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Identification of a Novel Putative Ran-Binding Protein and Its Close Homologue

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In the process of cloning genes at the breakpoint of t(5;14) (q34;q11), a recurring translocation in acute lymphoblastic leukemia, we isolated and characterized a novel gene at 5q34, and a close human homologue (66% amino acid identity) located at 8p11-12. The presence of an importin-β N-terminal domain at their N-terminus, their size of approximately 110 kD, their nuclear localization and the identity of the homologue to a gene of a recently submitted RanGTP binding protein (RanBP16), suggest that its protein is a novel member of the importin-β superfamily of nuclear transport receptors, therefore called RanBP17. Northern blot analysis of human tissues revealed a ubiquitous expression pattern of the RanBP16 gene and a very restricted expression pattern of the RanBP17 gene, showing high expression in testis and pancreas. Both genes are evolutionary conserved and show a high (99 and 94%) amino acid conservation with their murine counterparts and a striking similarity (40%) to a protein product of *Caenorhabditis elegans* (C35A5.8).

Key Words: **chromosomal breakpoint; leukemia; importin-β; RanGTP binding protein; t(5;14) (q34;q11); RNA expression; nuclear transport receptor; KIAA0745; RanBP16; RanBP17.**

In the present study we describe novel putative members of the importin-β superfamily of nuclear transport receptors. These genes were identified during an investigation of breakpoint sequences at the chromosomal translocation t(5;14) (q33-34;q11). This translocation is a recurring chromosomal aberration in a subset of acute leukemias (1).

In eukaryotic cells, cytoplasm and nucleoplasm are separated by the nuclear envelope (NE). Many proteins, some (RNA-protein complexes) RNPs and RNAs need to be transported between the two compartments in order to fulfill their normal cell functions. The exchange of macromolecules between the two compartments occurs through nuclear pore complexes (NPC) that are embedded in the nuclear membrane (for recent reviews, see 1–9). Transport between the cytoplasm and the nucleus is mainly mediated by members of the superfamily of importin β-related nuclear transport receptors also called karyopherins. The family of importin-β related nuclear transport receptors consists of proteins of similar molecular weight (90–130 kD) and isoelectric point (4.6–5.9) and most importantly a conserved region in their N-terminal part that mediates the binding of Ran (10, 11). The C-terminal regions of these proteins are frequently diverse and important for binding of cargo. In the last few years, many novel members of this superfamily of importin-β have been identified and some of them have been functionally characterized, like importin β, transportin, importin 5, importin 7, transportin SR, CRM1 (exportin-1), CAS, exportin-t and exportin 4 (reviewed in 9). Based on the direction in which these receptors carry their cargo, they are classified as importins or exportins. This directionality of nucleocytoplasmic transport is regulated by the energy state of Ran, a small ras-related GTPase, namely by the RanGDP and RanGTP concentrations. The asymmetric distribution of the two regulators of Ran, RanGTPase-activating protein (RanGAP1) and Ran nuclear guanine nucleotide exchange factor RanGEF (termed RCC1 in higher eukaryotes) in the cytoplasm and nucleus, respectively, ensures high nuclear and low cytoplasmic RanGTP

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concentration. Exportins bind to both their cargo and RanGTP, are transported into the cytoplasm where GTP is hydrolyzed and the complex dissociates. The exportin can then be reimported into the nucleus in an empty state. Importins bind cargo molecules initially in the cytoplasm, release them upon binding to RanGTP in the nucleus and return to the cytoplasm as RanGTP complexes without their cargo. The dissociation of importin-RanGTP complexes and exportin-cargo-RanGTP trimeric complexes in the cytoplasm is catalyzed by RanGAP that catalyzes the conversion of RanGTP to RanGDP. The activation of Ran's intrinsic GTPase activation by RanGAP is stimulated by RanBP1, a Ran binding protein (reviewed in 7–9, 12). Here we report the characterization of two putative Ran-binding proteins and their murine homologs, called *RanBP16* and *RanBP17*, respectively.

MATERIALS AND METHODS

DNA and RNA purification and analysis. High molecular weight DNA was prepared and analyzed by Southern blotting as described previously (13). Total RNA was isolated and purified by acid guanidinium isothiocyanate/phenol-chloroform extraction, as described (14). Northern blot analyses were performed as described by Shackleford and Varmus (15). Briefly, 10 μg of total RNA were resolved on 1.0% agarose formaldehyde gels, blotted onto Hybond-N membranes (Amersham Pharmacia Biotech, Freiburg, Germany), then hybridized as described for Southern blotting (Janssen *et al.*, 1991). Human and Murine Multiple Tissue Northern blots were obtained from Clontech (Clontech Laboratories, Heidelberg, Germany).

Molecular cloning. Human *ranBP16* and *17* cDNA clones were isolated from a Human Testis 5′-Stretch Plus cDNA library in the phage λgt11 (Clontech, Heidelberg, Germany), a Superscript Human Testis cDNA library in the plasmid pCMV-SPORT (Life Technologies, Karlsruhe, Germany) and from a Human Testis Large Insert cDNA library in the phage λTripIEx2 (Clontech, Heidelberg, Germany). The search for human *ranBP17* cDNA clones started with trapped exons from a cloned genomic region surrounding a t(5;14) translocation in all patients (Hansen-Hagge *et al.*, manuscript in preparation). The search for human *ranBP16* cDNA clones started with a PCR product that was obtained by amplifying human multiple tissue cDNAs (Human MTC Panels I and II, Clontech, Heidelberg, Germany) with the following primers (5′ ctctatggagacgatgccctg 3′ and 5′ gcctgctgcttctccggtg 3′). These primers were designed from a consensus sequence of *ranBP16* that was obtained by compiling various human ESTs (Accession Nos. H59369, H73639, N94067, AA062710, AA446810, and N76175) that showed approximately 65% homology to our incomplete *ranBP17* cDNA sequence. Murine *ranBP16* and *17* cDNA clones were isolated from a Mouse Testis 5′-Stretch cDNA library (Clontech, Heidelberg, Germany). Library plating, preparation of duplicate phage or bacteria filter lifts, hybridization and isolation of the positive clones were according to standard protocols or the manufacturer's instructions. Insert DNA of positive clones was cloned into the *Eco*RI site of the pT7T3 vector (Amersham Pharmacia Biotech, Freiburg, Germany).

GFP-*mRanBP17* was constructed by amplifying two *mRanBP17* overlapping cDNA fragments using four murine *RanBP17* specific primers (acgcgtcgacgcgctgcactttcagagtttg, gctcacatcttccccagtac, gcatgatgctatggatggag, cgcggatccacagcccacgctcaggagc, *Sal*I and *Bam*HI sites are underlined) of which the 5′ and 3′ primers contain a *Sal*I and *Bam*HI restriction site for cloning into the pEGFP-C1 GFP-expression vector (Clontech, Heidelberg, Germany), respectively. The two PCR products were cloned into a pUC57 T cloning vector (MBI Fermentas, St. Leon-Rot, Germany) and completely sequenced. Correctly amplified cDNA inserts were cut out of the T-cloning vector using the combination of restriction enzymes *Sal*I/*Nde*I and *Nde*I/*Bam*HI, respectively, purified and cloned into *Sal*I/*Bam*HI restricted pEGFP-C1. The final GFP-*mRanBP17* expression plasmid was checked by restriction enzyme analysis and by sequencing.

An eukaryotic expression vector containing a human *RanBP16* cDNA insert (PMT2-*hRanBP16*) was constructed by ligating three different cDNA fragments (~350 bp *Sal*I/*Eag*I 5′ fragment, ~1300 bp *Eag*I/*Bgl*II middle segment, and a 3′*Bgl*II/*Not*I KIAA0745 fragment) together into the *Sal*I/*Not*I restricted pMT2 expression vector.

A 3′ human *ranBP16* GST fusion construct was obtained by cloning a 468-bp PCR product of 3′ human *ranBP16* cDNA using primers (5′ cgggatccacaggctgctgctcctgcc 3′ and 5′ acgcgtcgacgcggccgcggatcc-gctctgtacaggtagagtcc 3′, the *Bam*HI sites are underlined) in the *Bam*HI cloning site of the GST vector pGEX-2T (Amersham Pharmacia Biotech, Freiburg, Germany).

DNA sequencing. For sequence analysis of PCR products or cDNA clones, DNA molecules were separated by agarose gel electrophoresis, purified using diethyl aminoethyl paper (Schleicher and Schuell; Dassel, Germany) or QIAEX II gel extraction kit (Qiagen, Hilden, Germany), and subcloned into pTZ19R or pT7T3 plasmid vectors (Amersham Pharmacia Biotech, Freiburg, Germany). Plasmid DNAs were sequenced either with a Thermosequenase fluorescent-labeled primer cycle sequencing kit with 7-deaza-dGTP (Amersham Pharmacia Biotech, Freiburg, Germany) on a LI-COR long Readir 4200 DNA sequencer (MWG-Biotech, Ebersberg, Germany) or with a T7 DNA polymerase sequencing kit with 7-deaza-dGTP or a dye terminator cycle sequencing kit on an ALF Express II (Amersham Pharmacia Biotech, Freiburg, Germany).

RNA in situ hybridization. Detailed protocols for RNA *in situ* hybridization have been described previously (16, 17). Briefly, whole embryos and adult organs were collected from B6 mice. The morning of plug detection was designated as day 0.5 p.c. The material or embryos were dissected in phosphate-buffered saline (PBS), fixed in 4% paraformaldehyde in PBS for 24 h, transferred to 0.5 M sucrose in PBS for 24 h, and frozen in liquid nitrogen. Tissues were cut into 10 μm sections at −20°C, collected on Super Frost slides (Menzel, Darmstadt, Germany), and stored at −70°C. A 942-bp murine *RanBP17* cDNA clone (position 1467–2409) inserted into the *Eco*RI site of pT7T318U (Amersham Pharmacia Biotech, Freiburg, Germany), was used as a probe. ^{35}S-labelled antisense RNA was synthesized using T7 RNA polymerase. The sense RNA control probe was synthesized using a clone with the opposite orientation. The probe length was reduced to 200 nucleotides by alkaline hydrolysis. Hybridization was performed in 50% formamide, 10 mM Tris, 10 mM sodium phosphate, pH 6.8, with 20 mM dithiothreitol, 0.2% Denhardt's solution, 10% dextran sulfate, 0.3 M NaCl, 0.1 mg/ml *Escherichia coli* RNA and 0.1 mM [α-^{35}S]UTP. Fifteen microliters of RNA labeled to 80,000 cpm/μl was added to the hybridization mix, applied to each section and incubated overnight in a humid chamber at 54°C. The slides were washed in hybridization salt solution with dithiothreitol at 54°C. After treatment with 30 μg/ml RNAse A, they were washed for 30 min in 2× and 0.1× SSC and dehydrated in increasing concentrations of ethanol. The slides were coated with a 1:1 (v/v) dilution of Ilford K5 emulsion in water and exposed for 3 weeks at 4°C. After development in Kodak D19b, slides were stained with Giemsa or haematoxylin/eosin. Photographs were taken under bright-field and dark-field illumination on a Zeiss CCD camera (Olympus DP 10).

Antibodies and immunofluorescence. RanBP16 polyclonal serum was obtained by immunizing rabbits with a carboxy-terminal peptide (MKNSTYGVNSNDMMS). Affinity purification was performed on a Sepharose-peptide column containing immobilized RanBP16 carboxy-terminal peptide.

HeLa cells were grown in DMEM (Life Technologies, Karlsruhe, Germany) supplemented with 10% FCS. HeLa cells were grown slides glasses in 4-well rectangular Quadriperm multidishes (Heraeus Instruments GmbH, Hanau, Germany) and transiently transfected with either the human *RanBP16* expression plasmid (pMT2-*hRanBP16*) or the GFP-murine *RanBP17* (GFP-*mRanBP17*) fusion plasmid using the transfection reagent Fugene 6 as per the manufacturer's instructions (Roche Biochemicals, Mannheim, Germany). Detection of the respective proteins followed approximately 48 h after transfection. For that purpose, adherent cells were washed twice with PBS (Life Technologies, Karlsruhe, Germany) in Coplin jars, drain off PBS, fixed in methanol/aceton (1:1) for 2 min at room temperature and rinsed in PBS twice. For nuclear staining slides containing GFP-*mRanBP17* transfected HeLa cells were directly incubated in 0.02 μg/ml 4′, 6-diamidino-2-phenylindole (DAPI) in PBS for 3 min, rinsed in deionized water, mounted in DABCO antifade medium (2.3% (w/v) 1,4-Diazabicyclo-[2.2.2]-octane/90% glycerol/20 mM Tris/HCl pH 8.0) and analyzed using conventional (Zeiss Axiophot, Oberkochen, Germany) or confocal (Leica Mikrosysteme, Bensheim, Germany) fluorescence microscopy. For pMT2-*hRanBP16*, cells were blocked in TBST (10 mM Tris/HCl pH 8.0, 150 mM NaCl, 0.2% Tween 20) containing 2% BSA for 60 min at room temperature. Primary antibodies were applied at 5 μg/ml in TBST/2% BSA for 1 h at room temperature. After washing with TBST three times for 10 min, the cells were incubated with FITC-conjugated goat anti-rabbit IgG (Sigma, Deisenhofen, Germany) at 10 μg/ml in TBST/2% BSA for 1 h at room temperature and washed four times with TBST for 10 min. Nuclear staining, mounting and observation were as described above.

Computer search and programs. Sequences were evaluated with the University of Wisconsin Genetics Computer Group (GCG) sequence analysis package (version 10.0-Unix) and with server facilities of the National Center for Biotechnology Information, National Library of Medicine, National Institutes of Health (Bethesda, MD; www.ncbi.nlm.nih.gov), as well as with the psort program of Kenta Nakai, Institute for Molecular and Cellular Biology, Osaka University (Osaka, Japan; http://psort.nibb.ac.jp/) and with the profileScan program of the Bioinformatics Group of Swiss Institute for Experimental Cancer Research (ISREC) (Epalinges, Switzerland; http://isrec.isb-sib.ch).

GenBank accession numbers. *Homo sapiens* mRNA for Ran binding protein 17 encoding the largest 1088 amino acid translation product, AJ271459; Mus musculus mRNA for Ran binding protein 17, AJ271458; Mus musculus mRNA for Ran binding protein 16, AJ297360,

RESULTS AND DISCUSSION

Isolation, Sequence, and Predicted Function of the RanBP16 and RanBP17 Genes

In the course of cloning the breakpoint sequences from a leukemia patient harboring a chromosomal translocation t(5;14) (q33-q34;q11), we recently identified the discovery of a pseudogenic variant of a novel deubiquitinating enzyme, *UBH1* (18). This variant is highly homologous, but not identical to *UBH1,* contains several premature termination codons and an insertion of an additional 63 bases at position 932, and therefore was omitted as a candidate gene for the t(5;14) chromosomal rearrangement. This prompted us to search for other gene-specific exons in this breakpoint region and enabled us to isolate the complete cDNA sequence of another novel gene located at the vicinity of the breakpoint, called *RanBP17.* Its exact location with respect to the breakpoint and its possible contribution to the development of t(5;14) positive all will be published elsewhere (Hansen-Hagge *et al.*, in preparation).

During the characterization of various *RanBP17* cDNAs, a comparison to the DNA GenBank sequence database showed homology to several ESTs located mainly in the 3′ end of the mRNA. In addition, we observed a ~65% homology to another set of ESTs, suggesting the presence of a distantly related human homologue. Using the latter EST sequence information we were able to asssemble a small contig, design primers from this contig and amplify a cDNA fragment of the RanBP17 homologue using cDNA of multiple human tissues, which was used as starting probe for the isolation of cDNA clones. After having finished most of the cDNA cloning and sequencing of this homologue, a comparison to the DNA GenBank sequence database revealed homology to the KIAA0745 sequence (Accession No. AB018288) submitted by the Kazusa DNA Research Institute (19). Our sequence showed 100% identity to the KIAA0745 sequence except for an additional 540-bp insert at position ~800 in the KIAA sequence and a few more bases at the 5′ end that contain the missing ATG codon in the KIAA0745 sequence (http://zearth.kazusa.or.jp). Using human-rodent hybrid panels the KIAA0745 gene has been mapped to chromsome 8p11-12. After having completed the cloning and sequencing of the RanBP17 homologue, a computer search in the beginning of 2000 revealed identity to the human RAN binding protein 16 mRNA, *RanBP16* (Accession No. AF064729) that also included the previously mentioned 540-bp insert.

With the aid of partial human *RanBP16* and *RanBP17* cDNA sequences and clones we simultaneously cloned the murine homologs of the two genes. Figure 1 depicts the sequence conservation between the human and murine RanBP16 and RanBP17 amino acid sequences and the predicted product of the *C. elegans* C35A5.8 gene. The human RanBP16 and RanBP17 proteins as well as their murine homologues show 66.8 and 66.0% identity to each other. The amino acid sequence conservation between human and murine RanBP16 and RanBP17 is high, 99.4 and 93.8%, respectively. Both human RanBP16 and RanBP17 proteins show homology to the predicted product of the *C. elegans* C35A5.8 gene, 40.3 and 39.1% identity, respectively. The Kazusa Institute could not find any motifs, profiles, or pfam domains in their KIAA0745 protein, but did find a transmembrane segment from amino acid 312 to 333 in the predicted KIAA0745 protein. However our

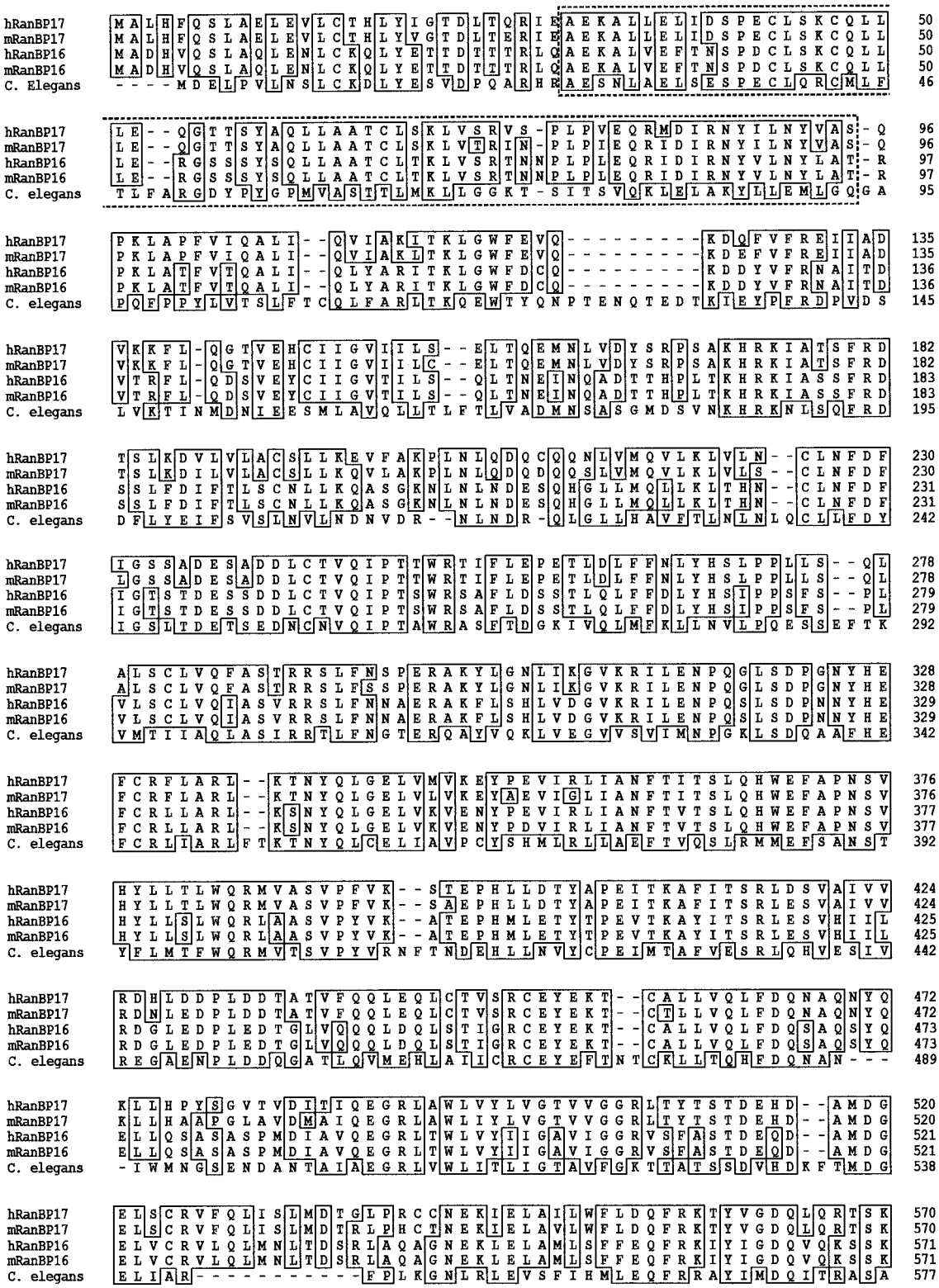

FIG. 1. Sequence conservation between the predicted amino acid sequence of human RanBP17, murine RanBP17, human RanBP16, murine RanBP16, and the predicted product of the *C. elegans* C35A5.8 gene. Sequence identities between the gene products are marked by boxes. The functional relevant importin-β N-terminal domain important for the binding of Ran protein is marked by a hatched box.

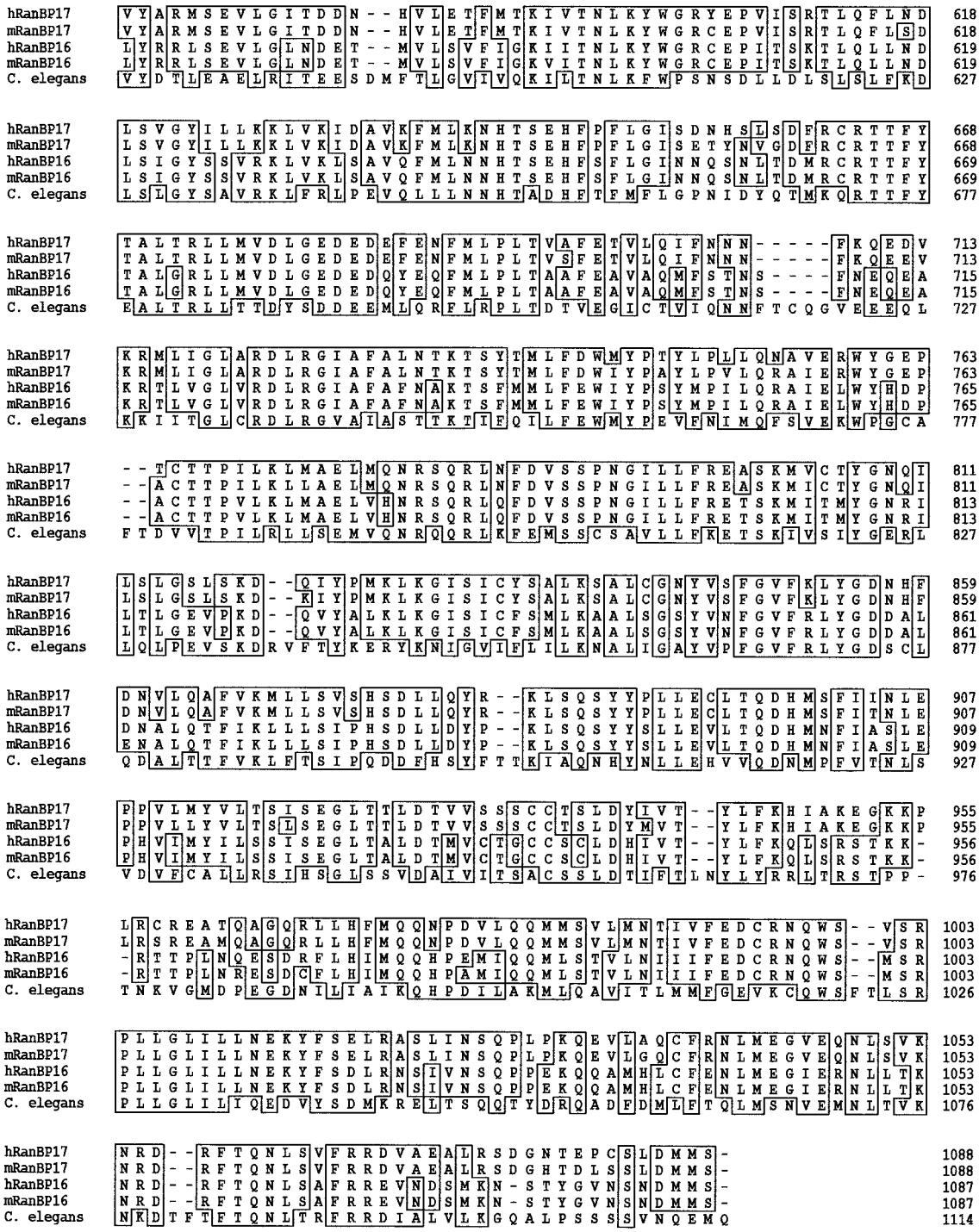

FIG. 1—*Continued*

latest computer profileScan search at ISREC revealed the presence of an Importin-β N-terminal domain in the N-terminal end of the RanBP16 and 17 proteins. This conserved region in the N-terminal part of Importin-β proteins is important for the binding of Ran protein (20). This finding and the size of approximately 110 kD of the two proteins strongly suggest that both proteins belong to the importin-β superfamily, components of the nuclear transport machinery (8, 9, 12).

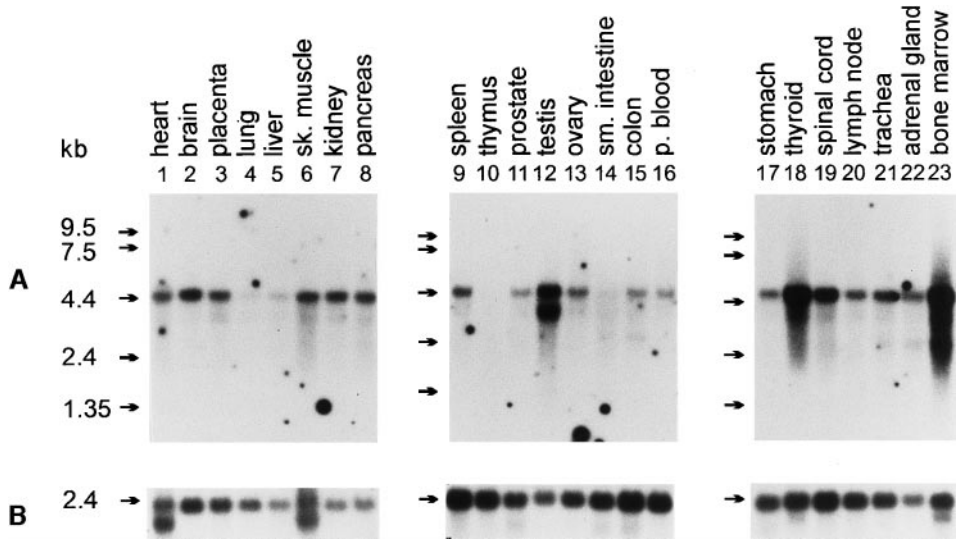

FIG. 2. Characterization of *ranBP16* transcripts in multiple human tissues by Northern blot analysis. Two micrograms of poly A^+ RNA from the indicated tissues were analyzed by Northern blot analyses. (A) Hybridization using a human 538-bp *ranBP16* probe. Molecular size markers are indicated by arrows at the left edges. (B) Control hybridization using a murine β-actin probe that cross-hybridizes with the human gene transcripts.

Expression Pattern of the Human RanBP16 and RanBP17 Genes

Northern blot analysis from different human tissues with the *RanBP16* gene showed a ubiquitous expression pattern (Fig. 2). A main transcript of ~4.8 kb was strongly expressed in testis, thyroid, and bone marrow, while many other tissues show a moderate *RanBP16* expression and some weak (lung, liver, and small intestine) or no expression (thymus). Our expression data do not resemble the expression profile of the KIAA0745 gene reported by the Kazusa Institute that report a very high expression in brain, a moderate expression in heart, lung, and ovary and low expression in liver, skeletal muscle, pancreas, testis, and spleen. The expression profile of the KIAA0745 gene was determined by a semiquantitative RT-PCR by enzyme-linked immunosorbent assay (RT-PCR ELISA). As discussed by the Kazusa Institute this RT-PCR ELISA method has a chance to include significant run-to-run variations which most likely explains the observed discrepancy in expression profiles. Two other transcripts with sizes of ~3.5 or 2.5 kb were strongly

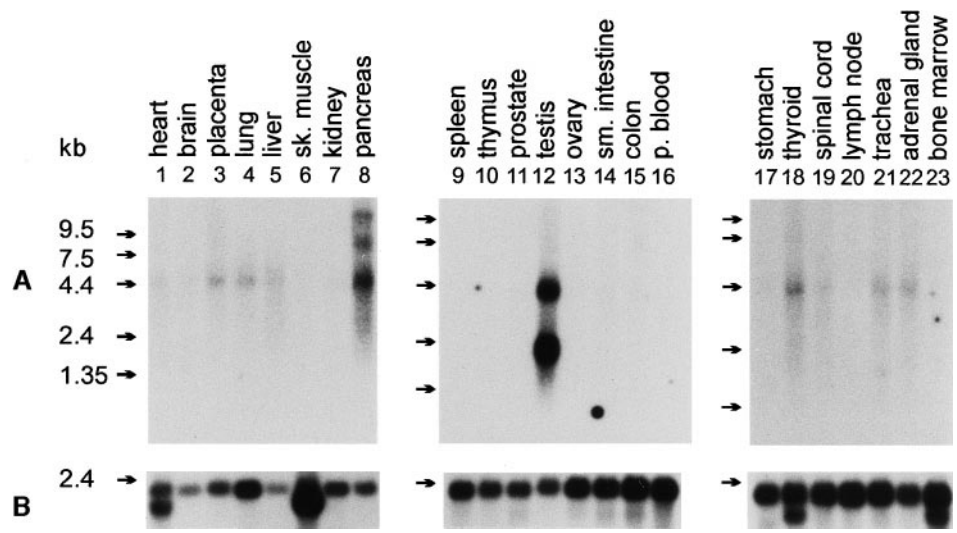

FIG. 3. Northern blot analysis of *ranBP17* in human tissues. Two micrograms of poly A^+ RNA from the indicated tissues were analyzed by Northern blot analyses. (A) Hybridization using a human 3′ 521-bp *ranBP17* probe (position 2439 to 2960). (B) Control hybridization using a murine β-actin probe.

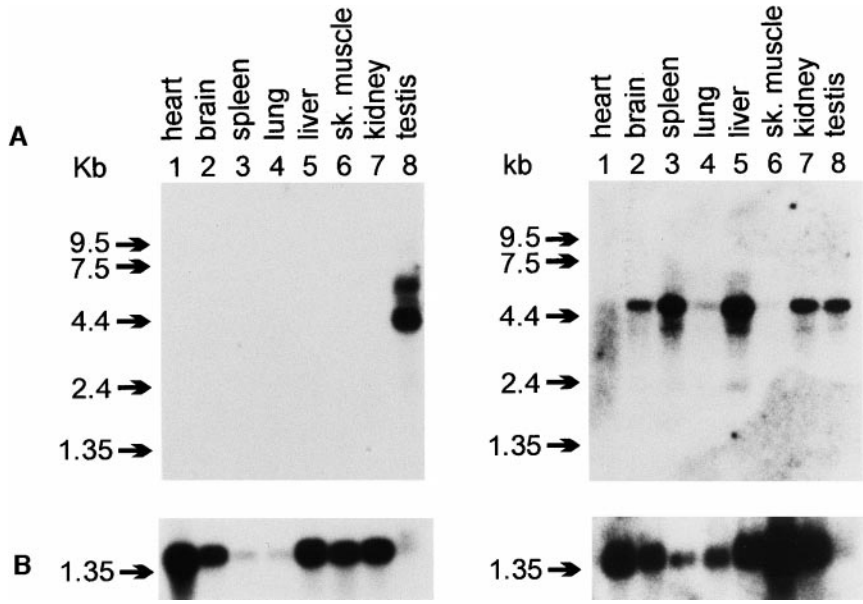

FIG. 4. Characterization of *ranBP16* and *17* transcripts in multiple murine tissues by Northern blot analysis. Two micrograms of poly A^+ RNA from the indicated tissues were analyzed by Northern blot analyses. (A) Hybridizations using a 940-bp murine *ranBP16* (position 1140 to 2080) probe (right side) and a 513-bp murine *ranBP17* (position 2448 to 2961) probe (left side). Molecular size markers are indicated by arrows at the left edges. (B) Control hybridizations using a rat glyceraldehyde 3-phosphate dehydrogenase (G3PDH) probe that cross-hybridizes with the murine gene transcripts.

expressed in bone marrow (both transcripts) and testis (3.5-kb transcript) and very weakly expressed in various other tissues. The size of the largest transcript corresponds with the size of the *RanBP16* cDNA sequence of approximately 4.8 kb.

The human *RanBP17* probe showed a remarkably restricted expression pattern. Two different transcripts with sizes of ~2.5, 4.5 kb were strongly expressed in human testis, whereas transcripts with sizes of ~4.5, 7.5, and 10 kb were moderately expressed in pancreas and weakly expressed in heart, placenta, lung liver, thyroid, spinal cord, trachea, and adrenal gland (Fig. 3). The size of the 4.5-kb transcript corresponds well with the size of the largest continuous cDNA sequence of 4463 bp isolated from a testis library that was submitted to the GenBank database.

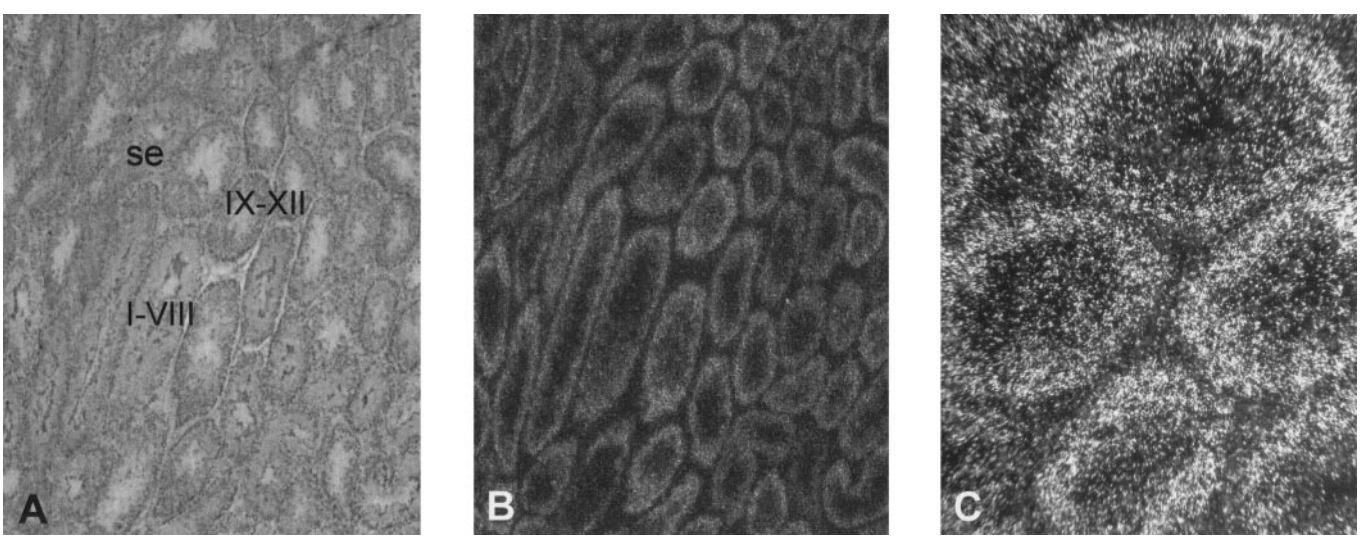

FIG. 5. RNA *in situ* hybridization with a 942-bp murine *ranBP17* probe (position 1467–2409). Giemsa staining (A) and dark field (B, C) illumination of a sagittal section of the testis of a 6-month-old male mouse. (C) Higher magnification. Se, seminiferous tubules; I–VIII, seminiferous tubules stadium I–VIII; IX–XII, seminiferous tubules stadium IX–XII.

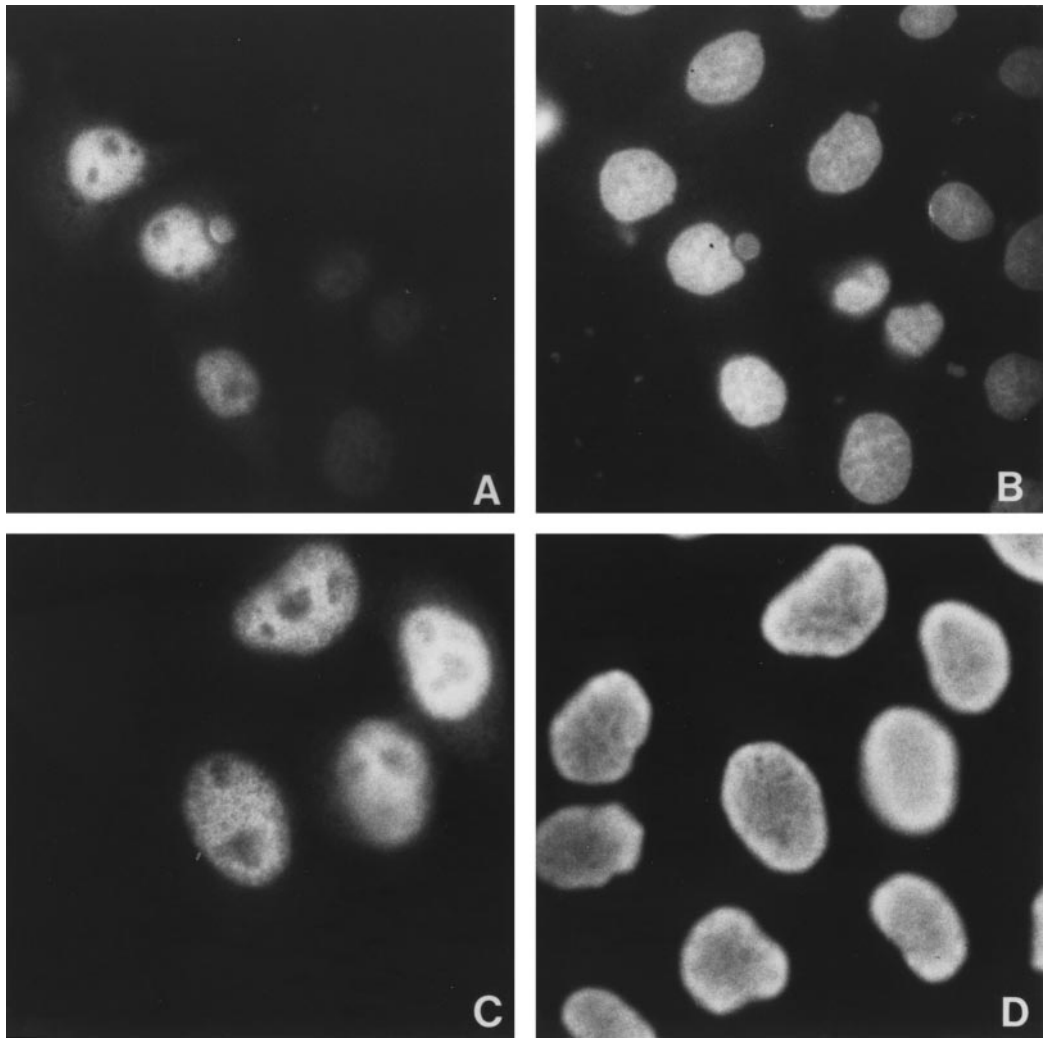

FIG. 6. Nuclear localization of ranbp17 and ranBP16. Detection of murine GFP-ranBP17 in HeLa cells approximately 48 h after transfection (A, magnification 630×) and a control nuclear DAPI-staining of the cells (B). Detection of human ranBP16 in HeLa cells about 48 h after transfection by immunostaining with affinity purified anti-ranBP16 antibody in conjunction with a FITC-conjugated secondary antibody (C, magnification 1000×) and its control nuclear DAPI-staining (D).

Northern Blot Analysis of the Murine RanBP16 and RanBP17 Genes

Figure 4 depicts the Northern blot analysis of polyA$^+$ RNA isolated from different murine tissues with murine *RanBP16* and *RanBP17* probes. As expected the murine *RanBP16* probe showed a rather ubiquitous expression in various murine tissues (Fig. 4, right panel). The size of the observed transcript (~4.7 kb) coincides with the size of the largest cDNA sequence of 4436 bp.

In accordance with the hybridization of human *RanBP17,* its murine homologue also shows a very restricted expression pattern with a marked signal in testis (Fig. 4, left panel). The sizes of the two different cDNAs isolated, sequenced, and submitted to the GenBank database (3458 and 4291 bp) correspond to two of the three transcripts with a size of ~4.0, 4.8, and 5.5 kb observed by Northern blot analysis, considering a poly A-tail of approximately 300 bp. Hybridization analysis of a 3′ probe with murine testis RNA revealed hybridization with the two largest murine *RanBP17* transcripts as expected (data not shown). This finding suggests that the largest transcript of ~5.5 kb is an 3′-extended form of the two smaller transcripts.

RNA in Situ Hybridization of Murine RanBP17

The murine *RanBP17* cDNA clone was used to investigate its expression by *in situ* hybridization of whole body sections. Strong expression was observed in testis and a very weak expression in pancreas (data not

shown). A sagittal section of testis of an adult mouse shows stronger *RanBP17* expression in stages IX–XII than in stages I–VIII, characteristic for an expression in primary spermatocytes (Figs. 5B and 5C). Such an expression pattern in testis is observed with many other genes and its possible function in testis development remains speculative.

Intracell Localization of the RanBP16 and RanBP17 Proteins

To determine the intracellular localization of the RanBP17 protein we constructed a fusion gene between the gene for the green fluorescent protein (GFP) in frame with the murine *RanBP17* gene (*GFP-mRanBP17*). Transient expression of the construct in HeLa cells shows a faint cytoplasmic staining and a strong nuclear signal (Fig. 6A). The intranuclear staining showed a somewhat speckeled distribution, with the signal in the nucleoli being less intense. A similar intracellular distribution can be observed by transient transfection of a human *RanBP16* expression plasmid and detection by indirect immunofluorescence using a peptide specific antibody (Fig. 6C). A comparable distribution using similar technical conditions were also reported for the localization of Exportin-t, another member of the importin-β superfamily of transport receptors (21).

In conclusion our data describe the characterization of a novel gene, *RanBP17*, located at the breakpoint of t(5;14) (q34;q11), its close human homologue, *RanBP16*, located at 8p11-12 and their respective murine counterparts. Bothe genes encode for two novel putative members of the importin-β superfamily of nuclear transport receptors

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