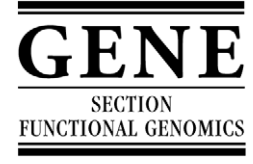


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Genomic structure, alternative splicing and tissue expression of *rFrp/sFRP-4*, the rat frizzled related protein gene[☆]

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Abstract

Secreted frizzled related proteins (sFRP) are regulators of Wnt signaling pathways that play central roles in developmental processes and oncogenesis. Various sFRP genes have been cloned from different tissues and implicated in diverse biological activities. rFrp, the rat homologue of sFRP-4, was initially identified as being upregulated in mutant p53-induced cellular transformation. Here, we report on the isolation of five novel splice variants, rFrp/sFRP-4 II, III, IVa and IVb. The complete rFrp/sFRP-4 genomic structure spans over 31 kb covering 9 exons. Except for the variant IVb, which was derived from IVa by alternative polyadenylation signal, variants I to IVa were alternatively spliced to different exons in the 3' end of mRNA and resulted in transcripts with truncated open reading frame. The deduced proteins of the variants had truncated C-termini, however, the two key functional protein domains, the cysteine-rich domain and the netrin-like domain of the isoforms, were not altered. In addition, different transcriptional initiation sites were found with variants II and IV, implying that these variants may be regulated differently from the rFrp/sFRP-4. RT-PCR analysis showed that these splice variants displayed different patterns of tissue-specific expression. Northern blot analysis revealed that the rFrp/sFRP-4 is most abundant in the ovary. Taken together, our findings suggest that alternative splicing of rFrp/sFRP-4 plays a role in regulating tissue-specific expression. The truncated C terminals of rFrp/sFRP-4 variants may confer structural specificity and hence exert different biological functions in different tissues. Characterization of these novel splice variants should help to elucidate the function of the sFRP family gene.

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Keywords: Ovary; Wnt signaling; Netrin-like domain; Cysteine rich domain

1. Introduction

Secreted frizzled related protein (sFrp) homologues have been independently isolated from bovine, Xenopus, mouse,

rat and human tissues by different laboratories (Hoang et al., 1996; Leyns et al., 1997; Wang et al., 1997; Rattner et al., 1997; Wolf et al., 1997; Zhou et al., 1998; Chang et al., 1999; Katoh, 2001; Finch et al., 1997). Frp protein contains a secreted signal peptide at the amino terminus and is followed by an extracellular cysteine-rich domain (CRD) which is highly homologous with the CRD present in the frizzled (Fz) family proteins (Hoang et al., 1996; Leyns et al., 1997; Rattner et al., 1997). The discovery of the CRD motif led to the finding that sFrp may act as an antagonist, competing with Fz for Wnt ligands, thereby modulating Wnt signals during development and carcinogenesis (Finch et al., 1997; Bafico et al., 1999; Dennis et al., 1999; Wada et al., 1999; Ladher et al., 2000; Üren et al., 2000; Bergwitz et al., 2001; Dann et al., 2001; Marvin et al., 2001). The C-

Abbreviations: sFrp, Secreted frizzled related protein; Fz, Frizzled; CDR, Cysteine-rich domain; NTR, Netrin-like; CNS, Central nervous system; PCR, Polymerase chain reaction; RT-PCR, Reverse transcriptase-PCR; R6, Rat 6 cell line; CREB, Cyclic AMP responsive element binding protein; CBP, CREB binding protein; RACE, Rapid amplification of cDNA ends.

[☆] Sequence data from this article have been deposited with the DDBJ/EMBL/GenBank Data Libraries under Accession Nos. AF140346, AF140347.

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terminal half of Frp conserves a netrin-like (NTR) domain that shares weak sequence similarity with the axon guidance protein, netrin. The NTR domain is also found in other proteins, including type I procollagen C-proteinase enhancer proteins, and tissue inhibitors of metalloproteinases. Evidence suggests the NTR domain of Frp may involve matrix stability, inhibit motility and promote growth of glioma cells (Roth et al., 2000). The precise interaction of each Frp protein in modulating Wnt signaling remains to be elucidated.

The physiological role of Frp is unclear. Involvement of Frp in apoptosis has been suggested by several investigators. One early study indicated that a Frp gene, *frpAp*, acts as a pro-apoptotic gene in rat corpus luteum (Wolf et al., 1997). Recently, an apoptotic role of sFrp2 was cited in the developing CNS (Ellies et al., 2000; Kim et al., 2001). Another study suggested that FrzB-2/sFrp3 may be involved in the pathogenesis of human osteoarthritic cartilage through its role in apoptosis (James et al., 2000; Ijiri et al., 2002). In human myocardium, expressions of sFRP3 and sFRP4 are correlated with the expressions of pro-apoptotic genes (Schumann et al., 2000). The involvement of Frp in human cancer has also been cited in several recent reports. For example, down-regulation of Frp expression has been observed in multiple human tumors, including those in kidney, breast and ovary, and also in mammary carcinoma cell lines (Zhou et al., 1998; Ugolini et al., 1999; Ugolini et al., 2001; Preiherr et al., 2000; Wong et al., 2002). In contrast, upregulation of FrzB has been found in primary malignant plasma cells (De Vos et al., 2001). Frp1 has also been found to promote tumor growth through the anti-apoptotic effect (Roth et al., 2000; Fukuhara et al., 2002). The contradictory roles of Frp proteins in apoptosis and carcinogenesis suggest that different Frps may exert different effects in different tissues or organs. Thus, the precise functional roles of the various Frps remain to be elucidated. In our previous study, a novel rat Frp (*rFrp*) gene was identified as one of the differentially expressed genes activated in Rat 6 fibroblast cell lines overexpressing p53^{val135} tumor suppressor gene. This *rFrp* gene is normally silent in the parental Rat 6 (R6) cells (Yam et al., 1999). Nucleotide sequence alignment indicates that *rFrp* appears to be the orthologue of human and mouse *sFRP-4* genes and is referred to as *rFrp/sFRP-4* in this study. In the attempt to understand the molecular basis of the transcriptional regulation of the *rFrp/sFRP-4*, a series of studies was conducted on the promoter region of the gene. We mapped the regions essential for the transcription of *rFrp/sFRP-4*, and this mapping revealed a number of putative consensus motifs for hematopoietic-specific and sex-determining transcriptional factors (Yam et al., 2001a). In a subsequent study, we further demonstrated that the cyclic AMP responsive element binding protein (CREB) transcriptional factor is crucial for the positive promoter activity of *rFrp/sFRP-4* demonstrated by transient reporter and site direct mutation assays. More importantly, CREB, phosphorylated

CREB, and the CREB binding protein (CBP) were found binding to the endogenous *rFrp/sFRP-4* promoter in vivo using chromatin immunoprecipitation assay (Yam et al., 2003).

As establishing the genomic organization of *rFrp/sFRP-4* and its expression patterns are necessary toward the understanding of various roles of *rFrp/sFRP-4*, we report here on the identification of five novel splice variants of *rFrp/sFRP-4*. The genomic origins and tissue specificity of each splice variant are described in this report. Our findings provide a framework for elucidating the biological significance of the *rFrp/sFRP-4* and its isoforms.

2. Materials and methods

2.1. cDNA library screening

A rat brain cDNA library constructed in λ Zap II (Stratagene) was screened using the ³²P-labeled 1.7 kb rFrp/sFRP-4 cDNA (GenBank accession no. AF140346) as probe. Filters containing 4×10^5 plaques were hybridized overnight at 42 °C in 50% deionized formamide, 10% dextran sulphate, 1% SDS and 1 M NaCl solution containing the ³²P-labeled probe. Filters were washed at 65 °C and exposed to autoradiography. One positive clone, rFrp/sFRP-4IV was isolated by in vivo excision according to the manufacturer's protocol.

2.2. 5'- and 3'-rapid amplification of cDNA ends (RACE)

Isolation of rFrp/sFRP-4 cDNA splice variants was performed using Marathon cDNA Amplification Kit (Clontech) with the T2 adaptor-ligated RACE cDNA library that we previously constructed (Yam et al., 2001b). The rFrp/sFRP-4 is highly expressed in T2 cells (Yam et al., 1999). First round PCR for 5'- and 3'-RACE was performed using library adaptor-specific primers AP1 (5'-CCATCCTAA-TACGACTCACTATAGGGC-3') or AP1A (5'-TAATAC-GACTCACTATAGGGCTCGA-3') and rFrp/sFRP-4IV gene-specific primers E5F (5'-GGATGATGCTTCTTGAAAATTGTTTAG-3') or E9R (5'-GCCACAGCAGTGCATACTGCCA-3'). The second round PCR was done using AP2 (5'-ACTCACTATAGGGCTCGAGCGGC-3', nested to AP1 or AP1A) and gene-specific primers nested to primers E5F and E9R. The 5'- and 3'-RACE products were directly sequenced by BigDye Terminator Kit using ABI PRISM 377 Automatic Sequencer (Applied Biosystems).

2.3. Determination of genomic structure

Genomic DNA was extracted from the tail of SD rat by proteinase K digestion. Sense and antisense primers were designed from each exon of the isolated rFrp/sFRP-4 cDNAs. Amplification of introns using primer pairs corresponding to the consecutive exons was performed by

using Expand 20 kb plus PCR system (Roche). PCR products were then sequenced directly. Determination of the exon/intron and intron/exon junctions were performed by sequence alignment between the amplified genomic DNA clones and the cDNA splice variants by Mac DNASIS v2 (Hitachi).

2.4. Determination of transcriptional start sites

Transcription initiation sites for splice variants were mapped using a RACE-based method in conjunction with the GeneScan Analysis using ABI PRISM 377 Automatic Sequencer (Applied Biosystems) (Yam et al., 2001b). Antisense gene-specific primers E5/E8R and E5/E9R were designed according to the specific exon/exon junction of rFrp/sFRP-4II and rFrp/sFRP-4IV cDNA, respectively. The 5' untranslated regions containing the transcriptional start sites were amplified using AP1 and gene-specific primers. Nested PCR was then performed using nested fluorescent labeled library adaptor primer 6-FAM AP2 and nested gene-specific primers E1R (5'-GCTACCAGGATGGAGAG-GAGCAT-3'). The nested PCR product was mixed with GeneScan 400HD [ROX] size standard (Applied Biosystems), denatured and electrophoresed on a 5% polyacrylamide gel at constant 3000 V at 51 °C for 2.5 h using ABI PRISM 377 Automatic Sequencer. Sizes of the PCR products were determined by GeneScan Analysis, version 3.1 software (Applied Biosystems). Single or multiple transcription initiation sites of each RACE cDNA were identified.

2.5. Tissue expression of rFrp/sFRP-4 by RT-PCR

Total RNA was isolated from rat tissues (brain, heart, thymus, lung, liver, spleen, kidney, adrenal gland, stomach, intestine, colon, uterus, ovary, testis, skeletal muscle and skin) of adult SD rat and cell lines (R6#13-8 and T2) (Yam et al., 1999). First-strand cDNA was synthesized from 5 µg of total RNA using SuperScriptTMII RNase H-Reverse Transcriptase (Gibco BRL) and random hexamer N6 (Gibco BRL) according to the instructions manual. Each splice variant was specifically amplified by the respective exon/exon specific primers using AmpliTaq Gold (Applied Biosystems) under the following conditions: 95 °C for 12 min and then 50 cycles of 94 °C for 30 s, 64 °C for 1 min, 72 °C for 30 s with a final extension of 5 min.

2.6. Northern blot analysis

Total RNA isolated from rat tissues and poly(A)⁺RNA isolated from R6#13-8 and T2 cells were fractionated on a 1% formaldehyde agarose gel and transferred to a Hybond N-plus membrane (Amersham). The membranes were hybridized overnight at 42 °C in 50% deionized formamide, 10% dextran sulphate, 1% SDS and 1 M NaCl solution containing the ³²P-labeled rFrp/sFRP-4 cDNA probes.

Filters were washed once in 2 × SSC and 0.1% SDS, and once in 1 × SSC and 0.1% SDS at 65 °C. Membranes were stripped and reprobed with actin to normalize RNA loading.

2.7. In situ hybridization

Rat ovaries and were fixed overnight in 4% paraformaldehyde at 4 °C, embedded in paraffin, subsequently sectioned and mounted on glass slides. The Sense and antisense rFrp/sFRP-4 containing plasmids were linearized with *Eco*RI and *Bam*HI, respectively. The [³⁵S]-uridine triphosphate (UTP)-labeled sense and antisense transcripts were generated using T7 and T3 RNA polymerase, respectively (Promega). Slides were dewaxed, rehydrated, and hybridized overnight in a humid box (containing 50% formamide, 5 × SSC) with a sense or antisense ³⁵S-labelled probe in hybridization solution (50% deionized formamide, 0.3 M NaCl, 5 mM EDTA, 20 mM Tris-HCl, 10% dextran sulphate, 2 × Denhardt's buffer, 0.5 mg/ml yeast tRNA, 10 mM DTT) at 55 °C. Following a series of washings under stringent conditions, the slides were dehydrated and subsequently dipped into a photographic emulsion (Amersham). The slides covered with emulsion were dried and stored in the dark at 4 °C. After 3 days of exposure, the sections were developed.

3. Results and discussion

3.1. Isolation of splice variants of rFrp/sFRP-4

Previously, the rFrp/sFRP-4 cDNA (GenBank accession no. AF140346) of 1715-bp had been isolated by 5'RACE from Rat 6 embryo fibroblast cell line transformed by mutant p53 (T2 cell line) (Yam et al., 1999). With Northern blot analysis of T2 mRNA, three sizes of transcript: 1.8 kb, 2.1 kb, and 3.3 kb in T2 cells have been identified (Yam et al., 1999). In order to identify the nucleotide sequence of each transcript, we screened an adaptor-ligated T2 cDNA library (T2 Library) that we had constructed earlier (Yam et al., 2001b). By PCR amplification of the T2 cDNA Library using various regions of 5' and 3' sequences of rFrp/sFRP-4 cDNA, we identified four rFrp/sFRP-4 splice variants, which are named as rFrp/sFRP-4I, II, III, and IVa. An additional splice variant, designated as rFrp/sFRP-4IVb, was isolated by screening a rat brain cDNA library using the full-length rFrp/sFRP-4 cDNA as probe (Fig. 1A). rFrp/sFRP-4IVa and rFrp/sFRP-4IVb are identical except that rFrp/sFRP-4IVb possesses an extended sequence at the 3' end, and both transcripts contain a putative poly(A) signal at the 3' end. These two transcripts are suspected to be generated by alternated poly(A) sites. Sequence analysis of the cDNA of all splice variants suggested that these transcripts share similar sequences at the 5' region, but differ at the 3' region of the transcripts (Fig. 1A). With the exception of variant IVb, which contains two adjacent non-

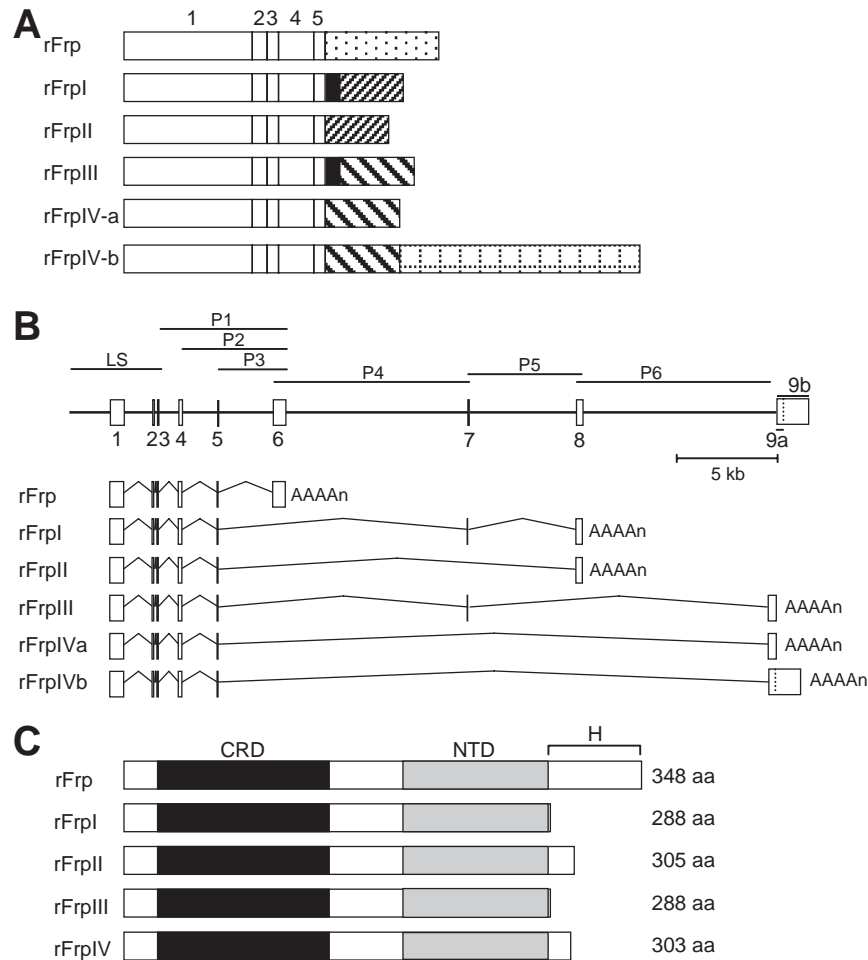


Fig. 1. Schematic representation of the *rFrp/sFRP-4* gene and its splice variants. (A) cDNA of splice variants of *rFrp/sFRP-4* showing the conserved 5'-end and the variable 3'-end regions. (B) Genomic organization of *rFrp/sFRP-4* gene. The 5' region of *rFrp/sFRP-4*, clone LS of 4.5-kb was obtained by genomic library screening using the 1.7-kb *rFRP* cDNA (GenBank accession no. AF140346) as a probe. Overlapping genomic fragments, P1-P6 were obtained by PCR using exon/intron-specific primers. Exons are numbered and indicated by boxes. Introns are represented by horizontal lines. Pattern of alternative splicing of each splice variants are shown below. (C) Schematic representation of the predicted protein structural domains of *rFrp/sFRP-4* and its splice variants. The cysteine rich domain (CRD) is located at residues 22–137 and the netrin-like domain (NTR) resides at residues 187–284, followed by a hydrophobic tail (H). Number represents the number of residues encoded by the each splice variant.

canonical poly(A) signals, UAUAAA, all variant transcripts contain the consensus AAUAAA poly(A) sequence at the 3' terminal. To our knowledge, no other variant of *Frp* family genes has ever been reported.

3.2. Genomic organization of *rFrp/sFRP-4*

In a previous study, a genomic fragment, LS of 4483-bp (GenBank accession no. AF140347) spanning the 5' untranslated region and exons 1 to 3 of *rFrp/sFRP-4*, was isolated by genomic library screening using 1.7-kb *rFrp/sFRP-4* cDNA as a probe (Yam et al., 2001a) (Fig. 1B). To map the remaining genomic structure, we designed primers based on exon sequences for amplifying genomic sequences in order to deduce the sizes of exons and introns. A total of 6 genomic fragments, P1 to P6 with sizes ranging from 0.5 kb to 9 kb were amplified (Fig. 1B). The exon–intron boundaries were determined by direct sequencing.

The sequences of the splice junctions are summarized in Table 1. All of the intron–exon boundaries contained the consensus splice donor–acceptor pair (GT-AG) except the

Table 1
Sequences of splice junctions of the *rFrp/sFRP-4* gene

Exon no.	Exon size	5' splice donor	3' splice acceptor	Intron size
1	692 bp	CGGAAG g taaga	tttcagATGTGA	1.2 kb
2	81 bp	GCCCTG g tgagt	tttcagATCGGT	0.1 kb
3	66 bp	GCTATG g taagt	tttcagTTATTC	0.8 kb
4	199 bp	TCAAG g taggt	ttttagGATGAT	1.6 kb
5	63 bp	TCCACAg g taagt	ccccagCAGTGG	2.5 kb
6	615 bp	CATGTG g ttggt	ttgaggGAGACC	8.9 kb
7	72 bp	AAACAG g taaca	tttcagGTTC	5.1 kb
8	349 bp	AACACTactttt	tttcagGATGTC	8.6 kb
9a	401 bp	–	–	–
9b	1379 bp	–	–	–

Exon and intron sequences are indicated by uppercase and lowercase letters, respectively. The consensus splice donor–acceptor sequences are bold.

5' splice donor of exons 6 (-TT), 8 (-AC) and the 3' splice acceptors of exon 7 (-GG). Intriguingly, the use of splice site in creating the variants, in this instance, seems to be conformed to the canonical GT-AG site, but not to the non-canonical sequences as found in the human genome (Chong et al., 2004). This is evidenced by the alternative splicing activities in generating the isoforms that we observed, i.e. the 5' splice donor of exon 5 spliced to three 3' splice acceptors of exon 7, 8, or 9; and 5' splice donor of exon 7, but not 8, spliced to 3' splice acceptor of exon 8 (Table 1). Sequence comparison of the isolated genomic fragments with the known cDNA sequences revealed the full rFrp/sFRP-4 genomic structure that spans over 31 kb covering at least 9 exons (Fig. 1B). Until this work, no genomic structure of Frp genes had been constructed based on the actual sequencing data. However, the intron–exon relationship of human sFRP4 was deduced based on the available Ensembl data by Jones and Jomary (2002). Interestingly, the genomic organization of the predicted sFRP4, which spans about 11 kb covering exons 1–6 of sFRP4 gene, closely resembles that of the rFrp/sFRP-4, which was deduced based on our actual sequence data. In the same report, the authors also predicted a second transcript variant lacking the 81-bp exon 2. However, we did not detect such a transcript variant among the rFrp/sFRP-4 splice variant forms.

3.3. Truncated C terminus of rFrp/sFRP-4 splice variants

Except for the rFrp/sFRP-4IVb transcript which was generated through alternative polyadenylation signal, all four alternatively spliced transcripts, rFrp/sFRP-4I, II, III and IVa were produced by perfect exon-skipping and resulted in in-frame, but truncated open reading frame (ORF) (Fig. 1C). The translational stop codon of rFrp/sFRP-4 exists in exon 6 and the ORF of rFrp/sFRP-4 encodes a full-length protein of 348 amino acids. Structurally, rFrp/sFRP-4 contains a signal peptide sequence comprising 20–30 amino acids for secretion at the amino terminus followed by a CRD at amino acid residues 22–137. A NTR domain is located at residues 187–284 (Banyai and Pathy, 1999) followed by a 60-amino acid hydrophilic carboxyl terminus. The CRD is responsible for binding to Wnt protein, and cysteine disulphide bonds are reported to form within the NTR domain of sFRP1 (Chong et al., 2002). Both rFrp/sFRP-4I and rFrp/sFRP-4III transcripts skipped exon 6 and spliced to exon 7 where translation terminated, and formed the identical truncated protein with 288 amino acids. Translation of rFrp/sFRP-4II terminates in exon 8 and has 305 amino acids, while translations of rFrp/sFRP-4IVa and b presumably terminates in exon 9 and yields same protein

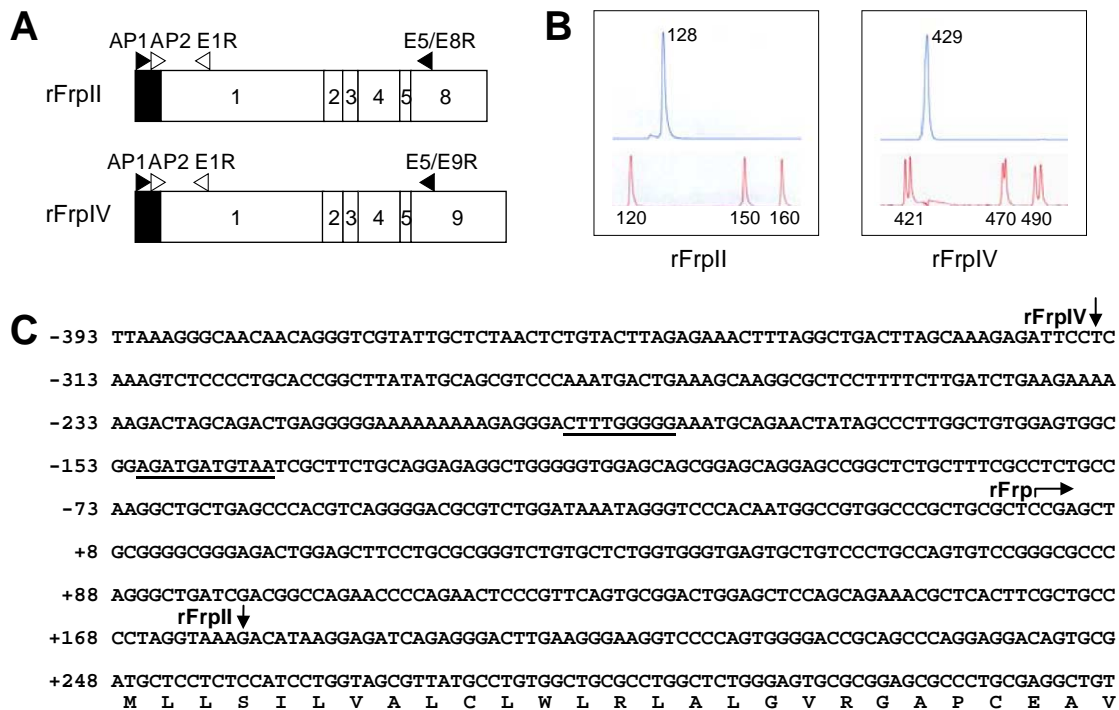


Fig. 2. Determination of transcriptional start sites of various splice variants. (A) Scheme of the RACE-based GeneScan[®] technique. Amplification of the adaptor-ligated cDNA was done using AP1 and exon/exon specific primers of each splice variant in the first PCR. The nested PCR was performed with 6-FAM AP2 and specific primer (close to the start codon) for each splice variant using the first PCR products as template. Primers used in the first PCR and nested PCR are indicated by filled and open arrowheads, respectively. (B) Electropherogram of nested PCR product of various splice variants analyzed by GeneScan[®] software. Locations of peak (upper panel) are aligned with the GeneScan[®] 400HD [ROX] size standard (lower panel). Number of transcriptional start sites is indicated by the number of peaks. (C) Locations of the determined transcriptional start sites of rFrp/sFRP-4. The major transcriptional start site (previously determined) of rFrp/sFRP-4 is designated as +1 and marked by an arrow (→). The determined start sites of rFrp/sFRP-4II and rFrp/sFRP-4IV are marked by arrows (↓). The positive *cis*-acting elements for the transcriptional regulation of rFrp/sFRP-4 are underlined (Yam et al., 2003).

with 303 amino acids. All splice variants have a shortened hydrophilic carboxyl tail compared with rFrp/sFRP-4, but preserve the CRD and NTR domains, thus revealing the functional significance of these two domains.

3.4. Determination of transcriptional start sites of rFrp/sFRP-4 splice variants

To determine the transcription initiation sites of different splice variants, we employed a RACE-based technique in conjunction with the GeneScan[®] Analysis (Yam et al., 2001b). As described in Fig. 2A, the 5' flanking region of each rFrp/sFRP-4 splice variant was amplified by first PCR using exon/exon specific primers (E5/E8R, E5/E9R) and an adaptor primer (AP1), followed by a second PCR with a fluorescence-labeled adaptor primer (6-FAM AP2) and a nested sequence specific primer (E1R). The size and number of the labeled PCR products was determined by using GeneScan[®] analysis. The sizes of the PCR products corresponding to the location of the transcriptional initiation site upstream from the nested gene specific primer are indicated as peaks precisely aligned with an internal fluorescent GeneScan 400HD [ROX] size ladder, while the number of peaks reflects the number of initiation sites. As shown in Fig. 2B, distinct PCR products were obtained for splice variants II and IV, and these PCR products suggest that each of these two transcripts is transcribed from a single transcriptional initiation site. The locations of transcriptional start site of variants II and IV relative to the previously determined transcriptional start site of rFrp/sFRP-4 (Yam et al., 2001b) are shown along the promoter region of rFrp/sFRP-4 (Fig. 2C). Two crucial transcriptional *cis*-acting elements of the rFrp/sFRP-4 promoter, CTTTGGGGG and AGATGATGAA are located in regions of –197 to –189 and –151 to –141, respectively (Yam et al., 2003). The transcriptional start sites of rFrp/sFRP-4II and rFrp/sFRP-4IV are located downstream and upstream to these positive elements, respectively. This finding suggests that rFrp/sFRP-4 and its splice variants are differentially regulated by the alternative usage of the promoter regulatory elements. The utilization of alternative promoters is a common scheme involved in regulating expression of gene in a tissue-specific manner. Examples can be found with genes encoded proteins such as neuronal nitric-oxide synthase (Newton et al., 2003), P450 aromatase (Payne and Hales, 2004 and connexins (Anderson et al., 2005). Of interest to our study, the expression of P450 aromatase, product of *CYP19* gene is found in the corpus luteum of the rat ovary as what we found with the *rFrp/sFRP-4* gene described below.

We were unable to determine the transcriptional start sites of variants I and III, due to the extremely low copy number of the variant transcripts present in the adaptor-ligated T2 cDNA library that was employed for the RACE reactions.

3.5. Tissue-specific expression of rFrp/sFRP-4 splice variants

In view of the complex splicing pattern of rFrp/sFRP-4 transcripts, RT-PCR analysis was done to examine rFrp/sFRP-4 expression in a panel of rat tissues using exon/exon specific primer pairs of each splice variant. Two cell lines, R6#13-8 and T2, from which rFrp/sFRP-4 was first identified by our lab, were also included as positive controls in the expression study. The RT-PCR analysis shows that rFrp/sFRP-4 was ubiquitously expressed in all tissues examined (Fig. 3). The four splice variants, rFrp/sFRP-4I to IV, displayed different patterns of tissue distribution.

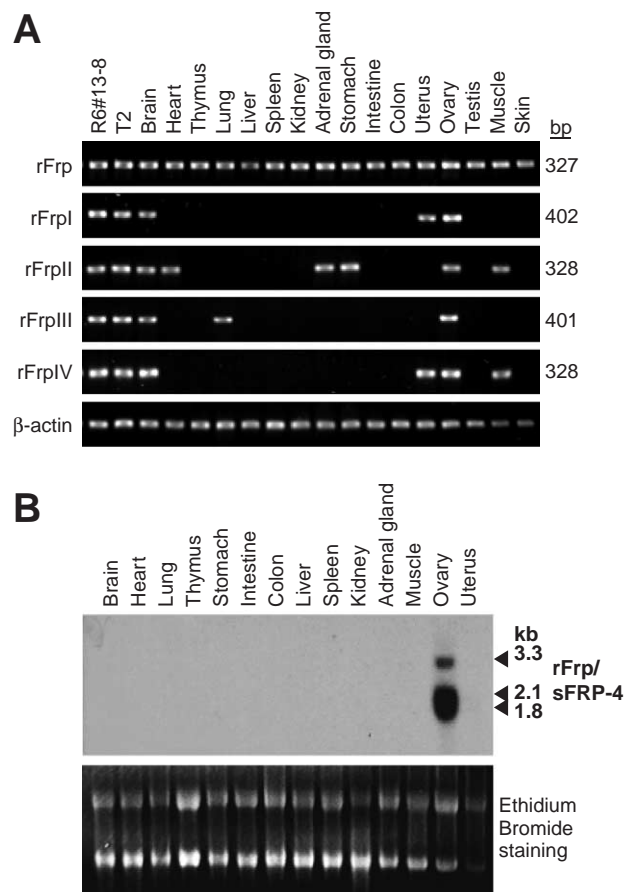


Fig. 3. Tissue expression of rFrp/sFRP-4 splice variants. (A) RT-PCR analysis of alternatively spliced rFrp/sFRP-4 transcripts in various rat tissues. Total RNA isolated from rat tissues of an adult Sprague-Dawley rat and cell lines (R6#13-8 and T2) were reverse transcribed and amplified by exon/exon specific primer pairs for each splice variant. Quality of the isolated RNA was revealed by amplification with actin primers. Primer pairs for each splice variant are shown as follows: rFrp/sFRP-4 (E3F, AGGTGAAGC-CAACTTTGGCAACGT; E5/E6R, GCCACCTGATGGACATCTGTGGA), rFrp/sFRP-4I (E3F, E7/E8R, CTGACACTGAGTTCGAGAACCCTGT), rFrp/sFRP-4II (E3F, E5/E8R, GACACTGAGTCGAGAACCCTGTGGA), rFrp/sFRP-4III (E3F, E7/E9R, TGCCACCTGATGGACATCTGTGTT), rFrp/sFRP-4IV (E3F, E5/E9R, GCCTCTCTCCACTGTGTGGA). (B) Total RNA extracted from various tissues of an 8-week-old female rat was probed with ³²P-radiolabeled rFrp probe. Ethidium bromide staining of total RNA was used for quantifying RNA on the gel.

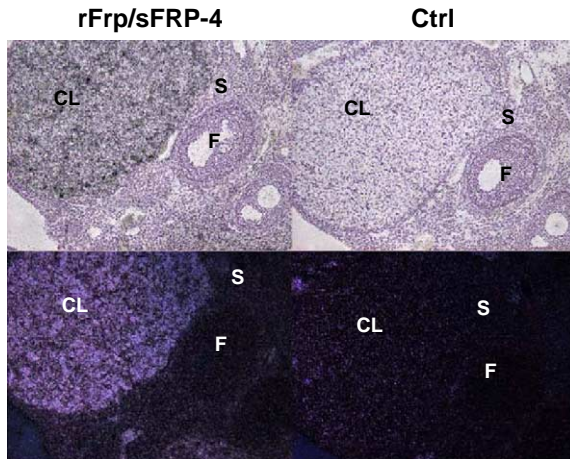


Fig. 4. Localization of rFrp/sFRP-4 in rat ovary. Upper and lower panels are bright-field and dark-field images, respectively, of hybridization in the ovary using antisense (left) and sense (right) ^{35}S -rFrp/sFRP-4 RNA probe. High levels of hybridization signals for rFrp/sFRP-4 mRNA are seen exclusively in the corpora luteum. CL: corpus luteum; F: follicle; S, Luteal stroma.

Brain and ovary are the only two tissues that express all five rFrp/sFRP-4 splice variants. Besides brain and ovary, rFrp/sFRP-4I and rFrp/sFRP-4III are expressed in uterus and lung, respectively. The rFrp/sFRP-4II is also expressed in heart, adrenal gland, stomach and muscles, while rFrp/sFRP-4IV is expressed in uterus and muscle. Although all splice variants were detected in different tissues by RT-PCR, transcripts of rFrp/sFRP-4 were only detected in ovary by Northern blot analysis using a full-length rFrp/sFRP-4 cDNA as probe (Fig. 3B). Expressions of rFrp/sFRP-4 poly(A)+RNA in tissues other than ovary were not detected using Northern blot analysis, suggesting that the copy number of rFrp/sFRP-4 mRNA are extremely low in non-ovarian tissues. The sizes of the ovarian transcripts (3.3, 2.1 and 1.8 kb) are identical to the sizes of transcripts found in mutant p53-transformed R6#13-8 and T2 cells (Yam et al., 1999). Based on the sequence analyses of the variants, the transcript sizes of rFrp/sFRP-4, variants I, II, III, IVa and IVb are 2062, 1765, 1518, 1818, 2063 and 3441bp, respectively, with the assumption that variants I and III contain the same transcriptional start site as the rFrp/sFRP-4. The 3.3 kb band on the Northern blot might be the variant IVb mRNA, the 2.1 kb band might represent transcripts of rFrp/sFRP-4 and rFrp/sFRP-4IVa, while the 1.8 kb band might represent transcripts of rFrp/sFRP-4 I, II, and III. Another sFRP-4 homologue, DDC-4 (Wolf et al., 1997) was also found to be expressed in ovary with three discrete transcripts ranging from 2.9 to 2.0 kb. Most likely, these transcripts were the products of alternative splicing present in the rat mammary gland of their study.

To verify the expression of rFrp/sFRP-4 in ovary, in situ hybridization with rFrp/sFRP-4 specific antisense riboprobe was performed. As shown in Fig. 4, rFrp/sFRP-4 was predominantly expressed in luteal cells of corpus luteum.

Only a weak hybridization signal is detected in follicles and stroma. This result is consistent with the upregulated sFRP4 found in the ovarian corpus luteum around the time of ovulation (Drake et al., 2003). During the ovulation cycle, corpus luteum undergoes profound morphological and functional changes. It is conceivable that the rFrp/sFRP-4 gene and its splice variants may be those of the luteal cell-specific genes underlying these molecular and structural changes.

To summarize, in this report, we presented the results of our work on isolating and characterizing five novel rFrp/sFRP-4 splice variants that were generated by exon-skipping at the 3' region of the mRNA. Tissue distribution of these splice variants suggests that these splice variants together with rFrp/sFRP-4 might have specific and overlapping functions in different tissues. This study provides a foundation toward the understanding of the tissue-specific regulation and functional significance of the gene.

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