Biochemical and Biophysical Research Communications **275,** 180–188 (2000)
doi:10.1006/bbrc.2000.3287, available online at http://www.idealibrary.com on IDEAL®

Alternative Exon Usage of Rat Septins

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Received July 17, 2000

Septins represent a family of phylogenetically conserved proteins required for cytokinesis. Their presence in pre- and postsynaptic neuronal membranes suggests a general function as scaffolds for membrane reorganization. The transcriptional regulation of all septins examined so far is complex, resulting in alternatively spliced variants. We focus here on the rat homologue of the gene for the human septin MSF, a truncated form of which, designated eseptin, had been described previously. It will be shown here that there is an alternative usage of the first exon by two forms, named exon r1a and r1b, respectively. Exon r1a, but not exon r1b, contains a part of the coding sequence while the start of translation for the remaining coding sequence resides in the second exon. The complete genomic organization was resolved and data on the temporal and spatial expression of this septins are presented. © 2000 Academic Press

Key Words: **septin; exon usage; MSF; Sint1.**

Septins are a family of phylogenetically conserved proteins (1–3) which share a common GTPase motif and can form homodimeric heteromers *in vitro* (4). They were first discovered in yeast (5) and subsequently detected in most eukaryotic organisms including other fungi (6), fruit fly (7, 8), rat (9–12), mouse (13–16) and man (17–24), but not in plants.

Septins are involved in defining the cleavage plane during cell division (1), and play a direct role in cytokinesis from yeast to mammals (2). Recent reports on the presence of septins in postmitotic cells (9) suggest that a more general role of this protein family has to be considered. The detection of septins in the exocytosis complex of presynaptic vesicles (9) and in postsynaptic membranes (25) gave rise to the assumption that septins rather act as active scaffolds for other proteins at sites of membrane remodeling. Because of the presence of a conserved GTP-binding domain, members of the septin family may be participants of a signaling network involving, for example, Nim 1-related kinases in yeast (26).

Clinical implications have been shown for the septin genes *MSF* (MLL septin-like fusion) (17–20) and *hCDCrel-1* (27) as fusion partner genes of *MLL* (28) in therapy-related acute myeloid leukemia. The corresponding fusion proteins are likely to affect cytokinesis and may play a role in the progression of the disease. Therefore, the genomic organization of both septins as well as differential splicing of either transcript was examined (19, 22). It was demonstrated that the major transcriptional form of *CDCrel-1* in rat neocortex is a fusion transcript of the adjacent genes for *CDCrel-1* and *GPIbβ* (platelet glycoprotein Ibβ) (12).

In the human, seven homologous septin genes have been described. One them, *MSF*, is alternatively spliced resulting in *MSF*, *MSF-A*, *MSF-B* and *MSF-C* (18, 19). We report here on the alternative exon usage of the corresponding rat septin (SLP, septin-like protein) and describe the characterization of two septin transcripts which were named *rSLP-a* and *rSLP-b*, respectively. It will be proposed that these transcripts yield proteins differing in their N-terminal sequence. Alternative splicing of non-coding sequences in the 3′ region, as reported for *MSF*, was not observed.

MATERIALS AND METHODS

Preparation and screening of rat DNA libraries. A cDNA library from cultured rat mesangial cells in λgt11 was prepared using the Librarian cDNA Library Construction System (Invitrogen) according to the manufacturer's instructions. Insert DNA of clones giving rise to fusion proteins reacting positively in an immunoscreening procedure described previously (29), was amplified by PCR using the primers 5′-GACTCCTGGAGCCCG-3′ and 5′-GGTAGCGACCGCCGC-3′, subcloned into pCRII (Invitrogen) and sequenced on an Alf Express automatic sequencer (Amersham Pharmacia Biotech). DNA from clones of interest was excised with *Eco*RI and labeled with α-[^{32}P]dCTP for screening using the Readiprime II Kit (Amersham Pharmacia Biotech). Immunoscreenings and screenings of libraries using DNA probes were performed according to the Lambda library user manual (Clontech). Positive clones were rescreened to homogeneity, and inserts were subcloned as described above. The screening

0006-291X/00 $35.00

FIG. 1. Sequence of rSLP. (A) Complete sequence of *rSLP-a* (GenBank Accession No. AF170253). Exon r1a resides upstream of bp 434. The beginning of exon 2 is boxed, the start codon for the translation of rSLP-a ($A_{412}..G_{414}$) is double underlined as well as the first start codon for the translation of rSLP-b ($A_{667}..G_{669}$). Consensus protein sequences of the GTP-binding motif (P loop motif) are underlined. (B) Sequence of the alternatively used exon r1b upstream of bp 308 resulting in rSLP-b (GenBank Accession No. AF173899). The beginning of exon 2 is boxed.

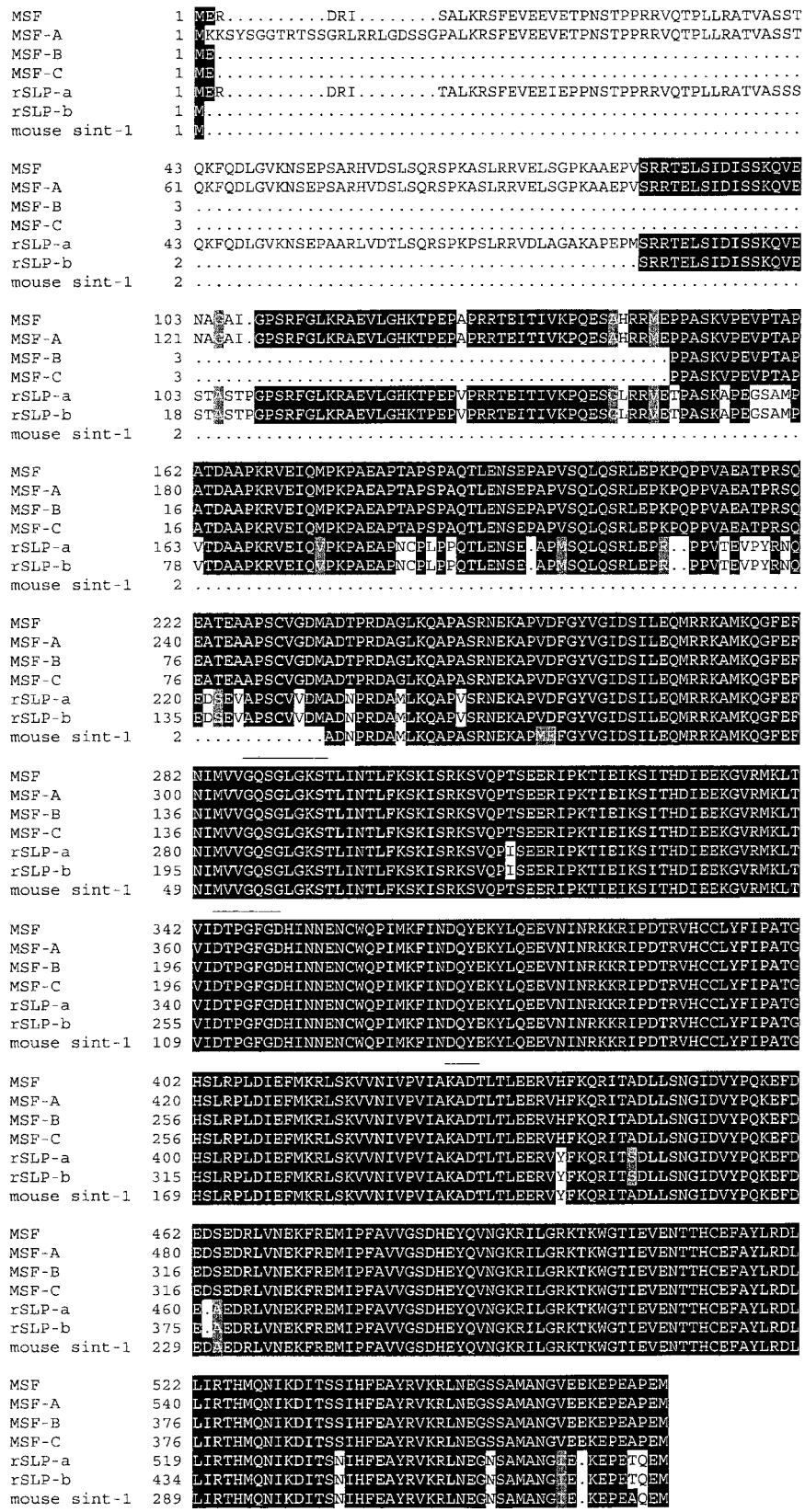

FIG. 2. Multiple sequence alignment of rSLP with human and murine MSF isoforms. Lines above the columns indicate the conserved GTP binding domain ($GX_4GKS\text{-}DX_2G\text{-}KXD$). White letters in black boxes represent a complete, white letters in gray boxes a partial amino acid identity between the proteins. Numbers refer to amino acid position of each protein.

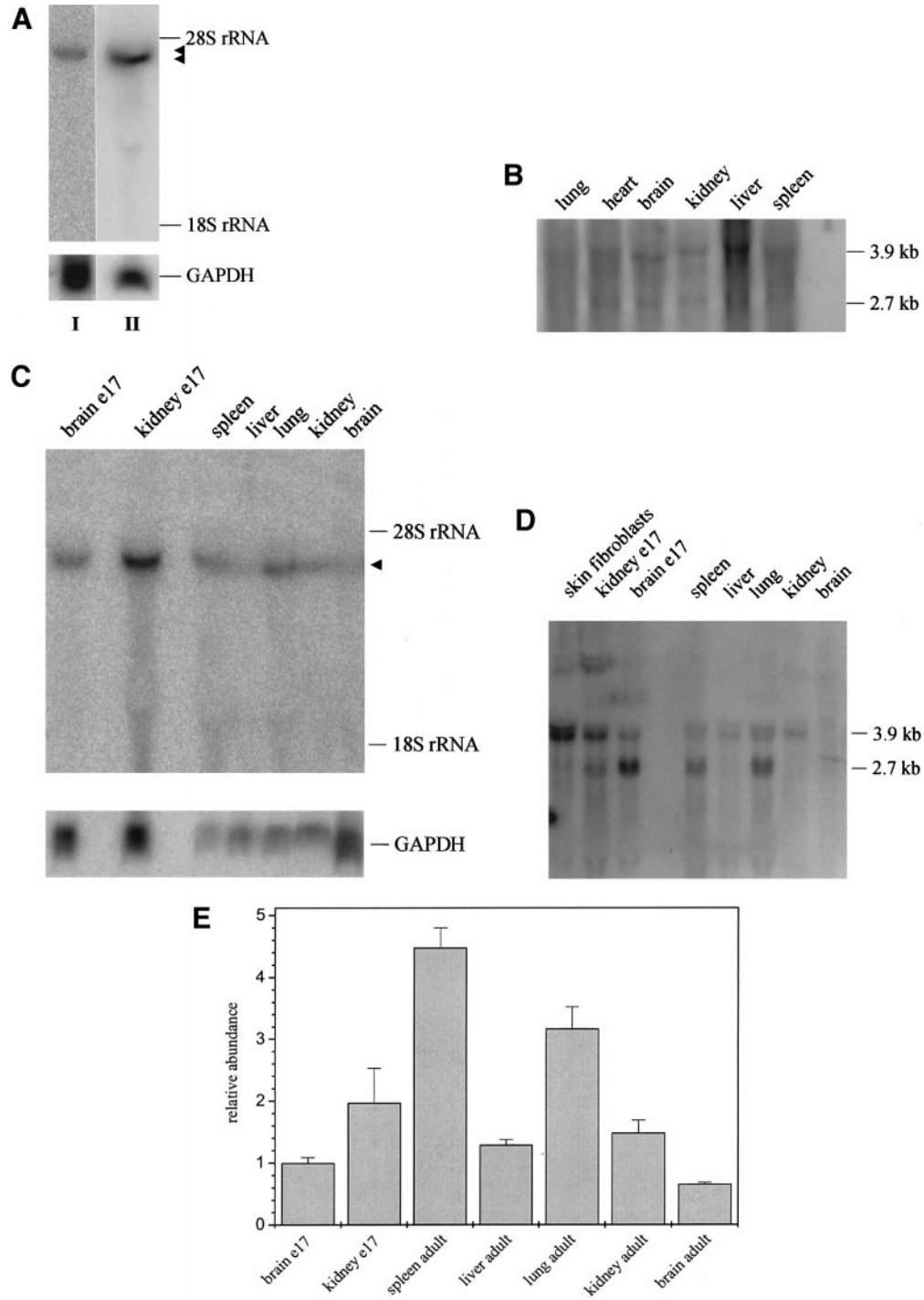

FIG. 3. Northern blot analysis of rSLP in cultivated skin fibroblasts and tissues. Northern blots were hybridized with probes comprising exon r1a, exon r1b and a part of the 3′ nontranslated sequence region, respectively. (A) Detection of *rSLP-a* and *rSLP-b* in RNA of rat skin fibroblasts. RNA in lane I was hybridized with a probe comprising exon r1a. One band with a size of 3.9 kb was detected. Probing of RNA with exon r1b (lane II) revealed a single band of 3.8 kb. Blots were rehybridized with a GAPDH probe. Arrowheads point to 3.8 and 3.9 kb, respectively. (B) RNA from multiple tissue sources of adult rats was hybridized with a probe comprising exon r1a. Two transcripts with sizes of 2.7 kb and 3.9 kb were detected in heart, brain, kidney, liver and spleen but not in lung. (C) RNA was hybridized with a probe comprising exon r1b. A single transcript with a size of 3.8 kb (indicated with an arrowhead) was detected in all examined tissues including brain and kidney from embryonic day 17. Blots were reprobed with GAPDH. (D) Hybridization with a probe comprising the sequence region $C_{2282}..C_{2697}$ of *rSLP-a*. Two transcripts with 3.8 and 3.9 kb were detected in cultivated rat skin fibroblasts (*rSLP-a* and *rSLP-b*). Transcripts with 3.9 kb were founding all examined tissues with the exception of adult brain, an additional transcript of 2.7 kb was detected in embryonic day 17 brain and kidney as well as in adult spleen and lung. (E) Relative amounts of *rSLP-b* in various adult and embryonic tissues. Data were obtained after Northern hybridization of RNA with exon r1b and normalization to GAPDH.

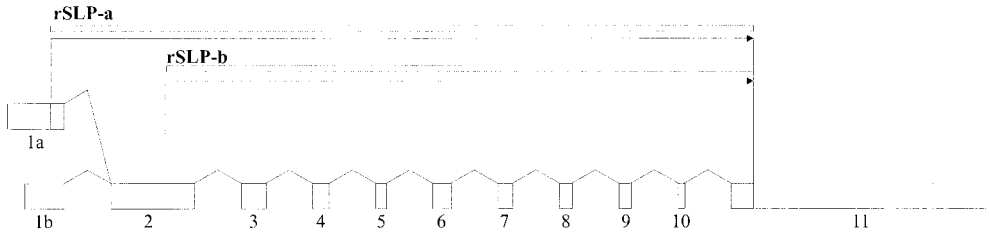

FIG. 4. Exon composition of different rSLP transcripts. Exons and introns are symbolized by boxes and lines, respectively. Exons are drawn to scale, for exon lengths see Table 2. The lengths of introns have not been determined. Coding regions in exons are filled gray. The lengths of the deduced amino acid sequences for rSLP-a and rSLP-b are represented by filled boxes above the intron/exon structure.

of a rat genomic DNA library in λEMBL3 SP6/T7 (Clontech) was performed accordingly, the inserts were strategically subcloned, first after *Xho*I digestion and subsequently by the alternative use of either *Apa*I, *Bam*HI, *Bgl*II, *Xba*I, *Xho*I or *Xma*I, and sequenced.

Cloning of rSLP-a1 cDNA. Total RNA from cultured rat skin fibroblasts and rat tissues was isolated using the Qiagen RNeasy Midi Kit (Qiagen) according to the manufacturer's instructions. Two micrograms of total RNA were reverse transcribed using display ThermoRTase (Appligene-Oncor) in a volume of 20 μl according to the manual. One microliter aliquots were subjected to a PCR in a final volume of 50 μl with 20 pmol of each primer, 50 μM dNTPs, reaction buffer containing 1.5 mM $MgCl_2$ and 2.5 U of HotStarTaq DNA polymerase (Qiagen). The PCR program was 60 s at 94°C, 60 s at annealing temperature and 120 s at 72°C plus a final extension for 20 min at 72°C. Further details are given in Table 1. The PCR products were analysed on 1% agarose TBE-gels, subcloned into pCRII TOPO or pCR4 TOPO (Invitrogen) and sequenced. To generate a template for 3′-RACE-PCR, 2 micrograms of total RNA were reverse transcribed using 5′-GACCACGCGTATCGATGTC-GAC(T_{22})V-3′ as a hybrid primer. 3′-RACE-PCR was performed as described (30).

Northern blotting. Thirty micrograms of total RNA were subjected to denaturing formaldehyde gel electrophoresis at 4°C overnight and blotted onto a nylon membrane (31). Probes were labeled

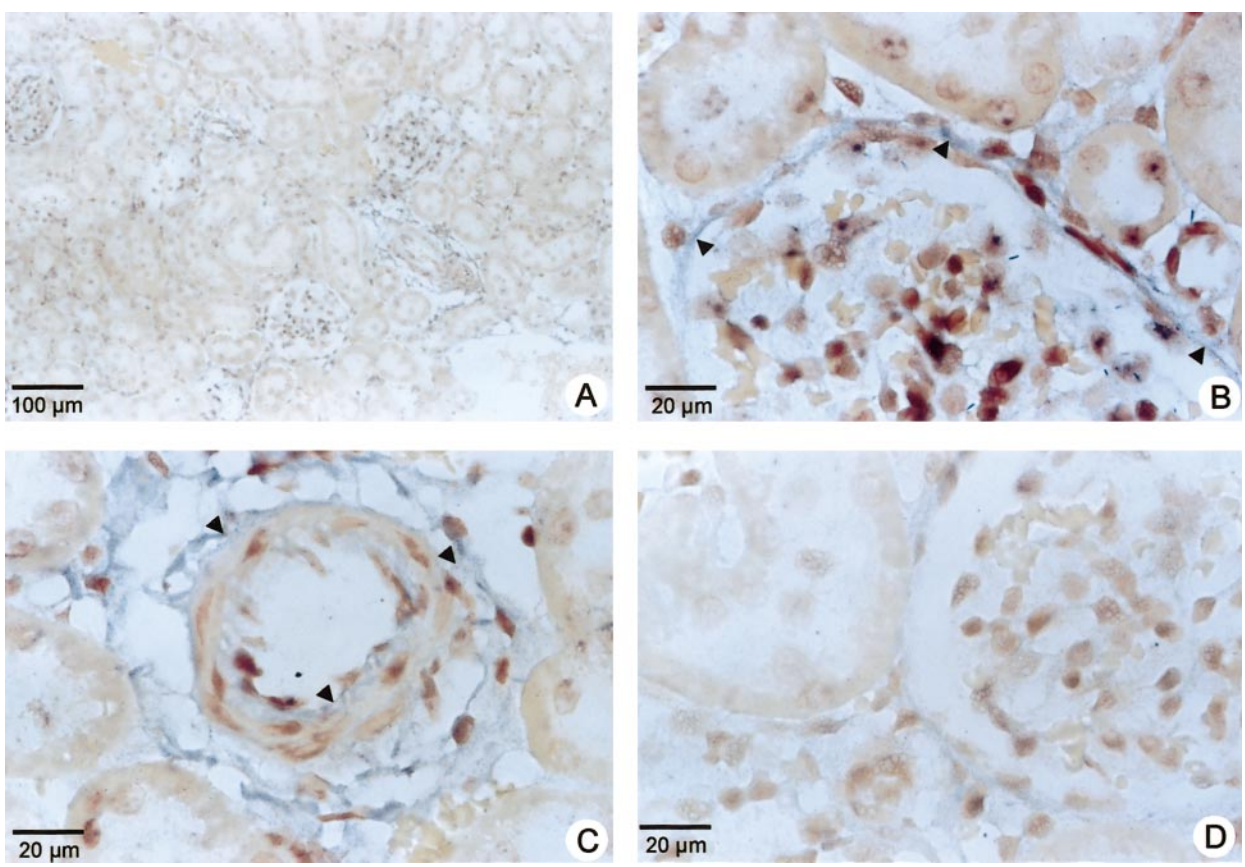

FIG. 5. *In situ* hybridization of rat kidney. (A) Overview of renal cortex (probed with *antisense* transcript). (B) Presence of *rSLP-a* mRNA in Bowman's capsules and absence in tubules (probed with *antisense* transcript). Arrowheads point to positive cells at the parietal side of Bowman's capsule. (C) Detection of rSLP-a mRNA in the adventitia of blood vessels (probed with *antisense* transcript). Positive cells are marked with arrowheads. (D) Control hybridization at high magnification, probed with *sense* transcript.

TABLE 1

Primers and Conditions Used for Generation of the Full-Length cDNA Sequence of *rSLP-a*

Upstream primer	Downstream primer	$T_{annealing}$
5′-GAGGGCGACGGGGTGAGAAA-3′ (323..342)	5′-CGCTCTGCCCGACCACCAT-3′ (1273..1255)	60°C
5′-CGGAGGACGGAAATCACCAT-3′ (802..821)	5′-ACGGTAGGCTTCAAAGTGGATGT-3′ (2031..2009)	55.8°C
5′-GGGGCACCATTGAAGTTGAGA-3′ (1907..1927)	5′-TCCACCCCCACCAGAAGC-3′ (3774..3757)	61.2°C
5′-GTGTAGTGGGAGGGGTAGGTGTGC-3′ (3421..3445)	5′-GACCACGCGTATCGATGTCGAC-3′ (adapter primer for 3′-RACE)	60°C

Note. Mismatch nucleotides in primer sequences are underlined.

with α-[^{32}P]dCTP as described above, and hybridization was performed in Quickhyb solution (Stratagene) at 60°C. The membrane was subsequently washed with 2× SSC/0.1% SDS (25°C, 2 min), 2× SSC/0.1% SDS (60°C, 20 min) and 1× SSC/0.1% SDS (60°C, 1 h). Signals were analyzed with the Storm 860 Phosphoimaging system (Amersham Pharmacia Biotech).

Sequencing of intron/exon boundaries. Genomic DNA was isolated from cultivated rat skin fibroblasts (31), resuspended in 10 mM Tris/EDTA, pH = 8.0 to a final concentration of 1 μg/μl. DNA was sheared by pressing several times through a 20 gauge needle and used as a template for PCR with appropriate primers under the conditions mentioned above. Screening of the rat genomic library 612 (kindly provided by the Resource Center and Primary Database of the German Human Genome Project, RZPD, Berlin) with a α-[^{32}P]dCTP labeled probe was performed according to the provider's instructions.

In situ hybridization. A cDNA probe comprising nucleotides 1-483 of *rSLP-a* in pCRII-TOPO (Invitrogen) was transcribed *in vitro* from the linearized plasmid to *sense* and *antisense* riboprobes using digoxygenin-labeled UTP and the SP6/T7-RNA polymerase kit (DIG RNA Labeling Kit SP6/T7, Roche). Tissue sections were obtained as described (32) and hybridized with the *sense* and *antisense* probes for 16 h at 58°C. The sections were then washed at ambient temperature for 30 min each with 50% formamide in 2× SSC and 50% formamide/1% 2-mercaptoethanol in 2× SSC followed by a wash with the latter solution at 50°C and a final wash with 1× SSC for 15 min at ambient temperature.

RESULTS

Cloning and Characterization of rSLP-a and rSLP-b

Immunoscreening of a rat mesangial cell cDNA library yielded a clone of 197 bp (clone 1; GenBank Accession No. AI943791) of which 51 bp were highly homologous to a region of the human septin *MSF* immediately downstream of the translocation breakpoint (17). An oligonucleotide probe derived from this clone was subsequently used to rescreen the same library. A clone of 485 bp (clone 2; GenBank AI943792) was found which comprised the homologous 51 bp mentioned above but contained a different 5′-sequence. The latter sequence showed 61.9% homology to the region of *MSF* located immediately upstream of the translocation breakpoint. Both clones were therefore considered to represent either alternatively spliced variants or isoforms with different exon usage. Starting from the sequence information of clone 2, a full length sequence was assembled under the conditions listed in Table 1 by using RT-PCR and 3′-RACE-PCR.

The cDNA sequence (AF170253) shown in Fig. 1A contains 3869 bp with a deduced amino acid sequence of 564 amino acids. This sequence was named *rSLP-a* and considered to encode rat septin-like protein-a with a predicted size of 63.8 kDa. Within the coding region there are three consensus sequences for nucleotide binding (33), representing a P-loop motif (34). GTP-binding domains have been found so far in all septins. Analysis for coiled coil regions (35), which were readily detectable in other members of the septin family, did not yield unequivocally a positive result.

As stated above, the 197 bp of clone 1 contained only 51 bp which were also found in *rSLP-a*. Starting from clone 1, further screenings of cDNA libraries (mesangial cells, rat brain) as well as 5′-RACE-PCR experiments did not result in additional information, presumably due to the high GC content (>70%) of this part of the sequence. Therefore, screening of a rat genomic DNA library in λEMBL3 SP6/T7 (Clontech) with clone 1 as probe was performed. The screening yielded one positive clone, clone 3 (AF179588). The insert of this clone with a length of 14044 bp was completely sequenced after subcloning. Computer based sequence analysis verified 146 bp of the probe sequence, but failed to identify the 51 bp which clones 1 and 2 have in common. Splice site prediction (SSPN, http://www.fruitfly.org/seq_tools/) for clone 3 revealed a putative intron/exon boundary 165 bp upstream of the probe sequence. On the basis of these data, the 311 bp identified in clone 3 were considered as an exon which was designated exon r1b (see Figs. 1B and 3). This conclusion was verified by RT-PCR experiments with RNA from rat brain and cultivated rat fibroblasts. Only combinations of primers located within exon r1b itself or spanning exon r1b and sequences further downstream of exon r1b, as supposed by the *rSLP-a* data, yielded the expected amplicons. All data were verified by DNA sequencing. Forward primers, hybridizing upstream of exon r1b in genomic clone 3 gave no

products. These data implicate a different usage of exon 1 in rat septins and define the existence of *rSLP-b* being characterized by the use of exon r1b (see Fig. 1B). The cDNA sequence of *rSLP-b* (AF173899) comprises 3741 bp. As there is no start codon in exon r1b, the open reading frame of 479 amino acid residues begins with the first appropriate codon downstream the exon/exon boundary (see Fig. 1B) encoding a protein, rSLP-b, with a predicted molecular weight of 54.4 kDa.

Multiple sequence alignments of the corresponding human and mouse proteins with rSLP-a and rSLP-b revealed a high degree of conservation (up to 87% between rat and man, up to 97% between rat and mouse) in the central portion and the C-terminus, whereas the isoforms show reasonable differences in their N-terminal region as a result of the distinct exon usage in the corresponding mRNAs (see Fig. 2). The N-terminal region of rSLP-a is highly conserved to MSF.

Using exon r1a and exon r1b, respectively, as a probe, Northern blot analysis of RNA from cultured rat skin fibroblasts showed a single band with the expected sizes of 3.9 kb and 3.8 kb, respectively (see Fig. 3A). In contrast, two bands of 2.7 kb and 3.9 kb were detected in rat brain, kidney and liver (see Fig. 3B), when hybridized with a probe comprising exon r1a, but only one band of 3.8 kb was observed when using exon r1b for detection (see Fig. 3C).

As two forms of human *MSF* with different 3′ non-translated regions are known, a probe comprising the sequence region $C_{2282}..C_{2697}$ was used for further hybridization experiments. Again, only bands of 3.8 and 3.9 kb were detected in RNA from cultured rat skin fibroblasts, whereas a second band of about 2.8 kb was visualized in embryonic kidney and brain as well as in adult spleen and lung (see Fig. 3D).

As an alternative splicing of the 3′ non-coding sequence was assumed to occur, RT-PCR experiments with RNA from multiple tissue sources were performed. However, carefully optimized PCR experiments with a number a different primer pairs, yielded no other amplicons from the 3′ non-coding region than the ones deduced from the sequence in Fig. 1A. Forward primers used for this purpose were 5′-ACTTCAAACAGCGGATCACC-3′$_{(1718..1737)}$, 5′-TGTCCAACGGTATTGACGTGTA-3′$_{(1748..1769)}$, 5′-ATTTGTCACCTGCCTCCATC-3′$_{(2196..2215)}$, reverse primers were 5′-GTGGAATGGCCTGGTGAG-3′$_{(3740..3723)}$ and 5′-GCTTCCTGGTGGGGGGTGGA-3′$_{(3777..3758)}$. Thus, the existence of a short sequence, as suggested from the human *MSF* data, could not be verified.

As only one band was detected in hybridization experiments with exon r1b as probe and the existence of a short form could not be verified, the relative amounts of *rSLP-b* in different organs and developmental stages could readily be quantified after normalization to GAPDH (see Fig. 3C). Comparing normalized data obtained from embryonic rat brain (day 17) and adult brain indicated an about 1.5-fold greater abundance of the *rSLP-b* message in the fetal organ than in the adult. No analogous developmental changes were found in kidney. It should be mentioned that there was an about seven fold higher expression of *rSLP-b* in adult spleen compared with brain (see Fig. 3E).

Exon Structure of rSLP-a and rSLP-b

Screening of a rat genomic library (RZPD library 612, created by Bento Soares) using exon r1b as a probe, yielded two positive clones (UI__p612M1536Q2 and UI__p612E0737Q2). The complete exon structure of the *rSLP* gene was resolved by sequencing PCR amplicons using cDNA-derived oligonucleotides as primers. Isolated rat genomic DNA or DNA from the two isolated clones were used as templates independently. Exon/intron boundaries were also obtained by direct sequencing of the two isolated clones whenever PCR was not successful. The results are listed in Table 2.

The rSLP gene consists of at least 12 exons. Their boundaries are highly conserved to the human *MSF* gene (19) and show characteristic splice donor and splice acceptor dinucleotides (36). As described above, exon 1a and exon 1b, respectively, are unique for each *rSLP* variant (see Fig. 4). Exon 1a contains an appropriate ATG codon for the start of transcription upstream of exon 2 while exon 1b contains 5′ UTR sequence only.

In Situ Hybridization of Rat Kidney

As the kidney represents an organ being composed of an especially large variety of differentiated cells that can be identified by light microscopy, paraffin sections of rat kidney were subjected to *in situ* hybridization procedure. However, the high GC content of the probe for the expression of exon r1b prevented the analysis of the expression of *rSLP-b*. The strongest expression of *rSLP-a* was seen in cells at the parietal side of Bowman's capsule and in the adventitia of blood vessels. Proximal and distal tubuli as well as mesangial cells exhibited only weak reactivity (Fig. 5).

DISCUSSION

We describe here for the first time the complete cloning and characterization of two cDNAs isoforms of rat septins, designated *rSLP-a* and *rSLP-b*. Because of alternative exon usage in the 5′ region, the two cDNAs encode for two proteins, rSLP-a and rSLP-b. Only one of the first two exons, exon r1a, contains an ATG sequence followed by an open reading frame. Thus, the two proteins have identical C-terminal sequences while rSLP-a is supposed to contain an additional sequence of 75 amino acids at the N-terminus to give a total of 564 amino acid residues. Our data contrast to

TABLE 2

Exon Structure of the *rSLP*-Gene

Exon	Acceptor splice site	Donor splice site	Size	Start *rSLP-1*	End *rSLP-1*	Start *rSLP-b*	End *rSLP-b*
1a	5′ UTR	...GAGATCGCATCACAG	433 bp	1	433		
1b	ctcc**ag**GCCCAGTTTTGACAG...	...TTCAGACTTTGAAGG**gt**gggt	308 bp			1	308
2	ggtc**ag**CCTTAAAGAGATCGT...	...TCGGAACCAGGAAGA**gt**gaac	640 bp	434	1073	309	948
3	ccca**ag**CTCCGAGGTGGCTCC...	...AACATCATGGTGGTT**gt**gagc	190 bp	1074	1263	949	1138
4	cccc**ag**GGGCAGAGTGGCCTC...	...AGTCCATCACTCACG**gt**gagt	130 bp	1264	1393	1139	1268
5	tctg**ag**ATATTGAAGAGAAGG...	...CAACAATGAGAACTG**gt**aggt	82 bp	1394	1475	1269	1350
6	gtgc**ag**CTGGCAGCCTATCAT...	...AGCCACCGGCCACTC**gt**aggt	138 bp	1476	1613	1351	1488
7	tttc**ag**ACTCAGGCCCCTGGA...	...CTACTTCAAACAGCG**gt**aggg	117 bp	1614	1730	1489	1605
8	ttcc**ag**GATCACCTCAGACCT...	...ACGAGAAGTTTCGGG**gt**gagt	95 bp	1731	1825	1606	1700
9	ttcc**ag**AGATGATCCCATTTG...	...GGGGCACCATTGAAG**gt**actg	96 bp	1826	1921	1697	1792
10	tgct**ag**TTGAGAATACCACTC...	...GGATCTCCTTATCAG**gt**gagt	52 bp	1922	1973	1793	1848
11	ctgc**ag**GACGCACATGCAGAA...	3′ UTR	1896 bp	1974	—	1849	—

Note. Uppercase letters represent coding exonic sequences; lowercase letters represent intronic or noncoding sequences. Conserved splice acceptor and splice donor dinucleotides are in bold.

those of Fung and Scheller (10), who described two incomplete variants of rat septins, named eseptins. The long form of the eseptins was considered to comprise the 404 C-terminally located amino acid sequences and the short form the 333 C-terminally located amino acids of rSLP. Additionally, an aspartic acid residue was located between residues 460 (glutamic acid) and 461 (alanine) of sequence reported here. It seems likely that the high GC-content of the 5′-cDNA sequence was the reason for the incomplete sequence information obtained by 5′-RACE as used by Fung and Scheller (10).

Analysis of the genomic structures revealed that the transcripts are composed from at least 12 exons. Further alternatively used exons in the 5′ region may exist, but have not yet been identified. Northern blot experiments showed that *rSLP-a* and *rSLP-b* transcripts with the expected size could be detected in cultures of rat skin fibroblasts, but an additional band of 2.8 kb was detected with the same probe designated to detect only the long forms. For human *MSF*, the existence of a polymorphic 3′ exon has been described, yielding a short form (*MSF*) and long forms (*MSF-A/-B/-C*). Attempts to verify a similar situation in the rat by RT-PCR experiments with forward primers residing within or upstream of exon 11 and reverse and complement primers from various positions of exon 11, were not successful. No sequences have yet been identified that hybridized solely to the 2.7 kb transcript. Thus, the identity of this transcript remains unclear. Our findings could be explained by alternative splicing of exons containing translatable information. This possibility has not been investigated further.

It also remains unclear what the function of the oligopeptide of 75 amino acids being unique for rSLP-a might be. It is tempting to speculate that this extra sequence is responsible for a specific targeting of rSLP-a. Computer-based searches (MOTIF, http://www.motif.genome.ad.jp/) for characteristic structural motifs did not yield conclusive results. Unfortunately, the tools to determine the localization of rSLP-a and rSLP-b on the ultrastructural level are not yet available. From the *in situ* hybridization experiments, a highly selective expression of *rSLP-a* can be deduced. Interpretation of these data will be possible when more information on the function of the mammalian septins is available. The factors that govern the expression of the septins as a family and their different subforms remain to be investigated.

ACKNOWLEDGMENTS

The authors thank Dr. L. W. Fisher, National Institute of Dental and Craniofacial Research, National Institutes of Health, Bethesda, Maryland for critically reading the manuscript and providing the rat genomic library. The expert technical help of Petra Blumberg and Sigmund Budny is gratefully acknowledged. This work was financially supported by the Deutsche Forschungsgemeinschaft (SFB 492, Project A6; Graduiertenkolleg 234). The rat genomic library 612 and all corresponding positve clones were provided by the Resource Center of the German Human Genome Project at the Max-Planck-Institute for molecular genetics, Heubnerweg 6, D-14059 Berlin.

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