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Journal of Cellular Biochemistry 108:304–314 (2009)

Journal of Cellular Biochemistry

SRrp37, a Novel Splicing Regulator Located in the Nuclear Speckles and Nucleoli, Interacts With SC35 and Modulates Alternative Pre-mRNA Splicing In Vivo

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ABSTRACT

We report here the identification and characterization of a novel SR-related protein, referred to as SRrp37, based on its apparent molecular weight and subcellular location. SRrp37 was identified through a yeast two-hybrid screen during the course of searching for proteins interacting with pNO40, a ribosomal 60S core subunit. SRrp37 exhibited two alternative spliced isoforms generated by differential usage of the translation start site with the longer one, SRrp37, initiating at first exon and the shorter, SRrp37-2, starting from exon 2. Three distinct motifs can be discerned in the SRrp37 protein: (1) a serine–arginine (SR) dipeptide enriched domain, (2) a polyserine stretch, and (3) a potential nucleolar localization signal comprising a long array of basic amino acids. SRrp37's message was translated in tissue-specific patterns with both isoforms expressed at comparable levels in tissues showing expression. Indirect immunofluorescence analysis with an anti-SRrp37 antibody, as well as an experiment using myc-tagged proteins, demonstrated that SRrp37 was localized in nucleoli and nuclear speckles. GST pull-down assay showed that SRrp37 interacted physically with SC35. Using adenovirus E1A and chimeric calcitonin/*dhfr* constructs as splicing reporter minigenes, we found that SRrp37 modulated alternative 5′ and 3′ splicing in vivo. Together, SRrp37 may participate directly in splicing regulation or indirectly through interaction with SC35. Studies on this novel splicing regulator may provide new information on the intricate splicing machinery as related to the RNA metabolism involving processing of mRNA and rRNA. J. Cell. Biochem. 108: 304–314, 2009.

KEY WORDS: SRrp37; ARL6IP4; pNO40; SC35; NUCLEAR SPECKLES; NUCLEOLI; ALTERNATIVE SPLICING

The availability of complete human genomic sequences has revealed that the primary source of human proteomic diversity, compared with unicellular organisms, is due not to a large increase in the number of protein coding genes, but rather to a relatively limited number of genes through the process of alternative splicing. Alternative splicing generates segments of mRNA variability that can insert or remove amino acids, shift the reading frame, or introduce a termination codon. It has been estimated that 75% of human genes generate multiple mRNAs by alternative splicing [Johnson et al., 2003] and ~80% of alternative splicing results in changes in the encoded proteins [Modrek and Lee, 2002].

There are a large number of factors known to be involved in alternative splicing, which could be roughly separated into two classes. One class consists of relatively widely expressed proteins, which seem to have wide-ranging roles in mRNA biogenesis. These come in two groups: SR proteins and hnRNP proteins, the former tends to promote exon inclusion while the latter usually has the opposite effect [Manley and Tacke, 1996; Smith and Valcarcel, 2000]. The others are factors with restricted expression patterns that are responsible for regulating tissue-specific alternative splicing events. These proteins, including Nova-1/2 and Hu proteins [Jensen et al., 2000; Zhu et al., 2006], have been identified by a variety of approaches and each of them shares a salient feature: each contains an RNA-binding domain of the KH or RRM type. In spite of this complexity, it is certain that attempts to explain genome-wide alternative splicing patterns will be impossible to realize without a complete list of alternative splicing regulators.

To search for additional splicing modulators, we sought to use yeast two-hybrid approach to identify a potential regulator that interacts with proteins displaying multiple isoforms derived from

Abbreviation used: SRrp, SR-related protein.

Grant sponsor: Ministry of Education; Grant numbers: EMRPD 170111, EMRPD170211; Grant sponsor: Chang Gung Memorial Hospital; Grant number: CMRPD170071; Grant sponsor: National Science Council; Grant number: NSC95-2320-B-182-040-MY3.

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Received 3 March 2009; Accepted 28 May 2009 • DOI 10.1002/jcb.22255 • © 2009 Wiley-Liss, Inc.
Published online 6 July 2009 in Wiley InterScience (www.interscience.wiley.com).

alternative splicing. Here, we report the identification and characterization of a novel splicing regulator SRrp37, which was found to interact and co-localize with SC35 in nuclear speckles and co-distribute with fibrillarin in nucleoli. We also demonstrated SRrp37 modulated alternative 5′ and 3′ splicing in vivo. Identification of SRrp37, a protein localized in both nucleoli and nuclear speckled domains, may provide us with new insights into the intricate splicing machinery as related to the RNA metabolism involving processing of mRNA and rRNA.

MATERIALS AND METHODS

REAGENTS

Dulbecco's modified Eagle medium (DMEM), Hank medium, and fetal calf serum (FCS) were purchased from Invitrogen Co. (Carlsbad, CA). Glutathione Sepharose 4B was purchased from Pharmacia Biotech Co. (Uppsala, Sweden). Molecular biology reagents, including restriction enzymes and Taq polymerase, were purchased from New England Biolab (Beverly, MA). All chemicals, unless otherwise specified, were purchased from Sigma Co. (St. Louis, MO).

TWO-HYBRID SCREENING

The yeast two-hybrid system-3 and a human kidney cDNA library were purchased from Clontech, Inc. (Palo Alto, CA). Competent yeast cells (strain AH109) were transformed with 6 μg of pNO40/pAS2-1 bait plasmid and 10 μg of library plasmid DNA and were plated onto minimal synthetic dropout (SD) agar lacking tryptophan, leucine, histidine, and adenine (QDO). The plates were incubated at 30°C for 7 days, after which 36 transformants were streaked onto fresh SD agar and tested further for their ability to hydrolyze X-Gal (5-bromo-4-chloro-3-indolyl-β-D-galactopyranoside). We also verified that the proteins encoded by prey plasmids did not interact with false baits (GAL4-binding domain-p53 and GAL4-binding domain alone).

5′-RAPID AMPLIFICATION OF cDNA ENDS (5′-RACE)

To determine the nucleotide sequence of 5′ region of human SRrp37 and SRrp37-2 cDNA (HY2-19 and HT41), respectively, 5′-RACE was performed using the 5′/3′ RACE kit, second generation (Roche, Indianapolis, IN) according to the manufacturer's instruction. The DNA sequence of a specific primer (arrows in Fig. 2A), used to amplify 5′ end of SRrp37 and SRrp37-2 were 5′-ttcttccggccatcactggacgaag-3′. The amplified PCR product was cloned into pGEM-T easy vector and then sequenced.

CELL CULTURE AND TRANSFECTION

CV1 and Cos7 cell lines of passages 10–60 were maintained in DMEM and supplemented with 10% FCS, 2 mM glutamine, and 200 U/ml each of streptomycin and penicillin G. Cells were passed with 0.1% trypsin and 0.04% EDTA in Hank's medium. Expression plasmids encoding myc epitope conjugated SRrp37 (myc-SRrp37) was constructed by ligating *Bam*H1–*Sal*I fragment derived from full-length cDNA clone in frame to the pCMV-Tag3A expression vector (Stratagene, La Jolla, CA). Transient transfection was performed at 37°C for 5 h using standard calcium phosphate method and transfected cells were assayed by immunofluorescent microscopy.

NORTHERN BLOT

A human multiple tissue Northern blot was purchased from Origene Biotechnologies, Inc. (Rockville. MD). Filter hybridization was carried out as described before [Chang et al., 2003]. The filter was prehybridized in 50% formamide, 5× SSC, 5× Dendhardt's solution, 0.2% SDS, and 200 μg/ml salmon sperm DNA for 10 h at 42°C. Hybridization was performed with the addition of ^{32}p-labeled *Apa*I fragment (302 bp) of HY2-19 at 42°C for 16 h. Filter was then washed twice in 2× SSC with 0.1% SDS for 30 min at room temperature and 1× SSC with 0.1% SDS at 55°C for 2 h and was exposed to X-ray film at −80°C with an intensifying screen.

IMMUNOFLUORESCENCE MICROSCOPY

CV1 cells cultured on coverslips or transfected with myc-SRrp37 or myc-pNO40 were treated for indirect immunofluorescence. Primary antibodies used were monoclonal 5E5 specific for SRrp37 (1:20; Abnova, Taipei, Taiwan), anti-pnn polyclonal serum, mouse anti-myc monoclonal antibody 9E10.2 (Balbco, Berkeley, CA), anti-SC35 monoclonal antibody (1:200; Sigma–Aldrich, St. Louis, MO), anti-fibrillarin polyclonal antibody (1:200; Novus Biologicals, Littleton, CO), and an antiserum against myc (1:400; Viogene, Taipei, Taiwan). Cultured cells were incubated with primary antibodies for 1 h at RT. After three 5 min wash in PBS, the samples were incubated with a mixture of FITC and rhodamine-conjugated goat anti-mouse and goat anti-rabbit IgG (Jackson Laboratory, West Grove, PA) 1:100 diluted for 1 h at RT. The samples were then washed extensively with PBS, mounted with 50% glycerol containing 0.4% *n*-propylgallate, and examined with a photomicroscope (Axioscope 2; Carl Zeiss) equipped with epifluorescence.

WESTERN BLOT

Whole cell extract was prepared by lysing cells in RIPA buffer (50 mM Tris, pH 8.0, 0.14 M NaCl, 0.2% SDS, 1% Triton X-100, and 0.5% sodium deoxycholate) containing 1 mM PMSF and 1 mg/ml each of pepstatin, leupeptin, and chemostatin. Samples were sonicated for 30 s on ice and boiled for 5 min prior to electrophoresis on a 10% polyacrylamide gel. Proteins were transferred from the gel to nitrocellulose paper. The paper was blocked with 5% non-fat dried milk in PBS followed by washing in PBS. Primary antibodies incubations, with 5E5 specific for SRrp37 (1:500), were carried out for 1 h at room temperature. After extensive wash in PBS, the paper was incubated for 1 h with HRP-conjugated goat anti-mouse IgG (diluted 1:1,000 in PBS; Jackson Laboratory). The peroxidase-labeled blots were reacted with 0.5 mg/ml diaminobenzidine and color reaction were developed using 0.01% hydrogen peroxide.

IN VIVO SPLICING ASSAYS

In vivo splicing was performed essentially as described by Caceres et al. [1994]. Briefly, 5 μg of the E1A reporter gene or pDC20 calcitonin/*dhfr* construct was transfected alone or together with an increasing amount (0.2, 0.5, and 2 μg) of SRrp37 expression plasmids (myc-SRrp37) into 2×10^5 Cos7 cells using the calcium phosphate method. Empty vector (pCDNA3; Invitrogen) was added

to ensure that equal amounts of DNA were transfected. Transfection was performed at 37°C for 5 h. RNA was isolated 17–24 h after transfection using Tri-Reagent (Invitrogen) as described in the manufacturer's instructions. Protein lysate was also isolated from one-third of transfected cells for immunoblotting by an anti-myc antibody, 9E10.2, to check for protein expression. For RT-PCR, 2 μg of isolated RNA was mixed with 2 μl (0.1 μg/μl) oligo-dT primer in reaction buffer containing 1 μl (40 U/μl) Stratascript reverse transcriptase (Stratagene). The tubes were incubated for 90 min in a 37°C water bath. Two microliters of the RT reaction was mixed with 2.5 pmol each of sense and antisense primers and PCR was carried out using the following conditions: initial denaturation for 2 min at 94°C, 25 cycles of 94°C denaturation for 30 s, 65 or 59°C (for pDC20) annealing for 30 s, and 72°C extension for 1 min. PCR products were separated on 2% or 2.5% (for pDC20) agarose gel, stained with ethidium bromide, visualized and quantified with a STORM fluorescence imagers (Molecular Dynamics). Primer pairs used for E1A splicing assay are 5′-cttttctcctccgagccgctccga-3′ (sense) and 5′-ctcaggatcaggttcagacacagg-3′ (antisense), those for pDC20 splicing assay are 5′-cgccaaacttgggggaagca-3′ (sense), 5′-cggaactgcctccaactatc-3′ (antisense 1) and 5′-ccagcatgcaagtactcaga-3′ (antisense 2).

RESULTS

MOLECULAR CLONING AND IDENTIFICATION OF SRrp37 AND SRrp37-2

Previous work from our lab has described the properties of a novel nucleolar protein pNO40 [Chang et al., 2003]. To extend these studies we used yeast two-hybrid screen to identify potential interacting proteins. Among those uncovered was a protein with features similar to SR-related proteins (SRrps). Two forms of this protein, HY2-19 and HY41, likely arising from alternative splicing, were recovered (Fig. 2A). The interaction of HY41 and pNO40 is specific and direct because only the full-length pNO40 interacts with HY41 upon yeast co-transformation (Fig. 1A) and expressed full-length HY41 (Fig. 1B, shown as EGFP-SRrp37), but not the EGFP alone, can be pulled down by GST-fused pNO40 in vitro (Fig. 1B). The nucleotide sequence of HY2-19 is identical to that of HY41 except at their 5′ termini. Using 5′-RACE and RT-PCR methods with total RNA isolated from HeLa cells, we determined an open-reading frame of 1,026 and 681 bp, respectively, for HY2-19 and HY41. Based on the apparent molecular weight (see below, Fig. 3A), a signature motif of SR domain in the protein domain (Fig. 2A) and gene structure relationship of the two cDNA clones (Table I), we called HY2-19 encoding protein as the long form SRrp37 (SRrp with molecular weight 37 kDa) and HY41 encoding protein the short form SRrp37-2 (sequences are available from GenBank under accession numbers EU624490 and EU624491).

The 1,587 bp full-length cDNA of SRrp37 contained an open-reading frame of 341 amino acids with 328 bp of 5′ untranslated region and 233 bp of 3′ untranslated region, while SRrp37-2 encoded a putative protein of 227 amino acids with 404 and 233 bp of 5′ and 3′ untranslated region, respectively. The 5′ untranslated region of SRrp37 contained an additional in-frame ATG initiation site 182 bp upstream of the authentic start codon. However, we found it may be inefficient for ribosome binding because its adjacent sequences were not conformed to the Kozak's consensus (purine nucleotide in position −3 and +4 relative to ATG start site) and there was little or no protein expression observed following cellular transfection (data not shown). Amino acid alignment of SRrp37 and SRrp37-2 revealed that the former is longer than the latter by 123 amino acids (Fig. 2B), and nucleic acid comparison (data not shown) indicated that sequence 13 bp upstream SRrp37-2 ATG start site was different between two proteins, indicating alternative use of translation initiation. Computer analysis of the genomic organization of SRrp37 and SRrp37-2 showed that both proteins were derived from a single gene product with diverse 5′ exons (exons 1a and 1b) and distinct initiation site; SRrp37 was at exon 1 and SRrp37-2 at exon 2 (Fig. 2A). The gene for *SRrp37* is 2,850 bp in length comprising six exons, with sequences in exons 1 and 3 differing from those of *SRrp37-2*. Nevertheless, the sequences of splicing donor and acceptor sites located at exon/intron boundary are all conformed to gt/ag rule (Table I). Blast comparison of the long (HY2-19) and short (HY41) isolated cDNAs against the EST database revealed the existence of two EST populations, EST20690 and BI915979, respectively, with 5′ end corresponding to the ends of the long and short cDNAs. Collectively, these observations indicate that SRrp37 can be translated as two alternatively spliced mRNAs, likely encoding two SRrp37 isoforms.

CHARACTERIZATION OF SRrp37

Based on the sequence analysis using the programs for the prediction of protein sorting signals and localization site in amino acid sequences (PSORT II, available at http://psort.ims.u-tokyo.ac.jp/) and database of protein families and domains (PROSITE, available at http://kr.expasy.org/prosite/), a number of interesting structure motifs were noted in the human SRrp37 (Fig. 2A,B). These included an arginine–serine dipeptide repeat region (RS domain, aa 132–140), a serine stretch region (poly S domain, aa 178–202), and a putative nucleolar localization signal (NoLS, aa 205–232). Additionally, PSORTII predicted that SRrp37 was a nuclear protein at the reliability of 82.6% with PI of 11.18. In general, no specific NoLSs have been described, but likely, a long array of basic amino acid flanked by basic amino acids, RXXR motifs (in which the X is preferably an arginine or leucine), or RGG motifs, may act as NoLS [Quaye et al., 1996; Liu et al., 1997; Das et al., 1998]. Many ribosomal proteins that transit to the nucleolus to be assembled with rRNA contain such a signal for nucleolar localization [Gueydan et al., 2002], and it should be noted that SRrp37 NoLS contains two such motifs (Fig. 2B) that might mediate SRrp37 nucleolar translocation. The RS domain is a characteristic feature of proteins involved in pre-mRNA splicing such as snRNPs and SR protein family members [Fu, 1995]. It may participate in the protein–protein interaction during the assembly of spliceosome complex and play a role in mediating SR protein localized to nuclear speckles, sites for storage/assemble/modification of splicing factors [Lamond and Spector, 2003]. In addition to the RS domain, SRrp37 contains a stretch of 25 consecutive serines (poly-S domain). Many splicing-related proteins, such as pinin, RNPS1 and SRrm300, like SRrp37,

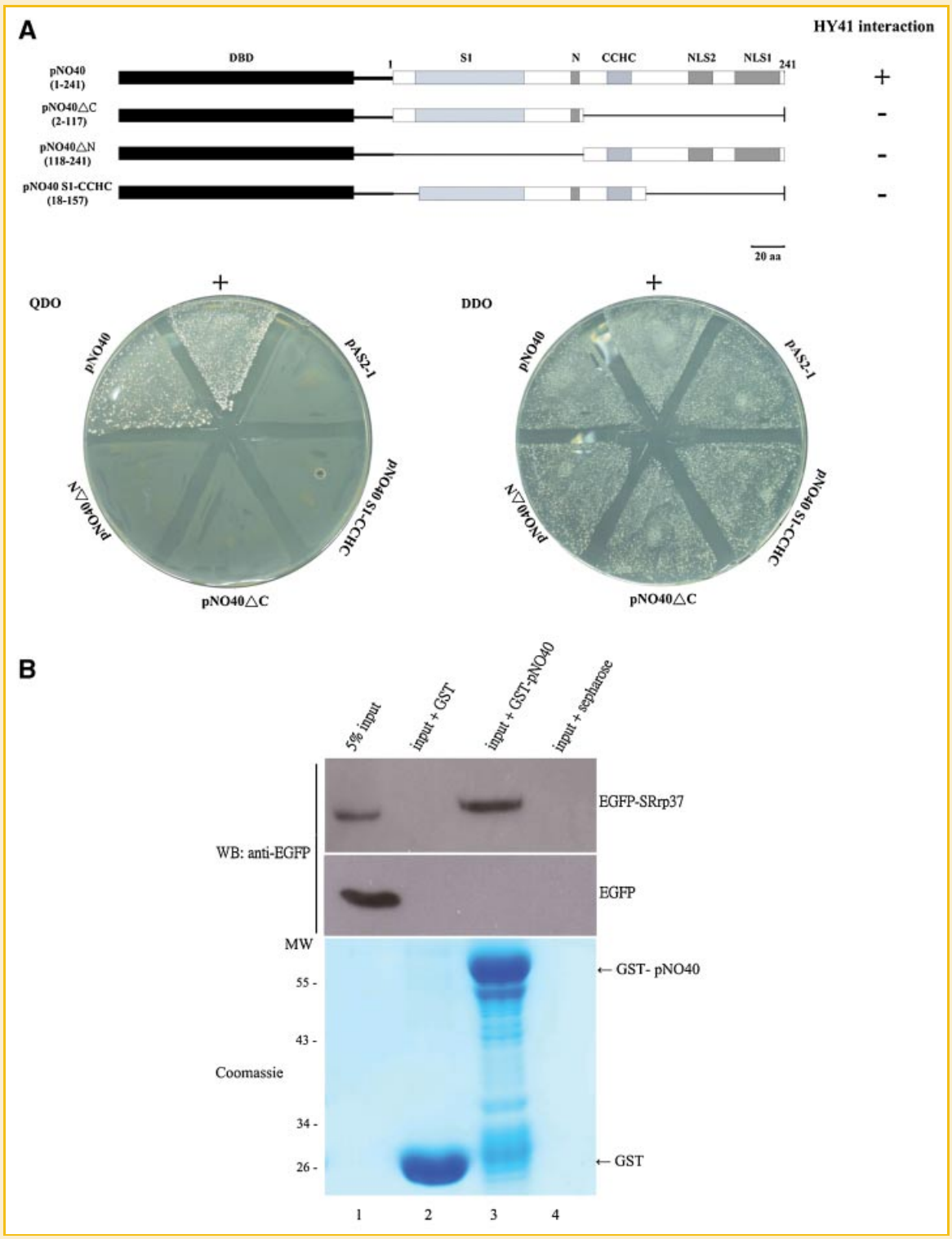

Fig. 1. Characterization of the interaction between SRrp37 and pNO40. A: Yeast two-hybrid confirmation of the interaction of pNO40 and SRrp37. Upper panel: Schematic diagrams of the full-length pNO40 or its deletion mutants fused in frame to the Gal4 DNA-binding domain (DBD) and results of their interactions with HY41, a partial cDNA clone of SRrp37. S1: S1 RNA-binding region; N, NLS: nuclear localization signal; CCHC: zinc finger motif. Lower panels are results of yeast co-transformation followed by plating out on DDO or QDO in which various bait plasmids containing full-length pNO40 or its mutants are transformed along with prey plasmid containing HY41 fused in frame to Gal4 DNA activation domain. + and pAS2-1 represent positive and negative control of co-transformation, respectively. DDO and QDO are yeast synthetic medium without Leu/Trp and Leu/Trp/Ade/His, respectively. Note that only full-length pNO40 interacted with SRrp37. B: GST pull-down demonstrating pNO40 interacts directly with SRrp37. EGFP-SRrp37 or EGFP was transfected into 293T cells and RIPA buffer-dissociated cell lysate was either subjected to Western blot with a monoclonal antibody against EGFP (line 1, input) or incubated with GST sepharose (lane 2), GST-fused pNO40 sepharose (lane 3) or sepharose alone (lane 4). The eluted proteins were then Western blotted with EGFP antibody. Expression of GST or GST-fused pNO40 can be identified with Coomassie blue staining. MW, molecular weight standard.

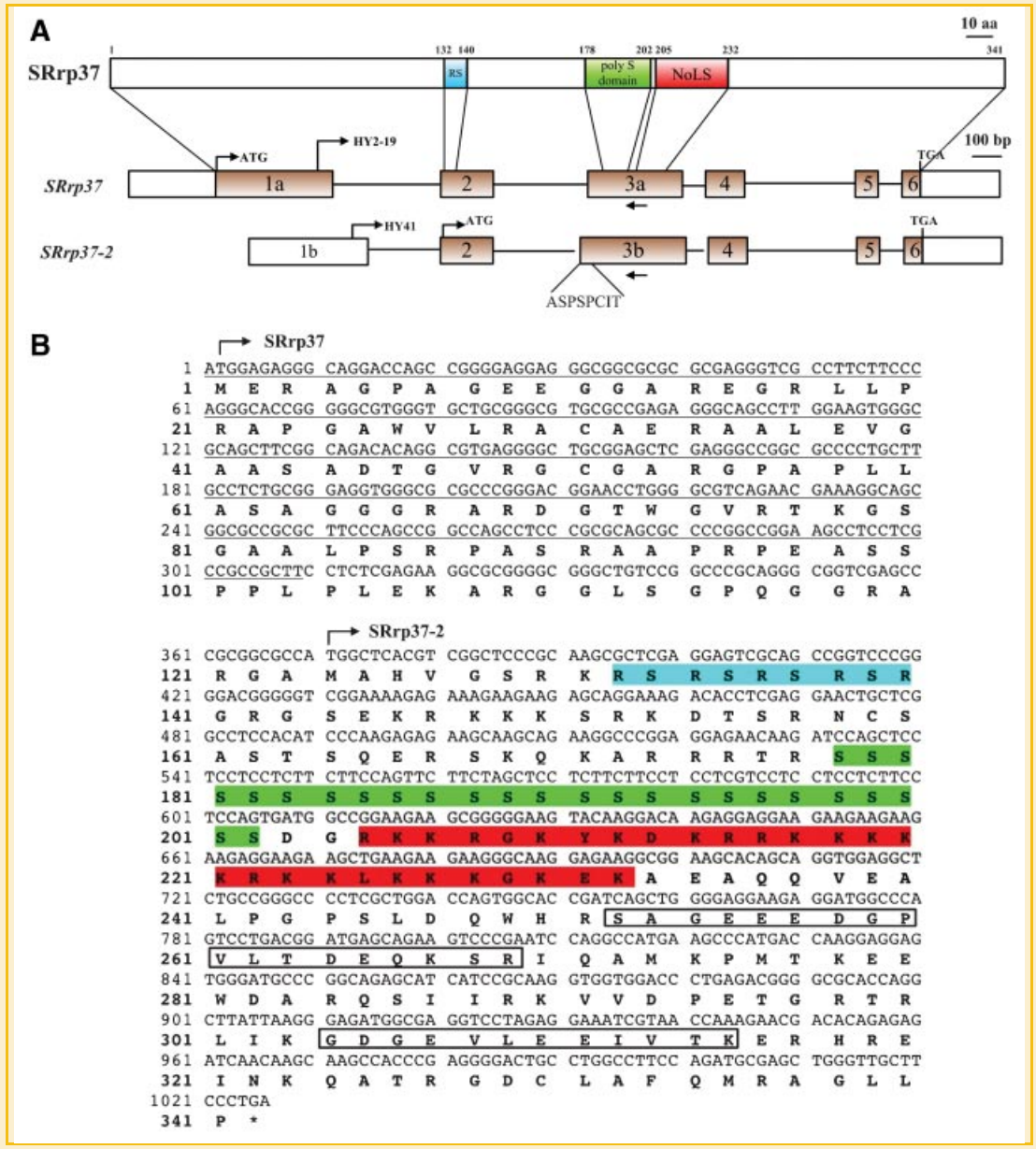

Fig. 2. Domain organization and predicted amino acid sequence of SRrp37 and SRrp37-2. A: Schematic representation of the domain structure of human SRrp37 protein (first panel) relative to its gene organization (middle panel). Gene structure of *SRrp37-2* is shown in the lower panel. Both *SRrp37* and *SRrp37-2* contain six exons with sequence difference at exons 1 and 3. Note the insertion of eight amino acids, ASPSPCIT, at the beginning of exon3b in *SRrp37-2*, while *SRrp37* and *SRrp37-2* coding region initiating (ATG) at exons 1 and 2, respectively. Rectangles with numbers represent exons. Empty rectangles denote 5′ and 3′ untranslated regions. HY2-19 and HY41 represent the upstream location of cDNA clones isolated by yeast two-hybrid screening. Arrows denote location of the primer used for 5-RACE. RS: arginine–serine domain; poly-S domain: serine stretch; NoLS: nucleolar localization signal. The cDNA sequences are deposited in GenBank under accession numbers EU624490 (SRrp37) and EU624491 (SRrp37-2). B: Deduced amino acid sequence of SRrp37 and SRrp37-2. Amino acids are shown by single letter code. Bent arrows represented translation initiation sites, while various motifs marked with different colors corresponded to the named structures shown in (A). Amino acids in rectangles represent sequences which can be recovered from proteomic analysis of human nucleolus purified from HeLa cells (databank available at http://www.lamondlab.com/Nopdb).

contain a successive array of serine residues ranging in number from 8 (RNPS1) to 42 (SRm30) [Ouyang and Sugrue, 1996; Blencowe et al., 1998; Mayeda et al., 1999]. Although it is not clear at present the exact function of the serine stretch, through the RS domain and the poly-S stretch, SRrp37 may engage in nuclear pre-mRNA splicing activity (see below).

As a first step toward characterizing the function of SRrp37, we used Western and Northern blot analyses to examine the expression pattern of this novel protein. A mouse monoclonal antibody, clone 5E5, raised against a partial GST-fused SRrp37 recombinant protein (261–341 aa) was employed for Western blot analysis. This antibody reacted with a single polypeptide band corresponding to molecular

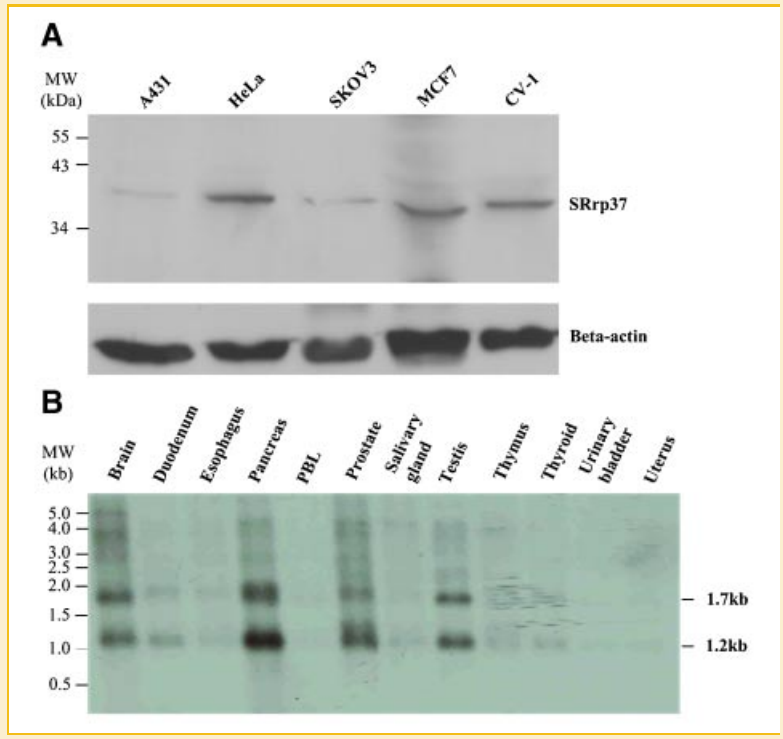

Fig. 3. Molecular characterization of SRrp37 and its isoform. A: Western blot analysis of total protein isolated from various culture cell lines with SRrp37-specific monoclonal antibody 5E5. A single peptide migrating at MW ~37 kDa can be identified upon immunoblotting. β-actin was used as a loading control. B: Northern blot analysis of SRrp37 transcripts. A multiple human tissue blot was hybridized with a 0.3 kb *Apa*I fragment derived from SRrp37 cDNA. Two transcripts migrating at 1.7 and 1.2 kb were identified in brain, pancreas, prostate, and testis, but little or no message can be detected in other tissues examined. Note that the two transcripts, each corresponding to SRrp37 and SRrp37-2, respectively, displayed comparable levels in tissues showing expression.

mass of ca. 37 kDa in the total protein prepared from various cultured cell lines (Fig. 3A). To examine the extent of SRrp37 expression, total RNA was isolated from a variety of human organs and subjected to Northern blot. SRrp37 transcripts were expressed in different tissues in distinct ways (Fig. 3B). In the brain, pancreas, prostate, and testes, two prominent bands can be identified at size of 1.7 and 1.2 kb, estimated to be corresponding to the messages representing SRrp37 and SRrp37-2, respectively. Neither the SRrp37 nor SRrp37-2 transcript was expressed at significant level in other tissues examined. These results suggest that SRrp37 message is expressed in tissue-specific patterns, while SRrp37 and its isoform SRrp37-2 display a comparable level in tissues showing expression.

DUAL LOCATION OF SRrp37 IN NUCLEAR SPECKLES AND NUCLEOLI

According to the predicted domains of SRrp37 from database analysis (Fig. 2A), it is assumed that this protein was localized in the nucleus with distinct subnuclear distribution. To examine the subcellular localization of SRrp37, we employed antibody 5E5 to detect the distribution pattern of endogenous protein in cultured cell lines. In CV1 cells, the main signal distributed to subnuclear structures throughout the nucleoplasm (Fig. 4a,d). The endogenous SRrp37 signals in the nucleus were distributed in nucleoli (arrows in Fig. 4a–f), as demonstrated by co-staining of nucleoli with fibrillarin (Fig. 4e), a snoRNP factor located in the granular compartment of the nucleolus. In addition, the dot-like SRrp37 staining within the nucleus is co-localized with pinin, a nuclear speckle-associated SRrp

TABLE I. The Exon and Intron Organization of the Human Gene for *SRrp37*

Exon	Exon size (bp)	5' splice donor	Intron/size (bp)	3' splice acceptor
1a	686	GTCGAGgtgggaac	1a/383	gtcgccagCCCGCG
1b	251	AGCCGGgtgcgcag	1b/241	gtcgccagCCCGCG
2	138	CCCAAGgtcgcaag	2a/328	catcacagAGAGAG
	138	CCCAAGgtcgcaag	2b/304	ccttccagCCTCAC
3a	285	GCCCAGgtactgtg	3/77	ccttccagTCCTGA
3b	309	GCCCAGgtactgtg	3/77	ccttccagTCCTGA
4	118	CACCAGgtggggag	4/394	acccccagGCTTAT
5	70	AACAAGgtgggtgt	5/81	ttccccagCAAGCC
6	270		N/A	

The sizes of exons and introns, the splice donor/acceptor sites (bold type) are listed. Nucleotides in capital letters are exonic sequence while those in small letters are of intron. Note that sequences of splicing donor and acceptor sites located at exon/intron boundary were all conformed to gt/ag rule.

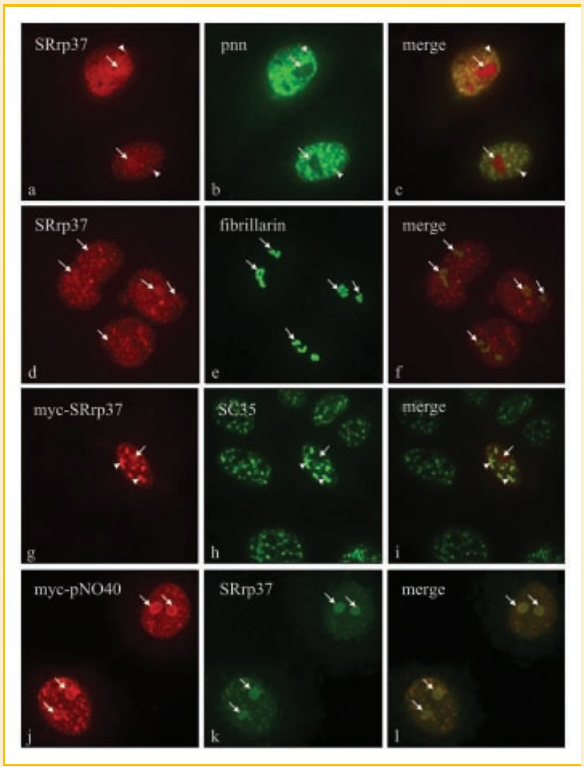

Fig. 4. Immunofluorescent micrographs showing the subcellular location of SRrp37 in nucleoli and nuclear speckles. CV1 cells were double immunostained with antibodies against SRrp37 and pinin (pnn, a–c) or against SRrp37 and fibrillarin (d–f). Endogenous SRrp37 can be found co-distributed with pnn in a speckled-localization pattern (arrowheads, a–c) and simultaneously co-localized with fibrillarin within nucleoli (arrows, d–f). Exogenously expressed SRrp37 (myc-tagged SRrp37), when overexpressed in CV1 cells, exhibited a prominent speckled pattern co-distributed with that of SC35 (arrowheads, g–i). Endogenous SRrp37 became prominent within nucleoli when CV1 cells were overexpressed with expression vectors encoding myc-tagged pNO40 (arrows, j–l). Arrowheads: nuclear speckles; arrows: nucleoli.

(arrowheads in Fig. 4a–c). To specify the respective location of SRrp37 in distinct subnuclear compartments, we transfected CV1 cells with expression vectors encoding myc-tagged SRrp37 or pNO40, a nucleolar protein interacting with SRrp37 (Fig. 1). When myc-SRrp37 was transfected into CV1 cells (Fig. 4g), prominent punctuates representing SRrp37 can be observed co-localized with SC35, hallmark protein for nuclear speckles (arrowheads in Fig. 4g–i). In addition to nuclear speckles, exogenously expressed SRrp37 was also faintly localized to nucleoli as evidenced by sphere structures lacking SC35 immunostaining within the nucleus (arrows in Fig. 4g–i). In contrary to exogenously expressed SRrp37, which showed prominent nuclear speckled patterns, endogenous SRrp37 was found predominantly in nucleoli (Fig. 4k) when pNO40 was overexpressed in CV1 cells. Upon overexpression, pNO40, a core component of 60S ribosome subunit [Gueydan et al., 2002; Chang et al., 2003], was concentrated in nucleoli (Fig. 4j) and induced marked SRrp37 nucleolar co-localization (arrows in Fig. 4j–l), thus confirming SRrp37 not only interacted physically with pNO40, as demonstrated before by GST pull-down assay (Fig. 1B), but also associated functionally with it.

SRrp37 INTERACTS WITH SC35 AND MODULATES ALTERNATIVE 5′ AND 3′ SPLICING *IN VIVO*

The co-localization of SRrp37 with pinin and SC35 in nuclear speckles prompts us to investigate whether this protein interacts physically with members of SR splicing factors. GST pull-down assay was performed using bacterially expressed recombinant SRrp37 proteins and whole cell lysate obtained from 293T cells overexpressing myc-tagged SC35 or myc-pinin (Fig. 5). Results showed that GST-fused SRrp37 interacted strongly with SC35 (Fig. 5, lane 3), but not with pinin or an unrelated proteins EGFP-tagged myosin light chain (Fig. 5). As a control, there was little or no interaction between SC35 and GST or with sepharose (Fig. 5, lanes 2 and 4), demonstrating the specificity of interaction for SRrp37 and SR family protein SC35.

We next wished to determine whether SRrp37 behaves like a typical SR protein family; for example, whether it can activate pre-mRNA splicing or regulate alternative splicing in a concentration-dependent manner [Fu, 1995; Manley and Tacke, 1996]. We thus carried out in vivo splicing assays to investigate the role of SRrp37 in regulating alternative splicing of pre-mRNA. We co-transfected Cos7 cells with SRrp37 expression vector and an adenovirus E1A reporter gene which is capable of producing multiple mRNAs (9S, 12S, and 13S) through the use of alternative 5′ splice sites (Fig. 6A). In general, transfection of the E1A minigene alone generated multiple RNA species characteristic of the utilization of alternative 5′ splice sites (Fig. 6A, upper right panel, lane 1), as has been shown previously [Caceres et al., 1994]. Addition of SRrp37 in small

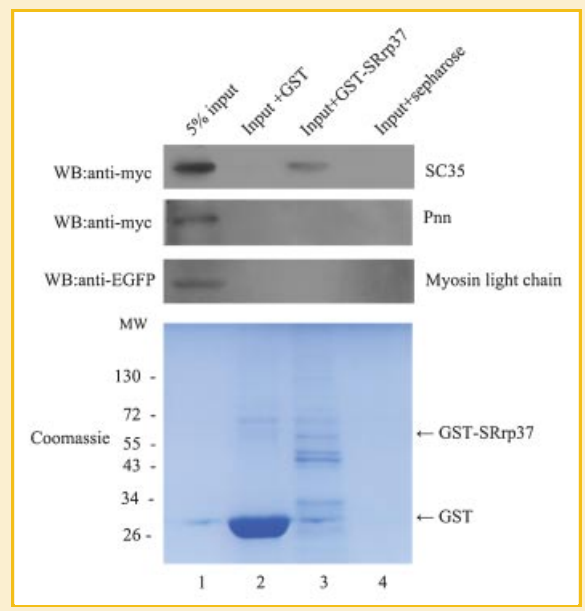

Fig. 5. SRrp37 interacts with SC35. Bacterially expressed GST-SRrp37 (lane 3), GST (lane 2), or sepharose (lane 4) was incubated with cell lysate (input, lane 1) derived from 293T cell overexpressing myc-SC35, myc-pinin, or EGP-myosin light chain. Proteins eluted from the bound sepharose were subjected to Western blot with anti-myc antibody or EGFP antibody. Coomassie blue denotes proteins separated by SDS–PAGE. Myc-pinin and EGFP-myosin displayed little or no reaction with GST-SRrp37 following parallel assay treatment.

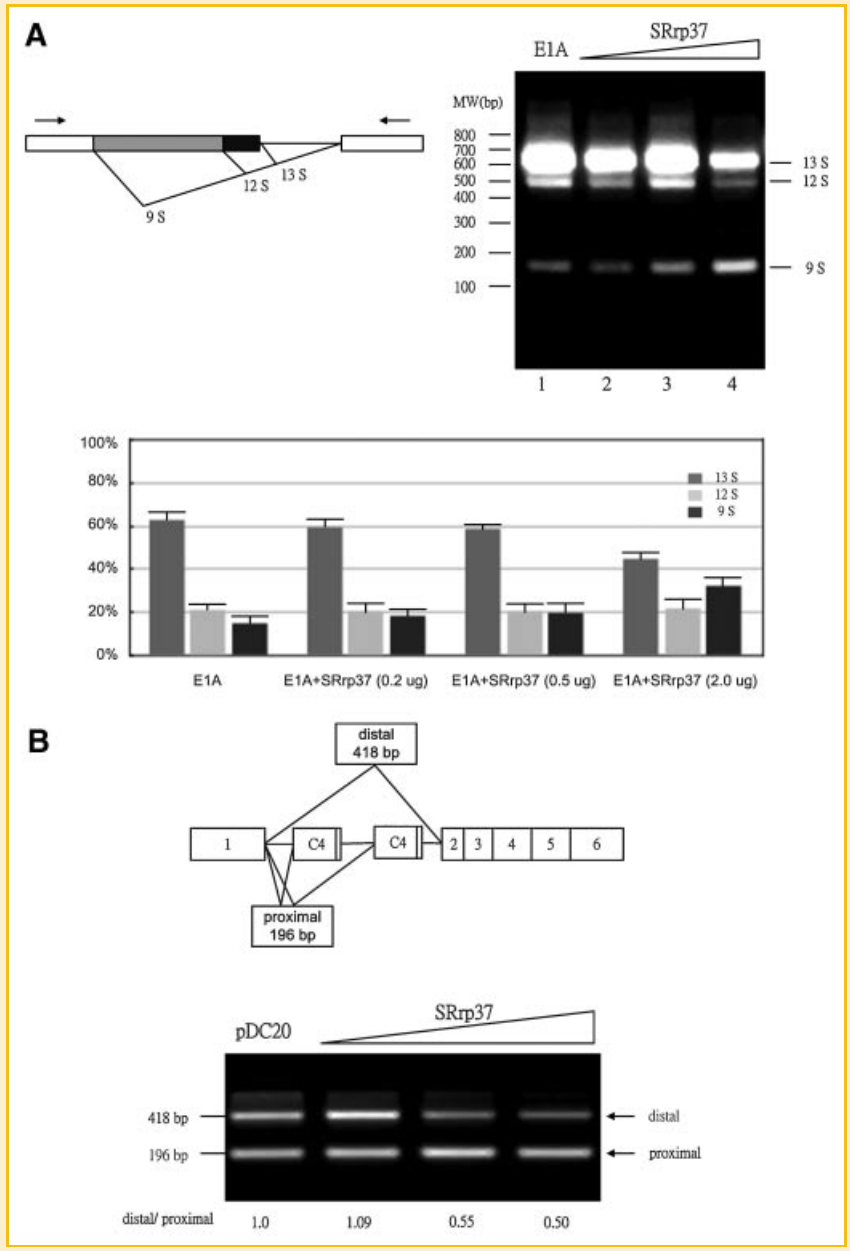

Fig. 6. SRrp37 modulates alternative splicing of model reporter minigene in vivo. Effects of SRrp37 transient overexpression on alternative splicing of adenovirus E1A pre-mRNA. Left panel: Diagram of the E1A reporter gene. The alternative 5′ splice sites and splicing events that generate 13S, 12S, and 9S mRNAs are shown schematically. The location of the exon primers used for RT-PCR is shown (arrows). Right panel: Pattern of E1A alternative splicing in Cos7 cells transfected with the E1A minigene (lane 1), or co-transfected with SRrp37 expression vectors in an increasing amount (lanes 2–4). Lower panel is the histogram representing the quantitative percentage of each E1A isoform upon transfection with increasing amount of SRrp37. Bars represent mean of at least three experiments, error bars indicate SD. B: Effects of SRrp37 on alternative splice-site choice of the chimeric calcitonin/*dhfr* pre-mRNA. Upper panel: Diagram of the 3′ alternative splicing patterns in the chimeric calcitonin/*dhfr* construct pDC20, which carries two copies of rat calcitonin exon 4 within the sole intron of a hamster *hfdr* minigene. The proximal and distal splicing products of different sizes are shown. Lower panel: Patterns of calcitonin/*dhfr* alternative splicing in Cos7 cells transfected with pDC20 minigene alone or co-transfected with SRrp37 in an increasing amount (0.2, 0.5, and 2 μg). The ratio of proximal to distal splicing product of each assay was shown at the bottom of each lane. Each experiment was performed at least three times in duplicates.

increments from 0.2 to 2 μg (Fig. 6A, upper right panel, lanes 2–4) resulted in a decreased use of the proximal (13S) site, from 63% to 45%, and a switch to the use of the distal (9S) site (from 15% to 33%, Fig. 6A, lower panel). As a control, co-transfection of E1A reporter with an empty vector, pcDNA3, did not change the splicing pattern (data not shown). In addition, E1A minigene splicing pattern did not change following co-transfection with an unrelated expression vector encoding myosin light chain (data not shown), therefore demonstrating the specificity of the splicing reaction and involvement of SRrp37 in splicing modulation was not an artifact.

To confirm that SRrp37 is indeed engaged in alternative splicing modulation, we utilized another splicing reporter minigene, pDC20,

to test the efficiency of SRrp37 in in vivo splicing assay. pDC20 was constructed by inserting as a tandem repeat the alternatively spliced terminal exon 4 of the rat calcitonin gene (C4) together with its flanking regions into the sole intron of a hamster dhfr minigene [Bai et al., 1999]. Differential splicing of calcitonin/*dhfr* pre-mRNA generated two major isoforms (Fig. 6B, upper panel): splicing at first or second C4 (a proximal 3′ spliced site) resulted in chimeric calcitonin/*dhfr* mRNA with C4 as a terminal exon (196 bp), while splicing at *dhfr* exon 2 resulted in the exclusion of C4 and generation of *dhfr* mRNA (418 bp). Overexpression of SRrp37 in an increasing amount from 0.2 to 2 μg gradually activated the proximal 3′ splice site choice of the chimeric calcitonin/*dhfr* pre-mRNA, yielding mainly mRNA that includes exon C4, when compared with the basal splicing pattern of pDC20 without the addition of exogenous SRrp37 (distal/proximal ratio decreases from 1.0 to 0.5, Fig. 6B, lower panel). Together SRrp37 may be involved in modulating alternative pre-mRNA splicing in vivo with either 5′ distal site activation or preferential use of 3′ proximal site.

DISCUSSION

We report here the identification and characterization of a novel splicing regulator protein SRrp37 whose expression shows tissue-specific expression patterns and alternative translation initiation. Endogenous SRrp37 protein was found to distribute in distinct subnuclear domains co-localized with fibrillarin and SC35 in nucleoli and nuclear speckles, respectively. We further delineated its function with regard to modulation of pre-mRNA alternative splicing based on its unique pattern of co-distribution and interaction with the authentic SR family splicing factor. Functional splicing assay for SRrp37 with reporter minigenes demonstrated that it modulated alternative 5′ and 3′ splicing in vivo, suggesting that SRrp37 may participate in pathways related to mRNA biogenesis/processing.

SRrp37 was identified previously by several different approaches. In a yeast two-hybrid screen searching for proteins interacting with ADP-ribosylation factor-like factor 6 (ARL6), a protein called ARL6IP4 (ADP-ribosylation factor-like factor 6-interacting protein) was identified [Ingley et al., 1999]. Subsequently, Lee et al. [2007] reported that ARL6IP4 was one of the two proteins that showed up-regulation in a proteomic analysis of targets that mediated the anti-apoptotic effect of the Rac1-dominant negative mutant (Rac1N17). Blast search and sequence comparison indicated that ARL6IP4 was identical to SRrp37 at both nucleotide and protein levels and SRrp37-2 was closely related in amino acids to both SR25, a protein obtained by random sequencing of a mouse insulinoma cell line (MIN6) cDNA library [Sasahara et al., 2000], and SR15, an early response protein induced by HSV infection capable of inhibiting HSV pre-mRNA splicing [Li et al., 2002]. However, neither ARL6IP4, SR25 nor SR15 was studied in great deal in terms of its expression patterns and functional significance.

SRrp37 transcripts were expressed in different tissues in distinct ways (Fig. 3B). In the brain, pancreas, prostate, and testes, two prominent bands can be identified at size of 1.7 and 1.2 kb, estimated to be corresponding to the messages representing SRrp37 and SRrp37-2, respectively. Several tissues and cell types, such as the esophagus, leukocytes, salivary gland, thyroid, urinary bladder, and uterus, however, displayed SRrp37 or SRrp37-2 transcripts at significantly lower level. These results suggest that SRrp37 messages, unlike that of other SRrps, such as pinin and RNPS1, which displayed ubiquitous expression patterns [Ouyang and Sugrue, 1996; Mayeda et al., 1999], are expressed in tissue-specific patterns and its variable levels of expression among different tissues do not seem to correlate with tissues metabolic activity.

Interestingly, there is a discrepancy between Northern (Fig. 3B) and Western blot analyses (Fig. 3A). Northern blot clearly demonstrated there were two populations of SRrp37 transcripts, consistent with the finding obtained from cDNA cloning (Fig. 2A), but there was only one peptide, corresponding to the longer form SRrp37, identified by Western analysis. The discrepancy can be explained by either the 5E5 antibody did not recognize the smaller SRrp37-2 isoform or SRrp37-2 is not expressed efficiently in cell lines examined (Fig. 3A). Because of the uncertainty of SRrp37-2 in protein expression, we have not pursued studies with SRrp37-2, and instead focused on the full-length SRrp37 in the reminder of this study.

To date, exhaustive proteomic analysis of subcellular compartment has been performed by various investigators using procedures involving a two-dimensional gel electrophoresis followed by mass spectrometric techniques. Andersen et al. [2002, 2005] reported that 126 uncharacterized novel proteins found in the categorized 715 proteins could be identified as nucleolar proteins of HeLa cells. Using either myc-tagged expression vectors (Fig. 4g,j) or an indirect immunofluorescence analysis with an anti-SRrp37 antibody (Fig. 4a,d), we demonstrated that SRrp37 was a novel protein localized in both nucleoli and nuclear speckles. This finding was substantiated by the fact that SRrp37 was detected as an uncharacterized protein in human nucleolus on the human nucleolar database at www.lamondlab.com/Nopdb [Leung et al., 2006]. Attempts to identify SRrp37 as a component of the nuclear speckle based on proteomic analysis of enriched IGC (interchromatin granule cluster) fraction purified from mouse liver [Saitoh et al., 2004], however, were not successful. Despite that we could not confirm from database search that SRrp37 is a component of the nuclear speckle, we provided strong evidence that transiently transfected cells revealed prominent SRrp37 in nuclear speckles (Fig. 4g). It has been reported that splicing factors for mRNA seem to follow a maturation pathway via nucleoli to their final destination. For example, Sm proteins (components of snRNPs) accumulated first in coiled bodies and nucleoli, and finally in speckles [Sleeman and Lamond, 1999]. We surmise that dual location of SRrp37 in nucleoli and nuclear speckles might reflect a maturation or traffic pathway for this protein within the nucleus. Alternatively, the presence of SRrp37 in nuclear speckles after transient transfection could be the result of elevated SRrp37 expression. Interestingly, a novel nucleolar pathway was described, in which newly synthesized NHPX protein was localized in the nuclear speckles before accumulation in nucleoli [Leung and Lamond, 2002]. NHPX was demonstrated to be involved both in late stage spliceosome assembly and in rRNA cleavage/modification by binding to U4 snRNA and box C/D snoRNA, respectively [Watkins et al., 2000].

Therefore, speckled localization of SRrp37 might well be caused by an overload of the same pathway as described for NHPX. After acute overexpression, SRrp37 can probably not be transported fast enough from the speckle and therefore accumulates. In light of our immunofluorescence results, it is possible that, like NHPX, newly synthesized endogenous SRrp37 is localized in speckles, whereas the mature protein is present in nucleoli. To address these questions, future experiments might examine synchronized cell populations by live cell microscopy in the presence or absence of protein synthesis inhibitors.

The subcellular localization of SRrp37 in nucleoli and nuclear speckled domains (Fig. 4) suggested a possible role in both rRNA and mRNA processing. Nucleolar accumulation of proteins is a common feature of either ribosomal proteins or proteins involved in the maturation of rRNA, while speckled localization pattern is highly diagnostic for proteins that are involved in pre-mRNA splicing. As ribosomal RNA synthesis and ribosome assembly constitute the main metabolic activities of nucleoli, we determined whether SRrp37 nucleolar localization was dependent on ongoing rRNA transcription. Low levels of actinomycin D cause the segregation of nucleoli and inhibit rRNA synthesis, but not pre-mRNA transcription. We found that SRrp37 nucleolar segregation did not occur after treatment of cells with actinomycin D (0.05 μg/ml, data not shown), suggesting that pol I transcription and/or ribosome biogenesis is not a prerequisite for the SRrp37 nucleolar localization.

To specify the role of SRrp37 in mRNA processing, we carried out in vivo splicing assays to investigate whether SRrp37 is involved in regulating alternative splicing of pre-mRNA. Our results demonstrate that SRrp37 favors not to use 5′ proximal splice sites (13S) in the E1A minigene but activates the use of proximal 3′ splice sites and stimulates inclusion of 3′ exon in pDC20 in vivo splicing assay. These changes are distinct and specific because transfection of SRrp37 readily results in a rapid increase in the use of either distal or proximal splice site of E1A and pDC20 reporter genes, respectively (Fig. 6A,B). In this respect, SRrp37 behaves like SR or SRrp proteins in pre-mRNA alternative splicing, such as ASF/SF2 or pinin, which tends to stimulate the use of both 5′ and 3′ proximal splice sites when multiple alternative splice sites are available [Fu et al., 1992; Wang et al., 2002]. However, SRrp37 is not completely identical to ASF/F2 in modulating pre-mRNA alternative splicing in vivo, because it favors not to use 5′ proximal splice sites (13S) in the E1A minigene, which is contradictory to ASF/F2 in selecting usage of the proximal 5′ splice site for the same reporter minigene [Caceres et al., 1994]. Interestingly, we additionally demonstrated that SRrp37 could interact directly with SC35 but not with pinin (Fig. 5), despite it co-localized with SC35 and pinin in nuclear speckles at subcellular level (Fig. 4). That said, SRrp37 may participate itself directly in alternative splicing modulation or indirectly through interaction with SC35, a bona fide splicing regulator. Further work to understand the mechanism by which SRrp37 regulates pre-mRNA splicing may explain its role in mRNA processing.

In conclusion, we identify a novel protein, SRrp37, with structure features similar to certain SRrps, and characterize its subcellular location in both nucleoli and nuclear speckles. We further delineate its function with regard to modulation of pre-mRNA alternative splicing in vivo based on its subnuclear speckled distribution pattern and physical interaction with SC35, a SR family splicing protein with functions involved in splicing regulation. Results from studies of this novel splicing regulator may provide us with new information relating to the RNA biogenesis and metabolism involving processing of mRNA and rRNA.

ACKNOWLEDGMENTS

The author thanks Ms. Ya-Li Chiu and Mr. Yang-Ming Lin and Bin-Shun Lee for technical assistance, Mr. George Piatnitski for English editing, and acknowledges supports from Chang Gung Memorial Hospital (CMRPD170071), Tope Center grant of the Ministry of Education (EMRPD170111 and 170211), and National Science Council, ROC (NSC95-2320-B-182-040-MY3).

REFERENCES

Andersen JS, Lyon CE, Fox AH, Leung AK, Lam YW, Steen H, Mann M, Lamond AI. 2002. Directed proteomic analysis of the human nucleolus. Curr Biol 12:1–11.

Andersen JS, Lam YW, Leung AK, Ong SE, Lyon CE, Lamond AI, Mann M. 2005. Nucleolar proteome dynamics. Nature 433:77–83.

Bai Y, Lee D, Yu T, Chasin LA. 1999. Control of 3′ splice site choice in vivo by ASF/SF2 and hnRNP A1. Nucleic Acids Res 27:1126–1134.

Blencowe BJ, Issner R, Nickerson JA, Sharp PA. 1998. A coactivator of pre-mRNA splicing. Genes Dev 12:996–1009.

Caceres JF, Stamm S, Helfman DM, Krainer AR. 1994. Regulation of alternative splicing in vivo by overexpression of antagonistic splicing factors. Science 265:1706–1709.

Chang WL, Lee DC, Leu S, Huang YM, Lu MC, Ouyang P. 2003. Molecular characterization of a novel nucleolar protein, pNO40. Biochem Biophys Res Commun 307:569–577.

Das A, Park JH, Hagen CB, Parsons M. 1998. Distinct domains of a nucleolar protein mediate protein kinase binding, interaction with nucleic acids and nucleolar localization. J Cell Sci 111(Pt 17):2615–2623.

Fu XD. 1995. The superfamily of arginine/serine-rich splicing factors. RNA 1:663–680.

Fu XD, Mayeda A, Maniatis T, Krainer AR. 1992. General splicing factors SF2 and SC35 have equivalent activities in vitro, and both affect alternative 5′ and 3′ splice site selection. Proc Natl Acad Sci USA 89:11224–11228.

Gueydan C, Wauquier C, De Mees C, Huez G, Kruys V. 2002. Identification of ribosomal proteins specific to higher eukaryotic organisms. J Biol Chem 277:45034–45040.

Ingley E, Williams JH, Walker CE, Tsai S, Colley S, Sayer MS, Tilbrook PA, Sarna M, Beaumont JG, Klinken SP. 1999. A novel ADP-ribosylation like factor (ARL-6), interacts with the protein-conducting channel SEC61beta subunit. FEBS Lett 459:69–74.

Jensen KB, Dredge BK, Stefani G, Zhong R, Buckanovich RJ, Okano HJ, Yang YY, Darnell RB. 2000. Nova-1 regulates neuron-specific alternative splicing and is essential for neuronal viability. Neuron 25:359–371.

Johnson JM, Castle J, Garrett-Engele P, Kan Z, Loerch PM, Armour CD, Santos R, Schadt EE, Stoughton R, Shoemaker DD. 2003. Genome-wide survey of human alternative pre-mRNA splicing with exon junction microarrays. Science 302:2141–2144.

Lamond AI, Spector DL. 2003. Nuclear speckles: A model for nuclear organelles. Nat Rev Mol Cell Biol 4:605–612.

Lee SC, Sim N, Clement MV, Yadav SK, Pervaiz S. 2007. Dominant negative Rac1 attenuates paclitaxel-induced apoptosis in human melanoma cells through upregulation of heat shock protein 27: A functional proteomic analysis. Proteomics 7:4112–4122.

Leung AK, Lamond AI. 2002. In vivo analysis of NHPX reveals a novel nucleolar localization pathway involving a transient accumulation in splicing speckles. J Cell Biol 157:615–629.

Leung AK, Trinkle-Mulcahy L, Lam YW, Andersen JS, Mann M, Lamond AI. 2006. NOPdb: Nucleolar Proteome Database. Nucleic Acids Res 34:D218–D220.

Li Q, Zhao H, Jiang L, Che Y, Dong C, Wang L, Wang J, Liu L. 2002. An SR-protein induced by HSVI binding to cells functioning as a splicing inhibitor of viral pre-mRNA. J Mol Biol 316:887–894.

Liu JL, Lee LF, Ye Y, Qian Z, Kung HJ. 1997. Nucleolar and nuclear localization properties of a herpesvirus bZIP oncoprotein, MEQ. J Virol 71:3188–3196.

Manley JL, Tacke R. 1996. SR proteins and splicing control. Genes Dev 10:1569–1579.

Mayeda A, Badolato J, Kobayashi R, Zhang MQ, Gardiner EM, Krainer AR. 1999. Purification and characterization of human RNPS1: A general activator of pre-mRNA splicing. EMBO J 18:4560–4570.

Modrek B, Lee C. 2002. A genomic view of alternative splicing. Nat Genet 30:13–19.

Ouyang P, Sugrue SP. 1996. Characterization of pinin, a novel protein associated with the desmosome-intermediate filament complex. J Cell Biol 135:1027–1042.

Quaye IK, Toku S, Tanaka T. 1996. Sequence requirement for nucleolar localization of rat ribosomal protein L31. Eur J Cell Biol 69:151–155.

Saitoh N, Spahr CS, Patterson SD, Bubulya P, Neuwald AF, Spector DL. 2004. Proteomic analysis of interchromatin granule clusters. Mol Biol Cell 15: 3876–3890.

Sasahara K, Yamaoka T, Moritani M, Tanaka M, Iwahana H, Yoshimoto K, Miyagawa J, Kuroda Y, Itakura M. 2000. Molecular cloning and expression analysis of a putative nuclear protein, SR-25. Biochem Biophys Res Commun 269:444–450.

Sleeman JE, Lamond AI. 1999. Newly assembled snRNPs associate with coiled bodies before speckles, suggesting a nuclear snRNP maturation pathway. Curr Biol 9:1065–1074.

Smith CW, Valcarcel J. 2000. Alternative pre-mRNA splicing: The logic of combinatorial control. Trends Biochem Sci 25:381–388.

Wang P, Lou PJ, Leu S, Ouyang P. 2002. Modulation of alternative pre-mRNA splicing in vivo by pinin. Biochem Biophys Res Commun 294:448–455.

Watkins NJ, Segault V, Charpentier B, Nottrott S, Fabrizio P, Bachi A, Wilm M, Rosbash M, Branlant C, Luhrmann R. 2000. A common core RNP structure shared between the small nucleoar box C/D RNPs and the spliceosomal U4 snRNP. Cell 103:457–466.

Zhu H, Hasman RA, Barron VA, Luo G, Lou H. 2006. A nuclear function of Hu proteins as neuron-specific alternative RNA processing regulators. Mol Biol Cell 17:5105–5114.