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Authors: Muto, Masahiro, Fujimori, Akira, Nenoi, Mituru, Daino, Kazuhiro, Matsuda, Yoichi, et al.

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# Isolation and Characterization of a Novel Human Radiosusceptibility Gene, *NP95*<sup>1</sup>

Masahiro Muto,<sup>a,2</sup> Akira Fujimori,<sup>a</sup> Mituru Neno,<sup>a</sup> Kazuhiro Daino,<sup>a,3</sup> Yoichi Matsuda,<sup>b</sup> Asato Kuroiwa,<sup>b</sup> Eiko Kubo,<sup>a</sup> Yasuyoshi Kanari,<sup>a,4</sup> Makoto Utsuno,<sup>a,5</sup> Hideo Tsuji,<sup>a</sup> Hideki Ukai,<sup>a,6</sup> Kazuei Mita,<sup>a,7</sup> Masahiko Takahagi<sup>a</sup> and Kouichi Tatsumi<sup>a,8</sup>

<sup>a</sup> Research Center for Radiation Safety, National Institute of Radiological Science, 4-9-1 Anagawa, Inage-ku, Chiba-shi, Chiba 263-8555, Japan; and <sup>b</sup> Laboratory of Cytogenetics, and Division of Bioscience, Graduate School of Environmental Earth Science, Hokkaido University, North 10 West 8, Kita-ku, Sapporo 060-0810, Japan

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The murine nuclear protein Np95 has been shown to underlie resistance to ionizing radiation and other DNA insults or replication arrests in embryonic stem (ES) cells. Using the databases for expressed sequenced tags and a two-step PCR procedure, we isolated human *NP95*, the full-length human homologue of the murine *Np95* cDNA, which consists of 4,327 bp with a single open reading frame (ORF) encoding a polypeptide of 793 amino acids and 73.3% homology to Np95. The ORF of human *NP95* cDNA is identical to the *UHRF1* (ubiquitin-like protein containing PHD and RING domain 1). The *NP95* gene, assigned to 19p13.3, consists of 18 exons, spanning 60 kb. Several stable transformants from HEK293 and WI-38 cells that had been transfected with the antisense *NP95* cDNA were, like the murine *Np95*-knockout ES cells, more sensitive to X rays, UV light and hydroxyurea than the cor-

responding parental cells. In HEK293 cells, the lack of *NP95* did not affect the activities of topoisomerase II $\alpha$ , whose expression had been demonstrated to be regulated by the inverted CCAAT box binding protein of 90 kDa (ICBP90) that closely resembles *NP95* in amino acid sequence and in cDNA but differs greatly in genomic organization. These findings collectively indicate that the human *NP95* gene is the functional orthologue of the murine *Np95* gene. © 2006 by Radiation Research Society

## INTRODUCTION

Np95 was identified as a single 95-kDa mouse nuclear protein that appeared only in the S phase of normal mouse thymocytes but that was aberrantly accumulated in all stages of the cell cycle in mouse thymic lymphoma cells (1). This protein was localized as dot-like foci in S-phase nuclei, and almost all of these foci were colocalized with chromatin-bound PCNA in early S phase, although the degree of overlapping became nonexclusive in mid S phase and late S phase (2, 3). The expression of Np95 is apparently essential for entry from G<sub>1</sub>/G<sub>0</sub> phase into S phase in adenovirus E1A-induced dedifferentiation of mouse myotube cells (4) but not in embryonic stem (ES) cells (5).

The Np95-encoding cDNA was cloned from a  $\lambda$ gt11 expression library using Th-10a mAb and was verified as a novel nuclear protein with an open reading frame consisting of 782 amino acids (6). Np95 contains an N-terminal ubiquitin-like domain, a leucine zipper motif, a central zinc finger motif of the PHD finger type, and a C-terminal zinc finger of the RING finger type. It also possesses consensus sequences for an ATP/GTP binding site, a cyclin A/E-Cdk2 phosphorylation site, two retinoblastoma (Rb) protein-binding sites in the PHD finger, and the ring finger domains (6). The numbers of structural and functional domains existing in NP95 suggest multiple functions of this protein through protein-protein interactions and/or DNA bindings as a transcription factor for some crucial components in DNA metabolism, i.e. repair, replication and recombination. In fact,

<sup>1</sup> The nucleotide sequences reported in this paper have been submitted to the GenBank/DBJ Data Bank with accession numbers AB177623, AB177624 and AB075601.

<sup>2</sup> Present address: Laboratory for Developmental Genetics Group, RIKEN Research Center for Allergy and Immunology, RIKEN Yokohama Institute 1-7-22 Suehiro-cho, Tsurumi-ku, Yokohama, Kanagawa 230-0045, Japan.

<sup>3</sup> Present address: Life and Bioresource, Graduate School of Science and Technology, Chiba University 33 Yayoicho-1-chome, Inage-ku, Chiba 263-8522, Japan.

<sup>4</sup> Present address: Department of Immunology, Kinki University School of Medicine 377-2 Ohno-Higashi, Sayama, Osaka 589-8511, Japan.

<sup>5</sup> Present address: Production Administration Department, SANKYO Co., Ltd. 3-5-1 Nihonbashi-Honcho, Chuo-ku, Tokyo 103-8426, Japan.

<sup>6</sup> Present address: Laboratory for Systems Biology, Center for Developmental Biology, RIKEN, 2-2-3 Minatojima-minamimachi, Chuo-ku, Kobe, Hyogo 650-0047, Japan.

<sup>7</sup> Present address: Insect Genome Laboratory, National Institute of Agro-biological Sciences, Owashi 1-2, Tsukuba, Ibaraki 305-8634, Japan.

<sup>8</sup> Address for correspondence: Research Center for Radiation Safety, National Institute of Radiological Science, 4-9-1 Anagawa, Inage-ku, Chiba-shi, Chiba 263-8555, Japan; e-mail: tatsumi@rea.or.jp.

NP95 has recently been shown to interact with histones *in vitro* and to function as E3 ubiquitin ligase for histones, preferentially H3 (7).

Homozygously *Np95*-inactivated ES cells (*Np95*-null cells) are more sensitive to X rays, UV light, *N*-methyl-*N'*-nitro-*N*-nitrosoguanidine (MNNG) and hydroxyurea (HU) than wild-type (*Np95*<sup>+/+</sup>) or heterozygously inactivated (*Np95*<sup>+/-</sup>) ES cells (5). The expression of the transfected *Np95* cDNA in the *Np95*-null cells restored the resistance to these DNA-damaging agents and DNA replication blockers to a level similar to that of *Np95*<sup>+/+</sup> cells. Moreover, the frequency of spontaneous sister chromatid exchange was significantly higher in *Np95*-null cells than in *Np95*<sup>+/+</sup> or *Np95*<sup>+/-</sup> cells (5). In view of these results, NP95 has been considered to function at least in ES cells as a common component in multiple DNA damage response pathways and to play a role in the maintenance of genomic stability (5), although the precise mechanism(s) remains largely to be defined. While it has been reported that DNA metabolism and cell cycle progression are controlled differently in ES cells than in somatic cells (8–13), no role of NP95 in maintaining genomic integrity has been suggested as yet in any kind of mouse somatic cells.

Whether NP95 and its human homologue serve similar functions in somatic cells from mice and those from humans, respectively, might be another question. The lack of the cross-reactivity of Th-10a with human cells (1) hampered our earlier characterization of human protein(s) corresponding to mouse NP95. Nonetheless, we had been successfully pursuing an attempt to isolate the full-length human homologue of the murine *Np95* cDNA, *NP95*, by using the databases for expressed sequenced tags (ESTs) and the two-step PCR procedure. The exons of the human *NP95* gene were partially sequenced and deposited as AB075601 in 2001. In this study we present the full-length cDNA sequence, genomic structure and chromosomal mapping of this gene. Furthermore, we generated several stable transfectants with human *NP95* cDNA in both antisense and sense orientations and observed the effect of suppressed transcription of the *NP95* gene on cellular resistance to DNA damages and DNA replication blocks including X rays in human somatic cells.

Human topoisomerase II $\alpha$  functions in growth-dependent processes such as DNA replication and chromosome segregation (14, 15) mainly by alleviating torsional stress ahead of replication forks (16). In 2000, Hopfner *et al.* used the one-hybrid system with an inverted CCAAT box as the DNA target sequence in screening human cDNA libraries and discovered ICBP90, an inverted CCAAT box binding protein of 90 kDa, as being involved in a transcriptional regulation of the topoisomerase II $\alpha$  gene expression in human cells (17–19). ICBP90 has similar domain structures with a 73.7% identity of amino acid sequences to those of mouse NP95 and has thus been considered as a candidate for a potential human counterpart of mouse NP95 (19). Inhibitors of topoisomerase II $\alpha$  including m-AMSA and eto-

poside have been shown to sensitize cultured cells to ionizing radiation (20, 21), and so reduced expression of topoisomerase II $\alpha$  could cause an increased sensitivity to ionizing radiation. We therefore examined whether knocking down of the *NP95* gene in fact suppressed topoisomerase II $\alpha$  gene expression together with the enzyme activity. Contrary to our expectation, the reduced expression of *NP95* did not affect the topoisomerase II $\alpha$  activity in human cells. The purified human NP95 protein indeed interacted with the critical ICB2 (inverted CCAAT box 2) element oligo-DNA, one of five CCAAT boxes in the inverted position of the topoisomerase II $\alpha$ , but in fact lacked the binding specificity to the CCAAT box.

Thus the combination of genomic structure of the human *NP95* being similar to that of the murine *Np95*, the resistance conferred by NP95-like murine NP95 against DNA insults and replication blocks, and a functional dissimilarity of human NP95 from ICBP90 in terms of the effect on topoisomerase II $\alpha$  led us to propose that *NP95* is the human functional orthologue of murine *Np95*.

## MATERIALS AND METHODS

### EST Database Screening and PCR-Based Full-Length cDNA Cloning

We searched for human homologues of the murine *Np95* gene by scanning a human EST database using tblastn (22, 23) of the Basic Local Alignment Search Tool (BLAST) network service at the National Center for Biotechnology Information (NCBI). We used sequence information from the resulting matches, AA811055, AA903902, AA306523, AA354253 and AA488755, to synthesize several primers and used them in two-step PCR to amplify the core cDNA, cDNA 5' end and cDNA 3' end from various human cDNA libraries (the detailed protocol of two-step PCR and sequences of primers are available on request).

### Isolation of Human Bacterial Artificial Chromosome (BAC) Clones

To isolate BAC clones containing the human *NP95* gene, PCR screening of the RPCI-11 library (24) was performed using the primers F2 (TGATCAGAACT CAC CAA CACC) and R3 (CTTGCCACCTT GAC ATTG) at a commercial facility (Nippon Technoservice Co, Chiba). The clones identified from the PCR screening were RPCI-11-165N13, RPCI-11-143D24 and RPCI-11-160C21.

### Chromosome Preparation and Fluorescence In Situ Hybridization (FISH)

The FISH method was used for chromosome assignment of the human *NP95* gene. Preparation of R-banded chromosomes and FISH were performed as described by Takahashi *et al.* (25, 26). The *NP95* cDNA (2.4 kb) in the pCR2.1-TOPO vector (E43 clone) or BAC clones were labeled by nick translation with biotin-labeled 16-dUTP (Roche Diagnostics, Tokyo). The hybridized probes were allowed to react with goat anti-biotin antibodies (Vector Laboratories, Burlingame, CA) and then stained with fluorescent anti-goat donkey IgG (Nordic Immunology). The hybridization signals were visualized with Nikon filter sets B-2A and UV-2A. Kodak Ektachrome ASA 100 film was used for microphotography.

### Generation of Expression Constructs Containing Human *NP95* cDNA in both Antisense and Sense Orientations

pCR2.1.E43.hNP95 plasmid was made by subcloning the full-length human *NP95* cDNA into the pCR2.1-TOPO TA cloning vector (Invitro-

gen). To insert NP95 cDNA into the mammalian expression vector in the antisense orientation, the DNA fragment was obtained by PCR using pCR2.1.E43.hNP95 as a template according to the manufacturer's protocol. This PCR product was inserted into the entry vector pDONR201 (Gateway system, Invitrogen) using BP clonase to create pDONR.AS.hNP95. The pDEST26.AS.hNP95 expression vector coding for NP95-antisense cDNA was created using the Gateway LR reaction with pDONR.AS.hNP95 and pDEST26 (Invitrogen). To construct the NP95 cDNA expression vector in the sense orientation, the reverse PCR product was converted to an entry clone by performing a BP reaction, and the pDEST26.hNP95 expression vector coding for NP95-sense cDNA was created using the Gateway LR reaction.

#### Cell Culture

HEK293 (JCRB9068) and WI-38VA13 (JCRB9057) cells were grown in DMEM/F12 medium (Sigma-Aldrich, St. Louis, MO) containing 10% FBS. The cells were obtained from the Japanese Cancer Research Resources Bank (JCRB).

#### In Vitro Treatment

**X irradiation.** Cells were trypsinized and plated on 100-mm dishes at a density of  $1.0 \times 10^3$  cells per dish. After incubation at 37°C for 16 h in DMEM/F12 medium supplemented with 10% FBS, cells were exposed to various doses of X rays from a Shinai-III X-ray generator (Shimadzu Seisakusho Ltd, Kyoto) at 0.76 Gy/min (200 kVp, 20 mA, with filters of 0.5 mm copper and 0.5 mm aluminum).

**UV irradiation.** Cells were trypsinized, plated on 100-mm dishes and cultured at 37°C for 16 h. Cells were washed twice and then overlaid with 5 ml of Dulbecco's PBS(-) (Nissui Pharm. Co. Ltd., Tokyo). The cells were irradiated with UV light emitted from germicidal lamps (15 W, GL-15, Toshiba, Tokyo) at room temperature. The fluence rate was 0.285 J/m<sup>2</sup>/s, as measured with a UV intensity meter (Topcon UVR-254, Tokyo Kogaku, Tokyo).

**HU treatment.** Cells were trypsinized and plated on 100-mm dishes at a density of  $1.0 \times 10^3$  cells per dish. After incubation at 37°C for 16 h, the medium was removed, and various concentrations of HU (Sigma-Aldrich) were added to the cultures. After treatment at 37°C for 24 h, cells were washed with 15 ml of Dulbecco's modified Eagle's medium and cultured further in DMEM/F12 medium supplemented with 10% FBS.

#### Colony Formation

After the treatments, cells were incubated with DMEM/F12 medium supplemented with 10% FBS for 10 days, and then colonies were scored. Plating efficiencies ranged from 15 to 20%. Relative survivals in terms of the loss of clonogenicity were based on at least two or three repeated experiments, which were carried out in triplicate.

#### Transfection

The day before transfection,  $5 \times 10^5$  HEK 293 or WI-38-VA13 cells per well were plated on six-well plates and cultured in DMEM/F12 medium containing 10% FBS. These cells were then transfected with pDEST26.AS.hNP95 or pDEST26.hNP95 plasmid DNA using Lipofectamine 2000 according to instructions provided by the manufacturer (Invitrogen). After incubation at 37°C for 24 h, the cells were trypsinized, seeded in 100-mm dishes, and cultured further for 10 days in DMEM/F12 medium with 10% FBS in the presence of Geneticin (300–500 µg/ml, Invitrogen). Thirty colonies resistant to Geneticin were isolated in each group. Stable transfected cells were obtained 3 weeks after transfection.

#### Production of an Anti-human NP95 Polyclonal Antibody

An antibody against a unique protein sequence in human NP95 was created by the subcutaneous immunization of rabbits with a synthetic

peptide (CFAPINDQE GAEAKDWRSKGK) that was coupled to keyhole limpet hemocyanin. The antibody was affinity-purified on a column containing the peptide crosslinked to sepharose.

#### Western Blot Analysis

Three million cells were seeded in 100-mm dishes, and after 12 h they were washed twice with PBS (++) and immediately lysed in lysis buffer (2) plus SDS sample buffer (New England BioLabs, Beverly, MA). Equal amounts of whole-cell lysates were separated by 4–20% gradient SDS-PAGE (DAIICHI PURE Chem. Co. LTD, Tokyo) and transferred to Immobilon-P (PVDF) membranes (Millipore, Bedford, MA). Membranes blocked in 5% skim milk (DIFCO Laboratories, Detroit, MI) in PBS for 1 h were rinsed and incubated with the anti-NP95 antibody in 2% (v/v) skim milk in PBS for 1 h at room temperature and washed three times with PBS-T (0.1% Tween in PBS). The bound primary antibody was allowed to react with horseradish peroxidase-coupled anti-rabbit IgG antibody (P0448, DAKO, Kyoto) diluted at 1:5000 in 2% skim milk in PBS for 1 h at room temperature and washed three times with PBS-T. Signals were detected by enhanced chemiluminescence (Lumi-Light Plus, Boehringer Mannheim, Germany).

#### Preparation of Nuclear Extract and Topoisomerase II Assay

Five million cells were collected by trypsinization, washed once in phosphate-buffered saline, and resuspended in 200 µl NE buffer [10 mM Hepes, pH 7.9, 10 mM KCl, 0.75 mM spermidine, 0.15 mM spermine, 0.2 mM EDTA, 0.2 mM EGTA, 0.5 mM dithiothreitol, and 1 mM phenylmethanesulfonyl fluoride (PMSF)] and incubated for 15 min on ice. Cells were then lysed by passage 10 times through a 26-gauge needle, and the lysate was centrifuged for 1 min in a microcentrifuge at 10,000 rpm. The resulting nuclear pellet was resuspended in 100 µl of NESG buffer [20 mM Hepes, pH 7.9, 0.4 M NaCl, 25% (v/v) glycerol, 0.75 mM spermidine, 0.15 mM spermine, 0.2 mM EGTA, 0.5 mM DTT, 1 mM PMSF] and incubated for 30 min on ice with frequent mixing. The extract was centrifuged for 20 min at 15,000 rpm at 4°C. The resulting supernatant (nuclear extract) was stored at –85°C, and the protein concentration was determined with a protein assay kit (Bio-Rad, Hercules, CA).

Topoisomerase II activity was assayed by the ATP-dependent decatenation of kinetoplast DNA (kDNA) (#1001, TopoGEN, Inc., Port Orange, FL). Human type II topoisomerase (p170 form, #2000H-1, TopoGEN) was used as a positive control. Decatenation assays were carried out using kDNA substrate in a final volume of 20 µl in topo II reaction buffer (50 mM Tris-HCl, pH 8.0, 120 mM KCl, 10 mM MgCl<sub>2</sub>, 0.5 mM ATP, 0.5 mM DTT, 30 µg/ml nuclease-free BSA) with 0.2 µg kDNA. Reaction products were analyzed on a 1% agarose gel containing 0.5 µg/ml ethidium bromide.

#### Purification of Recombinant Human NP95 Protein and Electrophoretic Mobility Shift Assay (EMSA)

To examine whether human NP95 binds with the critical ICB2, one of five CCAAT boxes in the inverted position of the human topoisomerase IIα promoter region (27), sense and antisense oligonucleotides; 5' ATAAAAGGCAAGCTACGATTGGTCTTCTGGACGGAGAC 3' and 5' GTCTCCGTCCAGAAGAACCAATCGTAGCTTGCCTTTAT 3', were synthesized and annealed. Mutated ICB2 (ICB2m) oligonucleotides, 5' ATAAAAGGCAAGCTA CGATTCTTCTTCTGGACGGAGAC 3' and 5' GTCTCCGTCCAGAAG AAGGAATCGTAGCTTGCCTTTAT 3', were also synthesized, annealed and then subjected to the test for binding specificity. These double-stranded oligo DNAs were <sup>32</sup>P-end labeled using T4 polynucleotide kinase (Takara, Tokyo) and (γ-<sup>32</sup>P)ATP (148 TBq/mmol, ICN Biomedicals).

For human NP95 purification, HEK293 cells were transfected with pCMV.Tag4A.hNP95 construct by using Lipofectamine Plus reagent (Invitrogen), and the cells were harvested 48 h after transfection. The nuclear extract was incubated with anti-Flag M2-agarose (H2220, Sigma) beads



for 1 h at 4°C followed by washing with 50 mM Tris-HCl (pH 8.0) and 0.15 M NaCl. Proteins were eluted in 0.1 M glycine-HCl (pH 3.5). The purity of the NP95 preparation was monitored with SDS-PAGE and Coomassie blue staining (>95% in purity) (Fig. 5B). EMSA was performed as described previously (28). In brief, the purified NP95 (330 ng) was mixed with 1.4 ng of <sup>32</sup>P end-labeled oligonucleotides in 12% glycerol, 20 mM Hepes (pH 7.9), 70 mM KCl, 5 mM MgCl<sub>2</sub>, 0.05% NP40, 1 mg/ml BSA, 0.5 mM DTT and 0.1 mg/ml poly(dI-dC) in 20 µl and incubated at 25°C for 20 min. For competition experiments, the indicated amounts of unlabeled oligonucleotides were first mixed with NP95 and incubated at 4°C for 25 min. The <sup>32</sup>P end-labeled oligonucleotides were added to the reaction mixtures, which were subsequently incubated at 25°C for 20 min. To verify the binding of the labeled oligonucleotides with NP95, affinity-purified anti-NP95 antibody (14 µg) was added to the above-mentioned reaction mixture and incubated at 25°C for 20 min. These reaction mixtures were loaded on 6% polyacrylamide gels and subjected to electrophoresis.

## RESULTS

### *Cloning of Human NP95 cDNA and Chromosomal Localization*

Using two-step PCR, we identified human NP95 cDNA, which is a 4327-bp cDNA from the human testis cDNA library, and a 3229-bp cDNA from the human fetal thymus cDNA library (Accession Nos. AB177623, AB177624). These contain a single open reading frame (ORF) encoding a polypeptide of 793 amino acids and shows 73.3% homology to mouse NP95 at the amino acid level. The chromosomal location of the human NP95 gene was determined by direct R-banding FISH. NP95 was localized to chromosome 19p13.3 in a region with conserved synteny to the mouse chromosome 17 D-E1.1 on which the mouse *Np95* gene was mapped (Fig. 1A). We searched the GenBank database using the human NP95 cDNA sequence as a probe and found two matches, accession numbers AC027319 and AC053467, which are localized to chromosome 19p13.3. These two DNA sequences are separated by a gap of approximately 5 kb genomic DNA. We cloned and sequenced the gap (exons 15–17 of human NP95) and submitted it to the database under accession number AB075601. Further analysis showed that NP95 includes 18 exons spanning 60 kb of genomic DNA (data not shown). After determining the sequence of the DNA fragment, we noted that the complete NP95 gene sequence had already been deposited by NCBI under the accession number NT\_011255. The genomic structure of the human NP95 is similar to the mouse *Np95* gene, which consists of 17 exons spanning 23 kb (5).

In the process of cloning the human NP95 gene from the RPCI-11 human male BAC library, we identified three clones, 165N13, 143D24 and 160C21, using a 138-bp cDNA probe (nucleotides 2068–2205 of the NP95 cDNA, Accession No. AB177623). Mapping by FISH, however, assigned those BAC clones to chromosome 12p12.2-p12.1 (Fig. 1B). We found that they all contained a DNA sequence 2.8 kb in length that was similar to a part of the human NP95 cDNA (nucleotides 1518–4327 of the 4327-bp human NP95 cDNA, Accession No. AB177623) and

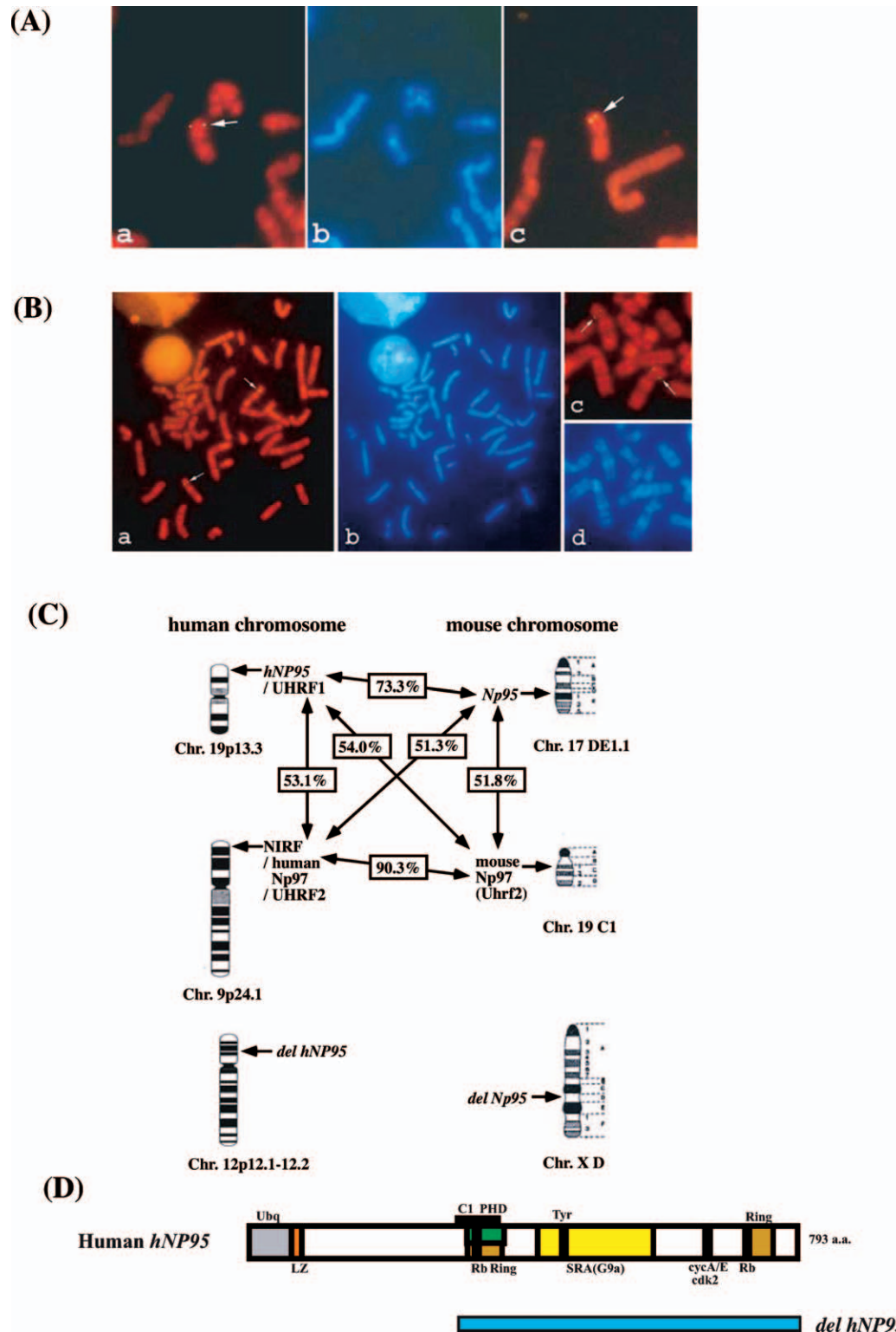
interestingly included polyadenylation signals followed by a poly-A tail at the 3' end. This 2.8-kb sequence was termed the *del hNP95* by us and was confirmed to exist in AC112777 of GenBank database, which had been assigned at 12p12.2-p12.1. Thus this sequence might have arisen from the integration into the chromosome of a naturally occurring cDNA sequence that had been generated as an RNA transcript by reverse transcriptase. In the mouse genome, a similar 2.7-kb sequence (an exonic part from exon 5 to exon 17 of the *Np95* cDNA) has also been recognized on chromosome XD (Fig. 1C). The deduced amino acid sequence of human NP95 was identical to that of UHRF1 (Accession No. AF274048). The NP95 protein contains a ubiquitin-like domain, a leucine zipper motif, a zinc finger motif, a putative cyclin A/E-CDK2 phosphorylation site, an RB-binding motif and a ring finger domain (Fig. 1D).

### *The 5' UTRs and 3' UTRs of Human NP95 Transcripts*

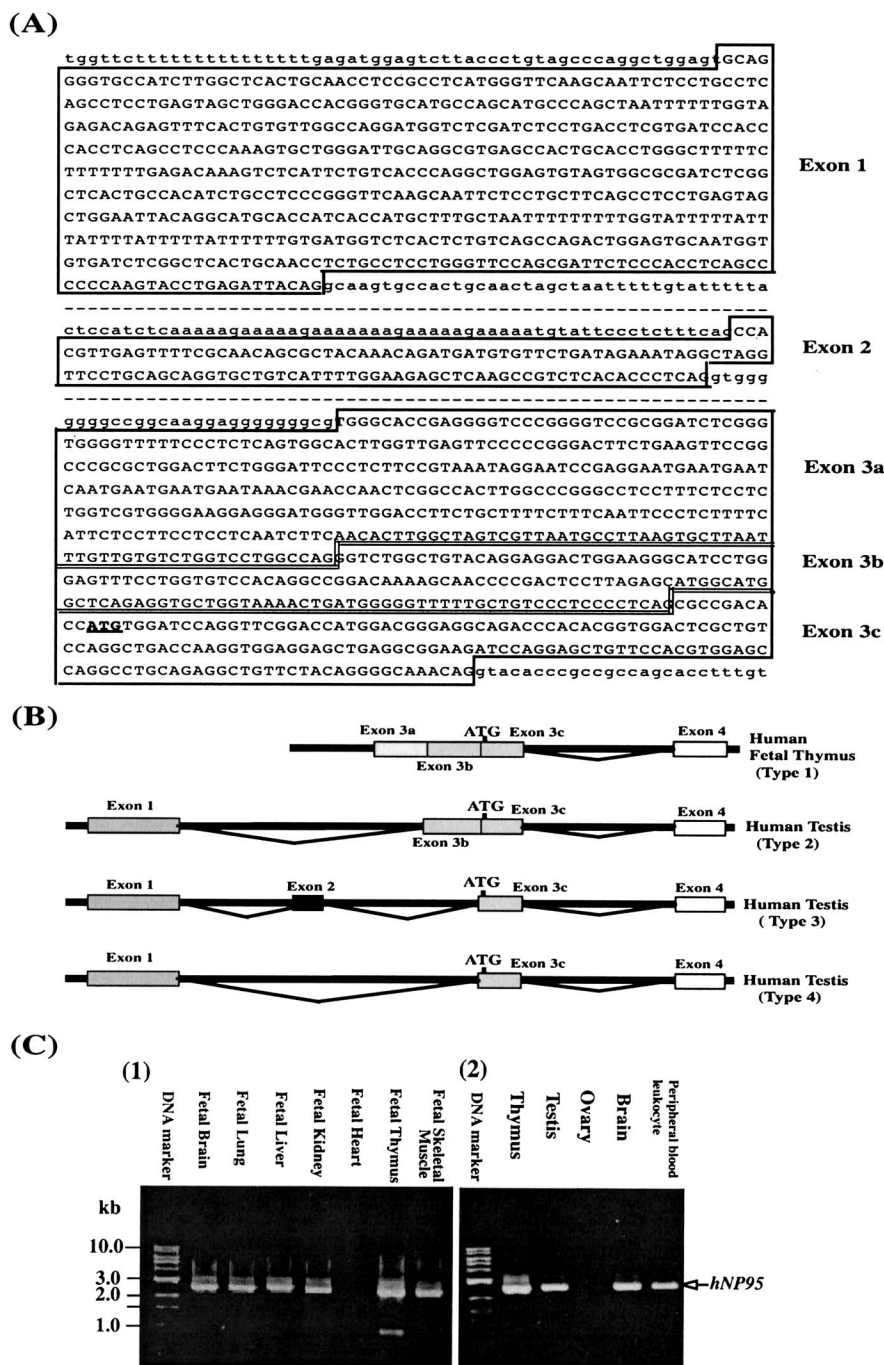
The 5'- and 3'-terminal regions of human NP95 cDNA were analyzed by RACE as described in the Materials and Methods. RT-PCR analysis was subsequently performed to further characterize the 5' UTRs of these transcripts in various tissues. The sequences of the two cDNA clones, E43.hNP95 and Q15.hNP95, as well as that of RACE and RT-PCR products obtained from different tissues, were matched against their corresponding genomic sequences (accession no. AC027319, AC053467, AB075601, NT\_011255). The results indicated that there are four non-coding exons (exons 1, 2, 3a and 3b) that undergo alternative splicing, forming divergent 5' UTRs (Fig. 2A and B). 3'-RACE products included two polyadenylation signals (AATAAA) at 1152 bp and 1328 bp downstream from a TGA translation stop codon and contained a poly-A tail (A6-A15), indicating that the 3' ends of the cDNA sequences are complete and that the polyadenylation signal is functional (AB177623, AB177624). To analyze the tissue specificity of different 5' UTRs, we performed two-step PCR using panels of normalized, first-strand cDNA preparations from human fetal tissues (K1425-1, Clontech) and human adult tissues (K1420-1, K1421-1, Clontech). When sense primers derived from the 5' UTR of exon 3b and antisense primers located in the 3' UTR of exon 18 were used, a full-length NP95 cDNA was detected in the brain, lung, liver, kidney, thymus and skeletal muscle of the human fetus (Fig. 2C-1) and also in adult brain, testis and thymus (Fig. 2C-2). When sense primers from the 5' UTR of exon 1 and antisense primers from the 3' UTR of exon 18 were used, NP95 cDNA was detected in the testis (data not shown).

### *Increased Sensitivities to DNA Insults and Replication Arrests after Human NP95-Antisense Expression in Human Cells*

We generated stable transformants from HEK293 and WI-38 cells that had been transfected with the human NP95 cDNAs in both antisense and sense orientations. Western

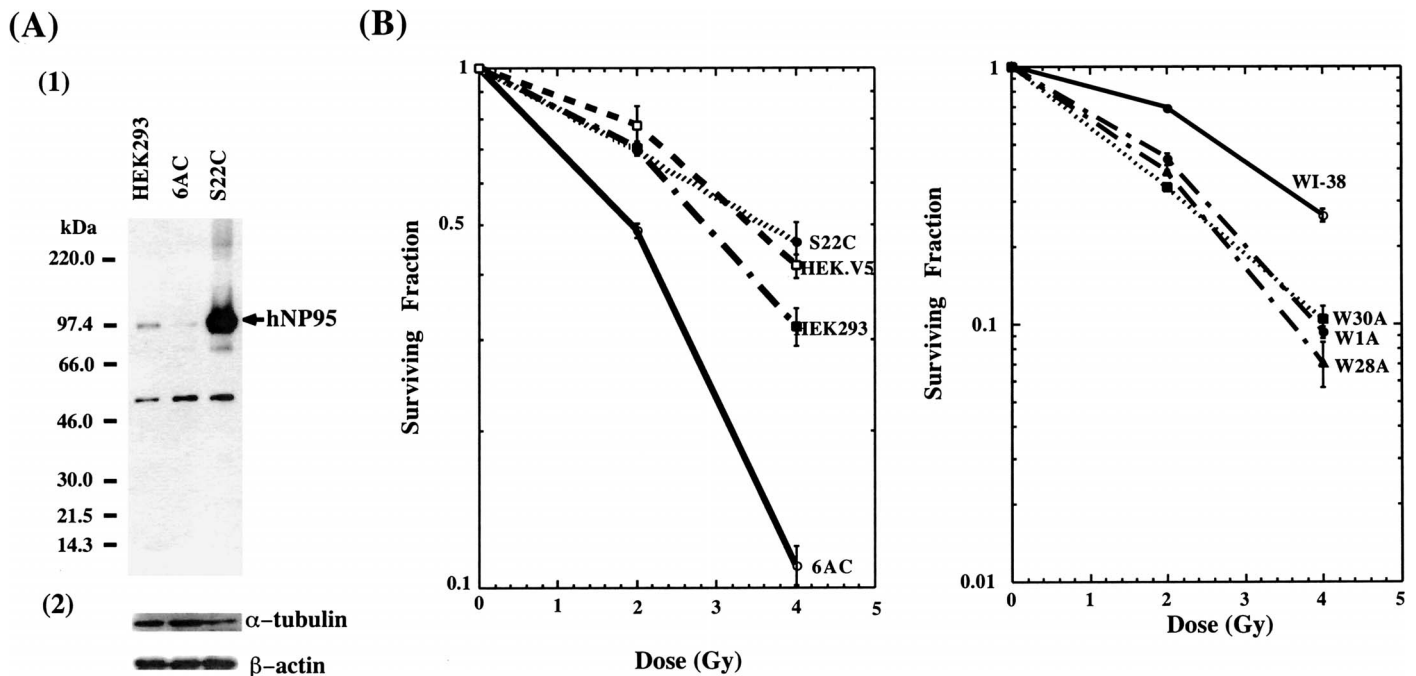


**FIG. 1.** Chromosomal localization of human *NP95* (*hNP95*) and *del hNP95* and structure of *NP95*. Panel A: The FISH was used for chromosomal assignment of the *NP95* gene. A 2.4-kb *NP95* cDNA fragment was used as a biotinylated probe. Arrows indicate the hybridization signals. The *NP95* gene was localized to Chr 19p13.3. The metaphase spreads were photographed with Nikon B-2A (a, c) and UV-2A (b) filters. R- and G-banded patterns are shown in (a, c) and (b), respectively. Panel B: BAC clone 160C21 was used as a biotinylated probe. Arrows indicate the hybridization signals. 160C21 clone (*del hNP95*) was localized to Chr 12p12.1–12.2. The metaphase spreads were photographed with Nikon B-2A (a, c) and UV-2A (b, d) filters. R- and G-banded patterns are shown in (a, c) and (b, d), respectively. Panel C: Distribution of the *Np95* gene family and the processed pseudogene on chromosomes. The figures in boxes are the percentages of amino acid sequence similarity between the ORFs of the corresponding pairs of genes. Panel D: Structure of *NP95* and *del hNP95*.



**FIG. 2.** Nucleotide sequence and alternative splicing in the 5' region of the human *NP95* (*hNP95*) gene. Panel A: Nucleotide sequence of the genomic region spanning the 5' end of the *NP95* gene. Exons (in capitals) are boxed. The ATG initiation codon of the ORF is underlined in bold. Panel B: Alternative splicing of the 5' UTR of the *NP95* transcripts. Exons and introns are marked by boxes and solid lines, respectively. Panel C: Expression of *NP95* mRNA in human fetal tissue. The full-length *NP95* cDNA was detected by two-step PCR using primers designed on the basis of sequence information of the 5' UTR in exon 3b and the 3' UTR in exon 18. The first PCR was performed using primers U221 (AGGTGCTGGTAAACTGATGG) and L3064 (AGGGAACGGAGGAAACACTAC), and human fetal tissue cDNA (K1425-1, Clontech, Palo Alto, CA) as a template. The second run used the first PCR product as a template and U36 (CTGATGGGGGTTTTTGCTGTC) and L3030 (GCCCACTAAGAACAAAATCAG) as primers.





**FIG. 3.** Human NP95 confers resistance against X radiation. Panel A: (1) Expression of NP95 protein in HEK293 cells and the *NP95*-antisense and -sense cDNA stably integrated HEK293 cells. Total cell extracts (6  $\mu$ l out of 300  $\mu$ l lysate prepared from  $1 \times 10^7$  cells) were immunoblotted with anti-NP95 antibody. Lane 1, untransfected HEK293 cells; lane 2, 6AC (the *NP95*-antisense cDNA transfected HEK293 cells); lane 3, S22C (the *NP95*-sense cDNA transfected HEK293 cells). (2) The same amounts of lysates from above cell lines were immunoblotted with anti- $\alpha$  tubulin (DM1A, Sigma-Aldrich) and anti- $\beta$  actin (AC-15, Sigma-Aldrich) as loading controls. Panel B: Right frame: Relative surviving fractions as a function of dose of X radiation in the *NP95*-antisense stably expressed HEK 293 cells, 6AC ( $\circ$ ), the *NP95*-sense cDNA expressed HEK 293 cells, S22C ( $\bullet$ ), untransfected HEK 293 cells ( $\blacksquare$ ), and the empty vector transfected HEK293 cells, HEK.V5 ( $\square$ ). Left frame: Relative surviving fractions as a function of X-ray dose in the *NP95*-antisense cDNA expressed WI-38 cells, W30A ( $\blacksquare$ ), W1A ( $\bullet$ ) and W28A ( $\blacktriangle$ ), and untransfected WI-38 cells ( $\circ$ ). Each data point denotes a mean from the experiment carried out in triplicate.

blot analysis verified that the amount of NP95 protein increased dramatically in the *NP95*-sense transfected S22C cells and markedly decreased in the *NP95*-antisense transfected cells, 6AC (Fig. 3A), W28A, W1A and W30A (data not shown). Diminished expression of NP95 resulted in a growth retardation; the population doubling times for the parental HEK293 cells, HEK.V5 cells, 6AC cells, and S22C cells were approximately 24, 24, 36 and 24 h, respectively. We examined whether the altered level of NP95 expression affects the sensitivity to various types of DNA damage using *NP95*-antisense cDNA transfected cells. The *NP95*-antisense transfectants, 6AC, W28AS, W1AS and W30AS, were more sensitive to X rays than their parental cells (HEK293, WI-38) or the *NP95*-sense transfectant, S22C cells, and the empty vector transfectant, HEK.V5 cells (Fig. 3B and C). 6AC cells were also more sensitive to UV light than S22C cells and the parental HEK293 cells (Fig. 4A). Moreover, these *NP95*-antisense transfectants were more susceptible to DNA replication blocks after treatment with hydroxyurea than S22C, the *NP95*-sense cDNA transformants, or the parental WI-38 and HEK293 cells (Fig. 4B). Thus the expression of NP95 in human somatic cells appears to confer the ability to cope with DNA damage and DNA replication blocks, as is also the case with NP95 in mouse ES cells (5).

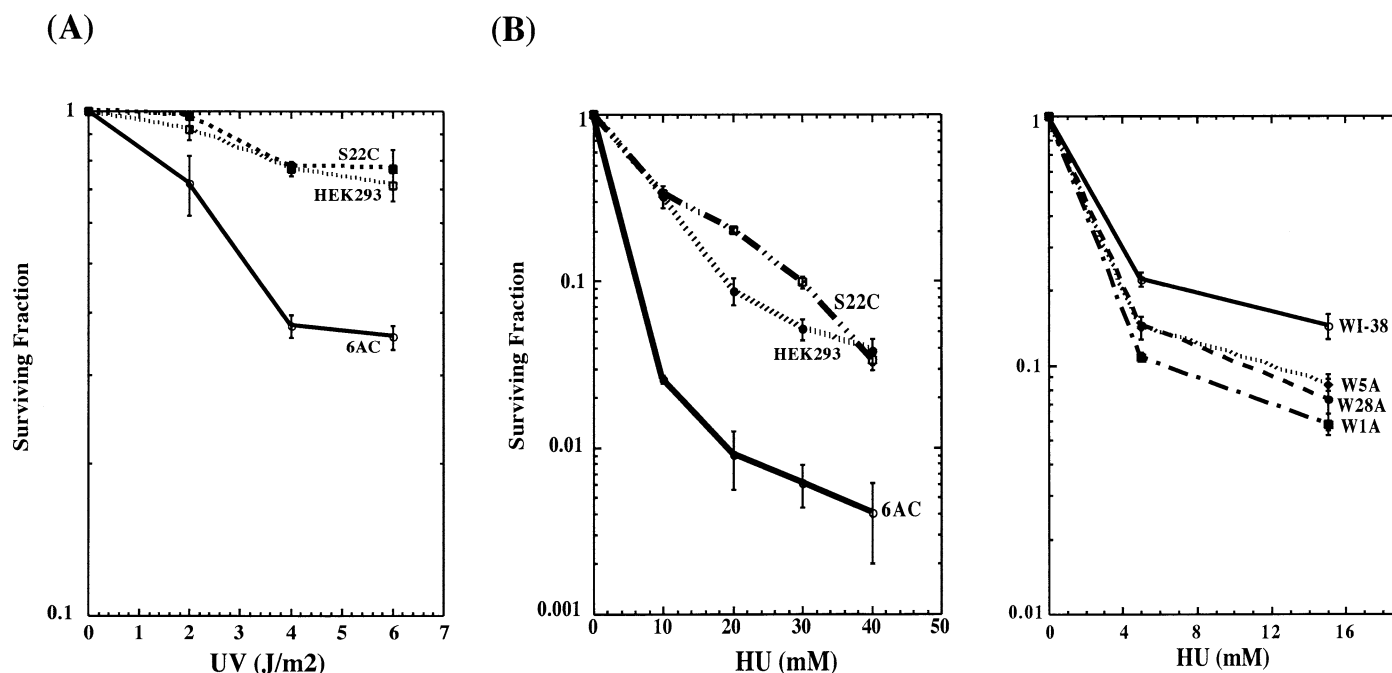
#### No Dependence of Topoisomerase II $\alpha$ Activity on Human NP95 Expression

The topoisomerase II $\alpha$  activities of the nuclear proteins extracted from HEK293, 6AC and S22C cells were compared using an assay of the ATP-dependent decatenation of kinetoplast DNA (kDNA). The topoisomerase II activities in these cells were equivalent to approximately 1 unit of human type II topoisomerase activity within the resolution of experimental condition (p170 form, #2000H-1, TopoGEN, Inc), and they did not correlate with the degree of NP95 expression (Fig. 5A-1 and 5A-2)

#### Nonspecific Binding of Human NP95 to the ICB2 Element of Topoisomerase II $\alpha$ Gene Promoter

We next tested whether NP95 binds specifically to the ICB2 element of the promoter region in the topoisomerase II $\alpha$  gene. Flag-tagged NP95 was expressed in HEK293 cells and purified to near homogeneity using anti-Flag M2-agarose beads (Fig. 5B). ICB2 oligo-DNA that contains CCAAT box or C $\rightarrow$ G mutated ICB2, i.e. ICB2m oligo-DNA that contains a GGAAT box, was  $^{32}$ P end-labeled as described by Hopfner *et al.* (19). The supershift of labeled ICB2 probe by anti-NFY antibody was used as a positive control for specific DNA binding, as shown in Fig. 5C. In





**FIG. 4.** Human NP95 confers resistance against UV radiation and against treatment with HU. Panel A: Clonogenic survival curves after UV irradiation. Relative surviving fractions as a function of UV-radiation dose were determined for the *NP95*-antisense cDNA expressed HEK293 cells, 6AC (○), the *NP95*-sense expressed cDNA HEK 293 cells, S22C (■), and untransfected HEK 293 cells (□). Data points denote means from the experiment performed in triplicate. Panel B: Increased sensitivity of the *NP95*-antisense cDNA stably integrated cells to continuous treatment with HU. Left: Relative surviving fractions in the *NP95*-antisense cDNA expressed HEK293 cells, 6AC (○), the *NP95*-sense cDNA expressed HEK293 cells, S22C (□), and HEK 293 cells (●) after treatment with HU for 24 h. Bars represent standard errors. Right: Relative surviving fractions in the *NP95*-antisense cDNA stably expressed WI-38 cells, W5A (◆), W28A (●) and W1A (■) cells and untransfected WI-38 cells (○) after treatment with HU for 24 h. Data points denote means from a representative experiment carried out in triplicate.

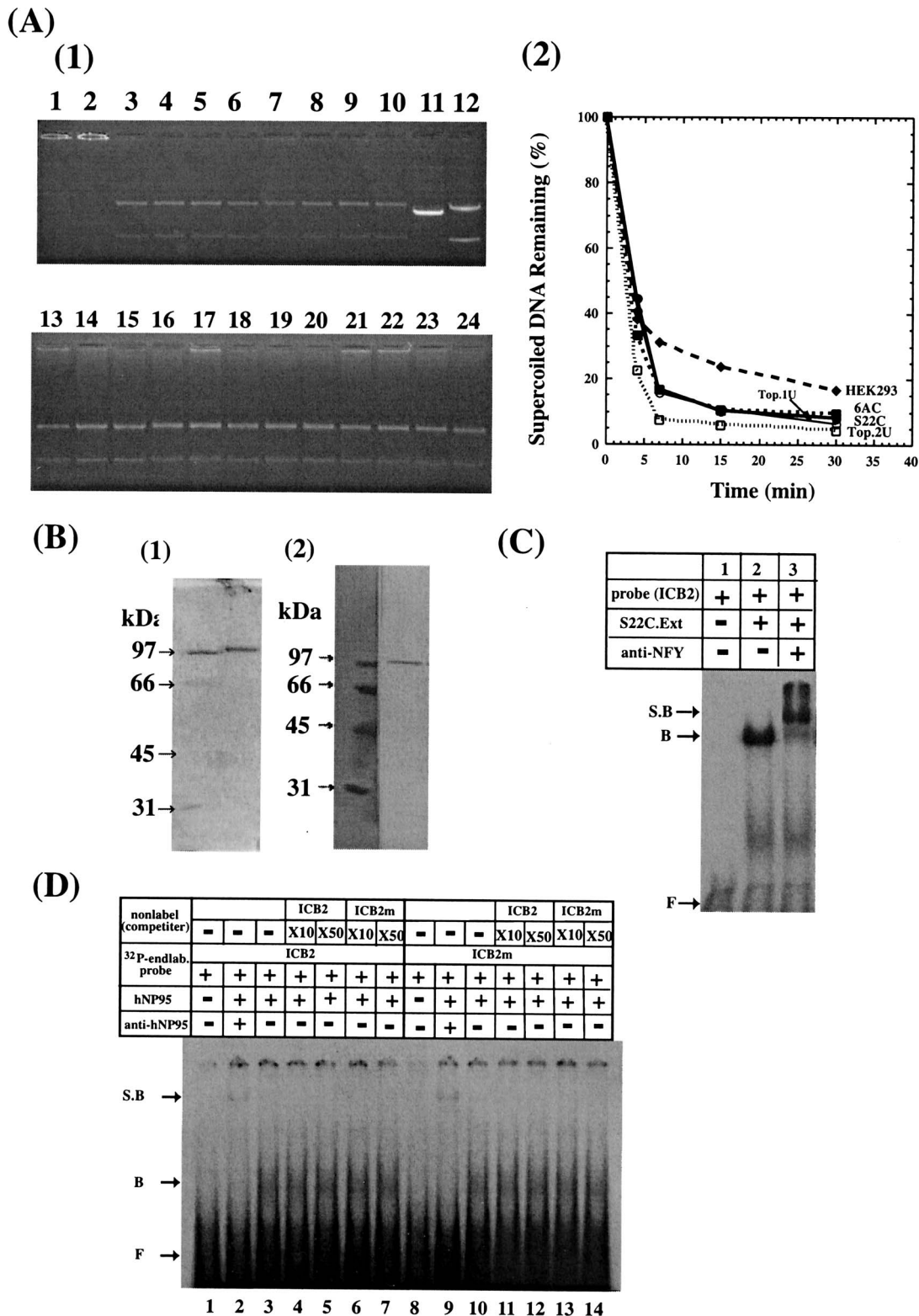
Fig. 5D, the purified NP95 was mixed with  $^{32}$ P-end labeled oligo-DNAs and the reaction mixture was loaded on 6% polyacrylamide gels. The intensity of the bands representing oligonucleotides bound to NP95 (denoted as B) indicated that NP95 interacted with both ICB2 (lane 3) and ICB2m (lane 10) to a virtually similar extent. In EMSA, the addition of affinity purified anti-NP95 antibody to a reaction mixture resulted in the appearance of super shift bands (denoted as SB) and the concomitant disappearance of B bands, and there was no difference in the intensity of SB bands between ICB2 and ICB2m (lanes 2 and 9). When NP95 was assumed to bind specifically to ICB2, the intensity of B bands in lanes 3–7 should have been much denser than those representing interaction between NP95 and ICB2m in lanes 10–14. These results indicate that NP95 binds to ICB2 but not in a specific manner.

## DISCUSSION

We have identified and characterized the *NP95* gene, which is the human homologue of the murine *Np95* gene. The *NP95* gene at 19p13.3 has 18 exons spread over 60 kb of genomic DNA, and its exon-intron structure is similar to that of mouse *Np95* (5). We characterized the 5' and 3' UTRs of the *NP95* transcripts in a few human cDNA libraries from fetal thymus and testis. Several alternative

splicings in the 5' UTR and differential polyadenylation in the 3' UTR were found in the *NP95* transcripts, although the ORF remained unaffected. These divergent mRNA leaders may play a role in the translational regulation of NP95 (Fig. 2B and C), as is the case for the differential *ATM* expression among different tissues (29).

Since we had noted the hypersensitivity of *Np95*-knock-out ES cells to X rays and UV light, MNNG and hydroxyurea compared with ES wild-type (*Np95*<sup>+/+</sup>) or heterozygously inactivated (*Np95*<sup>+/-</sup>) cells (5), we tested whether the same holds true for human NP95 expression in HEK293 and WI-38 cells. Under the experimental conditions used, it was found that the expression of the full-length antisense *NP95* cDNA led to an essentially total deprivation of NP95 protein (Fig. 3A). In accordance with our expectation, the *NP95*-antisense cDNA transfectant clones, 6AC, W28A, W1A and W30A, were all more sensitive to X rays than their untransfected parental cells (HEK293, WI-38), empty vector-transfected cells (HEK-V5), or the *NP95*-sense cDNA transfectant S22C cells (Fig. 3B). 6AC cells were also more sensitive to UV light than S22C or HEK293 cells (Fig. 4A). Moreover, these *NP95*-antisense transfectants were more susceptible to DNA replication blocks induced by hydroxyurea than S22C cells or untransfected parental HEK293 and WI-38 cells (Fig. 4B). Thus, just like our findings with *Np95* in mouse ES cells (5), the expression



**FIG. 5.** Irrelevance of human NP95 (hNP95) to expression of topoisomerase II $\alpha$ . Panel A: Influence of the level of NP95 expression on activities of topoisomerase type II. (1) Representative data from the decatenation assay. The nuclear extracts (150 ng) from 6AC (■), S22C (●), and HEK293 cells (◆) were prepared, and topoisomerase II activities were assayed by the ATP-dependent decatenation of kinetoplast DNA (kDNA). Human type II topoisomerase (p170 form) (TopoGEN, Inc.); 1 (○) and 2 units (□) were used as a positive control. The proportion of supercoiled kDNA remaining as a function of reaction time was determined. Panel B: (1) Coomassie staining of the purified Flag-hNP95 protein. (2) Immunoblot analysis with anti-Flag. Panel C: Electrophoretic mobility shift assays (NF-Y). Panel D: Electrophoretic mobility shift assays (hNP95). The purified human NP95 was reacted with <sup>32</sup>P end-labeled ICB2 or ICB2m. The positions of bound, B, and free, F, probes are indicated. To verify the binding of the labeled oligonucleotides with NP95, affinity purified anti-NP95 antibody was added to the reaction mixture (lane 2 and lane 9), and the position of super shift probe bound, S.B., was exhibited.

of NP95 in human somatic cells appears to confer the cellular capability to cope with both DNA insults and DNA replication arrests that could cause stalled replication forks in S-phase cells, a very vulnerable structure unless properly restored (30).

ICBP90, an inverted CCAAT box binding protein, has been demonstrated by Hopfner and his coworkers to be involved in the regulation of topoisomerase II $\alpha$  transcription (18, 19). Since several inhibitors of human topoisomerase II $\alpha$  have been reported to enhance cell killing by ionizing radiation (20, 21), the hypersensitivity to X rays of *Np95* knockout mouse ES cells (5) and of *NP95* knockdown human cells in this study may be attributable to a decreased expression of topoisomerase II $\alpha$ . However, the specific binding of NP95 to the CCAAT box was not assured in this study, since purified NP95 bound not only to ICB2 but also to the mutated ICB2 to a similar extent (Fig. 5D). Moreover, our results indicated that the topoisomerase II $\alpha$  activity in two different types of human cells employed were not correlated with the level of the expression of NP95 (Fig. 5A). Recently, the binding of this protein with methyl-CpG dinucleotides, especially methylated promoter region of some tumor suppressor genes, has been reported (31). No detectable amount of NP95 in the crude extracts from thymus tissue of C57BL/10 mice was demonstrated to bind to any of the seven CCAAT boxes in the upstream region of the mouse topoisomerase II $\alpha$  gene (32), whereas NF-Y, the ubiquitous transcription factor also known as CBF, ACF and CP1 (27), was unequivocally bound to three or four CCAAT boxes as a major binding factor (M. Neno *et al.*, data not shown). Thus NP95 and ICBP90 do not appear to share a common function in terms of the effect on topoisomerase II $\alpha$  activity.

Hopfner *et al.* have reported that the *ICBP90* gene spans approximately 35.8 kb on chromosome 19p13.3 and contains 6 coding exons named A to F (18). We have noted that *NP95* and *UHRF1* are identical in ORF and that *ICBP90* and *NP95* differ in ORF by only two amino acids (Lys<sup>383</sup>→Asn<sup>383</sup>, Ala<sup>457</sup>→Ser<sup>457</sup>) and seven nucleotides. However, we have been unable to recognize the sequence of exon F (2.6 kb) of the *ICBP90* gene in NT\_011255 of GenBank database at 19p13.3 (18). On the other hand, this exon F of the *ICBP90* gene is quite similar to the *del hNP95* sequence of 2.8 kb in our BAC clones (see AC112777). Exons through A to E of *ICBP90* do exist at 19p13.3 (see NT\_011255), not at 12p12.2-p12.1 (see AC129029 and AC112777). The gene encoding ICBP90 therefore appears to be distinct from the one encoding NP95.

NIRF (Np95/ICBP90-like ring finger protein)/human NP97/UHRF2 protein also resembles NP95 in possessing a ubiquitin-like domain, a PHD finger, an SRA (G9a) domain, an RB-binding motif, and a ring finger. Its gene is mapped at chromosome 9p24.1 (33). Since NP95/UHRF1 protein and NIRF/human NP97/UHRF2 protein have a similar organization, their corresponding genes found on

mouse chromosomes (Fig. 1C) probably derive from a common ancestral gene. If we compare their coding region amino acid sequences, NP95 is closer to NP95 (73.3% similarity) than to NIRF/human NP97/UHRF2 (51.3%), implying that the *NP95* mapped to 19p13.3 is the human orthologue of the mouse *Np95*.

In summary, judging from the similarity in the exon structure, the identity of deduced amino acid sequence, the hypersensitivity to X rays and other DNA insults and replication arrests in the NP95 knockdown cells, and the apparent lack of relevance to topoisomerase II $\alpha$  gene expression, we propose that *NP95* is the human functional orthologue of the murine *Np95*.

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