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Human Transformer-2-beta Gene (SFRS10): Complete Nucleotide Sequence, Chromosomal Localization, and Generation of a Tissue-Specific Isoform

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Htra2-beta is a human homologue of *Drosophila* transformer-2 and a member of the SR-like protein family. Here we report the isolation and characterization of the complete htra2-beta gene (HGMW-approved symbol SFRS10). The gene spans 21,232 bp and is composed of 10 exons and 9 introns. Radiation hybrid mapping localized the gene to chromosome 3q. The region upstream of the transcription initiation codon contains an *Alu* element and several potential transcription factor binding sites. RT-PCR and comparison with EST clones revealed five different RNA isoforms generated by alternative splicing. These isoforms encode three diverging open reading frames, and two of these, htra2-beta3 and htra2-beta4, lack the first SR domain. Htra2-beta3 is developmentally regulated and expressed predominantly in brain, liver testis, and weakly in kidney. Furthermore, the domain structure of htra2-beta3 resembles a variant found in the *Drosophila* male germline, indicating a remarkable conservation of alternative transformer-2 variants. Finally, we show that htra2-beta3 is expressed in the nucleus and interacts with a subset of SR proteins in a yeast two-hybrid system and *in vivo*. © 1998 Academic Press

INTRODUCTION

Pre-mRNA splicing is catalyzed in a macromolecular complex, the spliceosome (Krämer, 1996; Will and Lührmann, 1997). Major components of the spliceosome are the small nuclear ribonucleoproteins (snRNPs) (Will *et al.*, 1993), proteins associated with hnRNA (hnRNPs) (Dreyfuss *et al.*, 1993; Weighardt *et al.*, 1996), and a family of serine-/arginine-rich (SR)[2] proteins (Manley and Tacke, 1996). Based on their antibody reactivity and biochemical characteristics, this group is subdivided into SR and SR-like proteins (Fu, 1995; Valcárcel and Green, 1996). SR and SR-like proteins are characterized by a modular composition, consisting of one or more RNA recognition motifs and an arginine- and serine-rich domain (SR domain) in which the serine residues are phosphorylated. The phophorylation status is controlled by a number of SR protein kinases including a U1 70K associated kinase (Woppmann *et al.*, 1993), SRPK1 (Gui *et al.*, 1993), LBR kinase (Nikolakaki *et al.*, 1996), and a family of CDC2-like kinases (CLK) (Colwill *et al.*, 1996; Nayler *et al.*, 1997). In addition, phosphatase activities were shown to alter splice site selection, which is possibly mediated by SR-protein dephosphorylation (Mermoud *et al.*, 1994). SR proteins can complement splicing-deficient S100 fractions and are therefore considered essential splice factors. They have been implicated in 5′ splice site recognition and in the communication of splice sites caused by an exon bridging network of SR proteins (Wu and Maniatis, 1993). In addition, SR and SR-like proteins can bind to exon enhancer motifs (Watakabe *et al.*, 1993), which are often purine-rich sequences that can stimulate the usage of suboptimal splice sites (Xu *et al.*, 1993). The interaction with exon enhancers results in a concentration-dependent influence of SR proteins on alternative splicing decisions both *in vivo* (Cáceres *et al.*, 1994; Wang and Manley, 1995; Screaton *et al.*, 1995) and *in vitro* (Ge and Manley, 1990; Mayeda and Krainer, 1992).

The exact mechanism governing alternative splicing is still under investigation. In one model, different concentrations of spliceosomal proteins in different cell types and developmental stages cause alternative processing of pre-mRNAs. Evidence for this mechanism is provided by the observed antagonistic effects of hnRNPA1 and several SR proteins such as SF2/ASF, SRp40, and SRp55 on splice site selection *in vivo* (Cáceres *et al.*, 1994; Screaton *et al.*, 1995; Wang and Manley, 1995) and *in vitro* (Mayeda and Krainer, 1992). In addition, the expression levels of various SR proteins are variable among tissues (Ayane *et al.*, 1991;

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[2] Abbreviations used: EST, expressed sequence tag; 3′ UTR, 3′ untranslated region; RRM, RNA recognition motif; SR proteins, serine-/arginine-rich proteins; AT, 3-amino-triazole.

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Mayeda and Krainer, 1992; Zahler *et al.*, 1993; Screaton *et al.*, 1995).

Another possibility is the existence of cell- and/or developmental-specific splicing factors modulating splice site selection. The best studied example for this mechanism is the female-specific expression of *Drosophila* transformer (Boggs *et al.*, 1987). There, a functional transformer protein determines sexual fate by directing alternative splicing decisions. Further evidence for tissue-specific splice factors includes the observation that the neuron-specific RNA-binding protein ELAV can change splice site selection *in vivo* (Koushika *et al.*, 1996), the existence of tissue-specific splice factors such as SmN (Schmauss *et al.*, 1989), and a male germline-specific transformer-2 variant in *Drosophila melanogaster* (Mattox *et al.*, 1990) and *D. virilis* (Chandler *et al.*, 1997). However, as ELAV is involved in RNA destabilization, it is not clear whether the observed effect is mediated by changing the concentration of SR proteins by mRNA degradation (Morrison *et al.*, 1997) or by a direct involvement in the spliceosome. Furthermore, a functional role for the male germline-specific variant of transformer-2 (Mattox *et al.*, 1990) and SmN (Delsert and Rosenfeld, 1992) in alternative splicing was not observed.

In mammalian systems, the physiological targets of various SR proteins have not been determined. In contrast, genetic analysis in *Drosophila* revealed a direct influence of the SR-like proteins transformer and transformer-2 on exon 4 of the doublesex pre-mRNA, on an alternative 5′ splice site in fruitless (Heinrichs *et al.*, 1998), exuperantia (Hazelrigg and Tu, 1994), and on alternative testis transcript (Madigan *et al.*, 1996). Recently, two mammalian homologues of transformer-2 were isolated. The human alpha gene was identified as an EST clone and was shown to complement transformer-2 deficiency in *Drosophila* (Dauwalder *et al.*, 1996). The beta gene was isolated from rat (Matsuo *et al.*, 1995) and mouse (Segade *et al.*, 1996) using differential display and a subtractive library screen to identify genes involved in ischemia and silica induction. A biochemical analysis of both human transformer-2 proteins demonstrates that they are sequence-specific splicing enhancers binding to the purine-rich enhancer sequence A3 (Tacke *et al.*, 1998). We recently cloned the human tra2-beta homologue due to its interaction with the SR-protein SC35 in a yeast two-hybrid screen (Beil *et al.*, 1997). The characterization of the tra2-beta gene in rat, mouse, and human demonstrated that its expression is influenced by various physiological stimuli: in brain, ischemia and reoxygenation dramatically increased the message level (Matsuo *et al.*, 1995), in macrophages, silica particles increased levels twofold (Segade *et al.*, 1996), and stimulation of cultured human T-cells changed the expression level of its alternatively spliced variants (Beil *et al.*, 1997).

The genes of several SR and SR-like proteins were completely sequenced. In vertebrates, the genes of the essential SR proteins 9G8 (Popielarz *et al.*, 1995), SC35 (Sureau and Perbal, 1994), X16/SRp20 (Jumaa *et al.*, 1997), and of the SR-related protein U1 70K (Spritz *et al.*, 1990) have been characterized. In *D. melanogaster*, the genes of transformer-2 (Mattox *et al.*, 1990), transformer (Boggs *et al.*, 1987), suppressor of white apricot (SWAP) (Chou *et al.*, 1987), and RBP1 (=X16/SRp20) (Kim *et al.*, 1992) were sequenced. The transformer-2 gene was also cloned from *D. virilis* (Chandler *et al.*, 1997). A common feature among all these genes is the generation of multiple transcripts by alternative splicing. In the case of transformer-2 (Mattox and Baker, 1991), SWAP (Chou *et al.*, 1987), and X16/SRp20 (Jumaa and Nielsen, 1997), the alternative splice seems to be autoregulated. Finally, cDNA cloning from vertebrates revealed the existence of splice variants of SRp55, SRp40 (Screaton *et al.*, 1995; Snow *et al.*, 1997), ASF/SF-2 (Ge *et al.*, 1991), and the mammalian homologue of SWAP (Sarkissian *et al.*, 1996). With the exceptions of SC35, where alternative splicing occurs in the 3′ UTR (Sureau and Perbal, 1994), and an insert in the 3′ UTR of SRp55 (Screaton *et al.*, 1995), all other splicing events introduce stop codons leading to the creation of truncated protein isoforms lacking an RRM or SR domain.

Here, we report the characterization of the complete human tra2-beta gene[3] and demonstrate that it creates tissue-specific isoforms.

MATERIALS AND METHODS

Screening and sequencing. For screening, the primers 26f1 (gaccggcgacagcagccagg) and CAS010 (caaggagctaggaccacacag) were used to screen a human BAC library (kindly performed by the German Human Gene Center, Heidelberg). The same primers were used to determine the chromosomal localization using the Stanford Radiation Panel 4. The primers generate an amplicon of 107 nt. Double-stranded sequencing was performed on an Applied Biosystems sequencer using htra2-beta-specific oligonucleotides. A list of primers used for sequencing is available upon request.

PCR. Tissue-specific isoforms were detected by PCR using the primers traex4rev (ccggttcccaacatgacgccttcg) and traex1f (ggagtcatgagcgacagcggcgag). They produce expected amplicons of 340 nt (including exon III) and 206 nt (excluding exon III). cDNAs for human brain, fetal human brain, and brain tumor tissue were obtained from Invitrogen.

Constructs. PCR primers tradelSR1 (gggaattcaccatggattatcgtagacggcacagc) and 26-Bam (ttggatcccaaaacatttgagtgaaattgg) were used to construct traΔSR1, which uses the same start codon as the htra2-beta3 variant. To construct traΔSR2, we used primers tradelSR2 (gggatccttaataaatctgatccctgtcttgggc) and SC35_26 *Eco*RI (ttgaattcggagtcatgagcgacagcggcgagcag). A variant lacking both SR domains, traΔ1+2, was created using primers tradelSR1 and tradelSR2. The full-length variant was constructed using SC35_26 *Eco*RI and 26-Bam. After being subcloned in pCR3.1, the inserts were cloned into pEGFP-C2 (Clontech) to create EGFP tagged fusion proteins C2-traΔSR1, C2-traΔSR2, C2-traΔ1+2, and C2-tra2.

Two-hybrid analysis and immunofluorescence. Two-hybrid screening was essentially performed as described (Beil *et al.*, 1997).

[3] The HGMW-approved symbol for the gene described in this paper is SFRS10, splicing factor, arginine-/serine-rich (*Drosophila* tra2 homologue) 10.

Immunofluorescence was performed using EGFP-tagged cDNAs of the htra2-beta constructs. The constructs were transfected in HeLa cells using the calcium phosphate method as described (Nayler *et al.*, 1997). Counterstaining was performed using DiI (Molecular Probes).

Cell culture and immunoprecipitation. HEK 293 cells were transfected with 1 μg of EGFP-tra2-beta constructs as described (Nayler *et al.*, 1997). Cells were lysed in RIPA buffer (1% NP-40, 1% sodium deoxycholate, 0.1% SDS, 150 mM NaCl, 10 mM sodium phosphate, pH 7.2, 2 mM EDTA, 50 mM NaF, 5 mM β-glycerolphosphate, and freshly added 4 mM sodium orthovanadate, 1 mM DTT, 1 mM PMSF, 20 μg/ml aprotinine, and 100 U/ml bezonase) for 30 min on ice. Precipitates were cleared by centrifugation and diluted fourfold in RIPA rescue (10 mM sodium phosphate, pH 7.2, 1 mM NaF, 5 mM β-glycerolphosphate, 20 mM NaCl, and freshly added 2 mM sodium orthovanadate, 1 mM DTT, 1 mM PMSF, 20 μg/ml aprotinine). Immunoprecipitations were performed overnight at 4°C using anti-GFP antibodies (Boehringer) and protein A–Sepharose (Pharmacia) followed by three washes in HNTG (Nayler *et al.*, 1997).

Northern blot. htra2-beta3 full-length cDNA was labeled with radioactive [γ-^{32}P]dCTP using the random priming method (Amersham). A human multiple tissue Northern blot (Clontech) was probed following the manufacturer's instruction.

Antibody. A synthetic peptide corresponding to htra2-beta1/2 was coupled to KLH, and rabbits were immunized by standard procedures. A detailed description of the peptide synthesis and preabsorption experiments demonstrating the specificity of the antibody will appear elsewhere (R. Daoud *et al.*, manuscript submitted).

Computer analysis. Analysis was performed using the GCG package (Genetics Computer Group, 1994). Calculation of splice site scores was as described (Stamm *et al.*, 1994). This calculation can be performed at http://www2.imcb.osaka-u.ac.jp/splice/score.html.

RESULTS

The htra2-beta Gene Structure

To analyze the htra2-beta gene, we PCR screened a human BAC library employing two primers located in exon I and intron A. Southern blot analysis using gene-specific primers indicated that the obtained clone was full length (data not shown). A *Bam*HI fragment spanning the entire gene was then subcloned into pBluescript and sequenced. Comparison of the cDNA and the genomic sequence showed that the gene consists of 10 exons and 9 introns (Fig. 1). The exon size ranges from 60 bp (exon VIII) to 972 bp (exon X), whereas the intron sizes vary from 639 bp (intron I) to 5975 bp (intron A). Potential splice sites were individually compared with a splice consensus sequence and scored for their degree of homology (Stamm *et al.*, 1994). All splice sites follow the consensus sequence (Fig. 2). However, the 5′ splice site of exon II and the 3′ splice site of exon III deviate significantly and might therefore be regulated. The overall nucleotide composition is AT rich, the gene is composed of 28% A, 18% C, 21% G, and 33% T. Analysis of the dinucleotide distribution reveals clustered CG repeats at the 5′ UTR, as found for the mouse SRp20 gene (Jumaa and Nielsen, 1997) and human 9G8 gene (Popielarz *et al.*, 1995) indicating that their promoters are located in CpG islands.

Database searches identified *Alu* elements in introns D, E, and F and in the promoter region. Interestingly, the *Alu* element found in intron F, 200 bp downstream

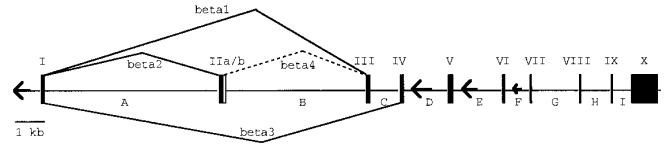

FIG. 1. Structure of the htra2-beta gene. Exons are indicated as vertical lines and numbered in roman letters. Introns are shown as vertical lines and numbered with letters. *Alu* elements are shown as arrows. The splicing events creating the htra2-beta1, beta2, beta3, and beta4 isoforms through alternative splicing are indicated.

of exon VI, bears high sequence similarity to an *Alu* element previously shown to cause exon skipping in the BRCA2 gene (Miki *et al.*, 1996). The element is flanked by an 8-bp (AAAATTTA) duplication. The poly(A) track found at its 3′ end is interrupted by several cytosine residues. The intron E *Alu* element is 88.5% identical in sequence to the *Alu*-Sp subfamily, and the intron D *Alu* element is 82% identical to the *Alu*-J subfamily consensus sequence (Jurka and Milosavljevic, 1991). The *Alu* element in intron J is flanked by an ACAAGAG repeat, but no repeat was found surrounding the element in intron E.

Like other members of the SR-protein family, transformer-2 has a modular structure reflected by its intron–exon organization. The first SR domain is composed of exons III and IV, the RRM is composed of exons V and VI, and the second SR domain is composed of exons VII and IX. The glycine-rich region that subdivides the second SR domain is encoded by exon VIII. Exons V and VI, which encode the RRM, show the highest homology to the human tra2-alpha and the *Drosophila* tra-2 gene (76%, 64% nucleotides are identical). The htra2-beta RRM displays weak sequence identity to the RRMs of other SR proteins (43% to SC35) and is more closely related to a family of glycine-rich cold stress response proteins found in plants and mammals (e.g., 66% identical to CIRP (Nishiyama *et al.*, 1997)). The RRM containing region also shows homology to hnRNPG (61%) and hnRNPA2/B1 (61%). A comparison between gene and domain structure is shown in Fig. 6.

Chromosomal Localization

Using the Stanford radiation hybrid panel with the primers 26-f1 and CAS010, we assigned the chromosomal localization of the htra2-beta gene to human chromosome 3. The probe was placed 8.77 cR from WI-6365, between WI-6365 and D3S1571. Several markers have been mapped to this interval (stSG10048, stSG2685, stSG2841, stSG3372, stSG396, stSG8306, and stSG9948), but no known transcripts were identified. Although radiation hybrid maps are not anchored to the cytogenetic maps, the most likely location is 3q26.2–q27 on the cytogenic map (Fig. 3). To our knowledge, no known phenotypes or diseases have been mapped to this region.

exon	5'S	exon sequence	intron sequence	exon sequence	3'S	exon
			(5' UTR) ...catttcggctctgagcggct	gggcgaccggcgcgtcgtgcg...		exon I 144 nt
I	7.5	..CAG AAC TAC GGC GAG CGG ..gln asn tyr gly glu arg	gtacgtagagctgcg.(A, 5975 nt) .....cctgttctttttctattaag	GTT AAT GTT GAA GAA GGA.. val asn val glu glu gly..	5.9	exon II 276 nt
II	4.7	..cag aaa gca cta cgc taa	gtaattactagtctg.(B, 4845 nt) .....cttttaacatctacgtgtag	GAA TCC CGT TCT GCT TCC.. glu ser arg ser ala ser..	2.8	exon III 134 nt
III	9.9	.G.TCC CGA TCT GAA TCT AG .g.ser arg ser glu ser ar	gtaagaaagactgtc (C, 978 nt) .....aggaggtctttgtcttgcag	G TCT AGA TCC AGA AGA AG. g ser arg ser arg arg se.	11.2	exon IV 163 nt
IV	8.9	..CGT CAT GTT GGG AAT CGG ..arg his val gly asn arg	gtaagatgaaaaact (D, 1479 nt) .....aaacttttttttttccctag	GCA AAT CCT GAT CCT AAC.. ala asn pro asp pro asn..	11.8	exon V 189 nt
V	8.7	..GTA GAT GAT GCC AAG GAA ..val asp asp ala lys glu	gtaagtaaaagcccg (E, 1670 nt) .....aatttatttacctttgaag	GCT AAA GAA CGT GCC AAT.. ala lys glu arg ala asn..	4.3	exon VI 116 nt
VI	7.4	.C ATG GGG AGA CCT ACC TA .r met gly arg pro thr ty	gtaagtttatttttca (F, 823 nt) .....gaatatttttttcttcatag	T GGC AGC TCT CGC CGT CG. r gly ser ser arg arg ar.	8.9	exon VII 84 nt
VII	12.4	.C TAT AGC AGA TCA TAC AG .r tyr ser arg ser tyr ar	gtaagttatgaggta (G, 1607 nt) .....ttgcctttctgtcttcacag	A GGA GGA GGT GGA GGA GG. g gly gly gly gly gly gl.	7.8	exon VIII 60 nt
VIII	6.6	.C AGG GAT CAG ATT TAT AG .p arg asp gln ile tyr ar	gtatgccaggagagg (H, 1004 nt) . .....atcttgacatttgtttatag	A AGG CGG TCA CCT TCT CC. g arg arg ser pro ser pr.	5.9	exon IX 74 nt
IX	6.6	.CT CGA TCA TAC TCA CCT C .er arg ser tyr ser pro a	gtaagtttttatctt (I, 639 nt) .....gttcattcttttcttcccag	GT CGC TAT TAA agc atg a. rg arg tyr OCH	13.2	exon X 972 nt

FIG. 2. Compilation of exon–intron boundaries. Coding exon sequences are indicated by uppercase letters, and intron and untranslated exon sequences are indicated by lowercase letters. Amino acids are indicated by a three-letter code. 5′ and 3′ scores (Stamm *et al.*, 1994) are given for each splice site. A perfect match to U1 snRNA would give a theoretical maximum 5′ score of 12.6, and the average score of constitutive exons is 8.0. Likewise, the theoretical maximum 3′ score is 14.2, and the average score for constitutive exons is 9.4.

Promoter Region

Analysis of the 5′ region of the htra2-beta gene revealed the presence of an *Alu* element located upstream of the putative promoter region (Fig. 4). This *Alu* element displays 82% sequence identity to the intron F *Alu* element and is flanked by a 11-bp target site duplication (TGACCTTTTTT). Another 5′ UTR region is 61% homologous to the promoter region of the POUF3 gene, which encodes the brain-specific brain-2/N-oct 3 protein (Atanasoski *et al.*, 1995). Computer searches revealed the presence of several putative transcription factor binding motifs, such as SP1 (GRGGCRGGW), CRE-BP1/c-Jun (TGACGTYA), SRY (AAACWAM), and HSF1/2 (NGAANNWTCK). Sequence comparison of the cDNAs derived from htra2-beta1 and beta2 (Beil *et al.*, 1997) showed that different initiation points could be used, and several EST clones, derived from different libraries, confirmed that the htra2-beta gene uses at least two start points.

Several mRNA Isoforms Are Generated by Alternative Splicing

We recently cloned the htra2-beta cDNA by virtue of its interaction with SC35 in a two-hybrid screen. In addition to the full-length cDNA htra2-beta1, we identified a shorter splice variant that was named htra2-beta2 (Beil *et al.*, 1997). Comparison of these two isoforms with the gene structure revealed that htra2-beta1 contained all exons except exons IIa+b. The shorter htra2-beta2 is generated by splicing exon I to exon IIa and using the polyadenylation signal present in exon IIa (Fig. 5A).

As other N-terminal variants have been described for human htra2-alpha (Dauwalder *et al.*, 1996) and the *Drosophila* gene (Mattox *et al.*, 1990), we searched the EST database (Lennon *et al.*, 1996) for alternative splice variants. Indeed, we found several EST clones that joined exon I directly to exon IV, thus skipping exon III (e.g., EST AA204749, AA205257, H11792, AA150074, and AA092783). Complete sequencing of two of these EST clones (AA204749 and AA205257) revealed that this isoform joins exon I to IV and uses all other downstream exons. We named this isoform htra2-beta3 (Fig. 5A). In addition, RT-PCR analysis (see below) revealed the existence of exon IIb, which is also present in the two mouse embryonic tissue EST clones AA675451 and W96993. This isoform, htra2-beta4, is generated by overreading the polyadenylation signal in exon IIa and using the 5′ splice site of exon

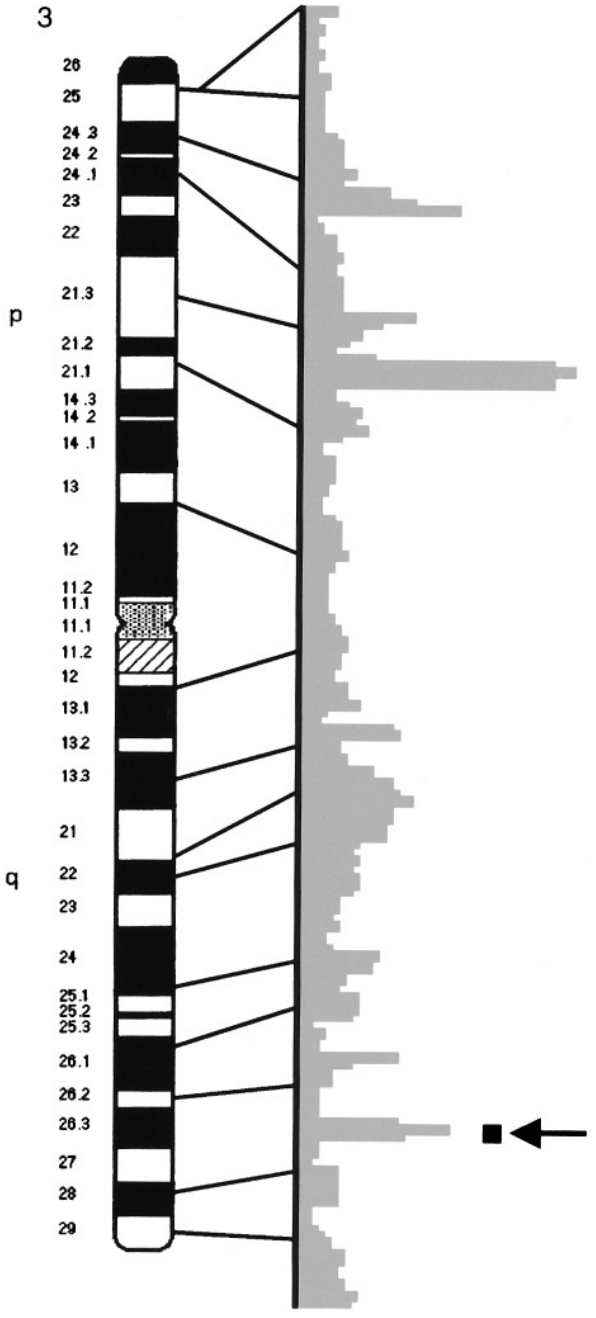

FIG. 3. Chromosomal localization of the htra2-beta gene. (**Left**) The cytogenic map of chromosome 3. (**Right**) The radiation hybrid map of chromosome 3. The location of htra2-beta mapping 8.77 cR from WI-6365 is indicated.

IIb. Finally, an EST clone derived from Jurkat cells (AA355058) started 72 bp upstream in intron B and was named htra2-beta5 (Fig. 5A). However, it is not yet clear whether this EST clone indicates the presence of a promoter in intron B or represents an incompletely spliced message.

Judged by RT-PCR (Fig. 5B) and the number of EST clones present in the database, htra2-beta4 and htra2-beta5 are minor transcripts. No ESTs indicating skipping of downstream exons were present in the database. However, in mouse, the htra2-beta gene uses several polyadenylation sites located in exon X (Segade *et al.*, 1996), which is also reflected by several human EST clones.

Tissue Distribution of tra2-beta Transcripts

To determine the tissue distribution of the htra2-beta1, 3, and 4 isoforms, we performed RT-PCR with primers located in exon I and IV using RNA from different rat and human tissues (Fig. 5B). The identity of the various PCR products was confirmed by subcloning into pCR3.1 (Invitrogen) and sequencing. In addition to the constitutively expressed product of 340 bp (htra2-beta1; Beil *et al.*, 1997), a shorter fragment was observed in brain, liver, testis, and kidney. In brain, this band appeared at embryonic day 20 (E20) and was not present at embryonic day 18 (E18). Sequencing of this fragment confirmed that it was the htra2-beta3 isoform. Similarly, we identified the larger band as the htra2-beta4 isoform and observed a weak expression in rat testis and a stronger expression in rat E18 brain and E18 total embryo, as well as in human brain, brain tumor, and fetal brain.

Summarizing, we conclude that the htra2-beta gene is expressed in various tissues in at least four isoforms, htra2-beta1–4, whereby the htra2-beta3 and htra2-beta4 isoforms appeared to be tissue-specific and developmentally controlled (Fig. 5B). This is in contrast to the htra2-beta1 and 2 isoforms, which were previously shown to be ubiquitously expressed, although their expression ratios varied (Beil *et al.*, 1997).

Generation of Two Different tra2-beta ORFs

The largest conceptual open reading frames encoded by the five RNA isoforms are indicated in Fig. 5A. The longest open reading frame is encoded by htra2-beta1 (Beil *et al.*, 1997). In the htra2-beta3 and beta4 isoforms, the start codon present in exon I is followed by stop codons created by a frameshift following exon III skipping (htra2-beta3) or by stop codons present in exon IIa (htra2-beta4). These isoforms, like htra2-beta5, use an alternative start site present in exon IV, thereby encoding a common protein lacking the first SR domain of htra2-beta1. The structure of the two ORFs encoded by the htra2-beta gene is shown in Fig. 6A. Comparison of their domain structure with htra2-alpha (Fig. 6B) and tra-2 of *Drosophila* (Fig. 6C) shows a structural resemblance between the proteins encoded by htra2-beta3 and beta4 and the dtra2^{179} protein. Due to an alternative splicing mechanism, dtra2^{179} is expressed exclusively in the male germline (Mattox *et al.*, 1990). In summary, we demonstrate that alternative splicing creates htra-2 isoforms that lack the first SR domain and that are specifically expressed in brain, liver, testis, and kidney.

Distribution of htra2-beta1 Protein in Various Tissues

Thus far, the expression of htra2-beta1 was studied only on the RNA level. Using PCR analysis (Beil

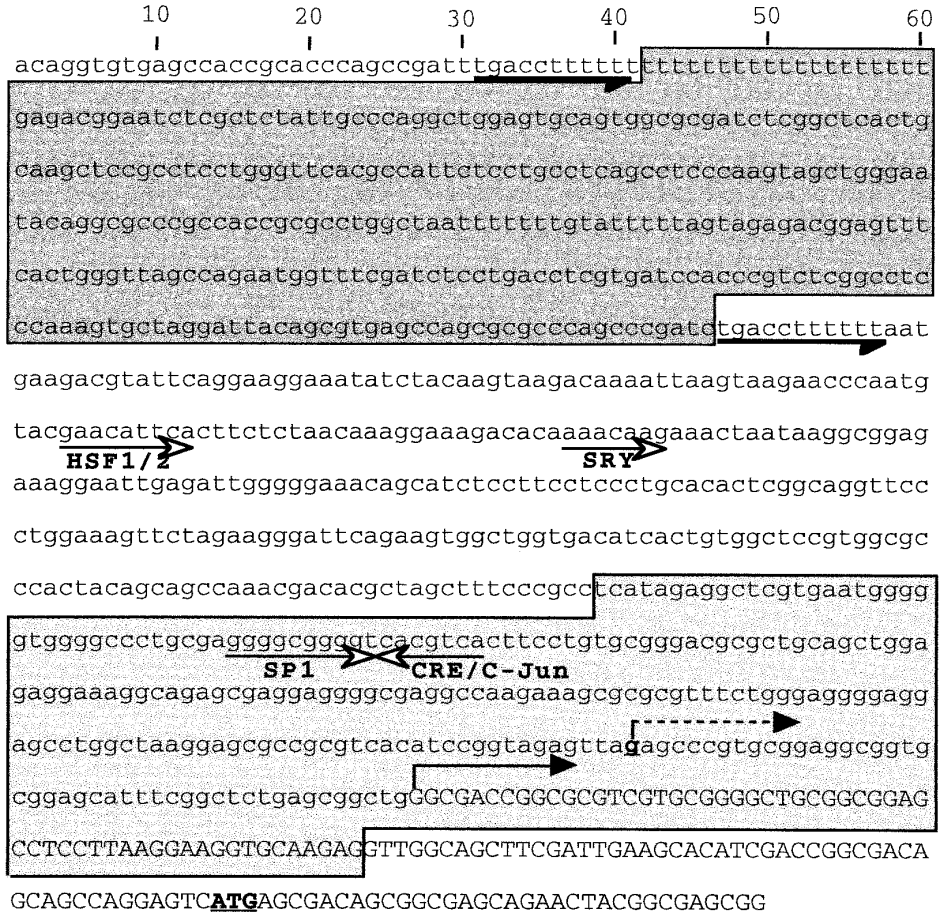

FIG. 4. 5′ untranslated region of htra2-beta. The *Alu* element is shaded in dark gray. The direct repeats marking the integration site are underlined. Light gray shading indicates the region with high homology to the human brain-2/N-oct gene. Potential binding sites for various transcription factors and the orientation of these elements are indicated with arrows. The two arrows on top of the sequence indicate the start of two classes of EST clones.

et al., 1997) it was found that htra2-beta1 is ubiquitously expressed (see also Fig. 5). However, quantitative PCR (Segade *et al.*, 1996) and RNase protection analysis (Beil *et al.*, 1997) revealed differences in RNA expression. Using quantitative PCR, the expression in rat was found to be highest in uterus, brain, and testis and lowest in kidney and liver (Segade *et al.*, 1996). To determine htra2-beta mRNA abundance more directly, we performed Northern blot analysis with human RNA and found that expression was highest in heart, skeletal muscle, and pancreas, less abundant in kidney, placenta, and brain, and lowest in liver and lung (Fig. 7A). As in rat, multiple bands that most likely reflect use of different polyadenylation sites are detected.

To test the protein distribution, we created an antiserum specific for htra2-beta1 and beta2 using an N-terminal peptide corresponding to the htra2-beta2 amino acid sequence (Daoud *et al.*, submitted for publication). With this antibody we observed the largest amounts of htra2-beta1 protein in rat testis and the developing brain (Fig. 7C). Expression in kidney and lung was observed only after prolonged exposure (data not shown). This contrasts with the htra2-beta mRNA levels detected by Northern blot (Fig. 7A) and RNase protection analysis (Beil *et al.*, 1997). Reprobing the blot with an anti-actin antibody revealed the presence of similar amounts of protein in these lanes (Fig. 7D). However, thus far we have not been able to detect a protein corresponding to the htra-2-beta2 isoform (Fig. 7C; and Daoud *et al.*, submitted for publication). We suspect that this isoform is not translated due to nonsense-mediated RNA decay (Ruiz-Echevarria *et al.*, 1996). Interestingly, we observed a band of roughly 100 kDa in extracts from brain and testis. This band could not be detected when the antibody was preabsorbed with peptide (data not shown) and might thus represent a related protein or another splice variant.

We conclude that the htra2-beta1 protein levels vary in different tissues and do not reflect the mRNA distribution. In addition, the protein in liver extracts migrated differently, which is indicative of posttranslational modification.

Intercellular Localization of tra2-beta Isoforms

As the htra-2-beta1 protein has a modular structure, we examined the influence of its various domains on the intercellular localization. We made several con-

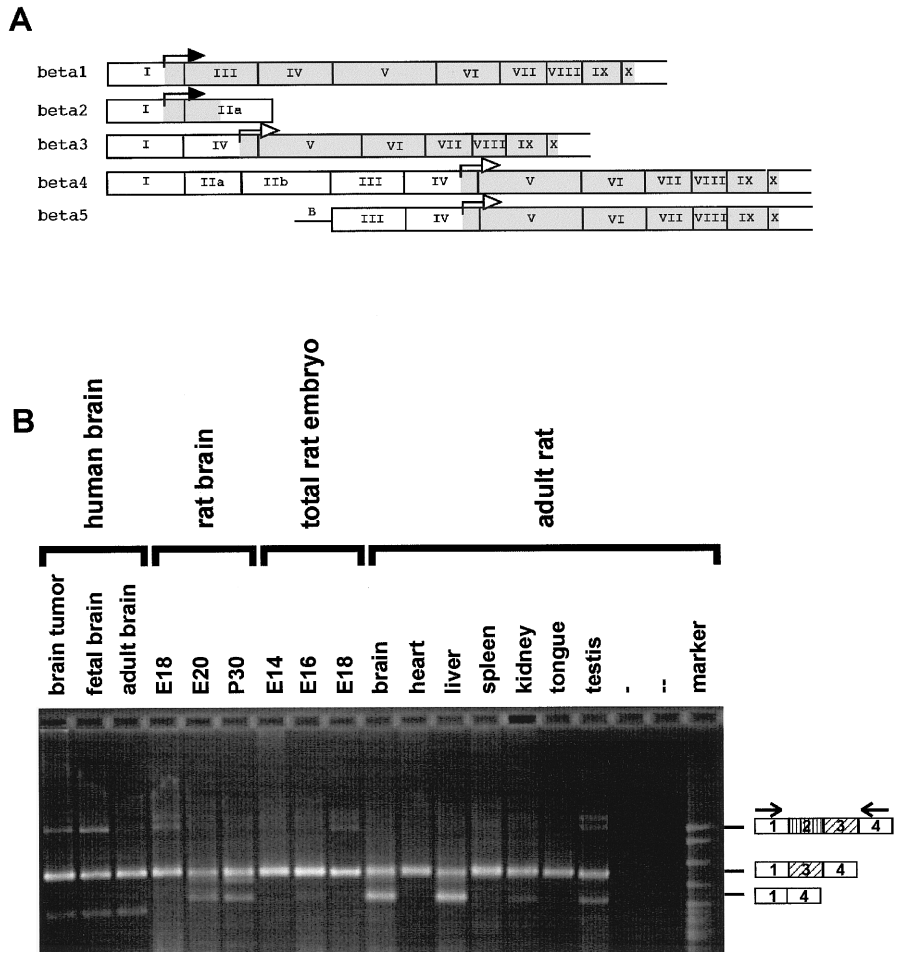

FIG. 5. htra2-beta variants and their tissue-specific expression. (**A**) Diagram of five alternatively spliced RNAs generated by the htra2-beta gene. Usage of exons in each isoform is indicated by roman numbers. The largest conceptual open reading frame is indicated by gray shading. Exons are to scale, and only the beginning of exon X is shown. The start site in exon I is indicated by an arrow; the start site in exon IV is indicated by an open arrow. (**B**) Analysis of isoform expression. RT-PCR was performed with primers specific for exons I and IV. Tissue and species are indicated. Primer location and the composition of the various PCR bands are indicated on the right.

structs lacking the first SR domain (ΔSR1), lacking the second SR domain (ΔSR2), and lacking both SR domains (ΔSR1+2). These constructs were fused to EGFP and expressed in HeLa cells. The construct lacking the first SR domain corresponds to the naturally occurring beta3 variant. Confocal microscopy analysis (Fig.) revealed that protein created by all constructs was present in the nucleus, forming a punctated pattern characteristic of splicing factors. Every construct containing at least one SR domain is present exclusively in the nucleus. In contrast, small amounts of protein from the construct lacking both SR domains were also present in the cytoplasma (Fig. 8G). We therefore conclude that, like the full-length beta1 isoform, htra2-beta3 is present in the nucleus and that this nuclear localization is due mainly to elements within the RRM.

Molecular Interactions of htra2-beta Proteins

We next investigated whether the first SR domain affected the binding properties of htra2-beta with other molecules. We tested this in the yeast two-hybrid system and show in Fig. 9 that htra2-beta1 interacts with SF2/ASF, SC35, SAF-B (Nayler *et al.*, 1998), and htra2-beta1 (Beil *et al.*, 1997) even in the presence of the histidine synthetase inhibitor 3-AT. Yeast growth was retarded but not abolished using a bait construct lacking the first SR domain. Retarded growth was not observed when the second or both SR domains were deleted. Furthermore, deletion of the SR domain of SF2/ASF abolished the interaction with all htra2-beta variants, indicating that the interaction was mediated by its SR domain. Similar results were previously obtained in a yeast two-hybrid assay and biochemical experiments using the *Drosophila* variant tra2^{179} (Amrein *et al.*, 1994), which structurally resembles the htra2-ΔSR1 construct.

Our finding that a construct lacking both SR domains showed a stronger yeast two-hybrid interaction than the construct lacking only the first SR domain is surprising. We therefore tested the interactions of the htra2-beta variants with SR proteins by immunoprecipitation. We overexpressed EGFP-tagged htra2 constructs in 293 cells and precipitated the formed complexes with an anti-GFP antibody. Benzonase was

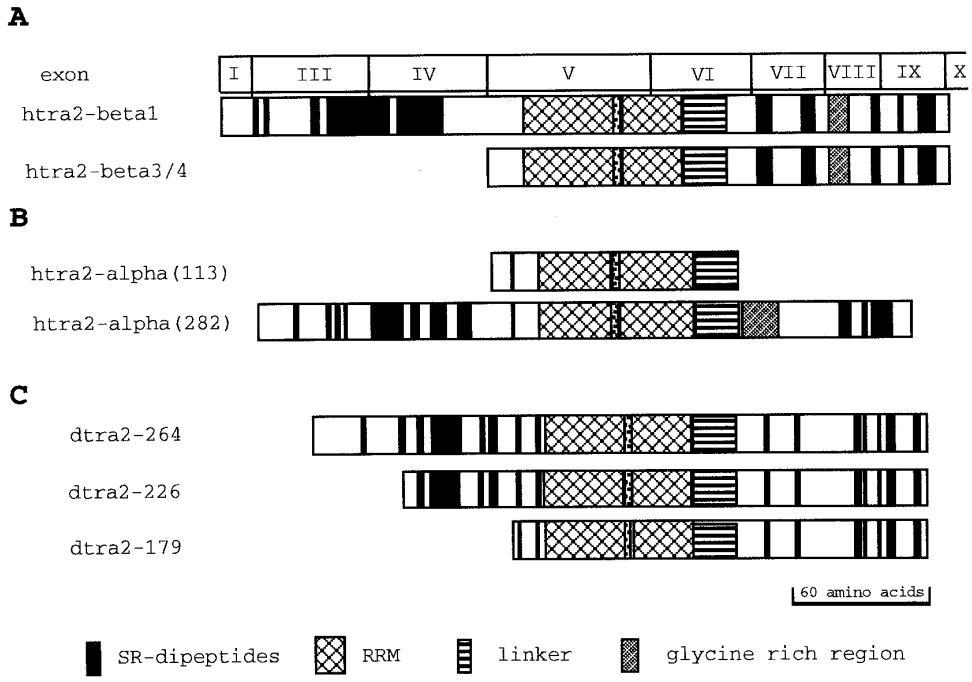

FIG. 6. Schematic representation of htra2-beta open reading frames. (**A**) Comparison of the exon–intron organization of the htra2-beta gene and the domain structure of its variants. The exon structure is shown at the top. Expressed proteins are shown below. SR dipeptides, the RRM, linker and the glycine-rich region are indicated. The beta2 variant is not shown because it is not detectable with an antibody. (**B**) The domain structures of the human tra-2 alpha gene variants. (**C**) The domain structures of the *Drosophila* gene variants.

included in the reactions to prevent detection of RNA-mediated interactions. SR proteins were detected with the SR protein-specific antibody mAb104. Interestingly, this antibody also reacted with both SR domains of htra2-beta1 (Fig. 10A). This indicates that both SR domains of htra2-beta1 are immunologically related to the SR-rich regions of the "classical" SR proteins. Furthermore, as shown in Fig. 10A, EGFP-htra2, EGFP-htra2-ΔSR1, and EGFP-htra2-ΔSR2, but not EGFP-htra2Δ1+2 bind to SR proteins in the range of 35 kDa. The last finding contrasts with the htra2Δ1+2 interactions observed in yeast, and we suspect that the two-hybrid data could be an artifact or indicative of an RNA-mediated protein interaction. Again, in contrast to our yeast data, we were not able to see a difference in binding between EGFP-htra2-ΔSR1 and other constructs containing SR domains. This underscores the fact that the yeast growth retardation observed with the construct lacking the first SR domain may reflect very subtle differences in binding to other known SR

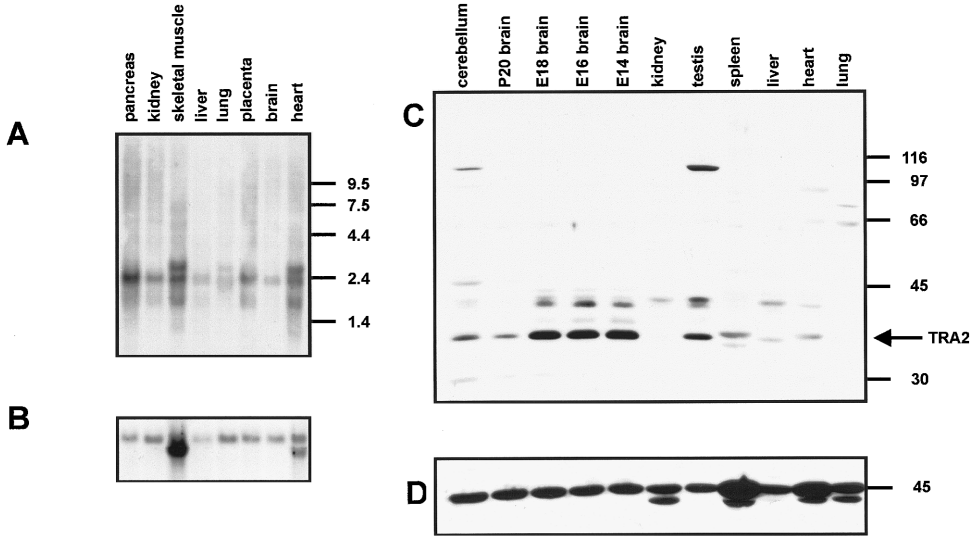

FIG. 7. Expression of htra2-beta1. (**A**) Northern blot analysis of htra2-beta. (**B**) The same blot was reprobed with actin. (**C**) Lysates from various tissues were separated on 10% SDS gels and probed with the anti-htra2 antibody. The band around 34 kDa (indicated with an arrow) corresponds to htra2-beta1. (**D**) The same filter was stripped and probed with anti-actin antibody, demonstrating that similar amounts of protein were loaded in each lane.

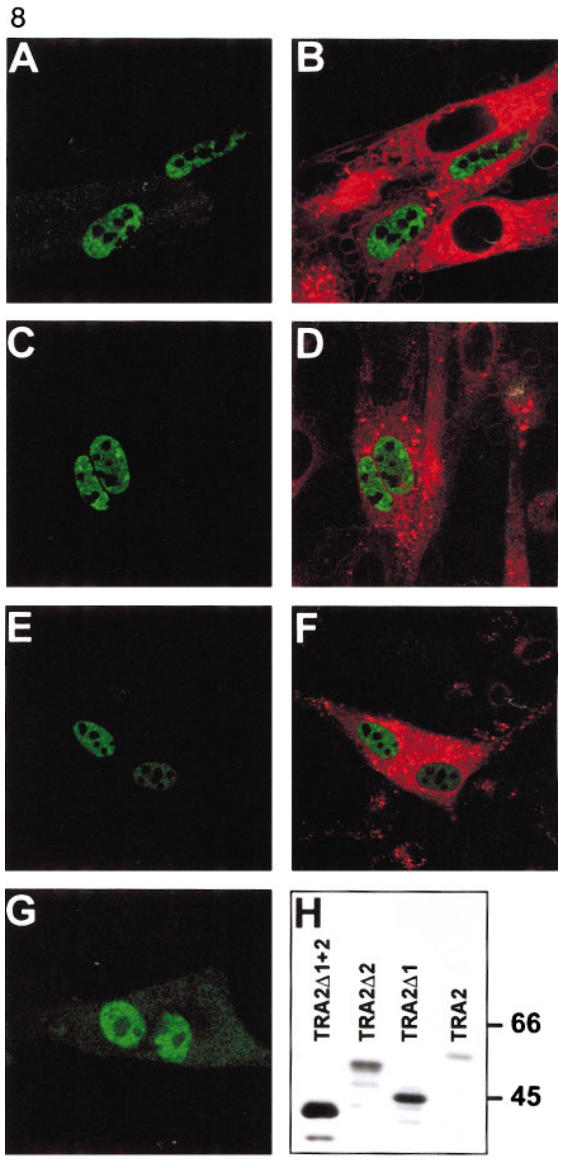

FIG. 8. Intracellular localization of htra2-beta1 variants. EGFP-tagged htra2-beta1 (**A, B**), htra2-ΔSR1 (**C, D**), htra2-ΔSR2 (**E, F**), and htra2-ΔSR1+2 (**G**) were transfected into HeLa cells and analyzed by confocal microscopy. (**A, C, E, G**) Green fluorescence generated by the EGFP fusion proteins. (**B, D, F**) Cell membranes were counterstained with DiI to highlight the nuclear localization; the overlay of the DiI and EGFP staining is shown. Magnification was 100×. (**H**) Western blot of cell lysates probed with anti-EGFP antibody demonstrates that the expected proteins were expressed.

proteins. We therefore conclude that the full-length tra2-beta1 isoform and the variants lacking the first SR domain (htra2-beta3–5) bind to a subset of SR proteins with similar affinity.

DISCUSSION

Although the human transformer beta gene and the *D. melanogaster* and *D. virilis* genes encode proteins of similar modular structure, they are different in size. The human gene is about five times larger than its *Drosophila* homologues. The protein domain composition is often reflected in gene structure, and the human tra2-beta gene is another example that this feature can be evolutionary conserved. However, the first SR domain is encoded by one exon (exon IV) in *Drosophila* and by two exons (exons III and IV) in human. Likewise, the second SR domain is encoded by one exon (exon VI) in *Drosophila* and by two exons (exons VIII and IX) in humans. Furthermore, a glycine-rich region present in the human alpha and beta genes but not in *Drosophila* is encoded by the single exon VIII. In both organisms, a tissue-specific isoform is generated by alternative splicing. In humans, using either exon II or skipping exon III creates a protein lacking the first SR domain that is expressed in brain, liver, testis, and kidney. A similar isoform is created by inclusion of intron MI in the male germline of *Drosophila*. This emphasizes the conservation of protein isoform generation in distant species and finds its reflection in the overall gene structure.

Another remarkable feature of the htra2-beta gene is its size. The coding region spans 21,232 bp, which is larger than the coding regions of SC35 (3028 bp (Sureau and Perbal, 1994)), 9G8 (7745 bp (Popielarz *et al.*, 1995)), and SRp20 (X16) (about 10 kb (Jumaa *et al.*, 1997)). However, U1 70K, another human SR-like protein, has been determined to be larger than 44 kb (Spritz *et al.*, 1990). The chromosomal localization of htra2-beta1 on chromosome 3 q26.2–q27 differs from the localization of other members of the SR-like and SR protein families: 9G8 was mapped to chromosome 2p22–p21 (Popielarz *et al.*, 1995), SC35 to 17q25, SRp40 to 14q24 (Snow *et al.*, 1997), and SF2/ASF to 17q21.3–q22. Mouse SRp20 (X16) was mapped to mouse chromosome 17 between markers D17Mit46

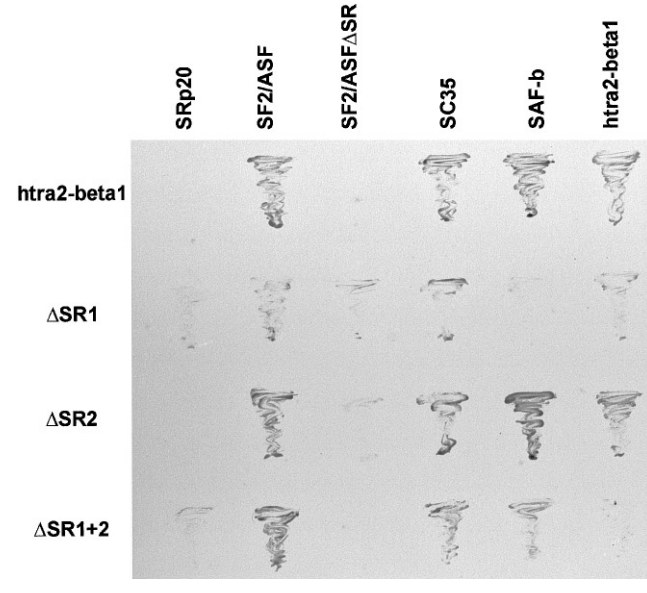

FIG. 9. Interaction of htra2-beta1 with other splicing components in the yeast two-hybrid system. htra2-beta1, htra2-ΔSR1, htra2-ΔSR2, and htra2-ΔSR1+2 were tested with SRp20 (X16), SF2/ASF, SF2/ASFΔSR, SC35, SAF-B, and htra2-beta1. Colonies obtained on His^- plates were restreaked on 10 mM AT plates.

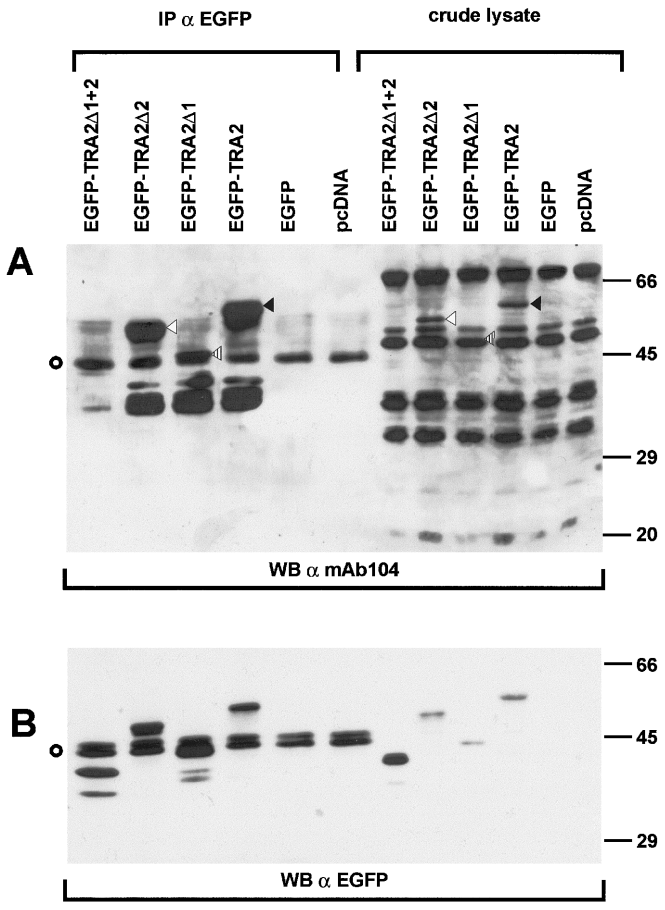

FIG. 10. Coimmunoprecipitation of htra2-beta variants and SR proteins. (**A**) The indicated htra2-beta variants were overexpressed in 293 cells and used for immunoprecipitations with an anti-EGFP antibody. Equal aliquots of the cell lysate (crude lysate) were also loaded as expression controls. The blot was probed with the SR protein-specific antibody mAb104. A small circle indicates the position of the immunglobulin bands. Triangles indicate the overexpressed htra2-beta variants that are recognized by the mAb104 antibody. (**B**) The filter was subsequently stripped and reprobed with the anti-EGFP antibody. A circle indicates the immunglobulin bands that are recognized by the secondary antibody.

and D17Mit80 (Jumaa *et al.*, 1997), and human U1 70K was mapped to chromosome 19q13.3 (Spritz *et al.*, 1990). These data clearly indicate that there is no clustering of genes belonging to the SR-protein family and further suggest that the expression of these genes cannot be coordinated by activation of one or a few chromosomal regions.

Like the SC35, 9G8, and SRp20 genes, the htra2-beta gene shows a clustering of CpG dinucleotides in the 5′ UTR, indicating that the SR protein promoters are located in CpG islands, which are frequently subject to regulation by methylation. In fact, the htra2-beta promoter has been isolated in a screen to identify nonmethylated and therefore transcriptionally active CpG islands (Cross *et al.*, 1994). It remains to be determined whether the htra2-beta gene can be imprinted like the genes of the splicing components SmN (Leff *et al.*, 1993) and U2AF-35 (Hayashizaki *et al.*, 1994).

The htra2-beta gene is regulated at several levels. RNA expression levels vary among tissues, which is most likely due to differential promoter usage. In addition, the promoter might be inactivated by methylation in certain cell lineages. RNA expression was highest in heart, muscle, and pancreas. The second level of regulation is alternative splicing. It creates at least four different isoforms, one of which is not translated and probably serves as an RNA storage pool. Furthermore, different 3′ UTRs are created by the use of at least four polyadenylation sites, resulting in mRNAs of different stability (Segade *et al.*, 1996). Finally, the protein might be subject to differential phosphorylation as indicated by the different SDS gel mobilities of the tra2-beta protein isolated from different tissues.

As the htra2-beta3 isoform is found only in certain tissues, it is unlikely to constitute a basic splice factor, which would be expected to be expressed ubiquitously. In *Drosophila*, the role of both dtra2 SR domains was studied (Amrein *et al.*, 1994). It was found that the first SR domain was dispensable, whereas the second SR domain and the RRM were necessary for the rescue of the female-specific phenotype in tra2$^-$ females. In agreement with these studies, our binding experiments show that the first SR domain of the human tra2-beta protein may have a subtle influence on the binding to other SR proteins. However, in contrast to the analysis in *Drosophila*, our yeast two-hybrid and immunprecipitation data indicate that the second SR domain is dispensable for binding to certain other SR proteins, indicating differences between human and *Drosophila* transformer-2 proteins. The function of the transformer-2 isoform lacking the first SR domain could thus reside in the modulation of spliceosomal activity in a tissue- or cell-specific way by interacting with proteins other than the previously known SR proteins.

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