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Multiple Alternative Transcripts of the Human Homologue of the Mouse *TRAD*/*R51H3*/*RAD51D* Gene, a Member of the *rec A*/*RAD51* Gene Family[1]

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Yeast *RAD51*, a homologue of *Escherichia coli recA*, plays a crucial role in mitotic and/or meiotic recombination and in the repair of double-strand DNA breaks. We have identified unique multiple alternative transcripts of a human *TRAD*/*R51H3*/*RAD51D* gene, a member of the *recA*/*RAD51* gene family. One of the transcripts encoded a 328-amino-acid protein with 83.0% overall amino acid identity and 98.2% similarity with the mouse *TRAD* gene and had two nucleotide binding consensus sequences, motif A and motif B, conserved among members of this family. Other transcripts encoded truncated proteins with a partial N-terminal region of the orthologue or short proteins lacking internal sequences which contain nucleotide binding motifs. Northern blot analysis revealed that multiple transcripts of the human *TRAD* gene were expressed in various tissues and their distribution was not ubiquitous.

DNA recombination plays an important role in the acquirement of gene diversity and in the survival of cells. Genetic recombination in meiosis produces genetic diversity. In somatic cells, V(D)J recombination generates diversity of immunoglobulin and T cell receptor genes. Recombinational repair removes DNA damage and suppresses oncogenesis and cell death. DNA recombination has been extensively studied in prokaryotes and lower eukaryotes. *Escherichia coli* (*E. coli*) RecA has been shown to be a major protein involved in DNA recombination. This protein recognizes homologous regions of double-stranded DNA and promotes DNA strand exchange (1). In *Saccharomyces cerevisiae* (*S. cerevisiae*), four gene encoding proteins homologous to RecA have been isolated, *RAD51*, *RAD55*, *RAD57*, and *DMC1* (2–5). Rad51 protein plays a crucial role in both mitotic and meiotic recombination and in the repair of DNA double-strand breaks. In mammals, seven *recA*-like genes, *RAD51*, *DMC1*/*LIM15*, *REC2*/*R51H2*/*RAD51B*, *RAD51C*, *XRCC2*, *XRCC3*, and *TRAD*/*R51H3*/*RAD51D*, have been isolated (6–17). The *TRAD*/*R51H3*/*RAD51D* gene has been reported independently by three groups including our laboratory. With the mouse gene, one group showed a human orthologue, *HsR51H3*, of mouse *TRAD* and another revealed an alternative transcript, *HsRAD51D*, encoding a truncated protein with a partial N-terminal region (~14%) of the orthologue and lacking two nucleotide binding motifs conserved in the *recA*/*RAD51* family (12, 17). In the present study, we identified several new transcripts of the human gene and report here a partial genomic sequence analysis of exon-intron structures of the gene and a unique expression pattern of the transcripts.

MATERIALS AND METHODS

cDNA cloning. A BLAST search of expressed sequence tags (EST) with an amino acid sequence of the mouse *TRAD* gene, was performed in the DDBJ/EMBL/GenBank databases and several EST candidates were identified. Sequence analysis revealed that one clone, I.M.A.G.E. clone no. 259579, which had the longest 5′ flanking sequence, lacked internal parts of the coding region corresponding to the mouse *TRAD* gene.

In addition, a human placenta cDNA library in λZAPII (total 5×10^5 pfu, Stratagene) was screened with a digoxigenin-labeled mouse *TRAD* gene probe. Digoxigenin-11-dUTP (Boehringer Mannheim) was incorporated into the probe by polymerase chain reaction (PCR), using the following primers; MMTRF (sense); 5′-TTAGTGTCCCTCACCTCGACTTGCATCCTCTTCCC-3′, MMTRR (antisense); 5′-TCTAGGTCTCTCTCACTGCAGCATCCCCTGGCCAG-3′. Non-RI chemiluminescent detection was performed according to the manufacturer's instruction manual (Boehringer Mannheim). To isolate a full-length cDNA clone, two oligonucleotides, HsTRADF (sense, 5′-TGTGTCCTCTCTAGGAAGGGGTAGGGGA-3′) and HsTRADR (antisense,

[1] The nucleotide sequence data reported in this paper are available in the DDBJ, EMBL, and GenBank databases with the accession numbers AB013341 (*TRAD*), AB016223 (*TRAD-d1*), AB016224 (*TRAD-d2*), AB016225 (*TRAD-d3*), AB018360 (*TRAD-d4*), AB018361 (*TRAD-d5*), AB018362 (*TRAD-d6*), AB01863 (*TRAD-d7*), and AB020412 (*TRAD-d8*).

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5′-TTTCGGGGAATTGCCTTTTTATATTTTTAAGAGC-3′), were synthesized based on the nucleotide sequences of the EST clone and the clones isolated from a human placenta cDNA library. PCR was carried out with the above primers, human adult brain QUICK-Clone cDNA (Clontech) as a template and ExTaq thermostable proofreading DNA polymerase (Takara). The products were fractionated by electrophoresis on a 0.7% agarose gel. The fragments were excised and purified using NaI and a FlexiPrep glass powder kit (Amersham Pharmacia Biotech). The DNA fragments were subcloned into the TA cloning vector pCR II (Invitrogen).

RT-PCR. Oligonucleotide primers for PCR were synthesized based on the nucleotide sequences of isolated cDNA clones as follows: HSTRF1 (sense); 5′-CTGCAGACCTGGAAGAGGTAGCTCAGAAAT-GTGG-3′, HSTRF5 (sense); 5′-TCCGGAGGATCCAGGTGGTGCA-TGCATTTGACATC-3′, HSTRDR (antisense); 5′-GTCATGTCTG-ATCACCCTGTAATGTGGCACTCTGC-3′, HSTRR1 (antisense); 5′-TCAAATGCATGCACCACCTGGATCCTCCGGAGAG-3′ and HSTRR4 (antisense); 5′-CTGCCAATGCCAGTGGACAGGATGGCAGTGGAGG-3′ (Fig. 3B). PCR was carried out with these primers, human adult brain QUICK-Clone cDNA (Clontech) or plasmids from human fetal brain SuperScript cDNA library (GIBCO BRL) as templates and ExTaq DNA polymerase (Takara). PCR was performed for 30 cycles of denaturation at 96°C, 30 sec, annealing at 68°C, 1 min, extension at 72°C, 3 min. The products were separated by electrophoresis on 5% polyacrylamide gels. The bands were excised and electroeluted, and the DNA fragments were subcloned into the pCR II vectors.

Genomic DNA cloning. A human genomic DNA library in λFIX II (total 1×10^6 pfu, Stratagene) was screened with the digoxigenin-labeled mouse TRAD cDNA probe described above. Hybridization was performed in 5 × SSC, 50% formamide, 7% SDS and 1% Blocking agent (Boehringer Mannheim) at 42°C. Washing was performed in 0.1 × SSC and 0.1% SDS at 50°C. Non-RI detection was performed as described above. Three clones were isolated and the Not I fragments of the clones were subcloned into the Not I site of the pCR II vector.

Northern blot analysis. A Human Multiple Tissue Northern Blot II (Clontech), containing ~2 μg poly A+ RNA on each lane, was hybridized with a digoxigenin-labeled human *HsTRAD* cDNA probe in 5 × SSC, 50% formamide, 7% SDS and 1% Blocking agent overnight at 50°C. The membrane was finally washed at 68°C in 0.1 × SSC and 0.1% SDS for 15 min twice. The probe was labeled with digoxigenin-11-dUTP using PCR with the cloned HsTRAD cDNA as a template and the primers HSTRF2, 5′-AACATGGGCGTGCTCAG-GGTCGGACTGTGCCCTGG-3′ (sense) and HSTRDR. Non-RI chemiluminescent detection was performed as described above. The same membrane was stripped and rehybridized with a human ribosomal protein S26 cDNA probe to standardize the amounts of poly A+ RNA applied on each lane (18). A 365 bp fragment of human ribosomal protein S26 cDNA was amplified by PCR using human adult brain QUICK-Clone cDNA (Clontech) as a template and the primers HSRPS26F2, 5′-ATGGTCGTGCCAAAAAGGGCCGCGGCCACG-TGC-3′ (sense) and HSRPS26R2, 5′-CTGTCTTCAGTCTTTAAGA-ACTCAGCTCCTTAC-3′ (antisense). The fragment was subcloned into the TA cloning vector pCRII, and the nucleotide sequence was confirmed by sequencing. The human ribosomal protein cDNA fragment was labeled with digoxigenin-11-dUTP by PCR with the same primers as described above and used as a control probe. Hybridization, washing and non-RI detection were performed as described above.

Sequence analysis. All sequence analyses were performed using an ABI 373A automatic sequencer and an ABI PRISM Dye terminator cycle sequencing ready reaction kit (Perkin Elmer) or Thermo Sequenase dye terminator cycle sequencing pre-mix kit, v2.0 (Amersham Pharmacia Biotech) by primer walking. Partial sequencing of exon-intron junctions of the genomic clones was carried out with primers corresponding to nucleotide sequences in exons.

RESULTS

cDNA cloning of human homologue of the mouse TRAD gene. Sequence analysis of I.M.A.G.E. clone no. 259579 which had the longest 5′ flanking sequence among several EST candidates examined revealed that this clone (named *HsTRAD-d3*) lacked internal parts of the coding regions corresponding to the mouse *TRAD* gene, but had the 5′-flanking region of the predicted initiation codon (Fig. 1, 3). In addition, two other clones were isolated from a human placenta cDNA library and sequence analysis showed that both clones defected the 5′ coding region corresponding to the mouse *TRAD* gene and had the 3′ flanking region of the predicted stop codon (data not shown). To isolate a full-length cDNA clone, PCR was performed using primers synthesized based on the identified clones. Four different fragments were separated by 0.7% agarose gel electrophoresis (Fig. 2A). Four clones were isolated by subcloning these fragments (Fig. 3). Analysis of the nucleotide sequences showed that one clone (named *HsTRAD*) had a complete open reading frame (ORF) of 328 amino acids corresponding to the mouse *TRAD* gene and two nucleotide binding motifs, motif A and motif B, conserved in the *rec A*/*RAD51* gene family (Fig. 1). Optimized alignment of the predicted human and mouse Trad amino acid sequences revealed that the human Trad protein had 83.0% overall sequence identity and 98.2% similarity with the mouse protein (16). The *HsTRAD* clone had the same ORF as *HsR51H3*, previously reported (12). The nucleotide sequence of the gene and other sequences presented in the study are available in the DDBJ/EMBL/GenBank DNA databases. These accession numbers are listed in the footnotes. In explanations of the results, exon numbers identified in the present study are used (Fig. 3). One clone, named *HsTRAD-d1*, lacked the sequence from nucleotide 251-369 of the *HsTRAD* cDNA (exon 3 and 4) and shared the nucleotide sequence of human *RAD51D* described recently (17). Another clone, named *HsTRAD-d2*, lacked the same sequence (exon 3 and 4) and nucleotides 452–586 (exon 6). The last clone, named *HsTRAD-d3*, lacked nucleotides 251–586 region (exon 3 to 6). *HsTRAD-d1* and *-d2* had a stop codon at the 254th nucleotide in exon 5 due to a frameshift and an ORF encoding a predicted protein of 49 amino acids. If *HsTRAD-d1* and *-d2* use an alternative initiation codon, they can encode 209 amino acids, which share amino acids 120 to 328, a partial C-terminal region of HsTrad (Fig. 1). *HsTRAD-d3* lacked one of the two nucleotide binding motifs, motif A (in exon 5), and had an ORF encoding 216 amino acids.

RT-PCR. PCR products amplified with the primers (sense, HSTRF1 in exon 2 and antisense, HSTRR1 in exon 7) flanking the region deleted in *HsTRAD-d1,-d2* and *-d3* were fractionated by 5% polyacrylamide gel electrophoresis and eight fragments were identified

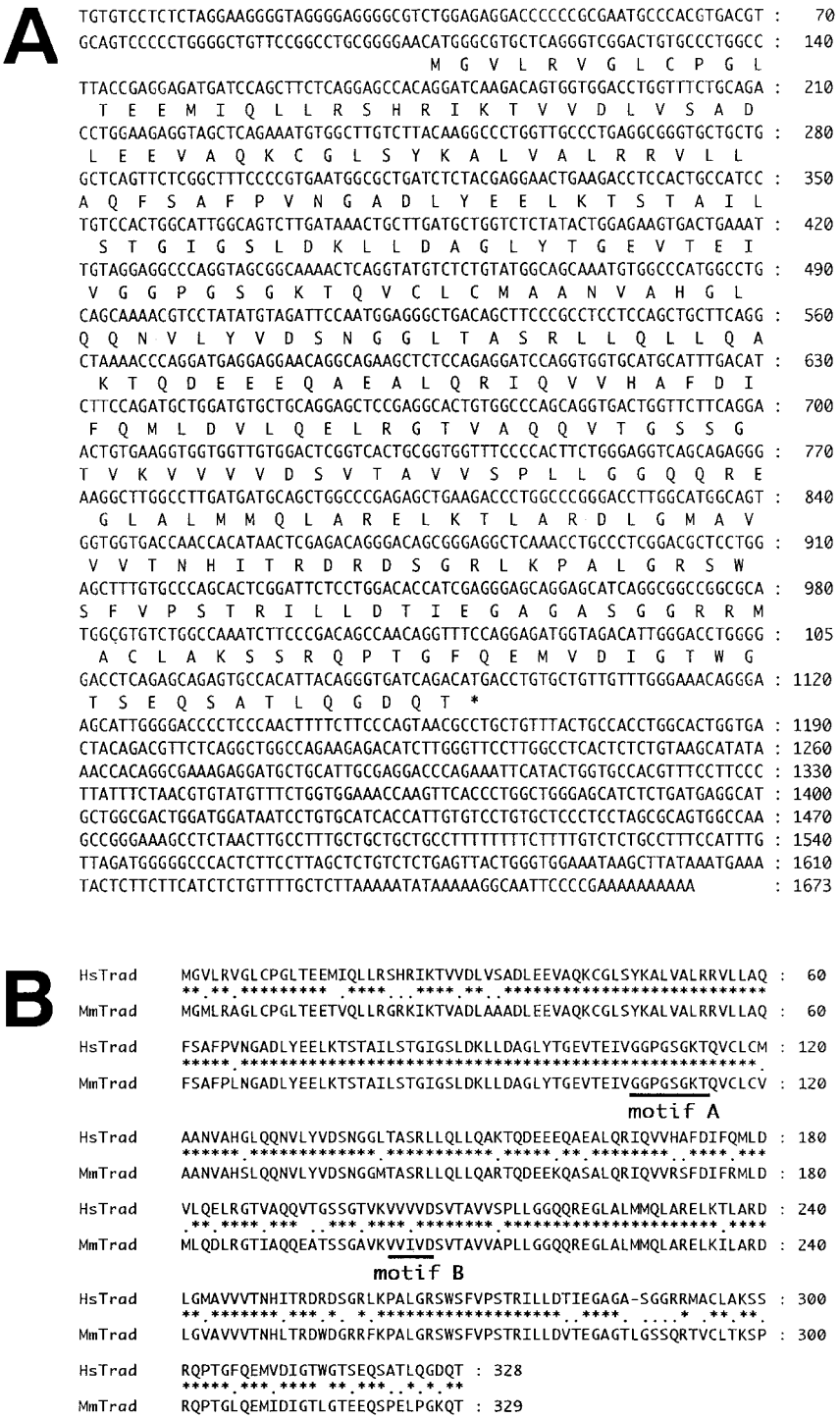

FIG. 1. (A) Nucleotide and predicted amino acid sequence of human *TRAD (HsTRAD)* and (B) amino acid alignment of the human (HsTrad) and mouse (MmTrad) Trad proteins. Asterisks and dots indicate identical and similar residues, respectively. Two nucleotide binding consensus sequences, motif A and motif B, are underlined.

(Fig. 2B). These fragments were subcloned into a TA cloning vector. Sequence analysis of the fragments revealed that four of eight were novel fragments, named *HsTRAD-d4*, *HsTRAD-d5*, *HsTRAD-d6* and *HsTRAD-d7*, while the other four were partial fragments of the cDNA clones described above (*HsTRAD*, *HsTRAD-d1*, *HsTRAD-d2* and *HsTRAD-d3*) (Fig. 3A). *HsTRAD-d4* lacked exon 4 and 6 and had a continuous reading frame with motif A (in exon 5) and B (in exon 9). *HsTRAD-d5* lacked exon 4 to 6 and had a frameshift in

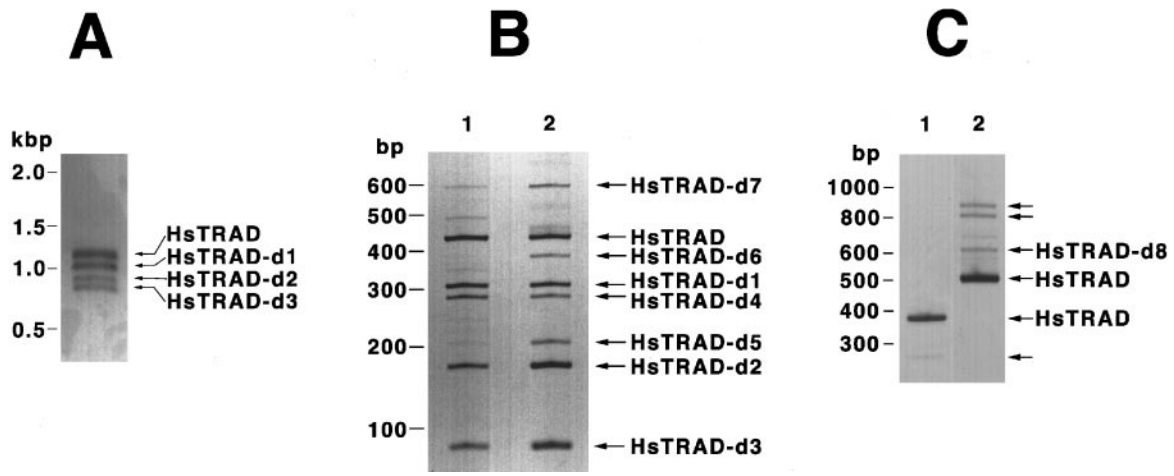

FIG. 2. RT-PCR analysis. PCR products were fractionated by electrophoresis on 0.7% agarose (A) or 5% polyacrylamide gels (B, C). Size markers are shown on the left. (A) To isolate a full-length cDNA clone, PCR was performed using human adult brain cDNA (~1 ng, QUICK-Clone, Clontech) and primers HsTRADF (at 5′-end) and HsTRADR (at 3′-end). (B) PCR using human adult brain cDNA (~1 ng) (lane 1) or plasmids (~1 ng) (lane 2) from a human fetal brain SuperScript cDNA library (GIBCO-BRL) with primers HSTRF1 (in exon 2) and HSTRR1 (in exon 7). (C) PCR using the human fetal brain cDNA and primer sets HsTRADF (in exon 1) and HSTRR4 (in exon 3) (lane 1), or HSTRF5 (in exon 7) and HSTRDR (in exon 12) (lane 2). The primer sites are schematically shown in Fig. 3B. Arrows indicate subcloned and sequenced fragments.

exon 7, generating a stop codon in exon 8 or 9. *HsTRAD-d6* lacked exon 5 and 6. *HsTRAD-d6* and *HsTRAD-d7* had a unique extra exon (exon 4) with a stop codon. Semi-quantitative densitometric assessment of the intensity of each band on the gel revealed that *HsTRAD*, *HsTRAD-d1*, *HsTRAD-d2* and *HsTRAD-d3* mRNA were expressed at largely equivalent levels, while the others were at less levels in the brain (Model GS700 Imaging Densitometer, Bio-Rad Laboratories, Fig. 2B).

Polyacrylamide gel electrophoresis of PCR products amplified with the primers HsTRADF (sense, in exon 1) and HSTRR4 (antisense, in exon 3) showed two fragments (Fig. 2C) and the nucleotide sequence of one of the subcloned fragments agreed with the *HsTRAD* gene, while the other was an unknown by-product (data not shown). At least four bands were clearly detected by 5% polyacrylamide gel analysis of PCR products with the primers HSTRF5 (sense, in exon 7) and HSTRDR (antisense, in exon 12), and these fragments were subcloned into a TA cloning vector, while other faint fragments failed to be subcloned (Fig. 2C). Sequence analysis of the fragments revealed that one was a predicted fragment of the *HsTARD* gene, two were by-products due to misannealing of the primers (data not shown), while the other one (named *HsTRAD-d8*) included a new exon (exon 8) with a stop codon (Fig. 3B).

Northern blot analysis. Multiple transcripts were detected with a human *TRAD* cDNA probe (Fig. 4). A 7.0 kb transcript was expressed in the colon and prostate, while a 4.8 kb transcript was clearly detected in the spleen and colon, and faintly in the prostate, testis, and ovary. Transcripts of 1.4, 1.8 and 2.5 kb were highly expressed in the testis, moderately in the spleen, and faintly in the thymus, prostate, ovary, small intestine, and colon. All of these transcripts were expressed at low levels in leukocytes.

Sequencing of the genomic DNA clones. Partial sequence analysis of exon-intron junctions of the human genomic clones using primers corresponding to nucleotide sequences in the exons showed that splice donor (GT) and acceptor (AG) signals were conserved at all junctions from exon 1 to exon 12 (Fig. 3B).

DISCUSSION

We identified multiple transcripts of a human homologue of the mouse *TRAD* gene. Partial sequence analysis of the genomic clones confirmed that the exon-intron structures agreed with the GT/AG splicing rule, indicating that the occurrence of multiple transcripts was due to alternative splicing. Sequence analysis showed that *HsTRAD* and *HsTRAD-d1* correspond to the previously cloned human *R51H3* and *RAD51D*, respectively (12, 17). Optimized multiple alignment analysis revealed that *HsTRAD*/*R51H3* may be an orthologue of mouse *TRAD*. *HsTRAD-d1*/*RAD51D* encodes a truncated protein with a partial N-terminal region of the orthologue and lacking the two nucleotide binding motifs conserved in the *recA*/*RAD51* family. The other seven, *HsTRAD-d2* to *-d8*, are novel alternative transcripts. Northern blot analysis showed that multiple transcripts were expressed in sev-

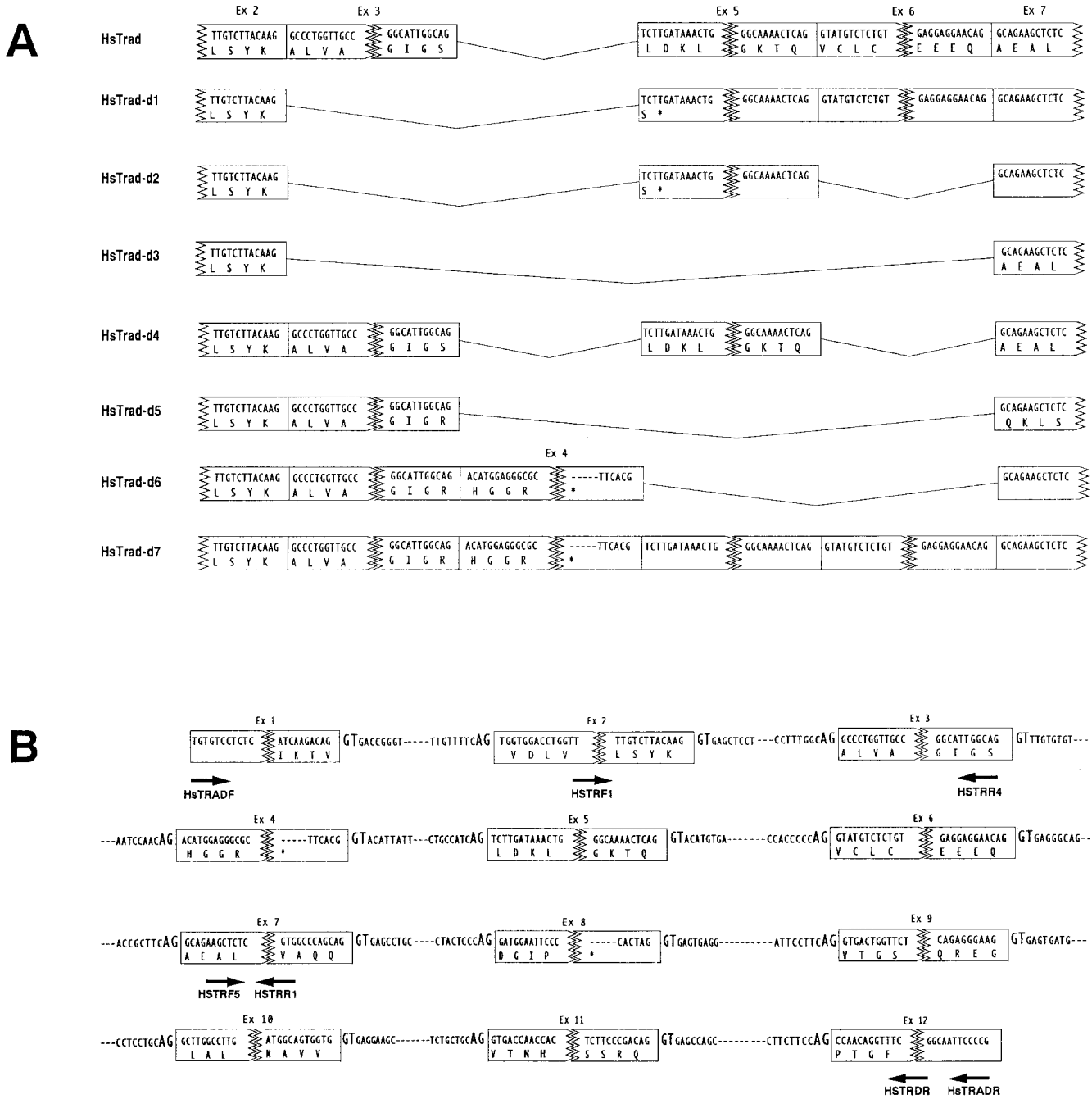

FIG. 3. Structure of *HsTRAD.* (A) Schematic representation of the multiple transcripts. Exons are boxed and jagged lines show omitted sequences. (B) Genomic sequence of exon–intron junctions. The top and bottom lines show nucleotide and amino acid sequences, respectively. Exons are boxed. Boldface indicates splice donor (GT) and acceptor sites (AG). Arrows show primer sites.

eral tissues, although it was uncertain which band represented to each of the identified transcripts. Previously, we reported that the mouse *TRAD* gene expressed transcripts of different sizes and their tissue distribution was largely ubiquitous (16). In contrast, in the present study human *TRAD* was shown to be expressed at highest levels in the testis, although its expression was also found in several other tissues. Previous study showed that 1.7 kb and 7.0 kb transcripts were detected using a human *R51H3* cDNA probe, which is the same gene of *TRAD,* while in the present study, transcripts of 1.4, 1.8, 2.5, 4.8 and 7.0 kb were detected with a human *TRAD* probe (17). As we performed hybridization under high stringency condition, detection of additional transcripts may have been due to higher sensitivity of the detection method used in the present study.

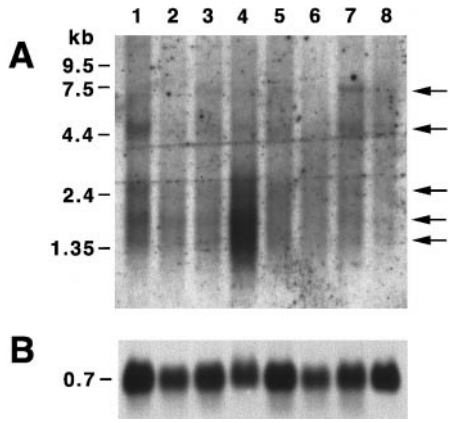

FIG. 4. Northern blot analysis. (A) Expression pattern of *HsTRAD* in human tissues: spleen (lane 1), thymus (lane 2), prostate (lane 3), testis (lane 4), ovary (lane 5), small intestine (lane 6), colon (lane 7), and leukocytes (lane 8). The human multiple tissue northern blot II containing ~2 μg poly A+ RNA/lane (Clontech) was hybridized with *HsTRAD* cDNA. RNA size markers are shown on the left. Arrows on the right indicate multiple transcripts (7.0, 4.8, 2.5, 1.8 and 1.4 kb). (B) The same membrane was stripped and rehybridized with a human ribosomal protein S26 cDNA to ensure loading of equal amounts of RNA.

Among the *recA*/*RAD51* family members, *DMC1* expresses two alternative transcripts. Although both transcripts have been shown to be translated into proteins, the role of the short form remains unknown (8). *REC2/RAD51B/R51H2* also has two alternative transcripts, while it is uncertain whether these transcripts are translated into proteins (12). The human *TRAD* gene is unique in expressing so many alternative transcripts. Like the mouse Trad, the amino acid sequence and domain structure of HsTrad protein are similar to those of Rad51 and other recA family members, suggesting that HsTrad protein has functions similar to those of Rad51/recA family proteins (16). In *S. cerevisiae,* four *recA* family members have been identified, *RAD51, RAD55, RAD57* and *DMC1* (2–5). Expression of Dmc1 protein is specific for meiosis (5). Rad51 protein forms nucleoprotein filaments on DNA (19). Rad51 protein interacts directly with Rad52 and Rad55 (20, 21). Rad55 and Rad57 proteins form a heterodimer that promotes DNA strand exchange by Rad51 with replication protein A (21). In mammals, seven *recA* family members have been identified: *RAD51/RAD51A, DMC1/LIM15, REC2/RAD51B/R51H2, RAD51C, TRAD/R51H3/RAD51D, XRCC2* and *XRCC3* (6–17). Strong interaction of Rad51B and Rad51C proteins was found using the yeast two-hybrid system, implying that they are mammalian counterparts of yeast Rad55 and Rad57 proteins (13). In yeast, mutants of any one of four recA members, *RAD51, RAD55, RAD57* and *DMC1,* are viable as *recA*$^-$ mutants of *E. coli* (2–5). In contrast, mammalian gene targeting of the *RAD51* gene is lethal in early embryogenesis (22, 23). Recently, conditional targeting of the *RAD51* gene in the chicken cell line DT40 revealed that inhibition of Rad51 expression led to chromosome breaks, cell cycle arrest and cell death (24). In the evolution from prokaryotes to higher eukaryotes, the number of *recA* family members has increased and mammalian members might have acquired more specialized roles than those in prokaryotes and lower eukaryotes (1). Especially, in the present study, we have shown that human *TRAD* gene expresses many alternative transcripts in contrast to other *recA* family members. Based on its structural similarity to Rad51, the predicted Trad protein may have a DNA binding domain and protein-protein interaction domain and function as part of the recombination machinery. Human and yeast Rad51 proteins have DNA binding and protein-protein interaction activity (25–29). If the N-terminal region is responsible for protein-protein interaction and the core region including the two nucleotide binding motifs is important for DNA binding, HsTrad-d1, -d2, -d5, -d6 and -d7 proteins lacking the core domain may competitively inhibit protein-protein interactions of Trad, Rad51 and other recA members. HsTrad-d3 protein lacks one of the two nucleotide biding motifs and HsTrad-d4 protein is missing 45 amino acids of intervening sequence between the two nucleotide binding motifs, suggesting that the predicted proteins may also have lost DNA binding activity and competitively inhibit protein-protein interactions of Trad, Rad51 and other recA members. In yeast, it was demonstrated by site-directed mutagenesis that one of the two nucleotide binding motifs was important for the function of Rad55 but not Rad57 (30). It remains to be determined whether these transcripts are translated into proteins and how the full-length Trad protein participates in the recombination machinery. We expect that identification of the multiple alternative transcripts of the human *TRAD* gene will contribute to elucidation of the biological roles of many *recA* members in mammals as well as providing insight into the mechanisms of DNA recombination and repair.

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