Isolation of XAB2 Complex Involved in Pre-mRNA Splicing, Transcription, and Transcription-coupled Repair*

Received for publication, August 10, 2007, and in revised form, October 29, 2007. Published, JBC Papers in Press, November 2, 2007, DOI 10.1074/jbc.M706647200

Isao Kuraoka†1, Shin suke Ito†, Tada shi Wada‡, Mi ka Hayashida§, Li li Lee..., Mas a fumi Saijo‡*, Yosh imichi Nakatsu†, Megumi Matumoto†, Tsu kasa Matsunaga†, Hiro shi Handa§, Jun Qin‡*, Yoshihiro Nakatani‡‡, and K i yo ji Tanaka§§

From the 1Laboratories for Organismal Biosystems, Graduate School of Frontier Biosciences, Osaka University and the 2Solution-oriented Research for Science and Technology, Japan Science and Technology Agency, 1-3 Yamadaoka, Suita, Osaka 565-0871, Japan, the 3Laboratory of Human Molecular Genetics, Graduate School of Natural Science and Technology, Kanazawa University, Kakuma-machi, Kanazawa 920-1192, Japan, the **Department of Biochemistry, Baylor College of Medicine, Houston, Texas 77030, and the ‡‡Dana-Farber Cancer Institute and Harvard Medical School, Boston, Massachusetts 02115

DNA carrying genetic information is continuously exposed to exogenous and endogenous DNA-damaging agents. The DNA damage interferes with DNA replication, transcription, and cell cycle progression and leads to mutations and cell death, which may cause cancer, inborn diseases, and aging (1, 2). However, a wide variety of DNA lesions, such as ultraviolet light-induced photo-lesions, intra-strand cross-links, and bulky adducts induced by various carcinogens and mutagens, are eliminated by nucleotide excision repair (NER)5 (3). NER is well conserved from Escherichia coli to mammals (4, 5) and consists of the consecutive steps of damage recognition, dual incisions on either side of the damage, excision of 24–32 oligonucleotides containing the damage, gap filling by repair DNA synthesis using the error-free strand as a template, and ligation (6, 7). It has been shown that NER operates via the following two pathways: global genome repair (GGR) and transcription-coupled repair (TCR). GGR can act on DNA lesions at any location in the genome, whereas TCR is involved in a rapid removal of the lesions on the transcribed strand and a resumption of transcription (1, 8).

The biological importance of NER in humans has been suggested by studies of autosomal recessive human genetic disorders as follows: xeroderma pigmentosum (XP), Cockayne syndrome (CS), and trichothiodystrophy, in which NER activity is impaired (9). XP patients are hypersensitive to sunlight and show an increased incidence of UV-induced skin cancers (9). Although CS patients are sensitive to sunlight, they have no predisposition to sunlight-induced skin cancer but instead show severe developmental and neurological abnormalities as well as premature aging (10). Seven NER-deficient complementation groups have been identified in XP (XP-A to XP-G) and two in CS (CS-A and CS-B) (3). In addition, XP-B patients and certain patients with XPD or XPB show features of CS in addition to symptoms of XP (XP-B/CS, XP-D/CS, and XP-G/CS). Recently, we reported that XPD stabilizes TFIIH. Mutations in XPD found in cells of patients with XP-G/CS result in the disassociation of CAK and XPD from the core TFIIH.

**This work was supported in part by a grant-in-aid for scientific research from the Ministry of Education, Culture, Sports, Science and Technology of Japan, and Solution-oriented Research for Science and Technology of Japan Science and Technology Corp. The costs of publication of this article were defrayed in part by the payment of page charges. This article must therefore be hereby marked "advertisement" in accordance with 18 U.S.C. Section 1734 solely to indicate this fact.

1 To whom correspondence may be addressed. Tel: 81-92-541-3231; Fax 81-92-542-8534; E-mail: ikuraoka@nk-cc.go.jp.
2 Recipient of an Advanced Biotechnology Scholarship from Ishihara Sangyo Kaisha, Ltd., in collaboration with the Singapore Economic Development Board.
3 Present address: Dept. of Medical Biophysics and Radiation Biology, Graduate School of Medical Science, Kyushu University, Fukuoka 812-8582, Japan.
4 To whom correspondence may be addressed. Tel: 81-6-6879-7971; Fax 81-6-6877-9136; E-mail: ktanaka@fbs.osaka-u.ac.jp.
5 The abbreviations used are: NER, nucleotide excision repair; TCR, transcription-coupled repair; XPA, xeroderma pigmentosum group A protein; XP, xeroderma pigmentosum; snRNA, small interfering RNA; CS, Cockayne syndrome; RNAP II, RNA polymerase II; TFIIH, transcription factor IIH; TPR, tetratricopeptide repeat; PMSF, phenylmethylsulfonyl fluoride; RT, reverse transcription; RS, RNA synthesis; RRS, RNA synthesis after UV irradiation; snRNA, small nuclear RNA; GGR, global genome repair.
quence, the phosphorylation and transactivation of nuclear receptors were disturbed in XP-G/CS cells. These results indicated that the features of CS in XP-G/CS are because of abnormal transcriptional activation by an unstable TFIH (11).

In human cells, XPC/HRR23B (12) and UV-DDB (13) are involved in the GGR-specific damage-recognition step, whereas in TCR, blockage of RNA polymerase II (RNAP II) at the DNA damage site on the transcribed strand is thought to trigger a TCR reaction (14–16). Following the damage-recognition step in each pathway, NER factors, including transcription factor IIH (TFIIH), XPG, XPA, and replication protein A, are recruited to the lesion, leading to the local unwinding of the DNA double helix. TFIIH, including XPB and XPD helicases, plays a critical role in the formation of this open complex. Two structure-specific endonucleases, XPF-ERCC1 and XPG, subsequently introduce single-stranded breaks on the 5' and 3' sides of the lesion, respectively, leading to excision of the oligonucleotides containing the damage.

We have performed yeast two-hybrid screening with XPA protein as bait and isolated a cDNA encoding a novel tetratricopeptide repeat (TPR) protein consisting of 855 amino acids and designated as XAB2 (XPA-binding protein 2) (17). It has been shown that XAB2 also interacts with the TCR-specific factors CSA, CSB, and RNA polymerase II and that the microinjection of anti-XAB2 antibodies into normal human cells specifically inhibited the recovery of RNA synthesis after UV irradiation as well as transcription but not UV-induced unscheduled DNA synthesis, and that XAB2 knock-out mice show preimplantation lethality (18). A recent in vivo cross-linking and chromatin immunoprecipitation study showed that XAB2 binds to RNA polymerase II in a CSA- and CSB-dependent manner (19). All these results have indicated that XAB2 is involved in TCR as well as transcription but not in GGR.

It is suggested that the TPR domains play a role in intra- and inter-molecular protein interactions. Therefore, we purified XAB2 as a multimeric protein complex from extracts of HeLa cells expressing FLAG-XAB2 fusion protein using anti-FLAG antibody beads. The XAB2 complex consists of hAquarius (IBP160), XAB2 (hSYF1), hPRP19, CCDC16, hISY1, and PPIE, which are known to be involved in pre-mRNA splicing, and bound to RNA but not DNA, indicating that this complex functions in pre-mRNA splicing. The down-regulation of XAB2 expression in normal human cells resulted in hypersensitivity to killing by UV light, and it decreased the rate of recovery of RNA synthesis after UV irradiation, nascent RNA synthesis, and pre-mRNA splicing. Moreover, in the cells treated with DNA-damaging agents, the association of XAB2 with RNA polymerase IIo (elongation mode) and XPA was enhanced. The DNA-damaging agents, the association of XAB2 with RNA polymerase II and XPA was enhanced. The fractions containing XAB2 complexes were collected and loaded onto a MiniQ column (Amersham Biosciences). The bound proteins were eluted with KCl buffer (50 mM to 1 M) containing 10 mM Tris- HCl (pH 8.0), 1% glycerol, 0.1% Tween 20, 10 mM β-mercaptoethanol, and 5 mM MgCl₂. The fractions containing the FLAG-XAB2 complex were pooled and dialyzed into buffer containing 20 mM Tris- HCl (pH 7.3), 100 mM KCl, 20% glycerol, 0.2 mM EDTA, 10 mM β-mercaptoethanol, and 0.25 mM phenylmethylsulfonyl fluoride (PMSF). The FLAG-XAB2 complex was stored at −80 °C.

**Mass Spectrometric Analysis**—The identification of proteins with mass spectrometry was conducted as described previously (21).

**Antibodies**—Anti-XAB2, anti-hPrp19, and anti-CCDC16 rabbit polyclonal antibodies were raised against peptides corresponding to amino acids 811–838 of XAB2, to amino acids 182–208 of hPrp19, and to amino acids 93–115 of CCDC16, respectively. Anti-hAquarius, hISY1, and PPIE rabbit antibodies were raised against the recombinant His-hAquarius (amino acids 1308–1421), GST-hISY1, and GST-PPIE proteins (amino acids 67–164), respectively. These antibodies were affinity-purified using a protein A-Sepharose column. Anti-FLAG monoclonal antibody (M2) was purchased from Sigma. Anti-RNAP II polyclonal antibodies (C-21 and N-20) were from Santa Cruz Biotechnology. Anti-RNAP II monoclonal antibodies (8WG16 and H5) were from Berkeley Antibody.

**Immunoprecipitation and GST Pulldown Assay**—For the co-immunoprecipitation analysis, extracts of HeLa cells stably expressing FLAG-tagged XAB2 protein were prepared as described previously (13, 17). The extracts (500 µl) were incubated with either anti-PPIE, hISY1, CCDC16, hPRP19, or hAquarius antibody (1 µg) at 4 °C for 2 h, and the mixtures were further incubated with protein G-Sepharose beads (Amersham Biosciences) for precipitation at 4 °C for 2 h. After the beads had been washed with NETN buffer (50 mM Tris- HCl (pH 7.8), 150 mM NaCl, 1 mM EDTA, 1% Nonidet P-40, 1 mM PMSF, 10 mM β-mercaptoethanol, and a protease inhibitor mixture (complete, Roche Diagnostics)), the proteins bound to the beads were eluted by boiling them in SDS sample buffer.

For the co-immunoprecipitation experiments in Fig. 1E, lane 8, and Fig. 2, B and C, HeLa cells (8 × 10⁶) were transfected with FLAG-tagged hPRP19 or a series of FLAG-tagged XAB2 in the expression vector pCAGGS (3 µg) using PolyFect (Qiagen) according to the manufacturer’s instructions. Twenty four hours after transfection, cells were collected and washed twice with phosphate-buffered saline (PBS). Cell extracts were prepared as described previously (13, 17). The extracts were incubated with anti-FLAG antibody (M2)-conjugated agarose (Sigma) and rotated at 4 °C for 2 h. After the agarose had been washed with NETN buffer, bound proteins were eluted with NETN buffer containing 0.5 mg/ml FLAG peptide (Sigma).

In Fig. 5A, FLAG-tagged XAB2 complexes were incubated with RNA polymerase II at 4 °C for 3 h and then rotated at 4 °C for 2 h with either anti-RNA polymerase II beads (C-21) (Santa Cruz Biotechnology) or anti-FLAG M2 beads (Sigma). After the beads had been washed with NETN buffer, the bound proteins...
were eluted by boiling in SDS sample buffer. In Fig. 5B, extracts of HeLa cells expressing FLAG-tagged XAB2 protein were incubated with anti-FLAG M2-agarose and rotated at 4 °C for 2 h. After the agarose had been washed with NETN buffer, the bound proteins were eluted with NETN buffer containing 0.5 mg/ml FLAG peptide (Sigma).

For the co-immunoprecipitation analysis in Fig. 5F, 293 cells stably expressing N-terminally 3xFLAG-His-tagged XPA were prepared using a pcDNA5/FRT/V5-His TOPO TA expression kit (Invitrogen) according to the manufacturer’s instructions. HeLa cells stably expressing FLAG-His-tagged RPB3 of RNA polymerase II were prepared as described previously (22). Cell extracts were prepared using NTN buffer (50 mM Tris-HCl (pH 7.8), 150 mM NaCl, 1% Nonidet P-40, 1 mM PMSF, 10 mM 2-mercaptoethanol, and a protease inhibitor mixture (Nacalai)). After incubation at 4 °C for 15 min, the lysates were centrifuged at 15,000 rpm for 30 min. The resulting supernatants were incubated with nickel-agarose (Qiagen) and rotated at 4 °C for 2 h. After the agarose had been washed with NTN buffer, proteins were eluted with NTN buffer containing 20 mM imidazole. The samples were incubated with anti-FLAG antibody (M2)-conjugated agarose (Sigma) and rotated at 4 °C for 1 h. After the agarose had been washed with NTN buffer, proteins were eluted with NTN buffer containing 0.5 mg/ml of FLAG peptide for RNAP II or 3xFLAG peptide (Sigma) for XPA. The GST-pulldown assay was done as described (17, 23).

Construction of Truncated XAB2 and PPIE Expression Plasmids—Expression constructs of the FLAG-tagged XAB2 cDNA with a C-terminal deletion in the mammalian expression vector pCAGGS were generated with the QuickChange mutagenesis kit (Stratagene) using oligonucleotides that contain a stop codon at the indicated sites. The truncation mutant of XAB2 (1–20 and 578–855) was made by restriction digestion of the full-length XAB2 cDNA with PpuMI. The N-terminal and C-terminal deletion mutants of XAB2 cDNA were generated by a PCR-based method. GST-PPIE proteins with various deletions were made as follows. PPIE cDNAs (3–301 and 67–164) were amplified by RT-PCR. PPIE cDNAs (3–81, 83–236, and 238–301) were obtained by restriction digestion of PPIE cDNAs (3–301) with MscI, and PPIE cDNAs (3–152 and 152–301) by restriction digestion with Nael. These PPIE cDNAs were amplified by RT-PCR. PPIE cDNAs (3–301) by restriction digestion with NaeI. These PPIE cDNAs (3–301) with MscI, and PPIE cDNAs (3–152 and 67–164) were amplified by RT-PCR.

RT-PCR Assay for Pre-mRNA Splicing—RNA was extracted from siRNA-transfected cells with an RNA purification kit (Qiagen) and then treated with RNase-free DNase I (Takara) as recommended by the supplier. Purified RNAs were employed to synthesize PCR products with a cMaster RT-PCR system and primers for the amplification of Bcl-x cDNA were described previously (31, 32).

Recovery of RNA Synthesis after UV Irradiation and Nascent RNA Synthesis—Cells (2 × 10⁵) were inoculated into 35-mm Petri dishes. After a 24-h incubation, cells were washed with PBS and treated with UV irradiation at 10 J/m². After another 24-h incubation, cells were washed with PBS and labeled for 1 h in Dublecco’s modified Eagle’s medium containing [³H]uridine (10 μCi/ml) to quantify the recovery of RNA synthesis after UV irradiation. After the labeling with [³H]uridine, 20 μl of 10 mg/ml sodium azide (NaN₃) was added to 1 ml of cell culture, and the cells were washed twice with PBS containing NaN₃ (200 μg/ml). The cells were suspended in 200 μl of sterilized water and then frozen. After the cells were thawed at room temperature, 400 μl of 1.2% SDS was added to the cell suspension, which was then incubated for 30 min at room temperature. After the addition of 400 μl of water, the cell lysates were collected into sample tubes and incubated for 30 min on ice. One milliliter of 10% trichloroacetic acid, 0.1 mM sodium pyrophosphate (NaPP₃) was added into each sample tube and mixed. After incubation for 30 min on ice, these mixtures were transferred onto GF/C.
glass microfiber filters (Whatman). The filters were washed with 5% trichloroacetic acid, 0.05M NaPi, then with ethanol, and dried. The filters were transferred into vials, and scintillation solution was added. Radioactivity was measured with an LS6500 liquid scintillation counter (Beckman Coulter). The radioactivity of the unirradiated cells was indicated as RNA synthesis (RS), whereas the percentage of radioactivity in the UV-irradiated cells divided by that in the unirradiated cells was indicated as recovery of RNA synthesis after UV irradiation (RRS).

Immunofluorescence Microscopy—Cells grown on coverslips were washed with PBS and fixed with PBS containing 0.2% Triton X-100 and 2% formaldehyde at room temperature for 10 min and then incubated in acetone for 5 min at -20 °C. The samples were blocked with PBS containing 5% normal goat serum at room temperature for 30 min. Cells were washed three times with PBS for 5 min, and incubated with anti-XAB2 antibody or antibody indicated in the legends for Fig. 5 in blocking buffer at room temperature for 1 h. Cells were then washed three times with PBS for 5 min, incubated with Alexa Fluor-conjugated antibody (diluted 1:500; Molecular Probes) in PBS at room temperature for 1 h, and washed three times with PBS. The samples were examined with a MRC-1024 fluorescence microscopy system (Bio-Rad).

Colony-forming Assay—Cells were inoculated into 10-cm dishes at a density of 1000 cells per dish. After a 6-h incubation, cells were washed with PBS and UV-irradiated at 0, 4, and 12 J/m². The cells were then incubated for 2–3 weeks. The colonies that formed were fixed with 10% formalin, stained with 0.1% crystal violet, and counted under a binocular microscope.

RESULTS

Purification of XAB2 Protein Complex—XAB2 is a TPR protein of 855 amino acids containing three stretches of acidic residues (Fig. 1A). Mutational and structural studies of some TPR proteins suggested that TPR domains play a role in intra- and inter-molecular protein interactions (24, 25). Consistent with these reports, our gel filtration analysis revealed that XAB2 is included in the ~0.5-MDa fraction (data not shown). Therefore, we purified XAB2 as a multimeric protein complex from nuclear extract of HeLa cells stably expressing FLAG-XAB2 fusion protein. First, the FLAG-XAB2 complex was isolated by immunoprecipitation with anti-FLAG antibody. The protein complex was further purified by gel filtration and MiniQ column chromatography (Fig. 1B). As a positive control (lane 1) and a negative control (lane 2), 5% of the input HeLa/FLAG-XAB2 extracts and 1 μg of rabbit IgG were used for binding, respectively. XAB2 was co-immunoprecipitated with anti-FLAG antibody (M2) from HeLa/FLAG-hPRP19 extracts (lane 8).

FIGURE 1. XAB2 is included in the protein complex. A, schematic representation of XAB2. Open boxes with numbers and closed boxes indicate TPR motifs and acidic regions, respectively. A FLAG tag was attached to the N-terminal region of XAB2 for protein purification. B, scheme to purify the FLAG-XAB2 complex. C, XAB2 was separated on a Superose 6 column. After fractionation, each fraction was resolved by SDS-PAGE and visualized by silver staining. Estimated molecular sizes are depicted in the lower panel. D, purified XAB2 complex was analyzed in a 5–20% SDS-polyacrylamide gel, and the proteins were visualized by silver staining (left). Immunoblotting of the XAB2 complex with anti-FLAG antibody and anti-XAB2 subunit antibodies are shown. Each subunit of the XAB2 complex is indicated on the right side of the gel. E, FLAG-XAB2 was co-immunoprecipitated with each anti-XAB2 subunit antibody (anti-PPIE (lane 3), anti-hISY1 (lane 4), anti-CCDC16 (lane 5), anti-hPRP19 (lane 6), and anti-hAquarius (lane 7)). As a positive control (lane 1) and a negative control (lane 2), 5% of the input HeLa/FLAG-XAB2 extracts and 1 μg of rabbit IgG were used for binding, respectively. XAB2 was co-immunoprecipitated with anti-FLAG antibody (M2) from HeLa/FLAG-hPRP19 extracts (lane 8).

XAB2 Complex Involved in Splicing, Transcription, and Repair
the key factor linking small nucleolar ribonucleoprotein formation to pre-mRNA splicing (26). The open reading frame of hAquarius contains the motifs of the DEXD box, p-loop NTPase, and leucine zipper and has weak homology with viral RNA-dependent RNA polymerase. PPIE is a nuclear cyclophilin containing two functional domains as follows: an RNA-binding domain at the N terminus and a peptidyl-prolyl cis-trans isomerase domain at the C terminus. It is suggested that PPIE could influence RNA processing, protein folding, protein transport, or protein interaction (27, 28), but its exact function is unknown. SYF1 (yeast homolog of XAB2), PRP19 (PSO4), and ISY1 are known to be involved in pre-mRNA splicing in yeast. Consistent with our findings, ISY1 was identified as a protein interacting with SYF1 in a two-hybrid screening of a yeast genomic library using SYF1 as bait. These results suggested that the XAB2 complex plays a role in pre-mRNA splicing in mammalian cells.

Antibodies against each subunit of the XAB2 complex were elicited, and immunoblot analysis using these antibodies identified all the subunits (Fig. 1D, right panel). To verify the interactions between XAB2 and other subunits, immunoprecipitations were performed using extracts of HeLa cells expressing FLAG-XAB2 fusion protein. Each antibody against hAquarius, hPRP19, CCDC16, hISY1, and PPIE co-immunoprecipitated FLAG-XAB2 (Fig. 1E, lanes 3–5 and 7), except for anti-hPRP19 antibody (Fig. 1E, lane 6). To confirm the interaction between XAB2 and hPRP19, we established HeLa cells stably expressing FLAG-hPRP19, and FLAG-hPRP19 was immunoprecipitated with anti-FLAG antibody. We detected XAB2 in the immunoprecipitant (Fig. 1E, lane 8). These results indicated that XAB2 interacts and forms a multimeric protein complex with these subunits (hAquarius, hPRP19, CCDC16, hISY1, and PPIE) in the cells.

Regions of XAB2 for Interaction with Other Subunits—Because XAB2 contains 15 tandem arrays of TPR, it could simultaneously interact with multiple proteins utilizing specific combinations of TPR motifs within the superhelix. Therefore, we identified the region of XAB2 involved in interactions with other subunits. We established HeLa cells expressing FLAG-XAB2 protein with deletions of various lengths (Fig. 2A), purified the XAB2 complex by immunoprecipitation with anti-FLAG antibody, and examined which subunits were retained in each XAB2 complex. All the FLAG-tagged truncated XAB2 proteins were expressed as expected in HeLa cells (Fig. 2B). In the complex of FLAG-XAB2(1–734), all the subunits were retained. In the FLAG-XAB2(1–699) complex, all the subunits except for hPRP19 were retained normally, whereas little hPRP19 was retained. In the FLAG-XAB2(1–699) complex, little hAquarius and no hPRP19 was retained. In the FLAG-XAB2(1–555) complex, neither hAquarius, hPRP19, nor CCDC16 was retained. Both hISY1 and PPIE were retained even in the FLAG-XAB2(1–295) complex. These results suggested that the N-terminal regions 1–734, 1–699, and 1–469 are required for the binding of hPRP19, hAquarius, and CCDC16, respectively, and that hISY1 and PPIE interact with the N-terminal region 1–295 of XAB2. On the other hand, none of the FLAG-XAB2 complexes with N-terminal deletions interacted with any of these subunits. These results suggested that the N-terminal deletion causes a disruption of the higher order structure of XAB2, leading to an inability of the truncated XAB2 to form the protein complex (Fig. 2C).

We have searched for XAB2-associated proteins using the yeast two-hybrid system, and we found that PPIE interacts with XAB2, suggesting that XAB2 directly binds to PPIE (data not shown). To identify the region of PPIE that interacts with XAB2, the in vitro translated XAB2 was incubated with truncated forms of GST-PPIE (Fig. 2D). XAB2 bound to PPIE-(3–81) and PPIE-(3–152) (Fig. 2, D and E), indicating that the residues 3–81, which contain the RNA recognition motif, are essential for binding to XAB2.

XAB2 Complex Binds to RNA but Not DNA—SYF1, a yeast homolog of XAB2, and ISY1 are known to be pre-mRNA splicing factors in yeast (29, 30). hAquarius, CCDC16, and PPIE have a DEAD-box helicase-like motif, U1-type zinc finger motif, and RNA-recognition motif, respectively. These findings suggested that the XAB2 complex has an affinity for DNA and/or RNA. To determine whether the XAB2 complex can bind to DNA and/or RNA, we performed gel mobility shift assays using purified XAB2 complex and a 32P-labeled DNA or RNA probe. The complex bound to RNA (Fig. 3A, lanes 2–4) but bound little to DNA (Fig. 3A, lanes 5–8). The binding to RNA was eliminated by an excess amount of cold RNA competitor (Fig. 3B, lanes 3–5) but not by DNA competitor (Fig. 3B, lane 6). Moreover, addition of anti-XAB2 antibody resulted in a supershift of the RNA/XAB2 complex, whereas addition of

| Table 1: Six peptides were isolated in the XAB2 complex |
|----------------|----------------|----------------|
| **M** | **Acc.** | **Amino acids** | **Protein** | **Yeast homolog** | **Motif** | **Comments** |
| p160 | NP_0555006 | 1421 | hAquarius (IBP160) (KIAA0560) | p-loop, leucine zipper, RNA-dependent RNA polymerase motif | DEXD |  |
| p100 | NP_064581 | 855 | XAB2 | SYF1 | Tetra-tricopeptide repeats | XPA-binding protein |
| p57 | NP_055317 | 504 | hPRP19 | prp19 | U-box, WD-40 | Pre-mRNA processing, E3 ubiquitin ligase activity, Intercross-link repair-related gene |
| CCDC16 | AAH02913 | 366 | hAquarius | D box, p-loop |  |  |
| p42 | NP_065752 | 351 | hISY1 (KIAA1160) | ISY1 |  |  |
| p35 | NP_006103 | 301 | PPIE | CPH1 | Cyclophilin, RNA recognition motif |  |

* Each subunit was identified on a 10% SDS-polyacrylamide gel.
* GenBank accession numbers are listed.
nonspecific IgG did not (data not shown). These results indicated that the XAB2 complex preferentially bound to RNA. To examine whether the XAB2 complex binds to RNA, purified XAB2 complex was incubated with glutathione-Sepharose beads, poly(U)-Sepharose beads, or poly(A)-Sepharose beads. The subunits that bound to each Sepharose bead were examined by SDS-PAGE. Results indicated that all the subunits were detected in both poly(U) and poly(A) beads (Fig. 3C, lanes 3 and 4) but not glutathione-Sepharose beads (Fig. 3C, lane 2). These results indicated that XAB2 binds to RNA as the XAB2 complex.

**XAB2 Knocked Down Cells**—When mixtures of the XAB2 siRNA duplex were transfected into HeLa cells, the XAB2 protein level was markedly reduced (Fig. 4A). In this cell line, normal amounts of hPRP19 and PPIE proteins were detected, but the amounts of hAquarius and hISY1 proteins were markedly decreased. Interestingly, the amount of CCDC16 was increased in the XAB2 knocked down cells compared with the control siRNA-transfected cells. The amount of the largest subunit of RNA polymerase II measured as a loading control was the same in both cell lines. On the other hand, when HeLa cells were transfected with hPRP19 siRNA, there were no effects on the expression level of hAquarius, XAB2, hISY1, and PPIE (data not shown). These results suggested that XAB2 is required for the expression or the stability of these subunits.

**FIGURE 2.** Regions of XAB2 and PPIE needed for interactions with other subunits. A, a schematic representation of a series of deletion mutants of FLAG-XAB2. For example, FLAG-XAB2-(1–734) has a deletion in the C-terminal region encompassing amino acid residues 735–855. B, production of deletion mutants of FLAG-XAB2. FLAG-XAB2 cDNAs with various deletions were expressed in HeLa cells, and the mutant FLAG-XAB2 proteins were detected by immunoblotting using anti-FLAG antibody (M2). The band indicated with an asterisk is a nonspecific band. C, interactions of various mutants of XAB2 with other subunits. The proteins that bound the mutant FLAG-XAB2 were analyzed by immunoblotting using anti-PPIE (diluted 1/5000), anti-hISY1 (1/1000), anti-CCDC16 (1/5000), anti-hPRP19 (1/10,000), and anti-hAquarius (1/5000). D, schematic representation of GST-PPIE with various deletions. A gray box and a black box in PPIE indicate an RNA recognition motif and a peptidy-prolyl cis-trans isomerase (Pro_isomerase) domain, respectively. For example, GST-PPIE-(67–164) has deletions in the PPIE region encompassing amino acid residues 1–66 and 165–301. The XAB2-binding region in PPIE is shown according to the results in E. **E,** in vitro pulldown of XAB2 with GST-PPIE. In vitro translated XAB2 was used for the GST-PPIE pulldown assay. The bound proteins were analyzed by immunoblotting with anti-XAB2 antibodies. As a control (lane 1), 1/25 of the in vitro translated XAB2 protein used for the binding assay was loaded onto the SDS-polyacrylamide gel.
To examine whether the down-regulation of XAB2 affects pre-mRNA splicing activity of the cells, splicing of Bcl-x mRNA was analyzed by RT-PCR of Bcl-x mRNA. Bcl-x mRNA is alternatively spliced to produce two distinct mRNAs, Bcl-xL and Bcl-xS (31, 32). The RT-PCR product of Bcl-xL mRNA decreased and that of the Bcl-xS mRNA increased in amount in the XAB2-siRNA transfected cells compared with control cells (Fig. 4B, lanes 2 and 4), suggesting that XAB2 is required for optimization of pre-mRNA splicing.

We have reported that the microinjection of anti-XAB2 antibody into normal human cells caused a decrease of transcription and TCR but not GGR. Consistent with these results, a colony-forming assay showed that the XAB2 knocked down cells were hypersensitive to killing by UV light, when compared with the control siRNA-transfected cells (Fig. 4C). These results suggested that the XAB2 knocked down cells have a defect in nucleotide excision repair. In addition, to corroborate the role of XAB2 in transcription and TCR, two different siRNAs, designated XAB2 siRNA-1 and -2, were transfected into HeLa cells. A decreased amount of XAB2 protein in the knocked down cells was detected by immunoblot analysis (Fig. 4D). No growth retardation of the XAB2 knocked down cells was observed under the present experimental conditions. The recovery of RNA synthesis after UV irradiation (RRS), which corresponds to TCR, and the nascent RNA synthesis (RS), which corresponds to transcription, were measured in these XAB2 knocked down cells and in the cells transfected with control siRNA. In two XAB2 knocked down cells (XAB2 siRNA-1 and -2), RS was decreased to 85.3 and 80.2% of that in the control siRNA-transfected cells, respectively (Fig. 4E). Notably, RRS was also decreased to 46.1 and 55.4% in the XAB2 siRNA-1...
and -2 cells, respectively (Fig. 4F). These results suggested that the XAB2 complex is involved in transcription and TCR. On the other hand, since the knockdown of XAB2 by siRNA caused a simultaneous down-regulation of hAquarius and hISY1 (Fig. 4A), we cannot completely rule out the possibility that hAquarius and/or hISY1 are involved in transcription and TCR.

**XAB2 Complex Directly Interacts with RNA Polymerase II and Contributes to Transcription in Vitro**—We have reported that XAB2 was co-immunoprecipitated with RNA polymerase II, and the microinjection of anti-XAB2 antibody into normal human cells resulted in a decreased uptake of \(^{3}H\)uridine (17). Here we examined whether the XAB2 complex directly binds to RNA polymerase II. A mixture of purified RNA polymerase II and FLAG-XAB2 complex was immunoprecipitated by either anti-RPB1 (largest subunit of RNA polymerase II) or anti-FLAG antibody. As shown in Fig. 5A, the XAB2 complex was co-immunoprecipitated with RNA polymerase II by the anti-RNA polymerase II antibody, and vice versa. In addition, immunoprecipitation of cell extracts from HeLa cells expressing either FLAG-XAB2 or FLAG-RPB3 by the anti-FLAG antibody revealed co-immunoprecipitation of polymerase Iio and XAB2 (Fig. 5B). The cellular distribution of RNA polymerase II and XAB2 was monitored with anti-RNA polymerase II monoclonal antibodies and anti-XAB2 antibody. H5 detects a hyperphosphorylated form of RNA polymerase II (IIo), whereas 8WG16 detects both hyperphosphorylated and hypophosphorylated forms of RNA polymerase II (Iio + IIA). XAB2 protein co-localized with the RNA polymerase II that was detected by H5, but only partially co-localized with the RNA polymerase II that was detected by 8WG16. These results suggested that the XAB2 complex interacts with the hyperphosphorylated form of RNA polymerase II in elongation mode (Fig. 5C).

**Enhanced in Vivo Interactions of XAB2 with RNA Polymerase II and XPA in the Presence of DNA Damage**—To examine the effect of DNA damage on the interaction between RNA polymerase II and the XAB2 complex, co-immunoprecipitation experiments were carried out using UV-irradiated cell extracts. Extracts from UV-irradiated or
nonirradiated HeLa cells expressing FLAG-RPB3 (3rd subunit of RNA polymerase II) were incubated with anti-FLAG M2 beads, and then the amounts of XAB2 and RNA polymerase II in the immunoprecipitants were analyzed by immunoblotting with anti-XAB2 and anti-RPB1 antibodies, respectively. The results showed that the association of XAB2 and RNAP II was weak in nonirradiated cells but increased dependent on the dose of UV (Fig. 5D). The association of XAB2 with RNAP II was also enhanced by the treatment of the cells with cisplatin and mitomycin C, but not with N-methyl-N’-nitro-N-nitroso-guanidine and actinomycin D (Fig. 5E). These results suggested that bulky DNA lesions introduced by UV light, cisplatin, and mitomycin C, which are subjected to NER, stimulate interaction between XAB2 and RNA polymerase II.

The interaction between the XAB2 complex and XPA in the UV light-damaged cells was also examined. Whole cell extracts from 293 cells expressing 3xFLAG-His-tagged XPA were immunoprecipitated by nickel beads or by nickel beads and then M2 beads. As shown in Fig. 5F, XAB2 was co-immunoprecipitated with XPA. However, when whole cell extracts from UV-irradiated 293 cells were used for co-immunoprecipitation, an increased amount of XAB2 was co-immunoprecipitated with XPA. Thus, the interaction of the XAB2 complex with XPA was also increased in the DNA-damaged cells.

**DISCUSSION**

We purified XAB2 as a multimeric protein complex from HeLa cells. The XAB2 complex consists of six subunits (hAquarius (IBP160), XAB2 (hSYF1), hPRP19 (hPSO4), CCDC16, hISY1, and PPIE), some of which are known to be involved in pre-mRNA splicing, and the XAB2 complex bound to RNA but not DNA. On the other hand, CSA, CSB, RNA polymerase II, and XPA were not included in the XAB2 complex, consistent with the notion that the interactions between the XAB2 complex and the CSA, CSB, RNA polymerase II, and XPA are transient, and only a small proportion of XAB2 binds to CSA and RNA polymerase II (17). However, the association of XAB2 with RNA polymerase IIo and XPA was enhanced in the cells treated with DNA-damaging agents such as UV, cisplatin, and mitomycin C, suggesting that the XAB2 recruits XPA to stalled RNA polymerase IIo. Knockdown of XAB2 with siRNA resulted in a hypersensitivity to killing by UV light, decreased recovery of RNA synthesis after UV irradiation, and nascent RNA synthesis in the cells. Taken together with our previous data (17), these results indicate that XAB2 is required for TCR in addition to pre-mRNA splicing and transcription.

**XAB2 Complex Consists of Pre-mRNA Splicing Factors**—The composition of the XAB2 complex is listed in Table I. Aquarius (IPB160) is a spliceosome component containing a DEAD or DEAD-like helicase domain and binds selectively to the region between small nucleolar RNA and a branch point in the splicing reaction C1 complex. Thus, Aquarius is a key factor linking the biogenesis of small nucleolar ribonucleoprotein to pre-mRNA splicing (33, 34). PRP19 is a spliceosome-associated protein and plays an essential role in pre-mRNA splicing (35–37). hPRP19 has been purified as a stable protein complex consisting of hCDC5, XAB2, hAquarius (IPB160), hISY1, PPIE, and additional proteins in the activated spliceosome (36, 38). These results suggested that XAB2 and PRP19, and probably other subunits of the XAB2 complex, are able to form their own stable protein complex with different combinations of subunits. The hPRP19 complex itself is part of a large ribonucleoprotein complex containing U2, U5, and U6 small nuclear RNA (snRNA), and is required prior to the first and/or second catalytic step(s) of pre-mRNA splicing. On the other hand, PRP19 is allelic to PSO4, which is known to be involved in the repair of DNA cross-linking damage (35, 37, 39). ISY1 associates with SYF1 (yeast XAB2) and snRNAs (U5 and U6 snRNAs) which are part of the spliceosome (29, 30, 40), and it is required for the optimization of pre-mRNA splicing (30) and cell cycle arrest through activation of the spindle checkpoint (29). Thus, hISY1 is thought to be a pre-mRNA splicing factor (41). CCDC16 belongs to a family of C_{2}H_{2}-type zinc finger proteins that are detected in matrin, U1 small nuclear ribonucleoprotein C, and other RNA-binding proteins (42, 43). PPIE is a peptidyl-prolyl cis-trans isomerase E (a nuclear RNA-binding cyclophilin) and appears to be involved in pre-mRNA splicing, transport or translation, although its exact function is not known (27, 28). Consistent with these findings, the XAB2 complex preferentially bound to RNA, and a knockdown of XAB2 by siRNA in HeLa cells resulted in a reduced rate of splicing of Bcl-x mRNA. Taken together, these results indicated that the XAB2 complex functions as a pre-mRNA splicing factor included in a spliceosome.

**XAB2 Complex Contributes to Transcription by RNA Polymerase II**—We found that the XAB2 interacts with RNA polymerase IIo, and knockdown of XAB2 by siRNA resulted in a reduced rate of nascent RNA synthesis. These results indicate that the XAB2 complex is required for transcription by RNA polymerase II. Transcription is coupled to several steps of gene expression, including pre-mRNA splicing, polyadenylation, and mRNA export (44–46). For example, the transcription elongation factors TAT-SF1 (47) and CA150 (48) and the transcriptional co-regulator SKIP (49), which are detected in the spliceosome complex, stimulate a transcription reaction. The XAB2 complex might be a similar pre-mRNA splicing complex that can contribute to transcription by RNA polymerase II. The knockdown of XAB2 by siRNA caused a simultaneous down-regulation of hAquarius and hISY1 (Fig. 4A). These results suggested that the XAB2, hAquarius, and/or hISY1 subunits are involved in the transcription.

Recently, it has been reported that overexpression of XAB2 inhibited all-trans retinoic acid-induced cellular differentiation in human rhabdomyosarcoma cells, and knockdown of XAB2 by siRNA enhanced the differentiation of HL60 cells (50). XAB2 was suggested to function as a co-repressor of the retinoic acid receptor/histone deacetylase 3 complex with an inhibitory effect on all-trans retinoic acid-induced cellular differentiation (50).

**Involvement of XAB2 Complex in Transcription-coupled DNA Repair**—We have previously reported that the micro-injection of anti-full-length XAB2 antibody into normal human cells inhibited RRS and nascent RNA synthesis, whereas the micro-injection of the anti-C-terminal 162 amino acid residues of XAB2 inhibited RRS after UV irradiation but not transcription. Neither antibody inhibited UV-induced DNA synthesis,
which corresponds to GGR (17). Consistent with these results, we showed that the knockdown of XAB2 with siRNA in HeLa cells resulted in a hypersensitivity to killing by UV light and a decreased RRS after UV irradiation, which corresponds to TCR, as well as decreased transcription and pre-mRNA splicing (Fig. 4). Taken together, these results suggested that the XAB2 complex is involved in TCR but not GGR.

We showed that the XAB2 complex bound to RNA but not DNA, and addition of anti-XAB2 antibody or XAB2 protein showed no effect on the in vitro dual incision reaction of NER (data not shown). Therefore, the XAB2 complex plays no direct role in the core NER reaction. However, we showed that XAB2 interacts with XPA and RNA polymerase IIo in vivo, and the interactions of XAB2 with XPA and RNA polymerase IIo were enhanced in the UV-irradiated cells (Fig. 5). These results suggested that the XAB2 complex has an affinity for the stalled RNA polymerase IIo, and XPA is recruited to the RNA polymerase IIo-stalled site via interaction with the XAB2 complex, leading to the assembly of core NER proteins at the RNA polymerase IIo-stalled site.

We have reported that the XAB2 interacts with CSA and CSB as well as XPA and RNA polymerase IIo (17). CSA and CSB are indispensable for TCR. We have shown that CSA is translocated to the nuclear matrix depending on the functions of CSB and TFIIF (51, 52) and associates with RNA polymerase IIo (13, 52) in UV-irradiated cells. The mutant CSA proteins derived from CS-A patients were not translocated to the nuclear matrix in UV-irradiated cells (51), and the association of CSA with RNA polymerase IIo in the irradiated cells was dependent on the function of CSB (19), suggesting that the UV light-induced translocation of CSA into the nuclear matrix and association of CSA with RNA polymerase IIo are relevant to TCR. We have found that CSA is included in the protein complex consisting of CSA, DDB1, Cullin4A, Roc1, and the COP9 signalosome, which has ubiquitin ligase activity (13), and that following UV irradiation, the CSB is ubiquitylated by the CSA complex and degraded in a proteasome-dependent manner (53). In the UV-irradiated cells, RNA polymerase IIo is also ubiquitylated in a CSA- and CSB-dependent manner (54). Thus, the UV-induced ubiquitylation of CSB and RNA polymerase IIo by the CSA complex appears to be required for the TCR reaction, although its biological significance to TCR is not known. We assume that the CSB function is required for recruiting the CSA complex to the RNA polymerase IIo that was stalled at the DNA damage site, and the CSA complex ubiquitylates both RNA polymerase IIo and CSB, which are then removed from the stalled site and degraded, respectively, so that the NER proteins can repair the DNA damage and/or transcription can be resumed.

To analyze the composition of UV damage-stalled RNA polymerase IIo complexes from human cells, in vivo cross-linking in concert with chromatin immunoprecipitation has been used (19). The results indicate that CSA, CSA, XAB2, other TCR-related proteins, and chromatin remodeling factors were associated with UV damage-stalled RNA polymerase IIo. CSA is required for the recruitment of p300, the CSA complex, and NER proteins. These results are consistent with the present findings. However, they indicate that CSA was dispensable for the recruitment of NER proteins to the DNA damage-stalled RNA polymerase IIo. On the other hand, they showed that the enhanced association of XAB2 with the stalled RNA polymerase IIo is dependent on both CSA and CSB. These results indicate that the recruitment of XAB2 to the stalled RNA polymerase IIo is mediated by CSB and CSA functions.

In summary, we purified XAB2 as a multimeric protein complex consisting of six subunits that are involved in pre-mRNA splicing (hAquarius, hPRP19, CCDC16, hISY1, and PPIE). Knockdown of XAB2 with siRNA in HeLa cells resulted in hypersensitivity to killing by UV light and a decrease in recovery of RNA synthesis after UV irradiation and total RNA synthesis. Enhanced interaction of XAB2 with RNA polymerase IIo or XPA was observed in cells treated with DNA-damaging agents, consistent with the recent finding that UV irradiation enhanced the association of XAB2 with the stalled RNA polymerase IIo complex depending on CSA and CSB. Taken together, these results indicated that the XAB2 complex is a multifunctional factor involved in transcription, pre-mRNA splicing, and TCR.

**Acknowledgment**—We thank Aya Yanagida for technical assistance.

**REFERENCES**

1. Hoeijmakers, J. H. (2001) *Nature* **411**, 366–374
2. Lindahl, T., and Wood, R. D. (1999) *Science* **286**, 1897–1905
3. Wood, R. D., Mitchell, M., Sgouros, J., and Lindahl, T. (2001) *Science* **291**, 1284–1289
4. Sancar, A. (1996) *Annu. Rev. Biochem.* **65**, 43–81
5. Prakash, S., and Prakash, L. (2000) *Mutat. Res.* **451**, 13–24
6. Araujo, S. T., Tirole, F., Coin, F., Pospiech, H., Syaooja, J. E., Stucki, M., Hubscher, U., Egly, J., and Wood, R. D. (2000) *Genes Dev.* **14**, 349–359
7. Guzder, S. N., Habraken, Y., Sung, P., Prakash, L., and Prakash, S. (1995) *J. Biol. Chem.* **270**, 12973–12976
8. Svejstrup, J. Q. (2002) *Nat. Rev. Mol. Cell Biol.* **3**, 21–29
9. Lehmann, A. R. (2003) *Biochimie* (Paris) **85**, 1101–1111
10. Nance, M. A., and Berry, S. A. (1992) *Am. J. Med. Genet.* **42**, 68–84
11. Ito, S., Kuraoka, I., Chymkowitch, P., Compe, E., Takedachi, A., Ishigami, C., Coin, F., Egly, J. M., and Tanaka, K. (2007) *Mol. Cell* **26**, 231–243
12. Sugawara, K., Ng, J. M., Masutani, C., Iwai, S., van der Spek, P. J., Eker, A. P., Hanaoka, F., Bootma, D., and Hoeijmakers, J. H. (1998) *Mol. Cell* **2**, 223–232
13. Groisman, R., Polanowska, J., Kuraoka, I., Sawada, J., Saijo, M., Drapkin, R., Kisselev, A. F., Tanaka, K., and Nakatani, Y. (2003) *Cell* **113**, 357–367
14. Inukai, N., Yamaguchi, Y., Kuraoka, I., Yamada, T., Kamijo, S., Kato, J., Tanaka, K., and Handa, H. (2004) *J. Biol. Chem.* **279**, 8190–8195
15. Mei Kwei, J. S., Kuraoka, I., Horibata, K., Uebukata, M., Kobatake, E., Iwai, S., Handa, H., and Tanaka, K. (2004) *Biochem. Biophys. Res. Commun.* **320**, 1133–1138
16. Donahue, B. A., Yin, S., Taylor, J. S., Reines, D., and Hanawalt, P. C. (1994) *Proc. Natl. Acad. Sci. U.S.A.* **91**, 8502–8506
17. Nakatsu, Y., Asahina, H., Citterio, E., Rademakers, S., Vermeulen, W., Kamiuchi, S., Yeo, J. P., Khaw, M. C., Saijo, M., Kodo, N., Matsuda, T., Hoeijmakers, J. H. J., and Tanaka, K. (2000) *J. Biol. Chem.* **275**, 34931–34937
18. Yonemasu, R., Minami, M., Nakatsu, Y., Takeuchi, M., Kuraoka, I., Matsuda, Y., Higashi, Y., Kondo, H., and Tanaka, K. (2005) *DNA Repair* **4**, 479–491
19. Fousteri, M., Vermeulen, W., van Zeeland, A. A., and Mullenders, L. H. (2006) *Mol. Cell* **23**, 471–482
20. Nakatani, Y., and Ogryzko, V. (2003) *Methods Enzymol.* **370**, 430–444
21. Wang, Y., Cortez, D., Yazdi, P., Neff, N., Elledge, S. J., and Qin, J. (2000) *Genes Dev.* **14**, 927–939
22. Hasegawa, J., Endou, M., Narita, T., Yamada, T., Yamaguchi, Y., Wada, T.,...
XAB2 Complex Involved in Splicing, Transcription, and Repair

23. Nagai, A., Saijo, M., Kuraoka, I., Matsuda, T., Kodo, N., Nakatsu, Y., Mimaki, T., Mino, M., Biggerstaff, M., Wood, R. D., Seijbers, A., Hoeijmakers, J. H. J., and Tanaka, K. (1995) Biochem. Biophys. Res. Commun. 211, 960–966

24. Das, A. K., Cohen, P. W., and Barford, D. (1998) EMBO J. 17, 1192–1199

25. Lamb, J. R., Tugendreich, S., and Hieter, P. (1995) Trends Biochem. Sci. 20, 257–259

26. Hirose, T., Ideue, T., Nagai, M., Hagiwara, M., Shu, M. D., and Steitz, J. A. (2006) Mol. Cell 23, 673–684

27. Kim, J. O., Nau, M. M., Allikian, K. A., Makela, T. P., Alitalo, K., Johnson, B. E., and Kelley, M. J. (1998) Oncogene 17, 1019–1026

28. Dahan, O., and Kupiec, M. (2002) Nucleic Acids Res. 30, 4361–4370

29. Sam, M., Wurst, W., Kluppel, M., Jin, O., Heng, H., and Bernstein, A. (1998) Proc. Natl. Acad. Sci. U. S. A. 95, 10602–10607

30. Bregman, D. B., Halaban, R., van Gool, A. J., Henning, K. A., Friedberg, E. C., and Warren, S. L. (1996) Proc. Natl. Acad. Sci. U. S. A. 93, 11586–11590