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A Double RING-H2 Domain in *RNF32,* a Gene Expressed during Sperm Formation

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The RING domain is a cysteine-rich zinc-binding motif, which is found in a wide variety of proteins, among which are several proto-oncogenes and the gene implicated in autosomal recessive juvenile parkinsonism, *Parkin*. The domain mediates binding to other proteins, either via their RING domains or other motifs. In several proteins, RING domains are found in combination with other cysteine-rich binding motifs and some proteins contain two RING domains. Recent evidence suggests that RING finger proteins function in the ubiquitin pathway as E3 ligases. A variant of the RING domain is the RING-H2 domain, in which one of the cysteines is replaced by a histidine. We have cloned and characterized a novel gene, *RNF32,* located on chromosome 7q36. *RNF32* is contained in 37 kb of genomic DNA and consists of 9 constitutive and 8 alternatively spliced exons, most of which are alternative first exons. A long and a short transcript of the gene are expressed; the short transcript containing exons 1–4 only. This gene encodes two RING-H2 domains separated by an IQ domain of unknown function. This is the first reported gene with a double RING-H2 domain. In humans, *RNF32* overlaps with a processed retroposon located on the opposite strand, *C7orf13*. RNF32 is specifically expressed in testis and ovary, whereas *C7orf13* is testis-specific, suggesting that its expression may be regulated by elements in the *RNF32* promoter region. *RNF32* is expressed during spermatogenesis, most likely in spermatocytes and/or in spermatids, suggesting a possible role in sperm formation.

Key Words: **RING-H2 domain; IQ domain; RNF32; testis; processed retroposon; C7orf13.**

RING finger domains (for Really Interesting New Gene) have generated a lot of interest in recent years, because of their widespread occurrence and their implication in human disease. The structure of this domain resembles that of the classical zinc finger, but instead of four cysteines, it contains seven cysteines (C1–C7) and one histidine (H1) (see Fig. 1) (1). This motif adopts a 'cross brace' structure in which one zinc atom is bound to C1, C2, C4 and C5 and another to C3, H1, C6 and C7 (2, 3). A subclass of the RING finger family is the RING-H2 domain, in which the fourth cysteine of the motif is replaced by a histidine residue (Fig. 1) (4). The domains have been found in animal, plant and virus proteins, proteins located in both the nucleus and the cytoplasm.

Several RING containing genes are involved in the ubiquitin proteasome pathway as E3 ligases, the proteins that specify targets for ubiquitination. One of these is the CBL protein, which is necessary for desensitization of the Epidermal Growth Factor (EGF) receptor. Mutations in the RING domain of CBL abolish ubiquitination of the EGF receptor (5, 6). These and other findings have led to the proposal that all RING containing proteins function in ubiquitination (7). However, not every reported RING protein contains a true RING domain; the 'RING' domains of two E3 ligases, RBX1 and APC2, do not follow the consensus C3HC4: RBX1 contains a cysteine-rich domain that can be described as C2HC3H2C2H, and the many cysteines and histidines in its homolog APC2 are even less conserved. It is unclear if these domains function in the same way as true RING domains.

RING proteins are often found in large protein complexes, and they are difficult to analyze because they tend to aggregate. This has led to the suggestion that they are involved in the formation of macromolecular complexes (8). RING domains can bind to other RING domains and thereby result in homo- or heterodimerization, or they can associate with non-RING domains. For example, the RING of BRCA1 interacts with the

The sequence data from this article have been deposited with the EMBL/GenBank Data Libraries under Accession Nos. AF43997, AF441222, AF441223, AF441224 and AF441225.

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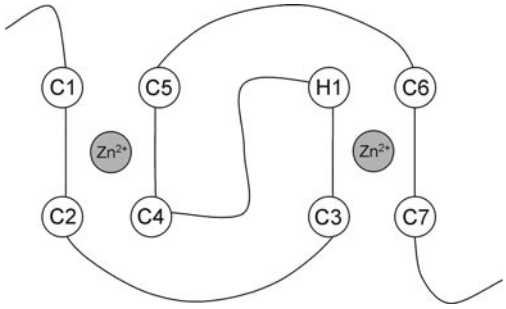

FIG. 1. Schematic representation of the RING domain. Two zinc ions are bound by four ligands each: Four cysteines bind the first zinc ion, three cysteines and one histidine bind the second zinc ion. In the RING-H2 domain, the fourth cysteine of the domain is replaced by a histidine.

BARD1 (BRCA1-associated RING domain 1) RING finger protein and with the BAP1 (BRCA1 associated protein-1) protein, which does not contain RING domains (9, 10).

Mutations in the RING domain of BRCA1 have been found in breast cancer patients (11, 12). Several other human proto-oncogenes contain RING domains, for example human c-Cbl becomes oncogenic after deletion or disruption of its RING finger (13, 14). The domain is also found in the promyelocytic leukemia protein PML and the transcriptional intermediary factor TIF1, which become oncogenic after chromosomal translocation resulting in their fusion to other proteins (15, 16). Not all RING-containing disease genes are proto-oncogenes: for example, mutations in the RING domains of Parkin result in autosomal recessive juvenile parkinsonism [MIM 600116] (17). Parkin also functions as an E3 ligase, although its target is unknown (18).

Parkin contains two RING domains separated by another cysteine rich motif, the IBR (for In Between Ring domains) (19). This motif is also found in the mouse Rbck1 (for RBCC protein interacting with Pkc1) protein and in the *Drosophila melanogaster* ariadne protein. The function of the IBR domain is unknown, but the integrity of the motif is necessary for Parkin function (19). Proteins with more than one RING domain all contain the IBR box. No proteins with a double RING-H2 domain have been reported.

We report the cloning and characterization of a novel human gene, *RNF32*. This gene is mainly expressed in testis. The putative protein of *RNF32* contains a novel motif: two RING-H2 domains separated by an IQ domain.

MATERIALS AND METHODS

Databases and software. PAC clones AC005534, AC007075, AC007097 and AC006357 were used for gene prediction, which was performed using the Nix analysis software of HGMP (http://www.hgmp.mrc.ac.uk/). This program uses the prediction programs GRAIL, Fex, Hexon, MZEF, Genemark, Genefinder and Fgene. Predicted genes were aligned with ESTs found in the region using the Vector NTI program package, which was also used for *in silico* translation of the transcripts. Putative proteins were screened for domains using Pfam, accessible through ISREC (http://www.isrec.ch/). Sequences homologous to the putative proteins were found using the BLAST program available at NCBI (http://www.ncbi.nlm.nih.gov/).

ESTs assigned to *RNF32* are AA968469, AA758018, AA412403, AI024391, AA904554, AA412402 AA581432, N51460, N34954, R60655, N48673, AA626330, N53600, R60654, N44021, AI190316, AI472192 and AI217423 (UniGene cluster Hs.26791).

C7orf13 ESTs are AA631112, AI693868, AA868311, AI492920, AI168080, AI139964, AA912620, AI468759, AA378476, AA826489, AI971242, AI827946 and AW104902 (UniGene cluster Hs.124854).

cDNA library screening. The cDNA libraries screened were derived from Human Testis (Clontech, HL1010b) and Mouse Developing Limb, kindly provided by J. P. Evans. For each library, 1×10^6 plaques were plated, transferred to nylon filters and hybridized with the probes of interest using standard procedures (34).

Sequencing. Sequence data were obtained using the Ready Reaction Dye Terminator Cycle sequencing kit (Perkin–Elmer) on an ABI 377 automatic sequencer. Sequences were aligned and analyzed using the DNASTAR program package.

RNF32 construct and transfection. A PCR product containing the ORF of *RNF32* was generated with primers that contained a *Bam*H1 recognition sequence (ringbamF: TGAAACCGCGGATCCAAATAAGGGTCACTCATCTAAGAAAGA and ringbamR: TGAAACCGCGGATCCATTCAACATTCAAGAATCTTCTTCTG). The product was cloned into the pCRII vector using the TA cloning kit of Invitrogen, digested with *Bam*H1 and cloned into the pcDNA3.1HisB vector (Invitrogen).

COS cells were cultured in Dulbecco's minimal Eagle medium (DMEM), and CHO cells were grown in α-MEM (Gibco BRL). Media were supplemented with 10% (v/v) fetal calf serum (Clontech), 100 IU/ml penicillin and 100 μg/ml streptomycin (both from Gibco BRL) and kept at 37°C in 5% CO_2.

Cells were transiently transfected with the *RNF32* construct using Lipofectamine reagent (Gibco BRL) according to the manufacturer's instructions.

Immunofluorescence. Cells were rinsed twice in cold phosphate-buffered saline (PBS), fixed and permeabilized in 100% methanol, 15 min at −20°C. The fixed cells were then incubated with mouse monoclonal antibodies against the Xpress epitope (1:100 dilution) (Invitrogen) for 1 h at room temperature and, after washing in PBS with 0.5% bovine serum albumin and 0.15% glycine, incubated with secondary fluorescein-conjugated rabbit anti-mouse antibody (1:100) (Sigma). Cells were mounted in Vectashield mounting media (Vector) and examined with a Leica DMXRA fluorescence microscope equipped with a digital camera.

Northern blots. Human multiple tissue Northern blots MTN-I and MTN-II and Mouse Embryo (Clontech) were hybridized with α-^{32}P-labeled probes and washed following manufacturer's instructions. The blots contained total RNA from the following tissues: MTN-I: heart, brain, lung, liver, muscle, kidney and pancreas; MTN-2: spleen, thymus, prostate, testis, ovary, small intestine, colon and peripheral blood leukocyte; Mouse Embryo: Total RNA from 7, 11, 15 and 17 dpc embryo.

For the mouse testes blot, mouse testes (C57/Bl6) were isolated at different days after birth (7, 14, 21, and 36 days) and RNA was extracted using TRIzol reagent (Life Technologies). For the testis fractions blot, spermatocytes, round spermatids, and elongated spermatids/cytoplasmic fractions were isolated from mouse testes (C57Bl/6) after collagenase and trypsin treatment, followed by sedimentation at unit gravity (StaPut procedure) (35). RNA was isolated by the LiCl/urea method (34). The amount of RNA was measured by

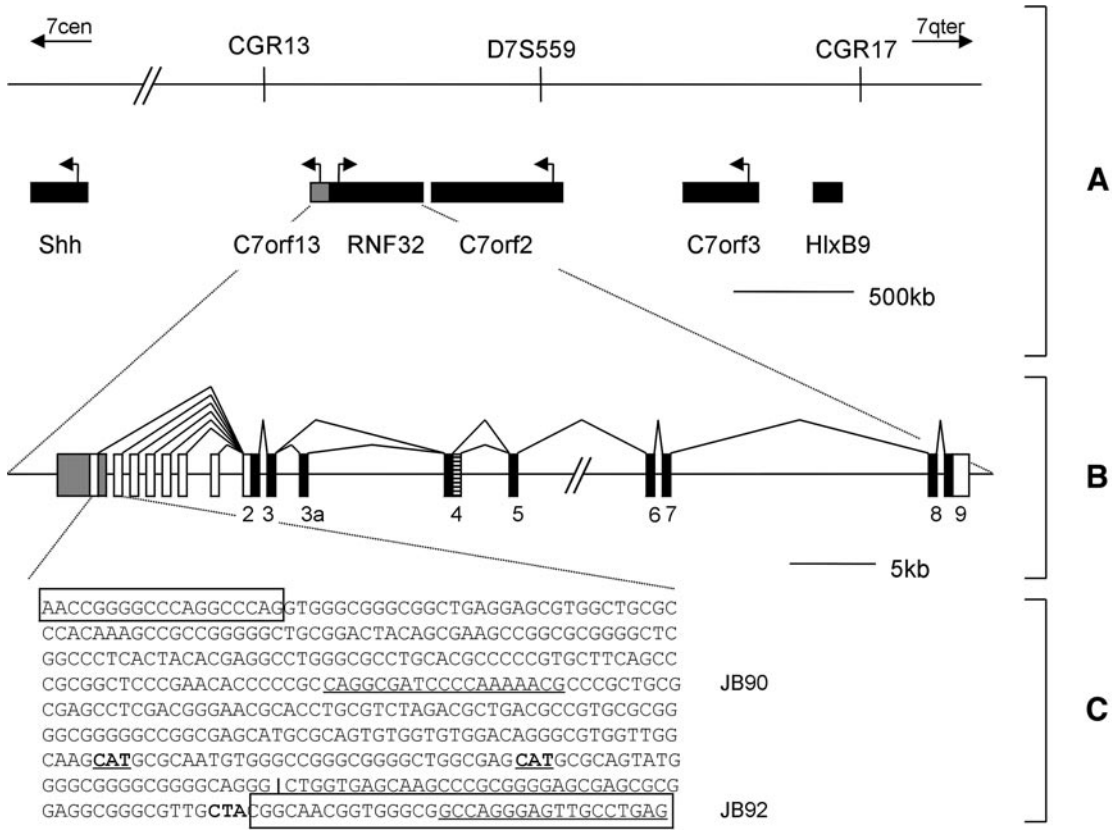

FIG. 2. Schematic representation of *RNF32* and *C7orf13*. (A) Localization of the genes on chromosome 7q36 with respect to other genes reported in this region [Heus, 1999, #57]. Orientation of genes is indicated by arrows. The orientation of HlxB9 is unknown. *C7orf13* overlaps one of the alternative exons 1 of *RNF32* and is indicated by a gray box. (B) Genomic organizaton of *RNF32* and *C7orf13*. The longer version of *RNF32* exon 4 is indicated by a hatched box; coding exons are in black, noncoding exons in white. For reasons of clarity, distances between alternative exons 1 are not drawn to scale; in reality they lie close together. *C7orf13*, in gray, overlaps the first alternative exon 1 of *RNF32* and is located on the opposite strand. (C) Sequence around the 5′ end of *C7orf13*. *RNF32* exons are boxed and primer sequences are underlined. The sequence between the two primers is shown with the in-frame methionine codons on the opposite strand in bold and underlined, and the preceding in-frame stop codon in bold. The putative splice site is indicated by a vertical line.

UV spectrometry and 20 μg of total RNA was applied to each lane on 1% agarose gels; the separated RNA was then transferred to Hybond-N^+ membranes and hybridized with the labeled probe.

Probes were prepared by PCR on cDNA (see below) and genomic DNA. Remaining template was removed by the Concert Rapid Gel Extraction System (Gibco BRL), running the PCR products on a 0.5% TAE gel, cutting out the bands and cleaning the DNA with glass beads (Bio101). *RNF32* probes were: an exon 3 probe (primers 3F: GTTGTATGTGCCATCAAGG and 3R: GCCCTAATAAGCCAAAAGATGA) and an exon 5–8 probe (primers 5F: CTTGTGCTGTGCTAAACAAACA and 8R: GCACTGATCGATTTCTGCAA). Probes from *Rnf32* and *C7orf13* were produced with T3 and T7 primers on IMAGE clones 515614 and 1158395 as templates, respectively.

cDNA production and amplification reactions. RNA was isolated from human lymphocytes with RNAzol (Campro Scientific) and 5 μg of RNA was used for reverse transcription with Superscript II RT (Gibco BRL) following manufacturer's instructions.

PCR amplifications were performed in a total volume of 50 μl containing 50 ng genomic DNA or 2 μl cDNA, 20 mM Tris HCl, pH 8.4, 1.5 mM $MgCl_2$, 50 mM KCl, 200 μM dNTPs, 0.5 U *Taq* polymerase (Gibco BRL) and 10 pmol forward and reverse primers. Cycling conditions were 5 min at 94°C, 35 cycles of 30 s at 94°C, 30 s at T_{an} (4× G/C + 2× A/T°C), 90 s at 72°C and a final step of 5 min at 72°C.

For confirmation of exon 3a, primers 3aF (GCCCTAATAAGCCAAAAGATGA) and 3aR (CTGGAACCTCTGGATGTTG) were used. Primer sets designed on mouse Rnf32 were used to identify a rat RNF32 homolog. These primers were 4F (TGCTTGGATTTTCTCCCAGT) and 8R (GCAGTTGCCTTACAGGACCA) in mouse exons 4 and 8, respectively.

For 5′ RACE PCR, the Marathon-Ready kit of Clontech was used following manufacturer's instructions. The probe specific primer for *C7orf13* was ACGGGAACGCACCTGCGTCTAGA, and the cDNA template was derived from Human Fetal Brain.

RESULTS AND DISCUSSION

In a screen for genes localized around marker *D7S559* on chromosome 7q36, we used exon trapping and cDNA selection (20). We extended this search by analysis of approximately 400 kb of genomic sequence, using several gene prediction programs in combinations with dbEST (see Materials and Methods). Here we describe two novel genes, *RNF32* and *C7orf13*. The genes are localized on opposite strands and overlap partially (Fig. 2).

Genomic Organization of RNF32

RNF32 (AF441222, AF441223, AF441224 and AF441225) comprises 37 kb of genomic DNA between markers *D7S168* and *D7S559* and consists of 9 consistent and 8 alternatively spliced exons (see below) (Fig. 2). Of the alternative exons, 7 are alternative first exons and the other is an exon that is sometimes found between exons 3 and 4, hence named exon 3a. All splice sites match the consensus (5′ AG/N, 3′ N/GT) with a polymorphism in exon 8; in 4 out of 42 alleles the less frequently used N/GC was found (21). A polyadenylation signal (AATAAA) was found 1372 nt into exon 9. The size of the putative cDNA is between 1658 and 1844 bp, with a 1086 nt open reading frame (ORF), starting in exon 2. The sequence around the putative start site, GGCATGT, contains the important purine in the −3 position, but does not otherwise fit the Kozak sequence (22).

Alternative Splices of RNF32

Based on the predicted sequence, we designed primer sets in exons 3 and 8 and performed PCR on cDNA derived from human lymphocytes. With the resulting PCR products we screened a human testis cDNA library and selected 19 clones that hybridized with both probes. These were sequenced and seven alternative first exons were found, all of which could be aligned to the genomic sequence. In addition, several Expressed Sequence Tags (ESTs) aligned with the predicted *RNF32* (see Materials and Methods). In two of the ESTs (aa 968469 and aa 758018) and one of our own clones, we found an additional exon 3a of 107 bp (AF441224). The two ESTs end after exon 4, including 219 and 250 nucleotides after the usual splice site and both contain a polyadenylation signal (AATAAA) 196 bp after the splice site. The clone contains all the normal RNF32 exons, including one of the alternative exons 1. To confirm that exon 3a is transcribed, we performed PCR on cDNA with primers in exons 3 and 3a and found a product of the correct size (data not shown).

Two other clones from the cDNA library (neither containing exon 3a) contain an additional 17 bp after exon 4, where an alternative splice site is located (AF441225). These clones do not contain a polyadenylation signal in exon 4 and they contain the other RNF32 exons.

Structure of the RNF32 Protein

The predicted protein is 362 aa in size and contains two RING-H2 domains separated by an IQ domain (Fig. 3A). Both RING domains follow the consensus $CX_2CX_{9-39}CX_{1-3}HX_{2-3}HX_2CX_{4-48}CX_2C$ (23). This is the first report of a gene that encodes a double RING-H2 protein. It is not known what the functional difference between the RING and RING-H2 domains is, but since RING containing proteins are important in protein-protein interactions, it is possible that a protein with a double RING domain acts as a scaffold for binding several proteins that function in the same pathway. This would bring these proteins close together, allowing them to act cooperatively.

The IQ domain (consensus: IQX_3RGX_3R) most resembles one of five IQ domains in mouse myosin heavy chain dilute, but in contrast to unconventional myosins such as this one, RNF32 contains only one IQ domain. Single IQ domains are also found in neuromodulin and neurogranin and, as in unconventional myosins, in these proteins the domain binds calmodulin (24). The motif has been found in other proteins that interact with calmodulin, but also in many other proteins that do not. Some of the non-calmodulin interacting proteins are GTP regulatory and cell cycle proteins, others are receptors or channel proteins (25). It is not known what function, if any, the IQ domain has in these non-calmodulin interacting proteins. It remains to be determined whether the IQ domain in RNF32 binds calmodulin, and what the functional importance of this domain may be.

The exon 1 splice variants of *RNF32* do not alter the predicted ORF, since the start codon is located in exon 2. However, exon 3a contains a stop codon, leading to truncation of the protein before the first RING domain. In the transcript that ends with the extended exon 4, a stop codon is present 16 bp after the 3′ splice site used in the normal exon 4, truncating the protein within the first RING-H2 domain This leaves an ORF of 145 aa (AF441223). The transcripts that use the alternative splice site after exon 4 result in a frameshift, which results in a stop codon in exon 7 and a 210 aa ORF. The frameshift occurs in the first RING-H2 domain.

RNF32 Homologs

The mouse ortholog of *RNF32* is *Lmbr2* (26), which is now renamed *Rnf32*. Alignment of the two predicted protein sequences is shown in Fig. 3B. The mouse protein is 76.6% identical to the human protein, the DNA sequence from the ORFs is 79.3% identical. No significant homology was found in the untranslated regions (UTRs). Using mouse primer sets on cDNA from rat brain and kidney tumours, we identified part of rat Rnf32 (data not shown).

A database search for sequences homologous to the RNF32 sequence revealed only one other sequence with a predicted double RING-H2/IQ structure. This sequence is an incomplete, single sequence read from *Giardia intestinalis*, a parasite in the human intestine (AC037512). Translation of this sequence predicts two RING-H2 domains separated by a single IQ domain, the same conformation as found in RNF32. There is no

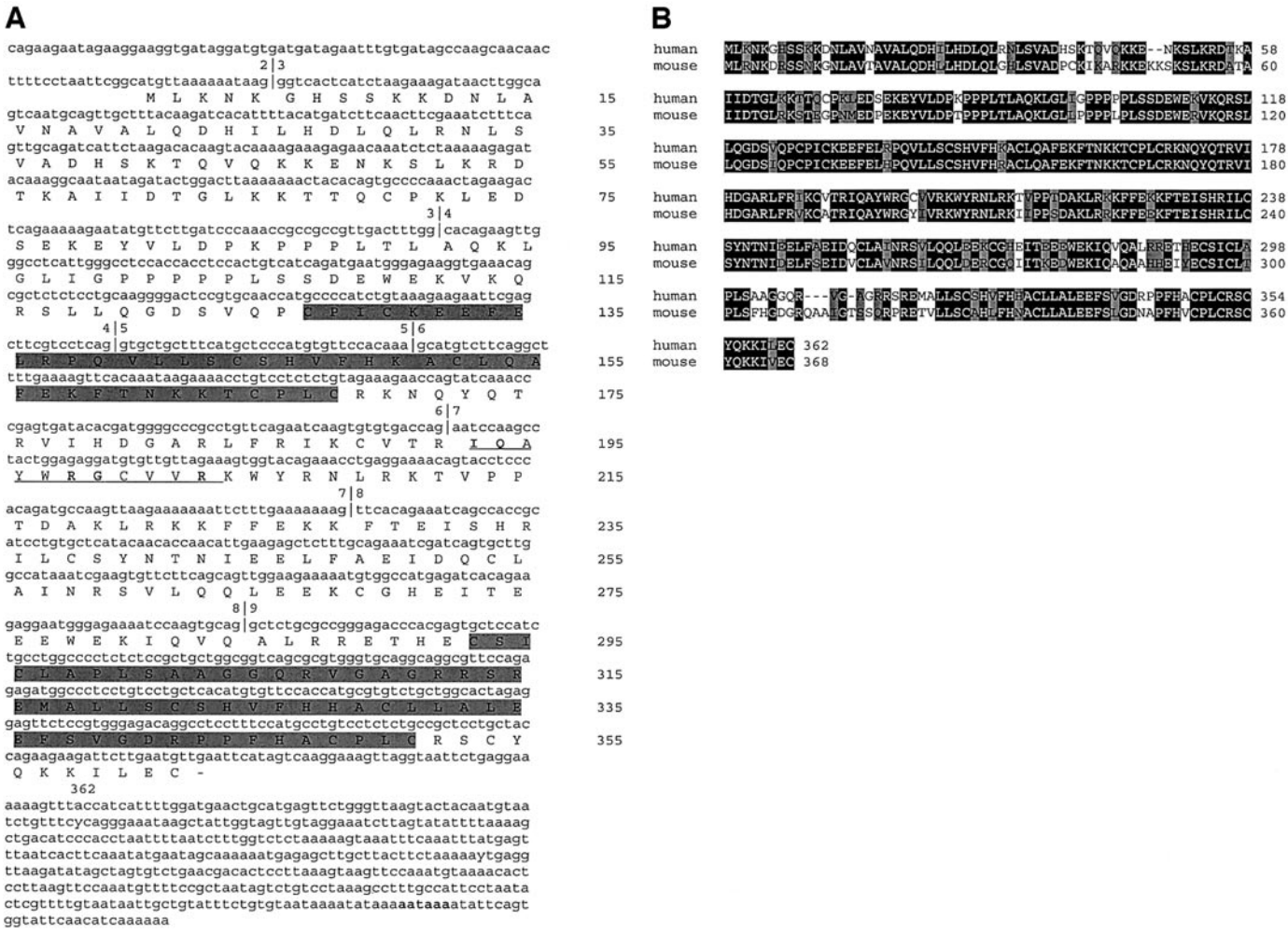

FIG. 3. (A) Human RNF32 protein and DNA sequence, starting with exon 2. RING-H2 domains are shaded, the IQ domain is underlined and the consensus residues are in bold. Splice sites are indicated by a vertical line. (B) Conservation of the predicted amino acid sequence between *RNF32* and mouse *Rnf32*. Identical residues are in black, similar residues are in gray.

sequence homology between RNF32 and this predicted protein outside the domains.

Expression of RNF32

The expression pattern of RNF32 was analyzed using Northern blots containing multiple human adult tissues. Two transcripts of 0.8 and 1.9 kb were detected in testis and, less abundant, in ovary (Fig. 4A). No expression was detected in any other tissue (see Materials and Methods). Exons 2–4 hybridized to both bands and exons 5–8 only to the larger 1.9 kb band, indicating that indeed a short and a long transcript of *RNF32* exist. The intensities of the bands seen in Fig. 4A indicate that both transcripts are present in equal amounts.

The found transcript sizes correspond to the size of the bands on the Northern blots, indicating that we have determined the full length transcript. We did not isolate the shorter transcript from the cDNA library because we did specifically select for clones that contained both exon 3 and exon 8, however, the finding of the smaller band is consistent with the ESTs that contain a polyadenylation signal after exon 4.

Additionally, a stop codon and a polyadenylation signal were found in exon 9, and we did not find any clone or EST with exons beyond exon 9, and no such exons were predicted by the gene prediction programs.

The expected size differences due to alternative splicing of the *RNF32* transcript are too small to detect on these Northern blots (107–250 additional nucleotides), therefore it is not possible to see if exon 3a is inserted and which version of exon 4 is used.

To further characterize testis expression of *RNF32*, we made use of its mouse homolog. In adult mice, spermatogenesis occurs continuously, with all stages of sperm development present at each time point. The first wave of spermatogenesis starts at birth. Meiosis is initiated around day 7 and the first haploid round

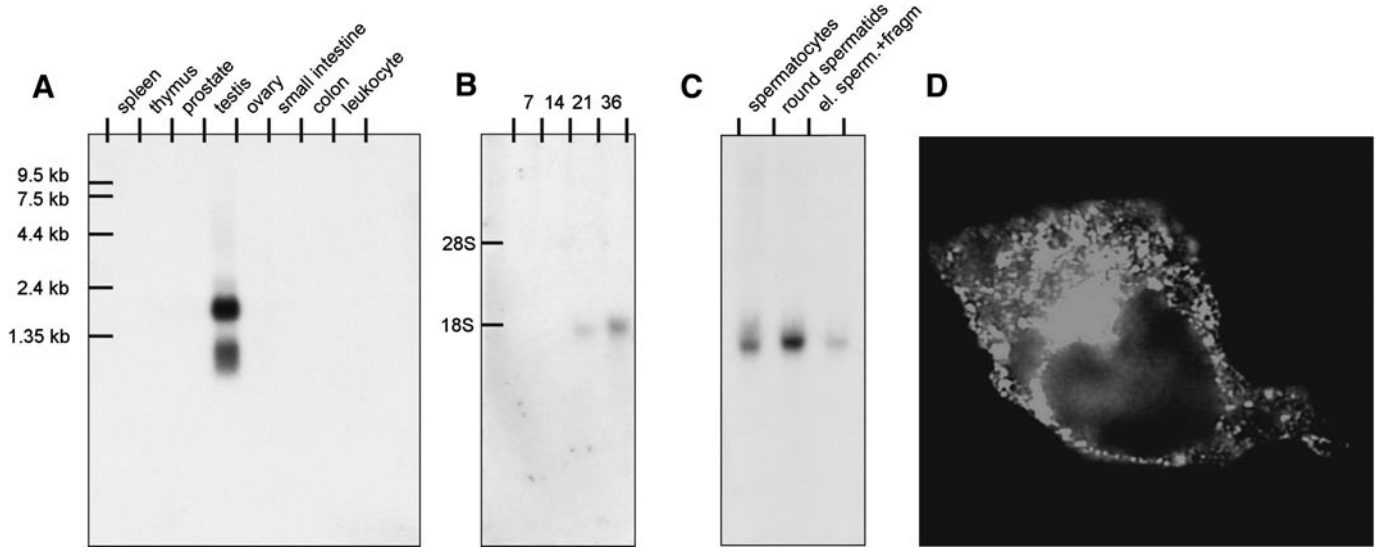

FIG. 4. Expression of *RNF32*. (A) Hybridization with exon 3 on human multiple tissue Northern blot. Two transcripts of *RNF32* are seen in testis. After prolonged exposure, a band in ovary is seen (not shown). (B) On a blot containing testis RNA of mice aged 7, 14, 21, and 36 days, *Rnf32* signal is seen in testis from 21- and 36-day-old-mice. (C) *Rnf32* is expressed in all three germ cell fractions: spermatocytes, round spermatids and the elongated spermatocytes/cytoplasmic fragments (el. sperm. + fragm). (D) His tagged *RNF32* localizes to the cytoplasm and forms aggregates in cultured COS cells.

spermatids can be detected around day 21. A Northern blot containing testis RNA of mice aged 7, 14, 21 and 36 days was used for hybridization with a mouse *Rnf32* probe. Signal was detected in testis aged 21 and 36 days (Fig. 4B), indicating that transcription of *Rnf32* starts late in the meiotic prophase or shortly after meiosis. Based on these results, it cannot be excluded that *Rnf32* is expressed only in somatic cells in the testis from postnatal day 21 onwards. Therefore, we isolated total RNA from different testicular cell fractions: one fraction highly enriched for spermatocytes going through the prophase of meiosis, one fraction containing postmeiotic round spermatids and one fraction containing elongated spermatocytes and cytoplasmic fragments. Northern blot analysis using the same Rnf32 probe, resulted in a signal in all fractions with the strongest signal in round spermatids (Fig. 4C). This shows that *Rnf32* is indeed expressed in germ cells.

To determine the intracellular localization of RNF32, we transfected COS and CHO cells with a RNF32-His tag construct containing the long transcript. After 24 h, the fusion protein was detected mainly in aggregates in the cytoplasm (Fig. 4D). These results confirm the findings of other groups that RING containing proteins are often found in aggregates (see Borden *et al.*, 2000). It remains to be determined if RNF32 functions in the ubiquitin pathway.

C7orf13

Several ESTs map proximal to *RNF32*. The sequence of these clones does not align with *RNF32* and they align to the genomic sequence without gaps, indicating that no intron is present. An open reading frame is predicted in the opposite direction from *RNF32*, indicating that a separate transcript maps to the other DNA strand. We designated this transcript *C7orf13* (AF439977) (Fig. 5).

To identify the start of the coding sequence, we used the genomic sequence to design primers proximal to the known sequence and used these to perform PCR on cDNA derived from human lymphocytes, with a primer located within the known sequence, JB69 as reverse primer. The most proximal primer that still gave a product was JB90, whereas primer JB92, located within the second alternative exon 1 of *RNF32*, did not produce a band (data not shown), indicating that the exon starts somewhere between those primers. Interestingly, JB90 is located within the genomic region of *RNF32*, after the most proximal alternative exon 1 (Fig. 2C). This means that *C7orf13* overlaps *RNF32*.

Two methionines are present between JB90 and JB92, and a stop codon is preceding the most proximal methionine. The sequence around both ATGs match the Kozak sequence in the important –3 position, indicating that one of these methionines may be the starting point of translation. However, a putative splice site lies between the most proximal ATG and the stop codon and therefore it is possible that an additional exon exists, preceding the known ORF. We tried to isolate additional 5′ sequence with race PCR, but we did not find additional exons.

The 216 aa ORF of *C7orf13* does not contain known domains and does not resemble any other proteins. A

polyadenylation signal is present 2257 nt downstream of the first methionine and all ESTs contain a poly(A) tail 17-114 bp after this signal.

A *C7orf13* probe hybridized to a human multiple tissue Northern blot showed a band of 2.3 kb in testis only (Fig. 6), indicating that we have determined the full-length transcript. The C7orf13 probe did not show any bands on a developing mouse Northern blot and we have not found any C7orf13 homologs in other species in GenBank or in our own libraries. Analysis of the mouse genomic sequence around exon 1 of *Rnf32* did not show a predicted *C7orf13* homolog and no homologies are present between the mouse and human sequences in this region. Because of this, and because C7orf13 is intronless, it could very well be derived from a retroposon (27). Still, the intact reading frame of *C7orf13* suggests that this is not a functionless pseudogene. It should be noted that the testis appears to express a relatively high number of functional retroposons (see (28) and references therein). Several of these genes encode variants of X-chromosomal genes, to compensate for the silencing of X (and Y) chromosomal genes in spermatocytes. In addition, a switch to the spermatogenic expression of retroposed genes is also observed for autosomal-to-autosomal retroposition (29).

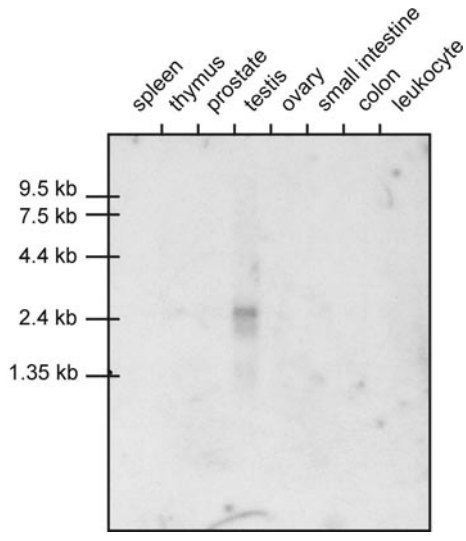

FIG. 6. Testis-specific expression of *C7orf13* mRNA in humans.

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atgctcgccagccccgcccggcccacattgcgcatgcttgccaaccacgccctgtccaca
 M  L  A  S  P  A  R  P  T  L  R  M  L  A  N  H  A  L  S  T      20
ccacactgcgcatgctcgccggccccccgcccgcgcacggcgtcagcgtctagacgcagg
 P  H  C  A  C  S  P  A  P  A  P  R  T  A  S  A  S  R  R  R      40
tgcgttcccgtcgaggctcgcgcagcgggcgttttggggatcgcctggcgggggtgttc
 C  V  P  V  E  A  R  A  A  G  V  F  G  D  R  L  A  G  V  F      60
gggagccgcgggctgaagcacgggggcgtgcaggcgcccaggcctcgtgtagtgagggcc
 G  S  R  G  L  K  H  G  G  V  Q  A  P  R  P  R  V  V  R  A      80
gagccccgcgccggcttcgctgtagtccgcagccccccggcggcttlgtgggcgcagccac
 E  P  R  A  G  F  A  V  V  R  S  P  R  R  L  C  G  R  S  H     100
gctcctcagccgcccgcccacctgggcctgggccccggttgtttccctgcggtggctgtg
 A  P  Q  P  P  A  H  L  G  L  G  P  G  C  F  P  A  V  A  V     120
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 V  V  P  V  P  G  S  R  A  H  R  P  F  A  A  L  L  V  E  G     140
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 S  F  L  G  D  P  P  I  P  P  R  R  S  G  V  L  A  R  G  S     160
gcaggcgccgactgcctggcgtcgtccgtgactcctggccctctctgtggatccctttg
 A  G  A  D  C  L  A  S  S  V  T  P  G  P  S  L  W  I  P  L     180
cttctcgttgcgggttgtgtttcctgcttcgtgggcctggccgtttgcgtttggatgcag
 L  L  V  A  G  C  V  S  C  F  V  G  L  A  V  C  V  W  M  Q     200
gcccgggtgagccctgcgtggcccgctggccttttcctgctccctaggtgactgagccgc
 A  R  V  S  P  A  W  P  A  G  L  F  L  L  P  R                 216
gtgtgtgacacatcggagtgaattgaggcaacgcgttcctttccaggcttgcagcgggcg
gtgtcgttcacgctcctgagcagagccttccggtgctttactcgacgccacaaatagtgc
agggttcctcccaggggctgtcgcgagctagaactcttccccgccctgtgagctcgggc
tgttcttcctcctccgcgtggctcactccagcctcggatgtttcctcgctgcacagacct
gctcgggaagactcgagaaggccacccccaggactccagctctccccggaagggcttcccc
tcggattgcgtcttggccctggaaccctccctctgtatcctctgctgttcctctcttgg
cctccatttgggttgtggcctggatactctggctcgctgcttccctctcttaaggatca
cagccctgcactgcctgtcgtccggtgcctggaaaccattgtatttttttggtctgatttt
gtagttgtttgagaacttaagtctggtccctcacactccatgcccagaagcagaaatgag
taaagtttgttttatcttttcttaatatgacaattattgtttggttcaacttatgttgt
actttaattagaagaaatttggccgaaaatacaaggaaaatatacaaatgcaagtaattt
tttttaaacttccctgaaagcagggtctaaagaaattaccaaccaacttagactggatct
agaagaaaaggaagggtctttgcagtcttaggactcttccgttccgcgacgtaggtgtta
ggataacagccataaatggttgtaagactttgggctcagataagtagacttaagttcaaa
ttttgacttatttacaagtgtgtgattttggcaagctcatcttcctaaaccatgagct
ccttatttgtaaaggggacattagccactctccagcaacagccctggtacttcttcagtc
ctgggatgggacgtatgattagcctaagcgaaccagaaaatccaggcccgtgaccagtga
cttgatcagggctggcgatatatctaggtaggccaaccaggtggactcagtatttttgtg
ggtgctactggaaaatttatttaattctaactgaatgtagaaacagcaacagacatgaaa
tggcagttgtattgctgtcttatcatgaggtgagggcctgaagctatggtagccaccctg
tgaaccttggaaggagggttctacaggaactggcagagctgagactgggacgcaacccat
gtcctggtgacgtatttgagtcctgaggctggccctcctgttagtcttttctcttactgg
cttcagccagatggggtctggttttctgttacttgcaacaggaaaaattggaatgatatc
tatgcatgctattaccgccttatggctgtggtgagattaaaacatgatactgtgggtgaa
ctgcttagtacagtgcctgctattctttatgttggttttaaggtatttttcttgaacttt
aaagttctaagaaaaattaccgttttaaaattcctaccctctgttcacatgtttaaattt
tcatgatagttttttctgtctgcatgttttctattcaaataaattggtcattcttcagtt
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FIG. 5. C7orf13 DNA sequence and predicted protein, starting from the first methionine after the stop codon (see Fig. 2C). The polyadenylation signal is underlined.

Both *RNF32* and *C7orf13* are mainly expressed in testis. Regulatory sequences are often found around the first exon of genes, and the physical overlap of *RNF32* and *C7orf13* may therefore cause the transcription of *C7orf13* in testis. The function of the alternative exons 1 of *RNF32* is unknown, but it is possible that they constitute a mechanism of regulation. In this respect, it will be interesting to see if the mouse homolog of *RNF32* also contains alternative splices.

RNF32 is the first protein reported with a double RING-H2 domain. Conventional RING domains have frequently been found in larger motifs that may contain other cysteine/histidine rich domains. Examples of these are the RBCC and TRAF motifs and the RING-IBR-RING motif (19, 30–33). All proteins with more than one RING domain reported so far contain the IBR box (19). In contrast, RNF32 contains and IQ domain between its RING-H2 fingers. This suggests that despite the similarities between RING-H2 and RING domains, these domains may have different functions.

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