeast two-hybrid vector. The interaction of the hHR23B fusion protein and a known dimerization partner XPC was confirmed with providing the histidine prototrophy to the yeast strain and an intense blue color upon *in situ* β-galactosidase assay. These results impaired that hHR23B was expressed and interacted specifically with a known natural target molecule in yeast. The yeast strain harboring the pGBT-hHR23B plasmid bait was transformed with a human WI38VA13 cell cDNA library in the pGAD vector, and histidine prototrophs were selected. The screening of approximately 1.5X 10^7 yeast transformants yielded 3 candidate His^+ clones. Analyses of nucleotide sequence revealed that all of obtained cDNAs were identical to human S5a (hS5a) protein, which is one of the regulatory subunits of 26S proteasome (Fig. 17), and they have different size in length. One clone contained the full-length of cDNA and other contained N-terminal truncated cDNAs which have different size in length each other. To confirm the specificity of the observed interaction, these clones were reintroduced into yeast with the various pGAD constructs. As expected, the histidine prototrophy and β-galactosidase activity were observed in yeast expressing both hS5a protein and hHR23B (data not shown). Interestingly, all of hS5a constructs also interacted with hHR23A. These results suggest that the C-terminal portion of hS5a may be sufficient to interact with hHR23 proteins in yeast. From the sequence comparison among the S5a homologs in eukaryotes (*Saccharomyces cerevisiae*, *Arabidopsis thaliana*, *Drosophila melanogaster*, mouse, and human), two copies of the motif composed with four amino acids, GVDP, were found in the C-terminal portion among higher eukaryotes (Fig. 7). Moreover, since it has been identified that hHR23 proteins have ubiquitin-related sequence

which highly conserved between hHR23A and hHR23B (Masutani *et al.* 1997), it was suggested that these sequences may be involved in the interaction with hS5a.

Determination of the hS5a-binding domain in hHR23B.

The result of yeast studies strongly suggested that hS5a might be one of the specific partner for the hHR23 proteins. However, it has been known that a component of the proteasome will often be isolated as a false-positive with yeast two-hybrid system. Therefore, to examine whether hS5a would interact directly with both the hHR23 proteins *in vitro*, hS5a was expressed from the obtained full-length cDNAs and purified. We previously identified the region of hHR23B that affects the interaction with XPC using various types of recombinant hHR23B (rhHR23B) mutants (Masutani *et al.* 1997). These rhHR23B mutants previously prepared were used to identify the region, which responsible for the interaction with hS5a, in hHR23B. A summary of the mutant hexahistidine-tagged hHR23B proteins is shown in Fig. 18A. The rhHR23B-6His with various deletions were adsorbed to nickel-chelating Sepharose beads, then incubated with non-tagged rhS5a proteins. The presence of rhS5a was assessed by immunoblotting. As shown in Fig. 18B, only rhHR23B laking 72 amino acids from the N-terminus failed to interact with rhS5a (Fig. 18B, lane 3), and the polypeptide constituted with only 87 amino acids from the N-terminus, designated as rhHR23B (1-87), was sufficient to precipitate the rhS5a molecule (Fig. 18B, lane 12). As we previously mentioned, N-terminal region (1 to 79 amino acids) shares the homology to ubiquitin. Then I examined whether the interaction between hS5a and hHR23B is somehow affected by either rhHR23B (1-87) or ubiquitin monomer. As shown in Fig. 19, rhHR23B was detected in the precipitated fraction incubated with GST-rhS5a (Fig. 19, lane 4) but not in the glutathione-Sepharose bound fraction (Fig. 19, lane 2). Interestingly, the amount of precipitated hHR23B by GST-rhS5a was dramatically decreased by the addition of rhHR23B (1-87) in a concentration-dependent manner (Fig. 19, lanes 6, 8, and 10), while ubiquitin monomer (designated as mono-ubiquitin) did not affect the interaction of rhS5a and rhHR23B (Fig. 19, lanes 12, 14, and 16). These results strongly suggest that the ubiquitin-

like domain of hHR23B is necessary and sufficient to interact with hS5a.

Determination of the hHR23 proteins-binding domain in hS5a.

To localize the region in hS5a that mediates the interaction with both the hHR23 proteins, several series of externally deleted rhS5a proteins were also prepared. A summary of the mutant recombinant hS5a (rhS5a) proteins is shown in Fig. 20A. To examine the binding activity with both the rhHR23 proteins, the hexahistidine-tagged rhS5a (full-length or truncated) proteins were incubated with either rhHR23A or rhHR23B. The tagged proteins were precipitated with nickel-chelating Sepharose beads, and the presence of both the rhHR23 proteins in bound fractions was assessed by immunoblotting. As shown in Fig. 20B and C, rhHR23 proteins itself did not bind to nickel-chelating Sepharose (Fig. 20B, lanes 2; Fig. 20C, lane 2). In the presence of the full-length 6His-rhS5a, detectable amount of the rhHR23 proteins were precipitated (Fig. 20B, lane 3; Fig. 20C, lane 3), indicating that the rhHR23 proteins formed a physical complex with rhS5a *in vitro*. When no more than 262 amino acids were deleted from the N-terminus, both the hHR23 proteins were still bound to the mutant rhS5a proteins (Fig. 20B, lanes 4 to 6; Fig. 20C, lanes 4 to 6). As for the deletion from the C-terminus, both hHR23 proteins were detected in the bound fraction with the 6His-rhS5a lacking amino acids from residue G^{344} onwards to the C-terminus (Fig. 20B, lane 7; Fig. 20C, lane 7). However, further deletion towards the N-terminus abolished the hHR23-binding activity almost completely (Fig. 20B, lanes 8 and 9; Fig. 20C, lanes 8 and 9). These results indicate that the hHR23-binding domain is located within the region covering amino acids M^{263} to P^{343} in hS5a. It is intriguing to note that this region does not include any GVDP motifs which found two times in the C-terminal portion.

Co-migration of the hHR23 proteins and the 26S proteasome during glycerol gradient centrifugation.

The results of *in vitro* binding assay identified that both hHR23 proteins interact with hS5a, and implied that both hHR23 proteins might be able to associate with the proteasome through

the interaction with hS5a *in vivo*. This can be analyzed by immunoblotting fractions of cell extract separated through glycerol density gradient centrifugation after preincubation of the extracts in either the presence or the absence of ATP, conditions which should promote either assembly or disassembly, respectively, of the 26S proteasome. Thus, the identification of both putative and regulatory subunits of the proteasome can be determined by comparing their distribution under these two conditions. When the distribution of both hHR23 proteins was analyzed with the extract preincubated with ATP, these proteins were found to migrate with hS5a peaking at fraction 9 (Fig. 21A, fraction 9; Fig. 21B, fraction 9), however the majority of both hHR23 proteins was found in top fractions (Fig. 21A and Fig. 21B). Under this condition, most of the proteasome activity was observed in fraction 9 (Fig. 21C). On the other hand, when the same analysis was repeated with the extract pre-incubated without ATP, the peak fractions of both hHR23 proteins were shifted from the fraction 9 to 13 although the pattern of hS5a did not show dramatic changes (Fig. 21D, fraction 13; Fig. 21E, fraction 13). They should contain both the 20S proteasome and the regulatory complex (Fig. 21F). These results were consistent with my hypothesis that both hHR23 proteins could be associated with the 26S proteasome at least through the interaction with hS5a in cell extract.

DISCUSSION

The hHR23 proteins belong to a growing family of the ubiquitin-like proteins, that contain N-terminal domains homologous but not identical to real ubiquitin (Masutani *et al.* 1994). Although these proteins have been identified in many organisms (Koken 1996), the functions of almost all of them are unknown. One of the major classes of ubiquitin-like proteins is involved in DNA repair, that includes the yeast Rad23 protein and its human homolog hHR23 proteins. *Rad23* was the first ubiquitin-like protein identified in yeast and was originally isolated in a screen for UV-sensitive mutants (Watkins *et al.* 1993). The ubiquitin-like sequence of Rad23 was shown to be important for nucleotide excision repair (NER) functions in yeast. However, a requirement for the ubiquitin-like sequence in hHR23B was unclear in the cell-free NER system (Masutani *et al.* 1997). Masutani *et al.* (1997) have examined interaction of hHR23B and XPC proteins by using several deletion mutants of hHR23B protein. The XPC-binding domain was mapped near the C-terminus of hHR23B, 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 XPC and hHR23B. Furthermore, the majority of hHR23 proteins are free from XPC in human cells (Sugasawa *et al.* 1996; van der Spek *et al.* 1996a). These results strongly suggest that the hHR23 proteins may have a novel role in cells other than complex formation with XPC.

In this thesis, I identified that hHR23B associates with hS5a, one of the regulatory subunit of the 26S proteasome using the yeast two-hybrid system. The first S5a protein was isolated as a multiubiquitin binding protein, MBP1, from *A. thaliana* (van Nocker *et al.* 1996a). So far, the MBP1 homolog has been isolated from *S. cerevisiae*, *D. melanogaster*, and human. The human homolog of MBP1, designated as hS5a in this thesis, shows high similarity to MBP1, and it has been appeared that hS5a is also a multiubiquitin chain binding protein (Ferrell *et al.* 1996). Moreover, Ferrell *et al.* (1996) proposed that S5a has two large repeats starting from the specific tetrapeptide, GVDP, and speculated that this proposed

repeats will be responsible for binding to ubiquitin polymers. Recently, the multiubiquitin chain-binding domain was mapped in hS5a to a C-terminal 45 amino acid region (196 to 241 amino acid) which includes one of two GVDP motifs of the protein (Deveraux *et al.* unpublished data). I demonstrated here that hHR23B interacts directly with hS5a through its ubiquitin-like sequence. Judging from the high homology in the ubiquitin-like domain between hHR23B and hHR23A, it is supposed that the interaction of hHR23A with hS5a will be also mediated through the ubiquitin-like domain in the hHR23A protein. Actually, I demonstrated that hHR23A binds to the hHR23B-binding domain in hS5a (Fig. 20). However, the domain required for the binding with the hHR23 proteins in hS5a is different from its multiubiquitin chain-binding domain. I reported that ubiquitin monomer cannot be competitive with hHR23B (Fig. 19, lanes 12, 14, and 16). Probably, this result is because of the difference of the binding domain. Although it has been appeared that hS5a has a little affinity for ubiquitin monomers (van Nocker *et al.* 1996a), my result is sufficient to indicate the ubiquitin-like domain monomer has a high affinity for hS5a compared to the ubiquitin monomer.

Furthermore, I demonstrate that the hHR23 proteins associate with the 26S proteasome during glycerol gradient centrifugation in an ATP-dependent manner (Fig. 21). Although I cannot exclude the possibility that the hHR23 proteins migrate with the 26S proteasome to be degraded, at present I suppose that hHR23 proteins associate with the 26S proteasome, probably, to regulate its function. It may be that the hHR23 proteins are involved in the determination of the substrate specificity or the regulation of speed of proteolytic pathway through the interaction with hS5a. Therefore, one possible explanation of the ubiquitin-like sequence of the hHR23 proteins is that this sequence may involved in some regulatory aspect in cell. One can speculate that the hHR23 proteins, directly or indirectly, affect DNA repair, DNA replication, and cell cycle control.

In this few years, several indications suggested that the function of the ubiquitin-like proteins are growing. It has been proposed that SMT3, which encodes an essential *S. cerevisiae* ubiquitin-like protein similar to the mammalian protein SUMO-1, is one of the

centromeric proteins (Meluh and Koshland 1995). *DSK2* gene encoding a ubiquitin-like protein in which the first 76 amino acids is required with Rad23 function for the spindle pole body duplication (Biggins *et al.* 1996). *NEDD8* gene encoding 81 amino acid polypeptide which shows high homology to ubiquitin, is supposed to be involved in developmental pathway in mouse (Kamitani *et al.* 1997). Quite recently, it was reported that the human virus type 1 (HIV1) *vpr* gene product interacts physically with hHR23A, and the vpr-binding domain in hHR23A is mapped to the C-terminus including one of two UBA domains (Withers-Ward *et al.* 1997). They demonstrated that overexpression of either the hHR23A or the vpr-binding domain of hHR23A alleviates the G2 arrest induced by Vpr.

These findings, including my data, will be good clues to investigate the function of these ubiquitin-like proteins. Although, in this thesis, I could not find any evidences to propose the function of UBA domain in the hHR23 proteins, this domain is thought to be involved in the proteolytic degradation of proteins, and in additional cellular processes, since this domain is homologous to a C-terminal extension of a Class II ubiquitin conjugating enzyme. Thus, I believe that the detailed analyses of UBA domain, and also the ubiquitin-like sequence, will provide us strong clues to elucidate the entire function of the hHR23 proteins.

CONCLUSION

In this thesis, I analyzed the function of both the XPC protein and the hHR23 proteins to elucidate their possible role in cell extract. In chapter 2, I demonstrated that XPC-hHR23B complex can directly interacts with TFIIH. Furthermore, I revealed that XPC-hHR23B complex is necessary to recruit TFIIH efficiently onto damaged DNA in cell extract. This is the first report that indicates the function of XPC-hHR23B complex in NER. In chapter 3, I indicated that the hHR23 proteins might be able to involve in the 26S proteasome dependent protein degradation pathway. Although this is still no more than circumstantial evidence, I believe that this information provide us strong clues to investigate the unknown function of the hHR23 proteins in near future.

EXPERIMENTAL PROCEDURES

Cell culture and media

Human 293, XP2OSSV (group A), XP7CASV (group A), XP4PASV (group C), XP6BESV (group D), XP3BRSV (group G), and CS2OSSV (CS-A) cells were grown at 37 °C in Dulbecco's modified Eagle's medium (Nissui) supplemented with 10% fetal bovine serum. A lymphoblastoid cell line (GM1629SV) from CS-B patient and GM2252A (group B) were grown in suspension with RPMI 1640 medium (Nissui) supplemented with 15% fetal bovine serum. HeLa cells were grown Dulbecco's modified Eagle's medium supplemented with 5% calf serum. For the preparation of whole-cell extracts, HeLa cells were grown in spinner flasks at 37°C.

Preparation of whole-cell extracts, and fractionation of HeLa S100 using the glycerol density gradients centrifugation

Whole-cell extracts which used in this thesis were prepared as described previously (Sugasawa *et al.* 1993). Cytoplasmic S100 fraction was prepared from HeLa cells was described previously (Ohkuma *et al.* 1995). HeLa S100 (7 mg) with either the presence or the absence of 2 mM ATP was incubated on ice for 1 h. After layered the extract onto 10-40% glycerol gradients, the centrifugation was performed at 22,500 rpm for 22 h at 4°C. The 30 ml gradients were fractionated into 1 ml aliquots from the bottom. Western blot analysis against fractionated samples was performed by anti-S5a, anti hHR23A, and anti-hHR23B antibody.

Preparation of NER proteins purified from cell extract

HeLa XPC-hHR23B complex was purified through four columns: phosphocellulose, ssDNA-cellulose, CM cosmogel, and Mono Q as described previously (Masutani *et al.* 1994). The TFIIH was purified from HeLa S100 fractions through five columns: phosphocellulose, DEAE-cellulose, HPLC-DEAE 5PW, HPLC-SP 5PW, and HPLC-

heparin 5PW as described previously (Ohkuma *et al.* 1995).

Preparation of the recombinant proteins

Non-tagged and the hexahistidine-tagged rhHR23B was expressed in BL21(DE3) following IPTG induction, and purified as described previously (Masutani *et al.* 1997). Recombinant human XPC (rhXPC) was expressed using baculovirus expression system, and purified as described previously (Sugasawa *et al.* 1996). To express the recombinant RPA, pET11a-RPA was constructed (Maekawa *et al.* manuscript in preparation). In briefly, rRPA was expressed in BL21(DE3) following IPTG induction, and was purified through two columns: ssDNA-cellulose, and Mono Q. The pT7-PCNA was kindly gifted from Dr. Tsurimoto. The recombinant PCNA was expressed in BL21(DE3) following IPTG induction, and was purified through three columns: HiTrap Q, Hydroxylapatite, Phenyl superose. Eight subunits of TFIIH (XPB, XPD, p62, p44, p34, MO15, cyclin H, and MAT1) were expressed as a GST-tagged proteins using *E. coli* expression system. The cDNA encoding each subunits are cloned into 6His-pET vector. All recombinants were expressed in BL21(DE3) following IPTG induction as described above. For the preparation of recombinant human S5a (rhS5a), PCR was performed with all of obtained cDNA for hS5a using oligomers (Table 6) which are designed to create both the *Nde*I site at the N-terminus and the *Eco*RI site at the C-terminus of hS5a. To prepare a series of the truncated proteins, PCR were performed with different primer combinations (Table 6). Each PCR products was cloned into 6His-pET11d vector. All recombinants were expressed in BL21(DE3) following IPTG induction, and were loaded onto nickel-chelating Sepharose column chromatography with 20 mM sodium phosphate [pH 6.8] containing 20 mM imidazole. The column was washed with same buffer containing 60 mM imidazole, and adsorbed proteins were eluted with buffer containing 250 mM imidazole. To obtain the GST-tagged hS5a protein, PCR product encoding the full-length S5a was cloned into pGEXIIT+ vector. GST-rhS5a was prepared using *E. coli* expression system as described above. To prepare the non-tagged rhS5a, PCR product encoding the full-length S5a was cloned into pGET3a vector, and

expressed as described above.

Co-immunoprecipitation

Anti-cyclin H polyclonal antibody were mixed with Immunopure Immobilized Protein G (Pierce) in buffer C (20 mM Tris-HCl [pH 7.9], 200 μM EDTA, 20 % glycerol, 0.2 M KCl, 0.5 mM ATP, 5 mM $MgCl_2$, 0.1% Nonidet-P40, 20 mM PMSF, 10 μM ß-melcaptoethanol) containing 200 μg/ml BSA at 4°C for 1 h with rotation. Then the mixture was washed three times with buffer C. The antibody bound-Sepharose beads were incubated with 80 μg of Manley's extract prepared from 293 human fibroblasts in buffer C at 4°C for 1 h with rotation. The mixture was centrifuged. The resin was washed with buffer C. The bound proteins were extracted by boiling in SDS sample buffer, and separated by 10% SDS-polyacrylamide gel electrophoresis (SDS-PAGE), and analyzed by immunoblotting with anti-XPC, and anti-p62 antibody.

***In vitro* binding assay: TFIIH and either the rhHR23B-6His-Sepharose or the rhXPC-rhHR23B-6His-Sepharose**

The purified rhHR23B-6His (2.2 mg) dialyzed against buffer containing 20 mM NaPi [pH 6.8], and 0.3 M NaCl was incubated with Activated CH-Sepharose 4B (Pharmacia Biotech) at 4°C for overnight followed washing with excess amount of buffer containing 0.1 M Tris-HCl [pH 8.0] and 0.5 M NaCl. Ten μl of rhHR23B-6His-Sepharose was incubated with either the presence or the absence of rhXPC (90 ng) in buffer C containing 200 μg/ml BSA on ice for 30 min, and the mixture was incubated with purified HeLa TFIIH at 4°C for 1 h with rotation. The mixture was centrifuged. The resin was washed with buffer C. The bound proteins were extracted in SDS sample buffer, and after boiling, separated by SDS-PAGE and analyzed by the immunoblotting with anti-p62 antibody. GST-hHR23B, which incubated with either the presence or the absence of rhXPC for 30 min on ice, was mixed with purified HeLa TFIIH, and incubated at 4°C for 1 h with rotation. The mixture was incubated with glutathione (GST)-Sepharose beads at 4°C for 1 h with rotation. The mixture

was centrifuged. The resin was washed with buffer C. The bound proteins were extracted by boiling in SDS sample buffer and separated by SDS-PAGE and analyzed by the immunoblotting with anti-p62 antibody.

In vitro binding assay: XPC-hHR23B complex and GST-tagged TFIIH subunits

The GST-tagged TFIIH subunits (100 ng) in *Escherichia coli* lysates were adsorbed to GST-Sepharose beads (Pharmacia Biotech) in buffer D (20 mM Tris-HCl [pH 7.5], 10% glycerol, 0.5 M NaCl, 2 mM DTT, 0.5 mM PMSF, 3X protease inhibitor) at 4°C for 1 h with rotation. After washing with buffer C containing 200 μg/ml BSA, purified GST-tagged TFIIH components were incubated with either XPC-hHR23B complex (50 ng), rhXPC (60 ng), rhHR23B (60 ng), rRPA (200 ng), or rPCNA (200 ng) in same buffer at 4°C for 1 h. The mixture was centrifuged. The resin was washed with buffer C. The bound proteins were extracted by boiling in SDS sample buffer, and separated by either 8 % (for detection of XPC and hHR23B) or 12% (for detection of RPA32 and PCNA) SDS-PAGE, and analyzed by immunoblotting with anti-XPC, anti-hHR23B anti-RPA32, and anti-PCNA antibody, respectively.

In vitro binding assay: rhS5a and a series of rhHR23B-6His mutants

A series of 6His-tagged rhHR23B mutants was prepared previously (Masutani *et al.* 1997). The 6His-tagged rhHR23B (full-length or truncated) proteins were incubated with non-tagged rhS5a proteins in buffer H (20 mM HEPES [pH 7.9], 0.2 M NaCl, 20% glycerol, 0.1% NP40, 200 μg/ml BSA, 0.25 M PMSF, 10 μM ß-melcaptoethanol) on ice for 1 h. Two fold suspension of nickel-chelating Sepharose with buffer H containing 20 mM imidazole was added, and incubated with for 1 h at 4°C. The mixture was centrifuged. The resin was washed with buffer H containing 60 mM imidazole and the bound proteins were extracted by boiling in SDS sample buffer, and separated by 8% SDS-PAGE, and analyzed by immunoblotting with anti-hS5a antibody.

Competition assay

The rhHR23B-6His protein (3 pmol) was mixed with either the presence or the absence of the competitor as indicated amounts in buffer H, and then incubated with GST-S5a (15 pmol) on ice for 1 h. Both the rhHR23B (1-87) protein, which contains the N-terminal ubiquitin-like portion of hHR23B, and the ubiquitin molecule was used as the competitor. Then GSH-Sepharose was mixed, and incubated at 4°C for overnight. The mixture was centrifuged, and the supernatant wa store as unbound material. The resin was washed with buffer H, and the bound proteins were extracted by boiling in SDS sample buffer and separated by 10% SDS-PAGE and analyzed by the immunoblotting with anti-hHR23B antibody.

Precipitation of proteins with DNA-cellulose

Three different kinds of cellulose solutions were prepared. For control experiments, cellulose (CF11; Whatman) was suspended in buffer C containing 200 μg/ml BSA. To precipitate the DNA-binding proteins, single-stranded and double-stranded DNA-cellulose (Sigma) were suspended in buffer C containing 200 μg/ml BSA, respectively. These cellulose solutions were mixed with same amount of Sepharose CL4B beads (Pharmacia Biotech) in buffer C containing 200 μg/ml BSA. Whole-cell extract (125 μg) was incubated with each kind of cellulose-Sepharose solutions (10 μl) in buffer C containing 200 μg/ml BSA at 4°C for 1 h with rotation. The mixture was centrifuged. The resin was washed with buffer C. The bound proteins were extracted by boiling in SDS sample buffer and separated by 10-14% gradient SDS-PAGE and analyzed by the immunoblotting with anti-XPC, p62, XPA, and RPA32 antibody. The calculated amount of DNA is 7 mg/g of solid in double-stranded DNA-cellulose. To examine the role of XPC-hHR23B complex in cell extract, 5 ng of HeLa XPC-hHR23B complex was added to the whole-cell extract prepared from XP-C cells.

Antibodies

The antibody for XPC, hHR23A, hHR23B, p62, PCNA, and RPA32 were obtained as described previously (Ohkuma and Roeder 1994; Sugasawa *et al.* 1996). Anti-XPA polyclonal antibody was kindly gifted from Dr. Kiyoji Tanaka. Anti-S5a antibody was obtained by immunization of rabbits with a hexahistidine-tagged rhS5a, and purified using affinity chromatography.

Preparation of PCR products for precipitation of NER proteins

The PCR (35 cycles) were performed with the T3 and the biotin-labelled T7 primer to amplify the human cDNA (0.8 kbp) cloned into pBS. PCR reactions were carried out in a total volume of 50 μl containing 1 ng of template DNA, 10 mM KCl, 20 mM Tris-HCl [pH 8.8], 2 mM $MgSO_4$, 10 mM $(NH_4)_2SO_4$, 0.1% Triton X-100, 0.1 mg/ml BSA, 200 μM dNTPs, 20 pmol each of the oligonucleotide primer, and 2.5 unit of Pfu DNA polymerase (Strategene) with GeneAmp PCR System 9700 (Perkin-Elmer Applied Biosystems). Each cycle consisted of 30 sec at 94°C, 30 sec at 62°C and 1 min at 72°C. After removing the excess primers by an ethanol precipitation, PCR products were adsorbed to 20 μl a twofold suspension of Streptavidin-agarose (Gibco BRL) in 10-1 TE [pH 8.0] containing 0.1 M NaCl at room temperature for 1 h with gentle agitation. The PCR products bound to agarose was used for precipitation of NER proteins from cell extracts. The calculated amount of adsorbed DNA is almost 2 mg/g of solid. To examine the role of XPC-hHR23B complex in cell extract, 5 ng of HeLa XPC-hHR23B complex was added to the whole-cell extract prepared from XP-C cells.

Preparation of damaged DNA substrate

Four to ten μg of PCR products (0.2 μg/ml) in 10-1 TE [pH 7.5] containing 20% ethanol were treated with 0.15 mM *N*-acetoxy-2-acetyl-2-aminofluorene (*N*-AAAF) at 37°C for indicated time followed diethylether extraction, chloroform extraction, and ethanol precipitation. *N*-AAAF treated PCR products dissolved in 10-1 TE [pH 8.0] were adsorbed

to 20 μl of a twofold suspension of Streptavidin-agarose in 10-1 TE [pH 8.0] containing 0.1 M NaCl and at room temperature for 1 h with gentle agitation. After washing with same buffer, DNA bound-agarose was incubated with buffer C including 200 μg/ml BSA at 4°C for 1 h. Then the mixture was incubated with cell extract in buffer C as described in "Precipitation of proteins with DNA-cellulose".

Yeast strains and plasmids

The yeast strain used for the histidine prototropy assays was HF7c (*MATa, ura3-52, his3-200, ade2-101, lys2-801, trp1-901, leu2-3, 112, gal4-542, gal80-538, LYS2::GAL1-HIS3, URA3::(GAL4 17-mers)3-CYC1-lacZ*), and SFY526 (*MATa, ura3-52, his3-200, ade2-101, lys2-801, trp1-901, leu2-3, 112, canr, gal4-542, gal80-538, URA3::GAL1 -lacZ*) was used for galactosidase assays. Both strains were obtained from Clontech Laboratories, Inc. (Palo Aito, CA). Plasmid vectors for the two-hybrid system (MATCHMAKER Two-Hybrid System) were also obtained from Clontech Laboratories, Inc. Complementing DNAs which encoding both the hHR23A protein and hHR23B protein was cloned in frame into the pGBT9 vector which encoding gal4 DNA-binding domain, and also into the pGAD424 vector which encoding gal4 activating domain.

Construction of expression library for two-hybrid screening

Poly (A)+ RNA (5 μg) from human WI38VA13 cells was converted to double-stranded cDNA by TimeSaver cDNA Synthesis Kit (Pharmacia Biotech). After treatment of *Eco*RI adaptor ligation, adaptor-ligated cDNA was digested with *Not*I at 37°C for 2 h. pGAD424 digested with *Sma*I following *Not*I-linker ligation (designated pGAD-N) was used. pGAD-N was digested with *Eco*RI and *Not*I simultaneously at 37°C. WI38VA13 cDNA (*Not*I-*Eco*RI) and pGAD-N/*Eco*RI-*NotI* were subjected to construct expression cDNA library. *E. coli*. DH5α was used to amplify the expression cDNA library.

Two-hybrid assays

Transformation of yeast with plasmid was performed as described by the manufacturer (Clontech). Approximately 1.5X 10^7 individual clones were screened, and the histidine prototrops were screened. His+ clones were then replica-plated onto nylon membrane (Amersham), grown for additional 2-3 days, and screened for ß-galactosidase activity using a filter lift assay as described by the manufacturer (Clontech). The complete nucleotide sequences of the cDNA inserts, which were obtained by two-hybrid assays, were determined for both strands by ALF sequencing system (Pharmacia Biotech).

Measurement of the peptidase activity of both the 20S and 26S proteasome

To measure the peptidase activity of both the fractionated 20S and 26S proteasome, fluorogenic peptide (Suc-LLVY-MCA) was used. Twenty µl of each sample which fractionated HeLa S100 by the glycerol density gradients centrifugation was incubated in reaction buffer (0.1 mM tetrapeptide substrate, 100 mM Tris-HCl [pH 8.0], 1 mM DTT) at 37°C for 15 min, and added 100 µl of SDS and 2 ml of 0.1 M Tris-HCl (pH 9.0) to stop the reaction. Fluorescence was measured on a Hitachi F-300 using an excitation wavelength of 380 nm and an emission wavelength of 440 nm. To identify the fraction which containing the 20S proteasome, 0.02% SDS was added in reaction buffer.

Other methods

SDS-PAGE was performed as described by Laemmli (1970). For the immunoblotting, proteins separated on SDS gels were elecrotransfered on to PVDF membrane (Immobilon-P; Milipore). Electrotransfer was performed at 8 V/cm for overnight 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 1 (1% Blocking reagent (Boehringer Mannheim) in 0.1 M maleic acid [pH 7.5], 150 mM NaCl) or blocking buffer 2 (5% skim milk in 25 mM Tris-HCl [pH 7.5], 0.15 M NaCl, 0.1% Tween 20), with first with antibody in blocking buffer, and then with anti-rabbit or anti-mouse $F(ab')_2$ antibody conjugated with horseradish peroxidase (Amersham). Detection was carried out with SuperSignal Substrate (Pierce)

according to the instructions. Protein concentration was measured according to the method of Bradford (1976), using Bio-Rad Protein Assay reagent (Bio-Rad Laboratories) and bovine serum albumin as a standard.

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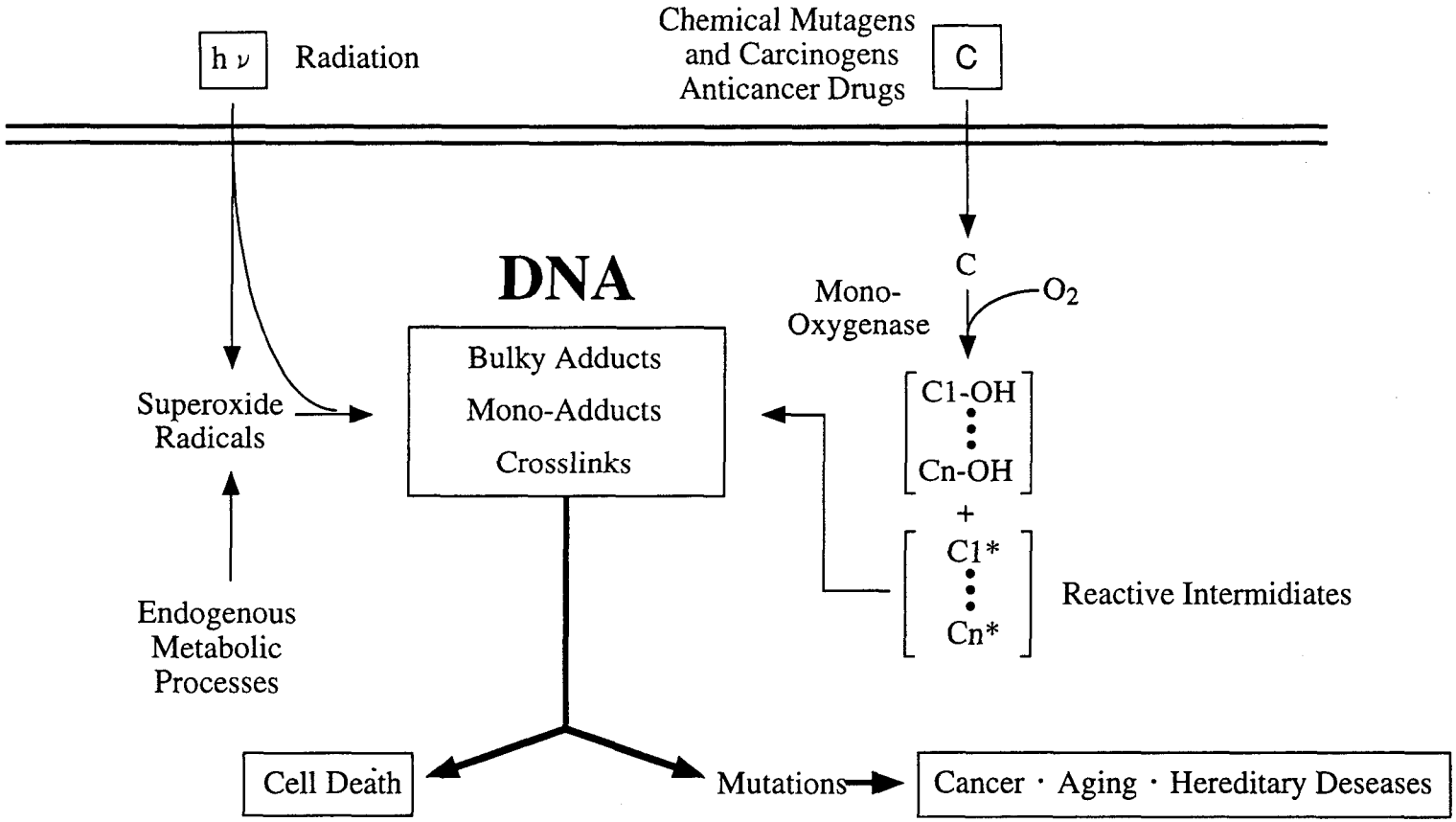


FIGURE 1. Mechanism of formation and biological processing of DNA damage.

Cn-OH: the first (water soluble) metabolite

Cn*: the second (electrophilic) metabolite

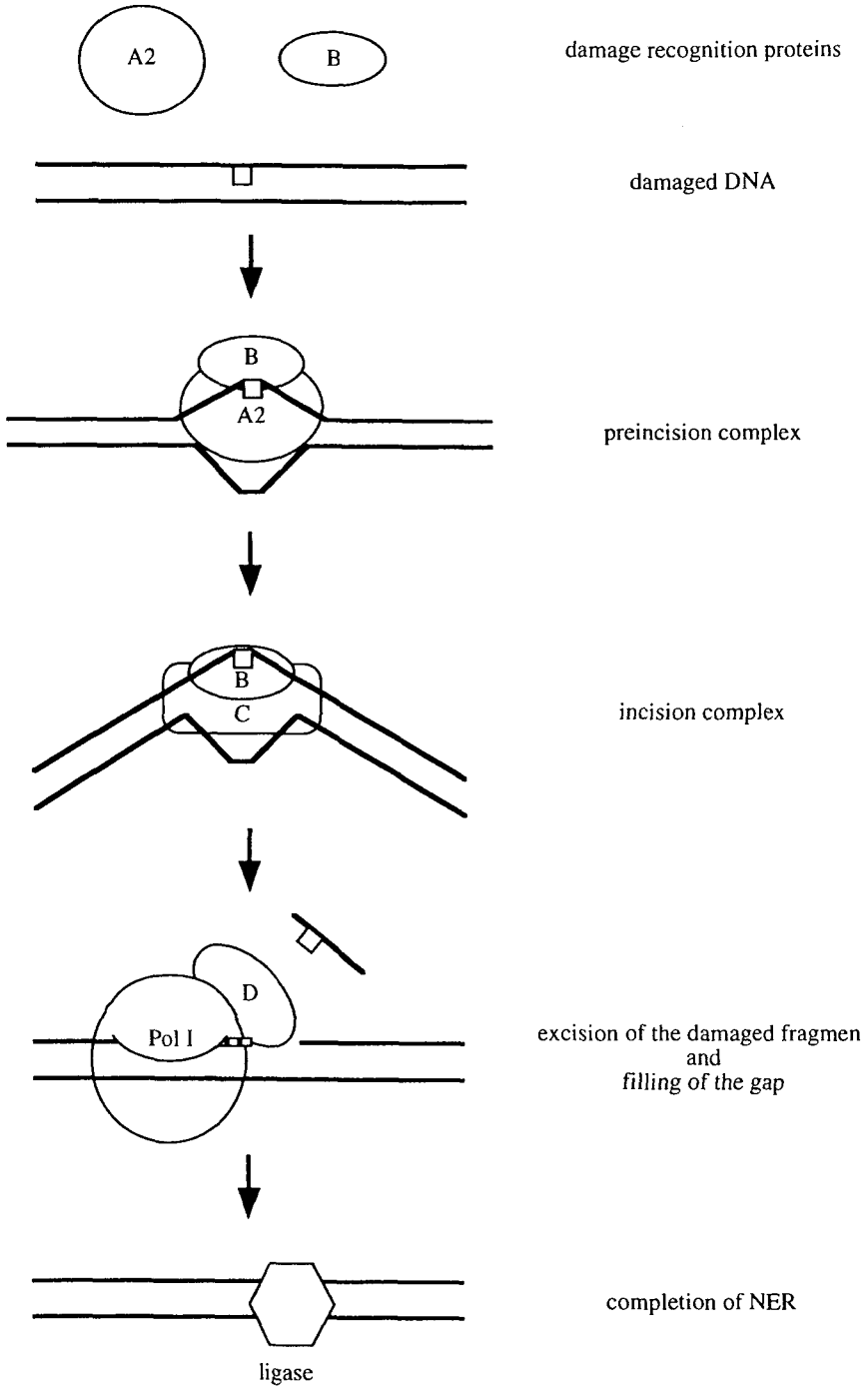


FIGURE 2. The NER model of *Escerichia coli*

A2: UvrA deimer B: UvrB C: UvrC
D: UvrD Pol I: DNA polymerase I

Table 1. Genes essential for the damage-dependent incision step of NER in yeast and mammalians

Yeast gene	human gene	remarks
RAD1	*XPF (ERCC4)*	protein complex (Rad1-Rad10/XPF-ERCC1) srtucture-specific endonuclease, 5' incision
RAD2	*XPG (ERCC5)*	structure-specific endonuclease, 3' incision
RAD3	*XPD (ERCC2)*	TFIIH subunit, 5'->3' helicase
RAD4	*XPC*	complex with Rad23 (in human hHR23B) phenotypes human/yeast mutants different XPC binds strongly to damaged DNA, interaction with TFIIH?
RAD10	*ERCC1*	see *RAD1*, ERCC1 interacts with XPA
RAD14	*XPA*	binds to damaged DNA
RAD23	*hHR23A, B*	see *RAD4*, ubiquitin fusion protein, Rad23 recruit TFIIH onto damage? Rad23 is involved in the replication of spindle pole body
RAD25	*XPB (ERCC3)*	TFIIH subunit, 3'->5' helicase
RAD26	*CSB (ERCC6)*	putative helicase motif, interaction with XPG
RAD28	*CSA (ERCC8)*	WD-repeat protein, interaction with p44
SSL1	*p44*	TFIIH subunit, DNA binding?
TFB1	*p62*	TFIIH subunit
TFB2, 3	*p52, ?*	TFIIH subunit
RFA1, 2, 3	*RPA1, 2, 3*	involved in pre-and post-incision steps of NER, ssDNA binding affinity for UV-damaged DNA, essential for viability

Table 2. Summary of main clinical symptoms of NER related disorders

Clinical symptoms	XP	XP/CS	CS	TTD
Sun-sensitivity	++	++	+	+
Abnormal pigmentation	++	+	-	-
Skin cancer	++	+	-	-
Neurodysmyelination	-	+	+	+
Bird-like facies	-	+	+	+
Growth defect	+/-	+	+	+
Hypogonadism	-	+	+	+
Brittle hair and nails	-	-	-	+
Scaling of skin	-	-	-	+

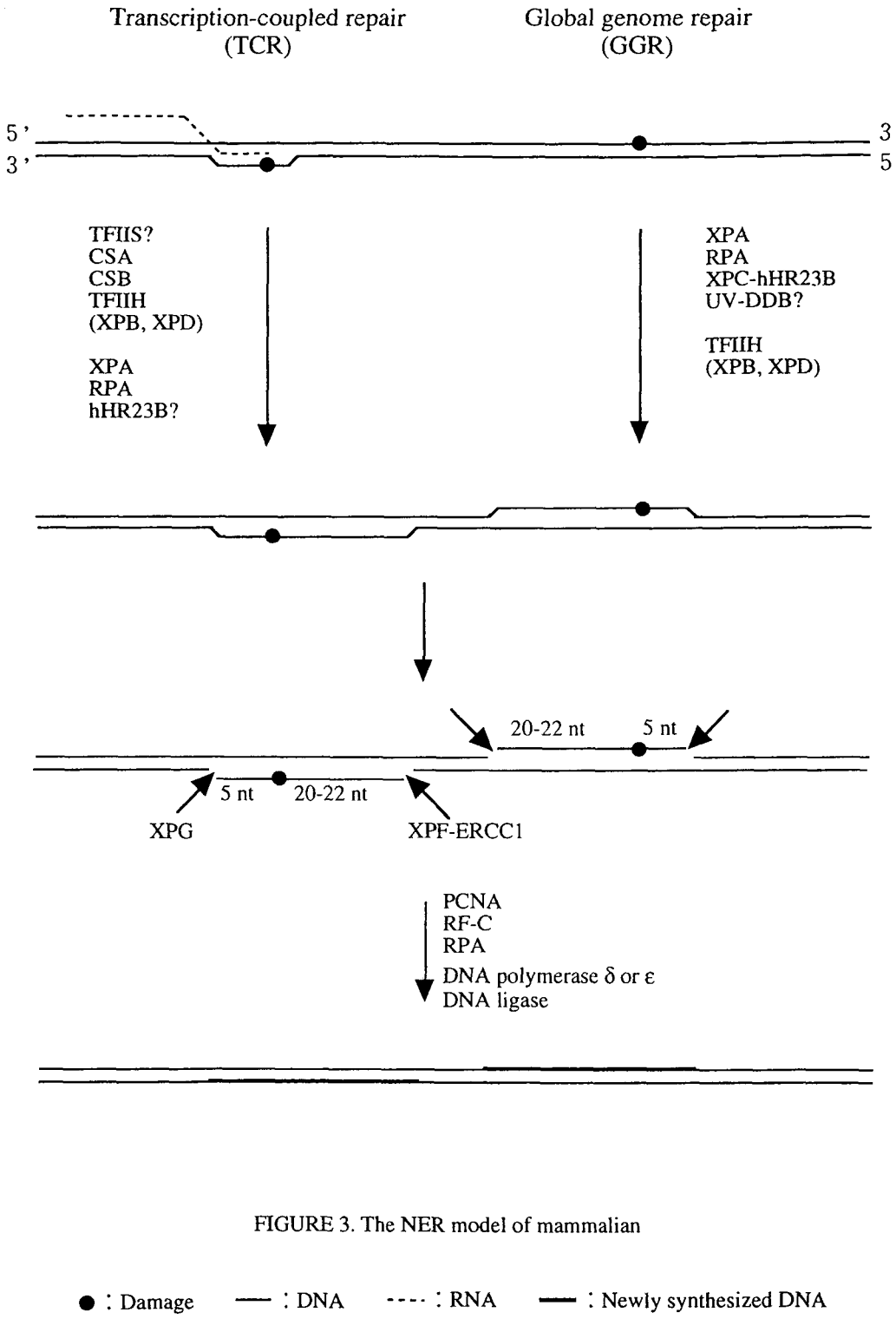


FIGURE 3. The NER model of mammalian

● : Damage — : DNA ---- : RNA ▬ : Newly synthesized DNA

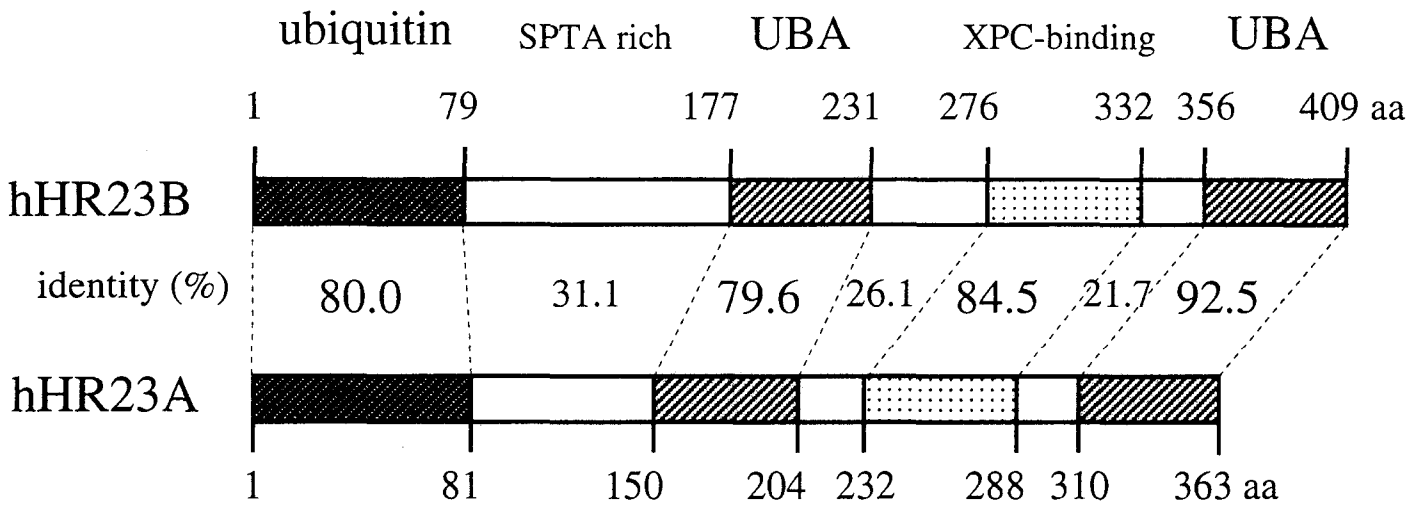


FIGURE 4. Domain structures of hHR23B and hHR23A

ubiquitin: ubiquitin-like sequence

SPTA rich: four kinds of amino acids (S, P, T, and A) are predominant in this region

UBA: ubiquitin-associated domain

XPC-binding: XPC-binding domain

aa: amino acids

Table 3. Genes for the subunits of the 20S proteasome

	Yeast gene	human gene	rat gene	essential
α-Type subunits	*PRE5*	*HC2*	*RC2*	+
		Pros30		
	Y7	*HC3*	*RC3*	-
	PRS1	*HC8*	*RC8*	+
	Y13	*HC9*	*RC9*	+
	PUP2	*Zeta*	*rZeta*	+
	PRS2	*Pros27 (Iota)*	*rIota*	+
	PRE6	*XAPC7-S*	*RC6-I-S*	+
		XAPC7-L	*RC6-I-L*	
β-Type subunits	*PRS3*	*HC5*	*RC5*	+
	PRE1	*HC7-I*	*RC7-I*	+
	PUP3	*HC10-II*	*RC10-II*	+
	PRE4	*HN3*	*RN3*	+
	PRE2	*X (MB1, Epsilon)*	*rX*	+
	PRE3	*Y (Delta)*	*rDelta*	+
	PUP1	*Z*		+
		LMP7-EI		-
		LMP7-E2		-
		LMP2		-
		MECL1		

Table 4. Genes for the subunits of the 19/22S regulator

	Yeast gene	human gene	essential
ATPase subunits	*YTA5, YHS4*	*S4 (p56)*	+
	CIM5, YTA3	*MSS1 (S7)*	+
	YTA1	*TBP1*	+
	YTA2	*TBP7 (S6, p48)*	+
	CIM3, SUG1	*p45 (TRIP1, S8)*	+
		p42	
Non-ATPase subunits	*SEN3*	*P112-L (S1)*	-
		p112-S	
	NAS1	*p97 (S2, TRAP2, 55.11)*	-
	SUN2	*p58 (S3)*	+
		p55	
	SUN1	*S5a*	
		S5b (p50.5)	
		p44.5	
		p44 (S10)	
		p40.5	
		p40 (S12, Mov-34)	
	NIN1 (MTS3)	*p31 (S14)*	+
		p28	
		p27	

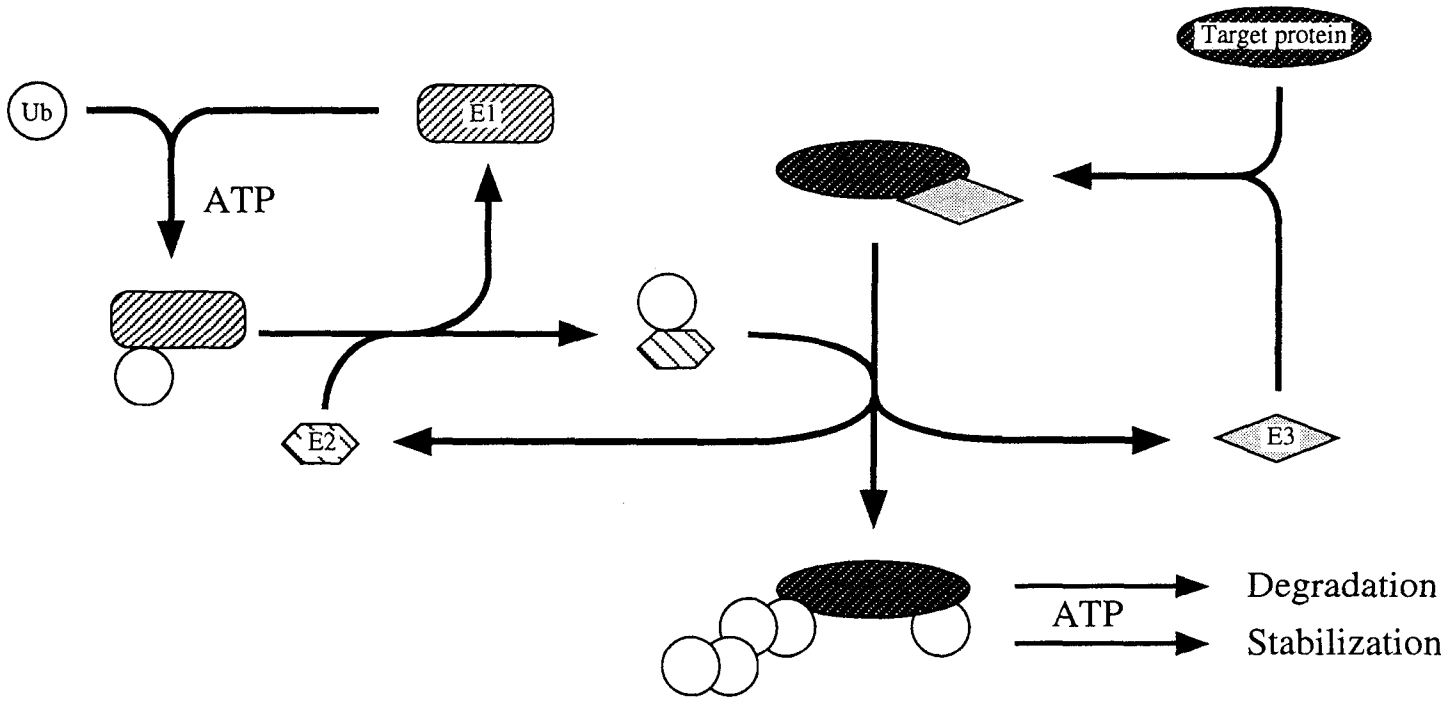


FIGURE 5. The model of ubiquitin conjugating pathway (simplified)

Ub: ubiquitin E1: the ubiquitin-activating enzyme

E2: the ubiquitin-conjugating enzyme E3: the ubiquitin ligase

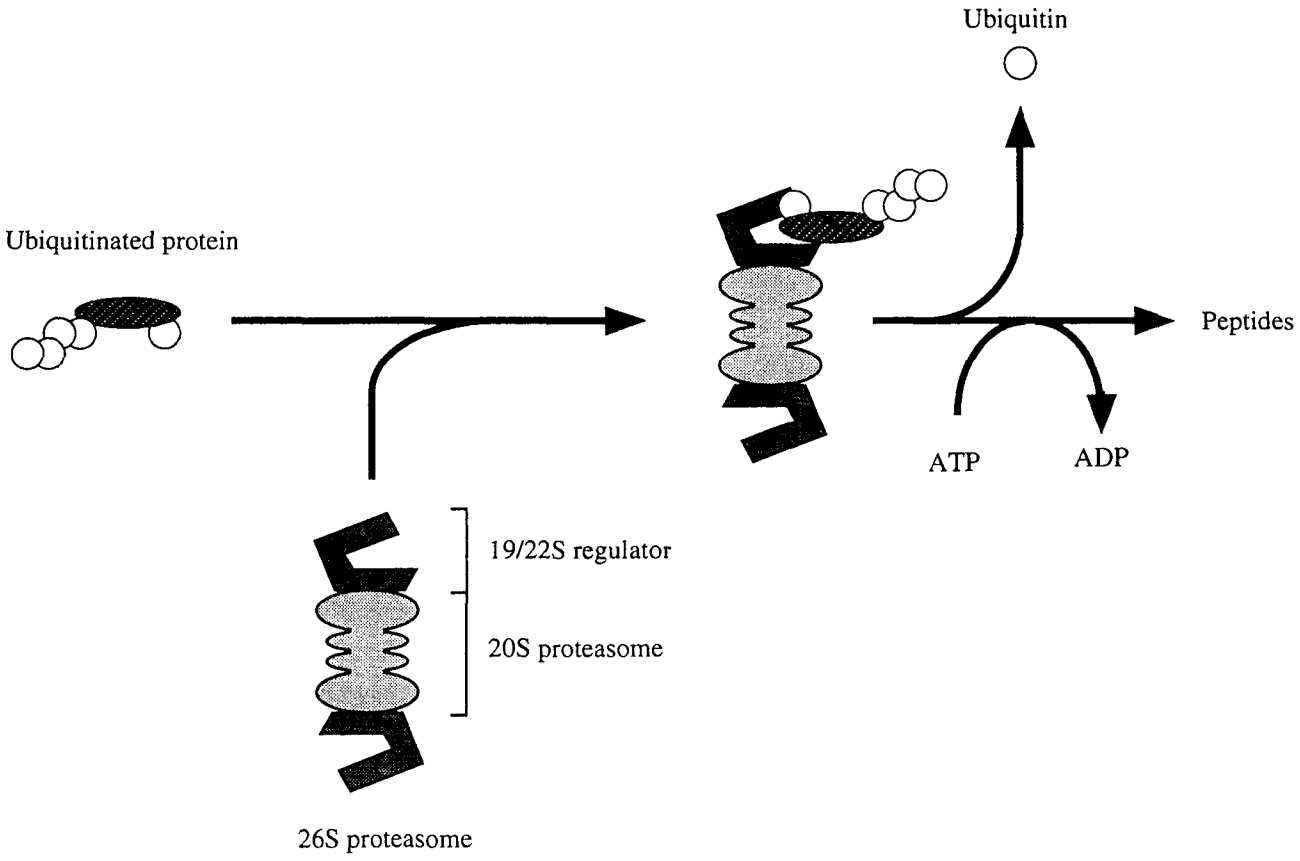


FIGURE 6. The model of ubiquitin-dependent proteolytic pathway (simplified)

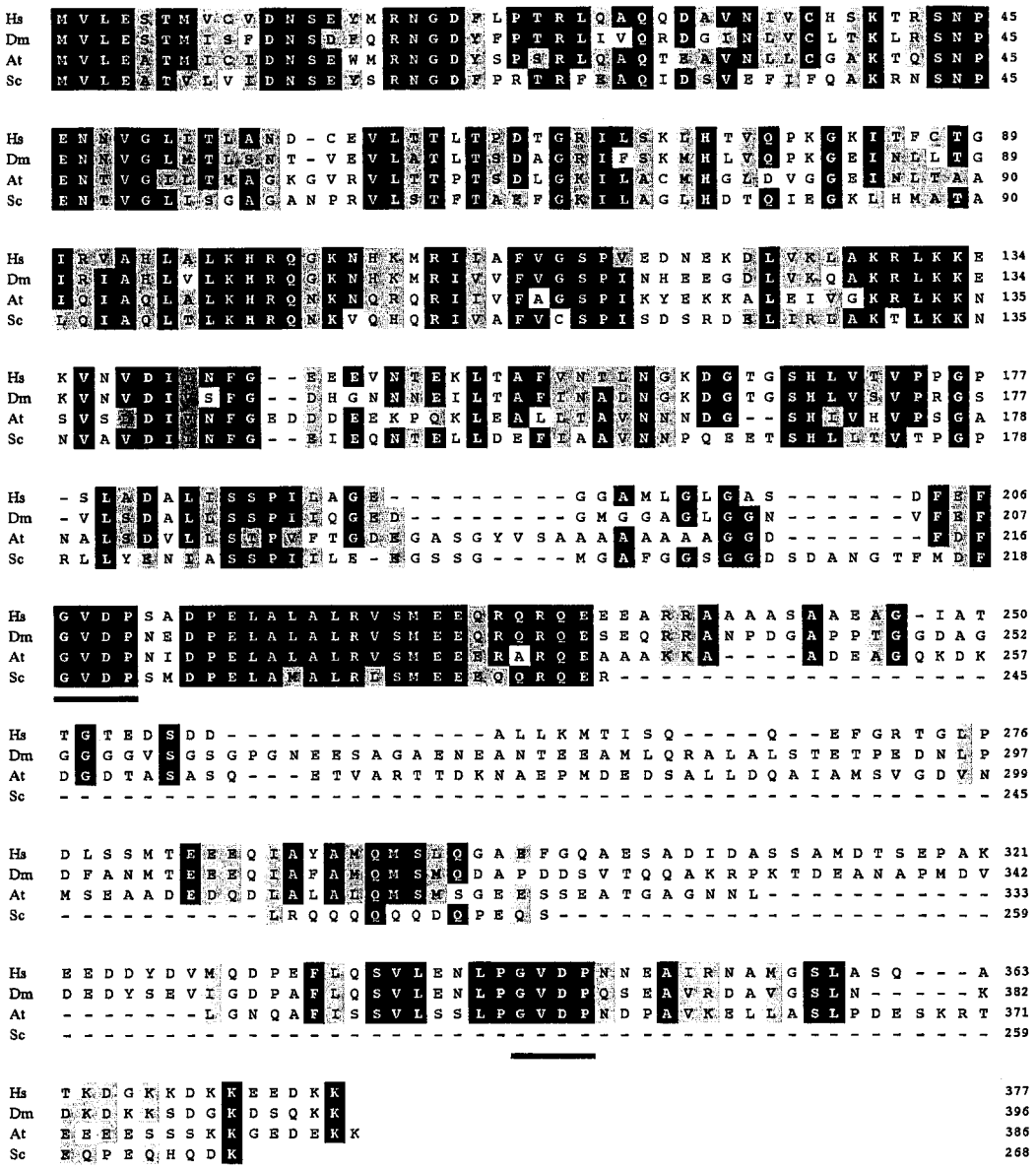


FIGURE 7. Alignment of S5a homolog

The amino acid sequences are derived from human (Hs), *Drosophila melanogaster* (Dm), *Arabidopsis thaliana* (At), and *Saccharomyces cerevisiae* (Sc). Identical amino acids are presented by black boxes, whereas similar residues are given in gray boxes. Gaps are indicated by dots. Conserved GVDP motifs are underlined.

Table 5. Ubiquitin-like proteins

CEP family

ribosomal proteins (CEP80 proteins, CEP52 proteins)

NEP family

splicing factors (SF3a120, PRP21p)

Ubiquitin-like proteins

15 kDa interferon-induced ISG15 gene product UCRP

NEDD8 protein

X-chromosomal GdX protein

BAT3 polypeptide

Baculovirus v-ubi protein

DNA repair proteins Rad23, hHR23 proteins

Positive refulator subunit p18 of the SIII general transcription/elongation factor

DSK2 protein involved in duplication of the microtubule organizing center

SMT3 protein

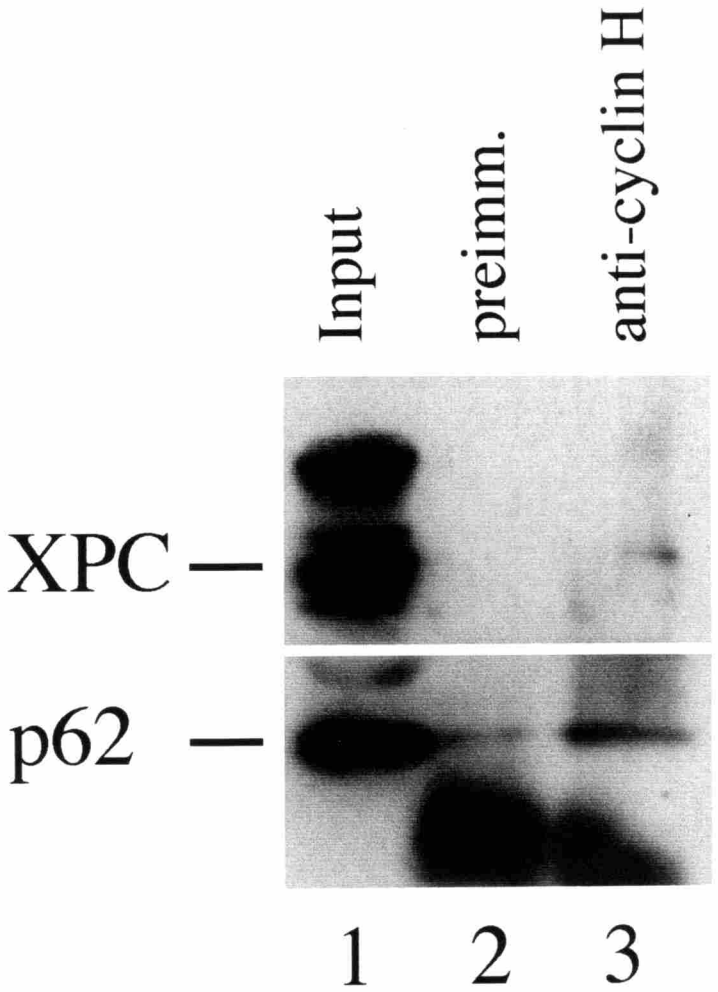


FIGURE 8. Co-immunoprecipitation of XPC-hHR23B with TFIIH by anti-cyclin H antibody. 10% inpu of the whole-cell extract prepared from 293 cells is shown in lane 1. The proteins bound to preimmune-Protein A-Sepharose are shown in lane 2. The proteins bound to anti-cyclin H-Protein A-Sepharose are shown in lane 3. The presence of TFIIH was assessed by immunoblotting with anti-p62 antibody. The presence of XPC-hHR23B was assessed by immunoblotting with anti-XPC antibody.

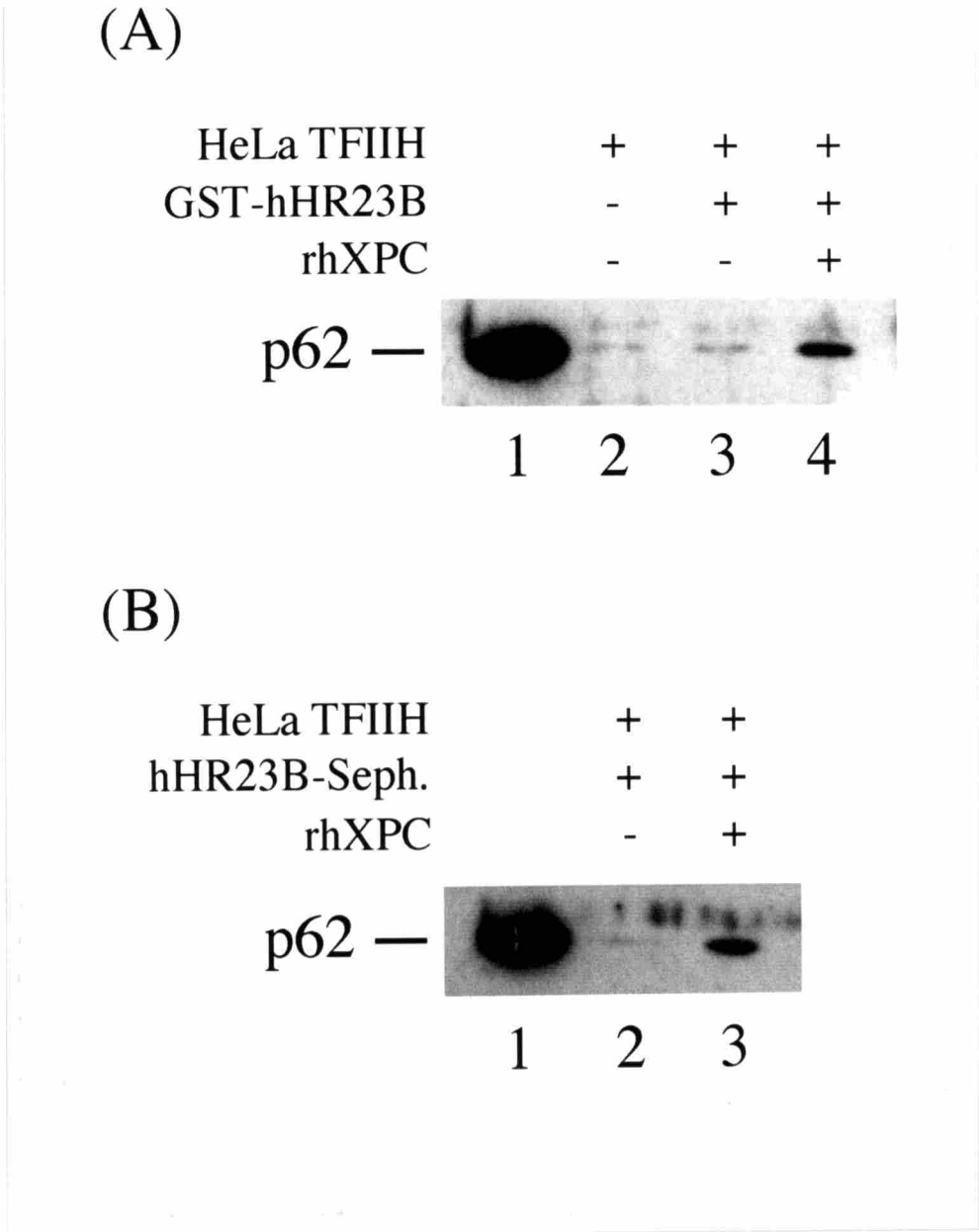


FIGURE 9. Physical interaction between TFIIH and XPC-hHR23B complex. (A) The GST-hHR23B was incubated with purified TFIIH with either the presence or the absence of rhXPC. 10% input of purified TFIIH is shown in lane 1. The proteins bound to GSH-Sepharose are shown in lane 2. The presence of precipitated TFIIH was assessed by immunoblotting with anti-p62 antibody. (B) The rhHR23B-Sepharose was incubated with purified TFIIH with either the presence or the absence of rhXPC. 10% input of purified TFIIH is shown in lane 1. The proteins bound to Sepharose and rhHR23B-Sepharose are shown in lanes 2 and 3, respectively. The proteins bound to XPC-rhHR23B-Sepharose are shown in lane 4. The presence of precipitated TFIIH was assessed by immunoblotting with anti-p62 antibody.

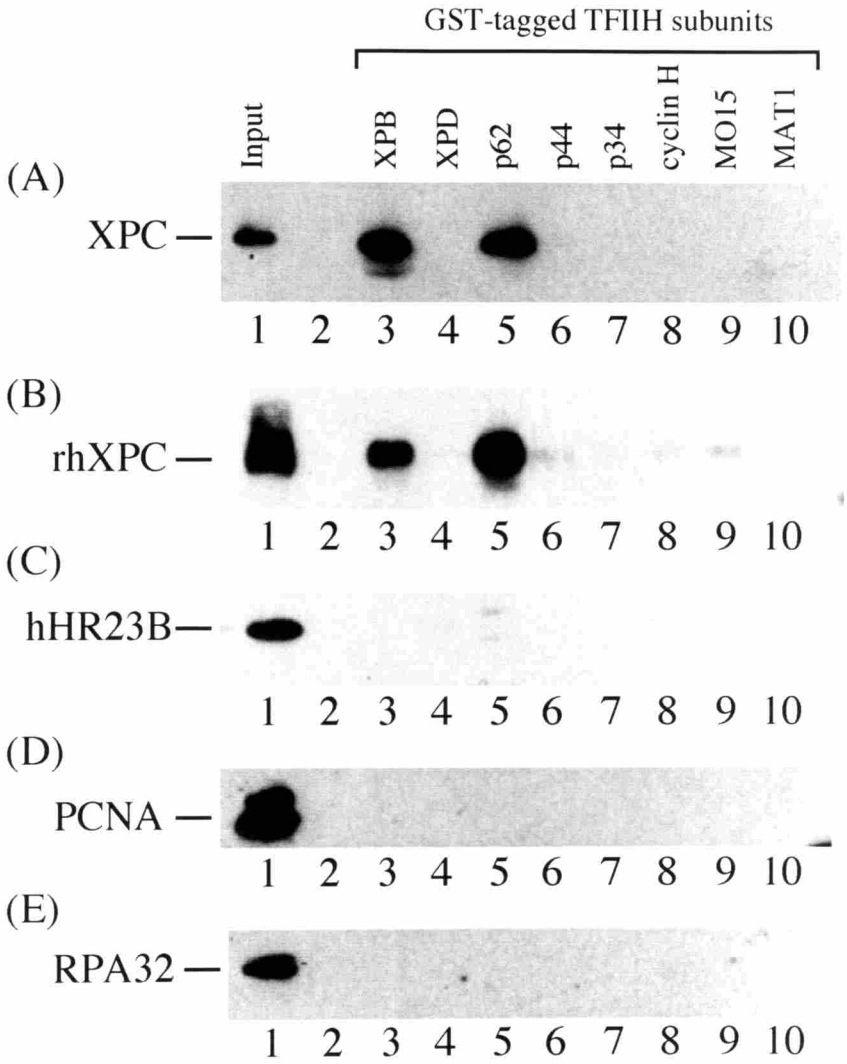


FIGURE 10. Co-precipitation of XPC and hHR23B with GST-tagged TFIIH subunit. (A) HeLa XPC-hHR23B complex was precipitated by each subunits. Precipitated complex was detected by immunoblotting with anti-XPC antibody. (B) The rhXPC was precipitated by each subunits. (C) The rhHR23B was precipitated by each subunits. Precipitants were detected by immunoblotting with anti-hHR23B antibody. (D) The rPCNA was precipitated by each subunits. Precipitants were detected by immunoblotting with anti-PCNA antibody. (E) The rRPA was precipitated by each subunits. Precipitants were detected by immunoblotting with anti-RPA32 antibody. 10% input of each substrates are shown in lane 1 of each figures.

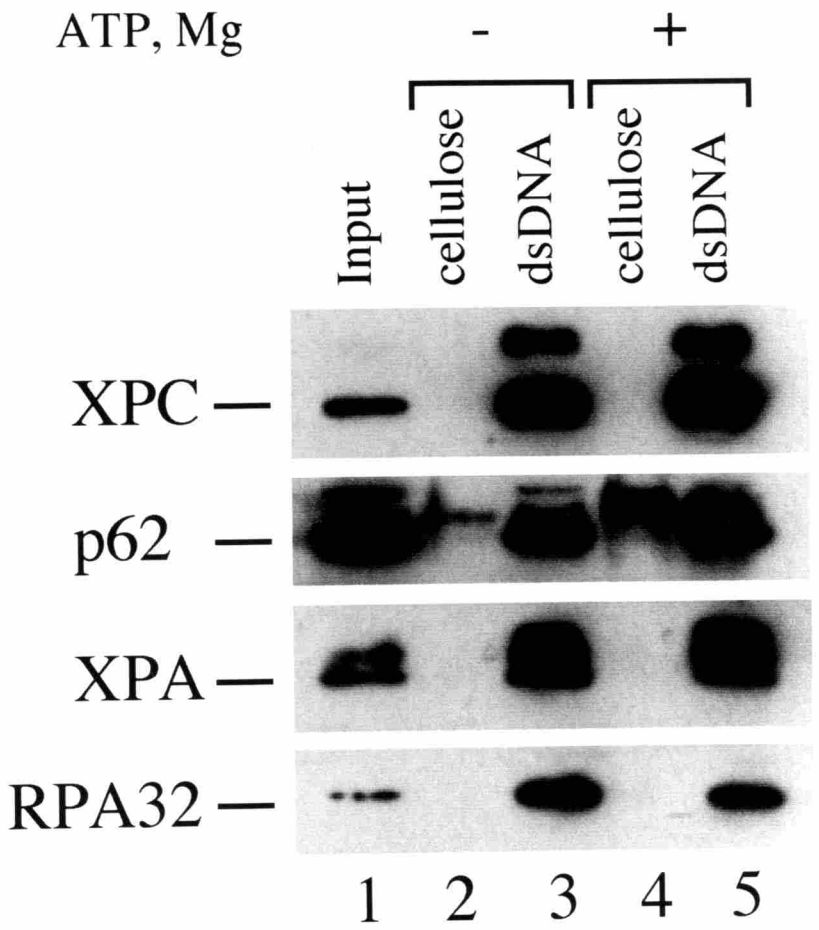


FIGURE 11. Precipitation of NER factors with DNA-cellulose from cell extract. 10% input of the whole-cell extract prepared from 293 cells is shown in lane 1. DNA-cellulose and cell extract were mixed, and incubated under two different conditions (± ATP). The proteins bound to cellulose are shown in lanes 2 and 4. The proteins bound to dsDNA-cellulose are shown in lanes 3 and 5. The presence of NER factors was assessed by immunoblotting.

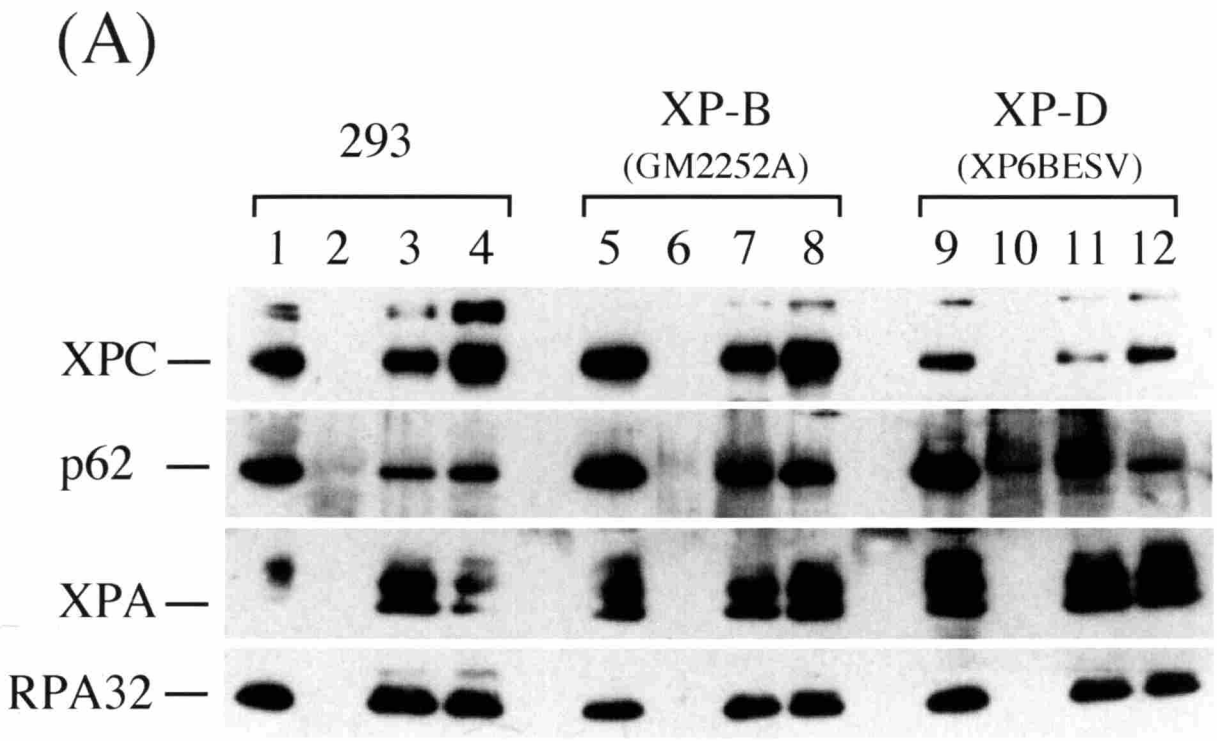
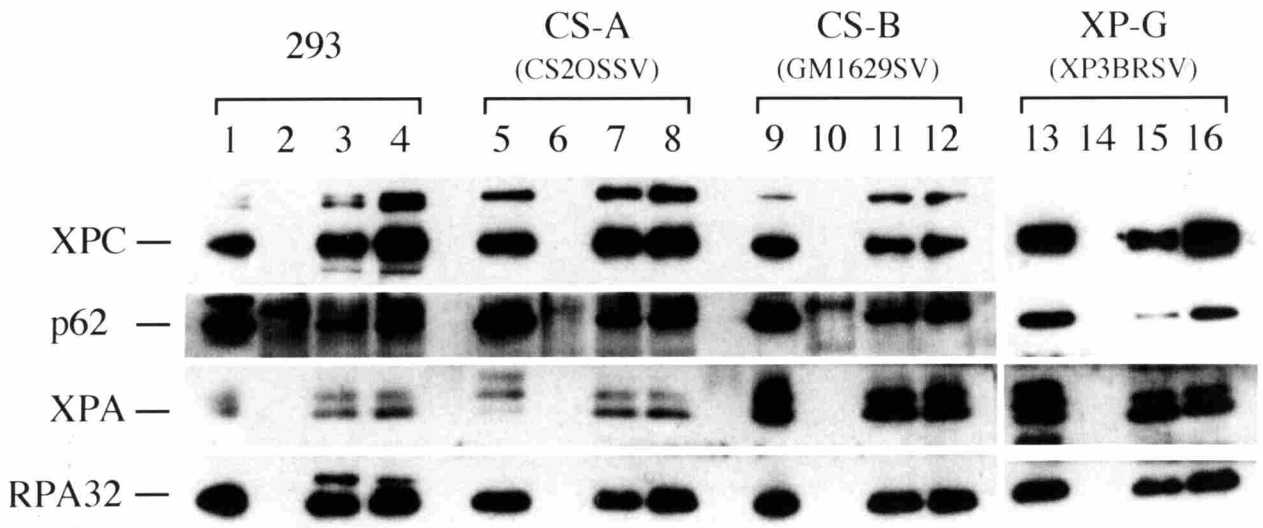


FIGURE 12. Precipitation of NER factors with DNA-cellulose from cell extracts derived from NER-deficient cell lines. (A) 10% input of whole cell extracts prepared from 293, XP-B, and XP-D are shown in lanes 1, 5, 9, respectively. The proteins bound to cellulose are shown in lanes 2, 6, 10. The proteins bound to the single-stranded DNA-cellulose are shown in lanes 3, 7, 11. The proteins bound to the double-stranded DNA-cellulose are shown in lanes 4, 8, 12. (B) 10% input of whole cell extracts prepared from 293, CS-A, CS-B, and XP-G are shown in lanes 1, 5, 9, 13, respectively. The proteins bound to cellulose are shown in lanes 2, 6, 10, 14. The proteins bound to the single-stranded DNA-cellulose are shown in lanes 3, 7, 11, 15. The proteins bound to the double-stranded DNA-cellulose are shown in lanes 4, 8, 12, 16. The presence of NER factors was assessed by immunoblotting.

(B)


293
CS-A
(CS2OSSV)
CS-B
(GM1629SV)
XP-G
(XP3BRSV)
1 2 3 4
5 6 7 8
9 10 11 12
13 14 15 16
XPC
p62
XPA
RPA32

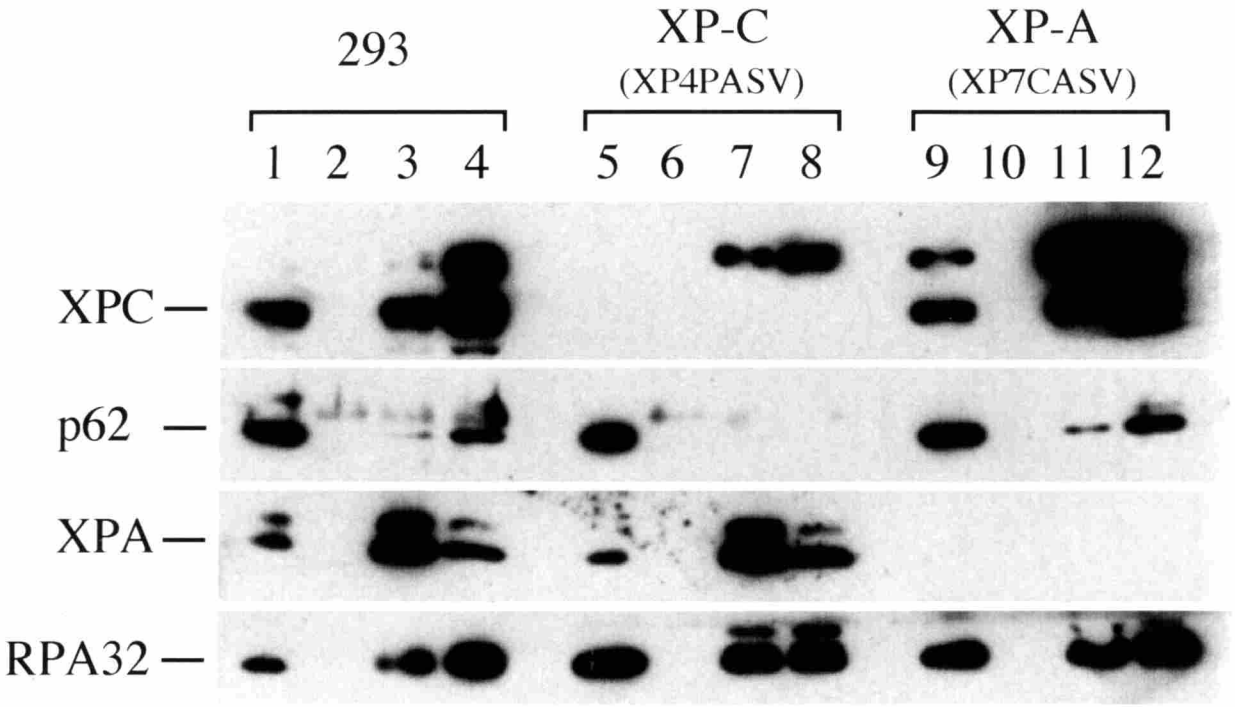


FIGURE 13. Precipitation of NER factors with DNA-cellulose from cell extracts derived from XP-C and XP-A cells. 10% input of whole cell extracts prepared from 293, XP-C, and XP-A cells are shown in lanes 1, 5, 9, respectively. The proteins bound to cellulose are shown in lanes 2, 6, 10. The proteins bound to the single-stranded DNA-cellulose are shown in lanes 3, 7, 11. The proteins bound to the double-stranded DNA-cellulose are shown in lanes 4, 8, 12. The presence of NER factors was assessed by immunoblotting.

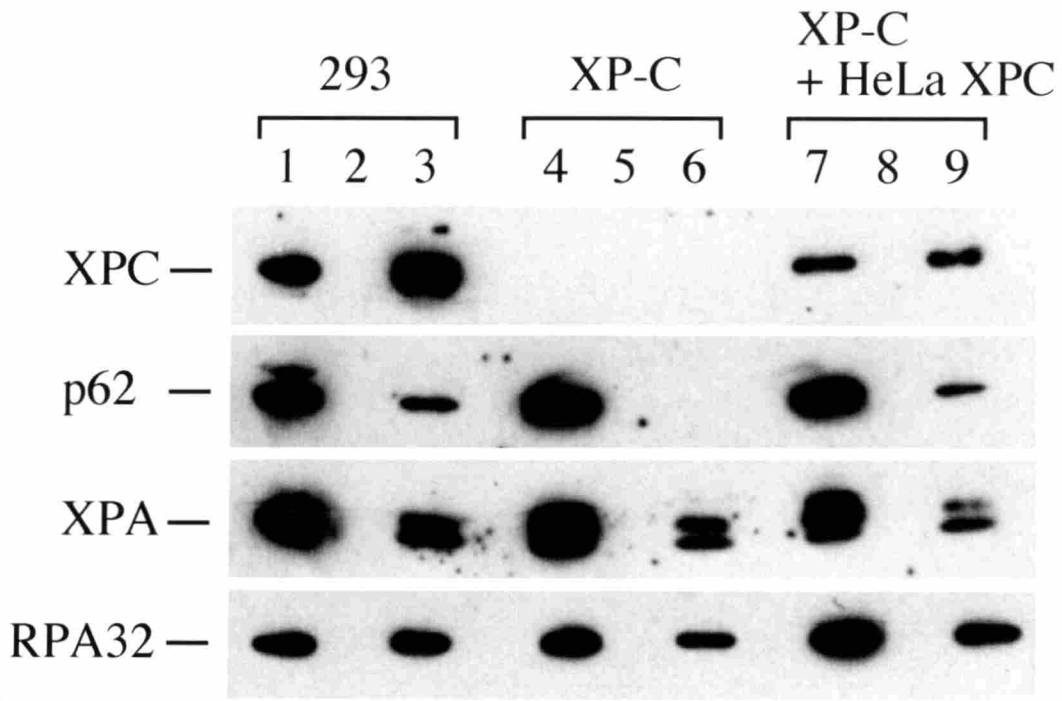


FIGURE 14. XPC-hHR23B complex dependent precipitation of TFIIH by DNA cellulose from cell extract. 10% input of 293 cell extract, XP-C cell extract, and XP-C cell extract presence with HeLa XPC-hHR23B complex are shown in lanes 1, 4, 7, respectively. The proteins bound to cellulose are shown in lanes 2, 5, 8. The proteins bound to the double-stranded DNA-cellulose were shown in lanes 3, 6, 9. The presence of NER factors was assessed by immunoblotting.

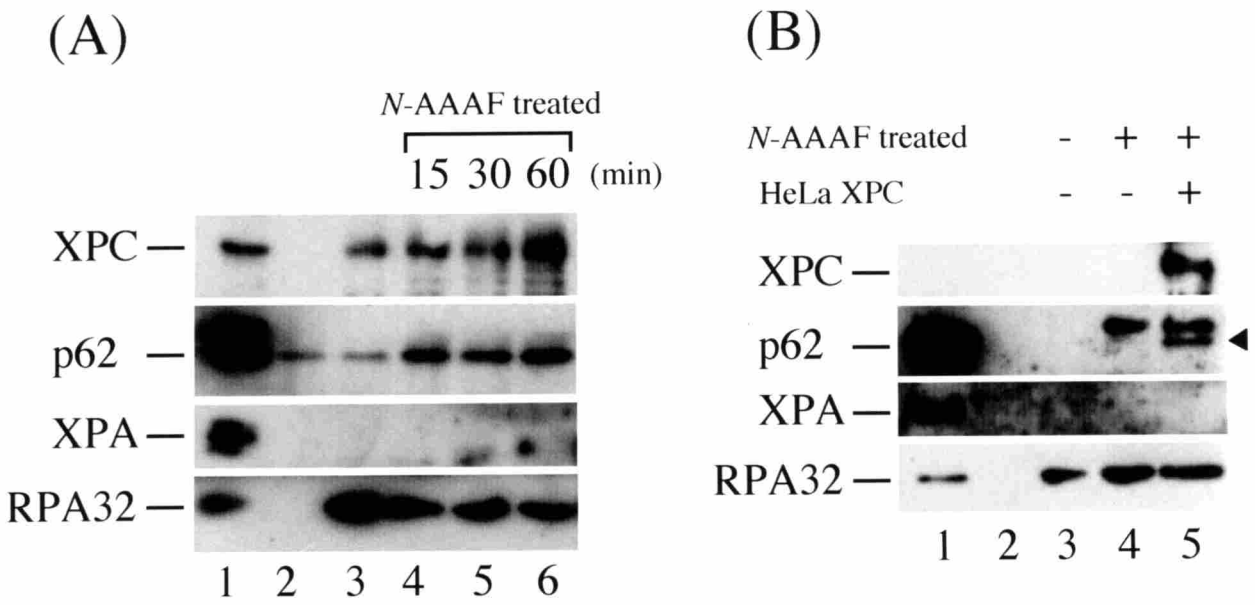


FIGURE 15. XPC-hHR23B complex dependent recruitment of TFIIH onto the damaged DNA. (A) Precipitation of NER factors from 293 cell extract was performed using damaged DNA. 5% input of 293 cell extract is shown in lane 1. The proteins bound to Streptavidin agarose are shown in lane 2. The proteins bound to the adduct-free DNA are shown in lane 3. The proteins bound to the *N*-AAAF treated DNA are shown in lanes 4 to 6. PCR product was treated with *N*-AAAF as indicated time. The presence of NER factors was assessed by immunoblotting. (B) Precipitation of NER factors from XP-C cell extract was performed using the damaged DNA, which treated with *N*-AAAF for 60 min. 5% input of XP-C cell extract is shown in lane 1. The proteins bound to Streptavidin agarose are shown in lane 2. The proteins bound to the adduct-free DNA are shown in lane 3. The proteins bound to the *N*-AAAF treated DNA are shown in lanes 4 and 5, whereas lane 5 shows the influence of XPC-hHR23B complex. The presence of NER factors was assessed by immunoblotting.

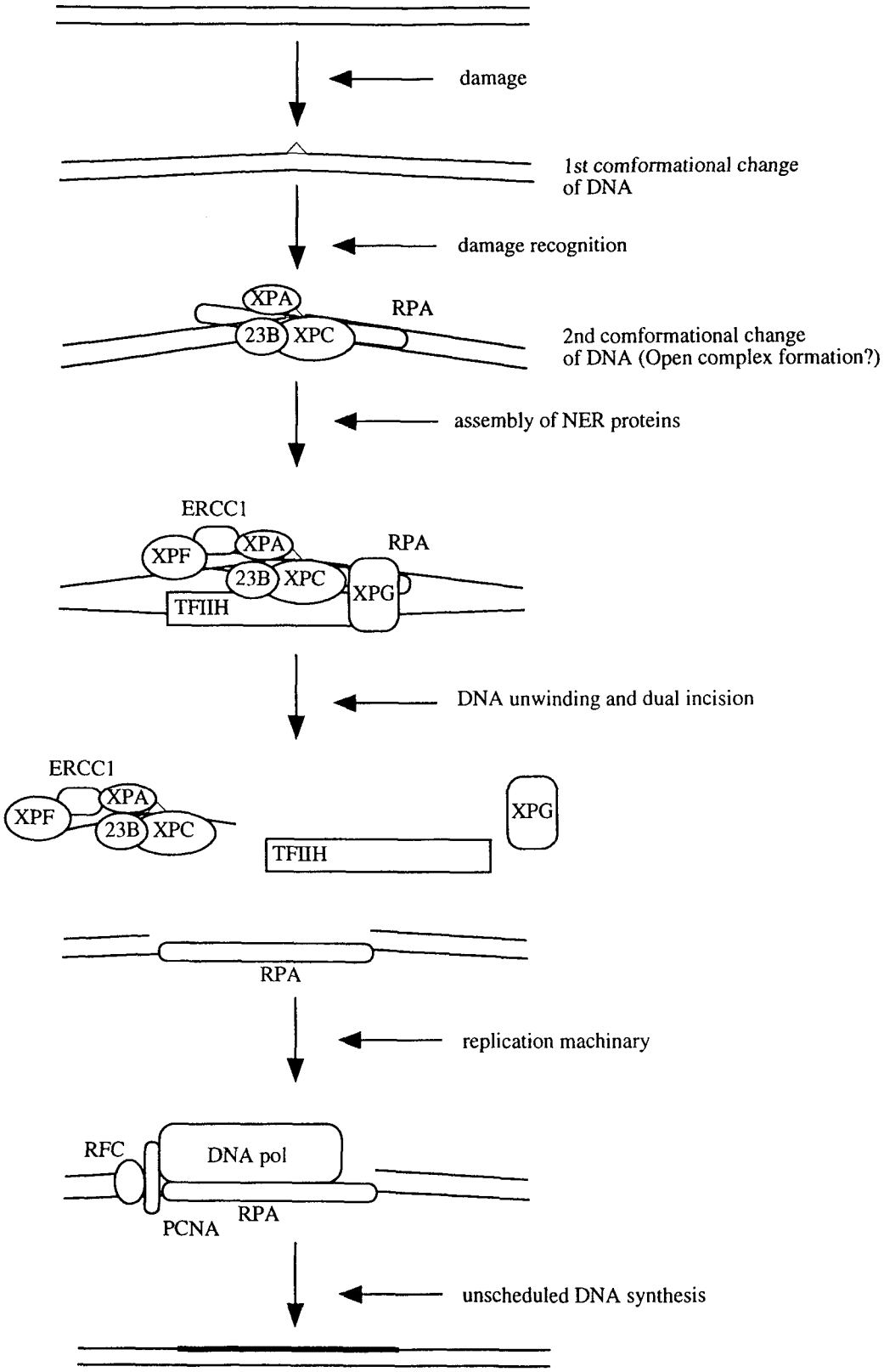


FIGURE 16. The GGR model of mammalian

```
ATGACCTTTT ATTTCATACA GAGATACAAA GGCAACTATG TGCAGCAACA ATCTGATGGG CAGTCCAAAC TCTTG  -150
CAGTCCAAAC TCTTGGGAGG AAGTAAATTC ATGGTAAATG TCATGATGGC GGTCGGGAGG GAGGAAGGTG GCAAG   -75
ATGGTGTTGG AAAGCACTAT GGTGTGTGTG GACAACAGTG AGTATATGCG GAATGGAGAC TTCTTACCCA CCAGG    75
M  V  L  E   S  T  M   V  C  V   D  N  S  E   Y  M  R   N  G  D   F  L  P  T   R     (25)

CTGCAGGCCC AGCAGGATGC TGTCAACATA GTTTGTCATT CAAAGACCCG CAGCAACCCT GAGAACAACG TGGGC   150
L  Q  A  Q   Q  D  A   V  N  I   V  C  H  S   K  T  R   S  N  P   E  N  N  V   G     (50)

CTTATCACAC TGGCTAATGA CTGTGAAGTG CTGACCACAC TCACCCCAGA CACTGGCCGT ATCCTGTCCA AGCTA   225
L  I  T  L   A  N  D   C  E  V   L  T  T  L   T  P  D   T  G  R   I  L  S  K   I     (75)

CATACTGTCC AACCCAAGGG CAAGATCACC TTCTGCACGG GCATCCGCGT GGCCCATCTG GCTCTGAAGC ACCGA   300
H  T  V  Q   P  K  G   K  I  T   F  C  T  G   I  R  V   A  H  L   A  L  K  H   R    (100)

CAAGGCAAGA ATCACAAGAT GCGCATCATT GCCTTTGTGG GAAGCCCAGT GGAGGACAAT GAGAAGGATC TGGTG   375
Q  G  K  N   H  K  M   R  I  I   A  F  V  G   S  P  V   E  D  N   E  K  D  L   V    (125)

AAACTGGCTA AACGCCTCAA GAAGGAGAAA GTAAATGTTG ACATTATCAA TTTTGGGGAA GAGGAGGTGA ACACA   450
K  L  A  K   R  L  K   K  E  K   V  N  V  D   I  I  N   F  G  E   E  E  V  N   T    (150)

GAAAAGCTGA CAGCCTTTGT AAACACGTTG AATGGCAAAG ATGGAACCGG TTCTCATCTG GTGACAGTGC CTCCT   525
E  K  L  T   A  F  V   N  T  L   N  G  K  D   G  T  G   S  H  L   V  T  V  P   P    (175)

GGGCCCAGTT TGGCTGATGC TCTCATCAGT TCTCCGATTT TGGCTGGTGA AGGTGGTGCC ATGCTGGGTC TTGGT   600
G  P  S  L   A  D  A   L  I  S   S  P  I  L   A  G  E   G  G  A   M  L  G  L   G    (200)

GCCAGTGACT TTGAATTTGG AGTAGATCCC AGTGCTGATC CTGAGCTGGC CTTGGCCCTT CGTGTATCTA TGGAA   675
A  S  D  F   E  F  G   V  D  P   S  A  D  P   E  L  A   L  A  L   R  V  S  M   E    (225)

GAGCAGCGGC AGCGGCAGGA GGAGGAGGCC CGGCGGGCAG CTGCAGCTTC TGCTGCTGAG GCCGGGATTG CTACG   750
E  Q  R  Q   R  Q  E   E  E  A   R  R  A  A   A  A  S   A  A  E   A  G  I  A   T    (250)

ACTGGGACTG AAGACTCAGA CGATGCCCTG CTGAAGATGA CCATCAGCCA GCAAGAGTTT GGCCGCACTG GGCTT   825
T  G  T  E   D  S  D   D  A  L   L  K  M  T   I  S  Q   Q  E  F   G  R  T  G   L    (275)

CCTGACCTAA GCAGTATGAC TGAGGAAGAG CAGATTGCTT ATGCCATGCA GATGTCCCTG CAGGGAGCAG AGTTT   900
P  D  L  S   S  M  T   E  E  E   Q  I  A  Y   A  M  Q   M  S  L   Q  G  A  E   F    (300)

GGCCAGGCGG AATCAGCAGA CATTGATGCC AGCTCAGCTA TGGACACATC CGAGCCAGCC AAGGAGGAGG ATGAT   975
G  Q  A  E   S  A  D   I  D  A   S  S  A  M   D  T  S   E  P  A   K  E  E  D   D    (325)

TACGACGTGA TGCAGGACCC CGAGTTCCTT CAGAGTGTCC TAGAGAACCT CCCAGGTGTG GATCCCAACA ATGAA  1050
Y  D  V  M   Q  D  P   E  F  L   Q  S  V  L   E  N  L   P  G  V   D  P  N  N   E    (350)

GCCATTCGAA ATGCTATGGG CTCCCTGGCC TCCCAGGCCA CCAAGGACGG CAAGAAGGAC AAGAAGGAGG AAGAC  1125
A  I  R  N   A  M  G   S  L  A   S  Q  A  T   K  D  G   K  K  D   K  K  E  E   D    (375)

AAGAAGTGAG ACTGGAGGGA AAGGGTAGCT GAGTCTGCTT AGGGACTGCA TGGGGGAATT CGACTGGAGG GAAAG  1200
K  K  *                                                                              (377)

GGTAGCTGAG TCTGCTTAGG GGACTGCATG GGAAGCACGG AATATAGGGT TAGATGTGTG TTATCTGTAA CCATT  1275
ACAGCCTAAA TAAAGCTTGG CAACTTTTAA AAAAAAAAAA AAAAAAAAAA AAAAAAAAAA AAAAAAAAAA AAAAA  1350
AAAAAAAA                                                                            1358
```

FIGURE 17. Nucleotide and deduced amino acid sequences for human S5a

Top numbers of the right are nucleotides, and lower ones are amino acids numbered from A of the initiation codon, and methionine 1, respectively. The astarisk indicates the termination codon, TGA.

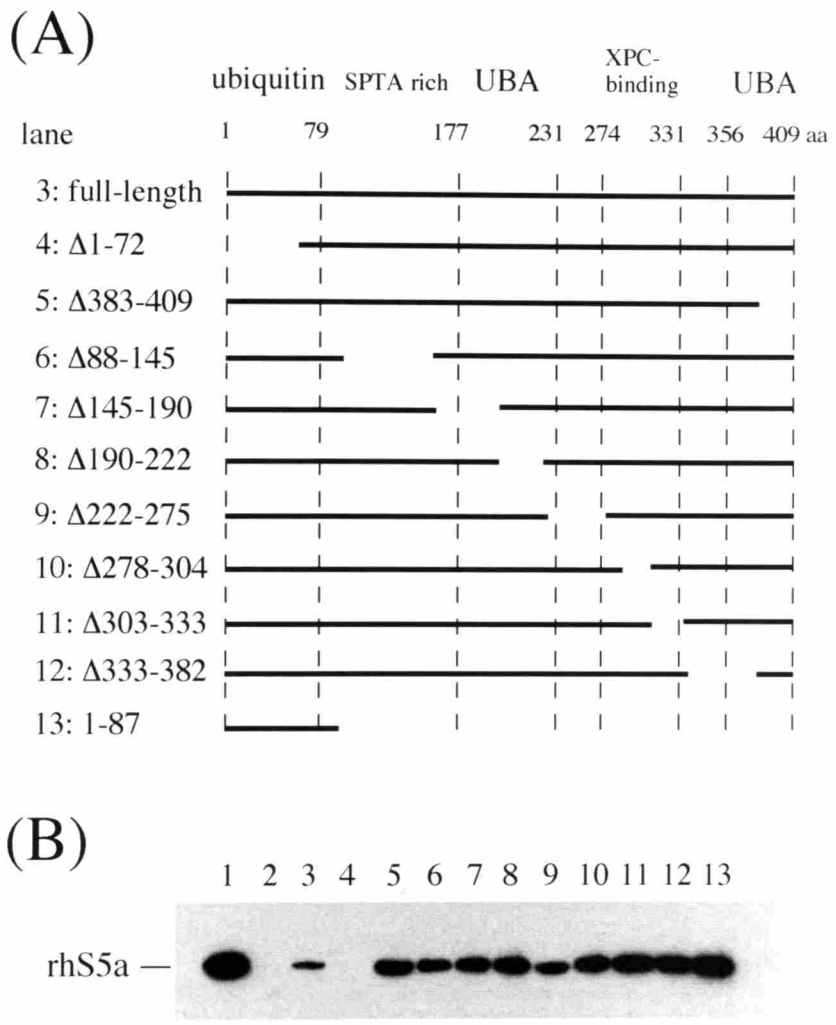


FIGURE 18. rhS5a-binding activities of truncated rhHR23B-His proteins. (A) A summary of the mutant hexahistidine-tagged rhHR23B proteins is indicated. ubiquitin, ubiquitin-like region; SPTA rich, four kinds of amino acids (S, P, T, and A) are predominant in this region; UBA, ubiquitin-associated domain; XPC-binding, XPC-binding domain; aa, amino acids. (B) The presence of precipitated rhS5a was assessed by immunoblotting. 10% input of rhS5a is shown in lane 1. The proteins bound to the nickel chelating Sepharose are shown in lane 2.

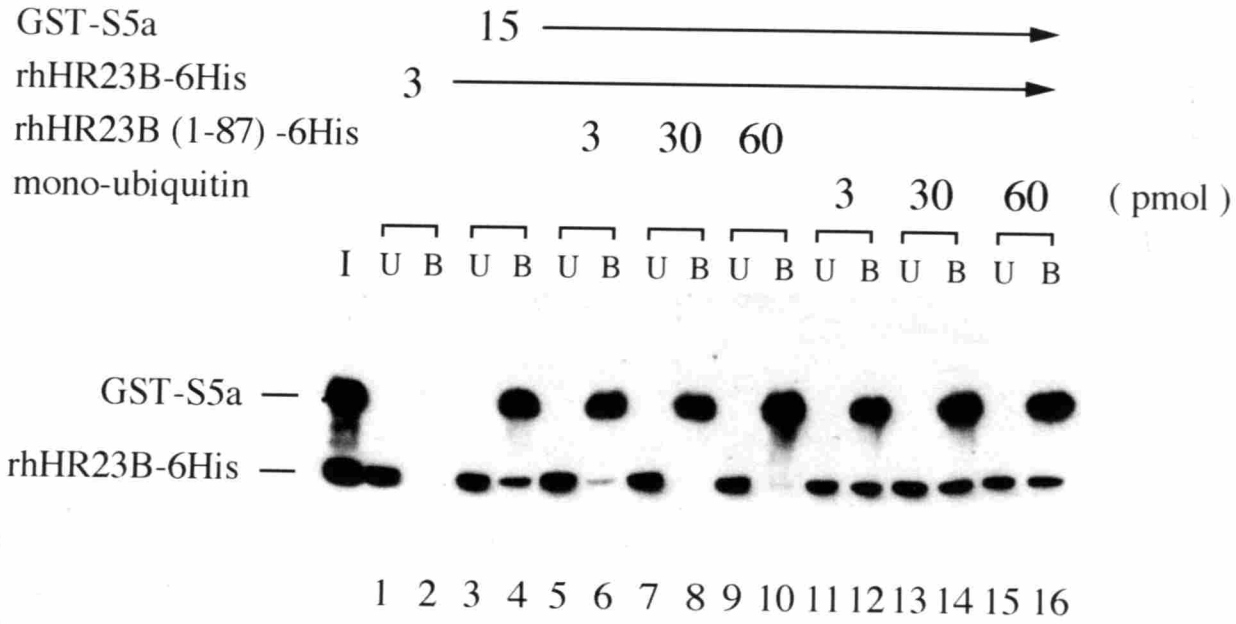


FIGURE 19. The interaction between rhHR23B and rhS5a was inhibited by rhHR23B (1-87) but not by ubiquitin monomer. rhHR23B-6His (3 pmol) and GST-tagged rhS5a (15 pmol) were mixed, and materials unbound and bound to GSH-Sepharose beads were recovered as described in EXPERIMENTAL PROCEDURES. 10% of unbound (U) and 100% of bound (B) are shown in lanes 1 to 16. 50% input of GST-rhS5a and 20% input of rhHR23B-6His are shown in lane I. (1-87)-6His or ubiquitin monomer (designated as mono-ubiquitin) was used as competitor with indicated amount.

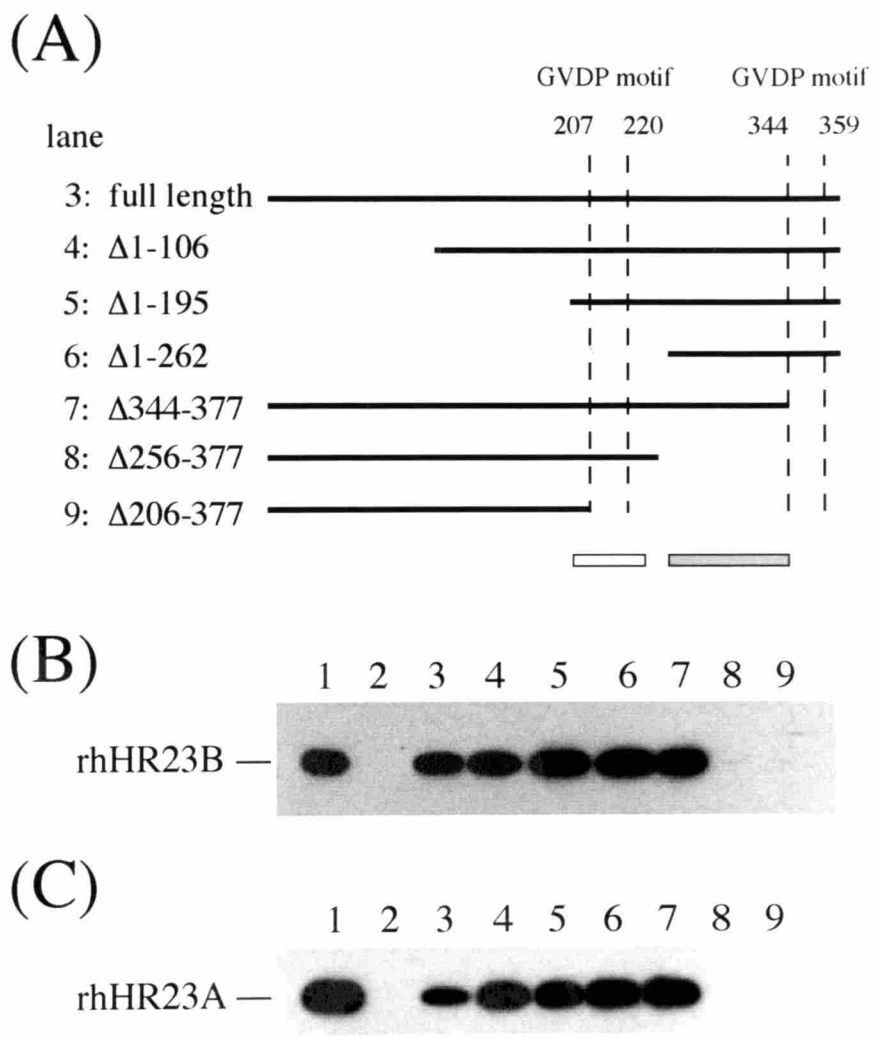


FIGURE 20. rhHR23 proteins-binding activities of truncated 6His-tagged rhS5a. (A) A summary of the mutant hexahistidine-tagged rhS5a proteins is indicated. White bar represents the multiubiquitin-binding domain. Gray bar represents the hHR23 proteins-binding domain. aa, amino acids. (B) The presence of precipitated rhHR23B was assessed by immunoblotting. 20% input of rhHR23B is shown in lane 1. The proteins bound to the nickel chelating Sepharose are shown in lanes 2 to 9. (C) The presence of precipitated rhHR23A was assessed by immunoblotting. 20% input of rhHR23A is shown in lane 1. The proteins bound to the nickel chelating Sepharose are shown in lanes 2 to 9.

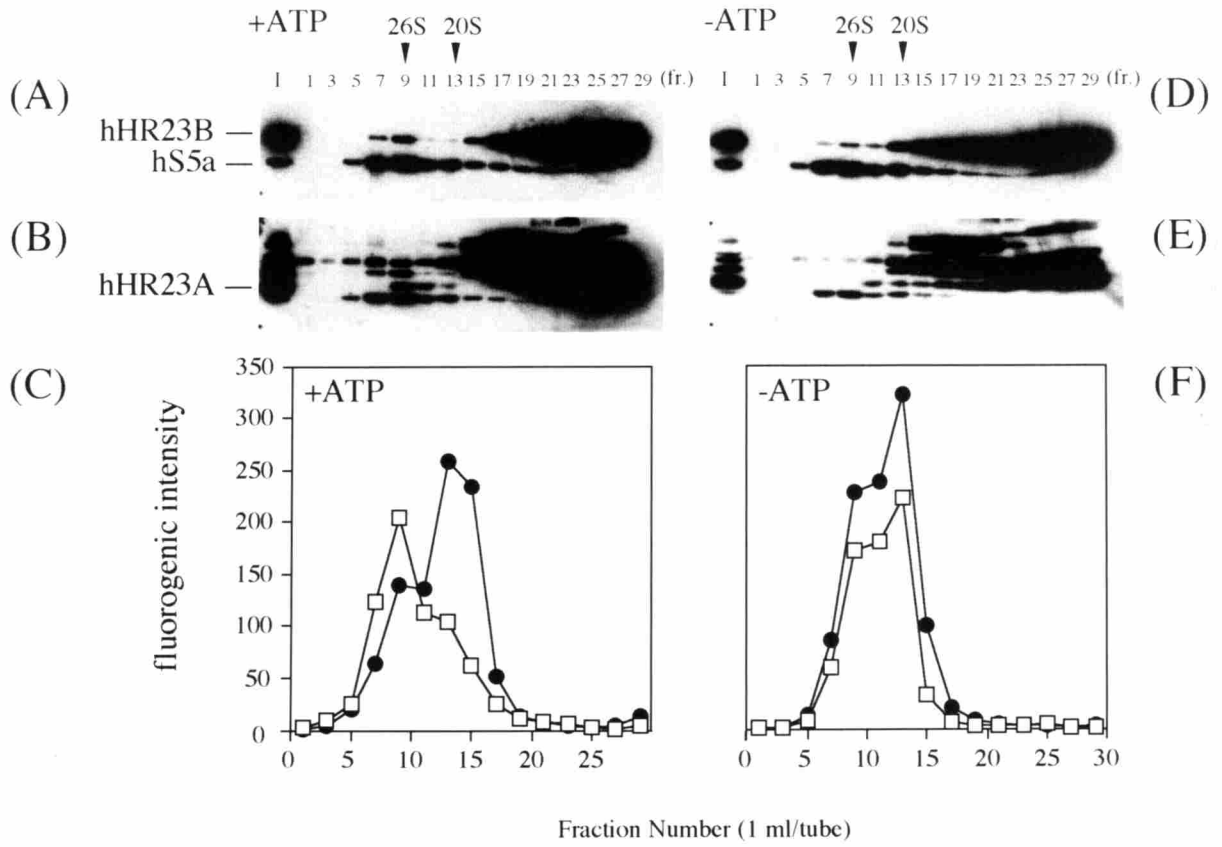


FIGURE 21. Co-fractionation of the hHR23 proteins with the 26S proteasome during glycerol density gradient. HeLa S100 was fractionated on glycerol density gradient in two different conditions (± 2 mM ATP), and fractions were collected. (A) The distribution of hHR23B and hS5a in +ATP condition was assessed by immunoblotting. (B) The distribution of hHR23A in +ATP condition was assessed by immunoblotting. (C) The proteasome activity in +ATP condition was measured. (D) The distribution of hHR23B and hS5a in -ATP condition was assessed by immunoblotting. (E) The distribution of hHR23A in -ATP condition was assessed by immunoblotting. (F) The proteasome activity in -ATP condition was measured. Molecular weight are indicated with arrow head. fr, fraction; I, 10% input.

Table 6. Sequence of oligonucleotides used for PCR

Oligonucleotide	Sequence
Primers for the full-length	
S5aN	5'-GGCGCCATGGACCATATGGTGTTGGAAAGCACTATGGTGTG-3'
S5aC	5'-GTCGAATTCGTCGACCCTTTCCCTCCAGTCTCACTTCTTGTCTTCCTCC-3
Primers for the N-terminal truncation	
S5a Δ 344-377	5'-CCGGAATTCATGGGAGGTTCTCTAGGACACTCTG-3'
S5a Δ 256-377	5'-GCTTGAATTCAGTCTTCAGTCCCAGTCGTAGC-3'
S5a Δ 206-377	5'-CCGGAATTCATTCAAAGTCACTGGCACCAAGACC-3'
Primers for the C-terminal truncation	
S5a Δ 1-106	5'-GGGAATCCATAGCGCATCATTGCCTTTGTGGG-3'
S5a Δ 1-195	5'-GGGAATCCATATGCTGGGTCTTGGTGCCAGTGAC-3'
S5a Δ 1-262	5'-GGGAATCCATATGACCATCAGCCAGCAAGAGTTTGGC-3'