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The *trans*-spliced variants of Sp1 mRNA in rat☆

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Abstract

trans-Splicing is the biological reaction that generates a mature mRNA from separate strands of pre-mRNAs. Previously, we reported that the *trans*-splicing between the two Sp1 pre-mRNA strands produced an mRNA with the exon 3–2–3 alignment in human HepG2 cells. Here we describe the rat counterpart as well as a newly identified variant with the exon 3–3 alignment in cultured rat cells. A qualitative evaluation of such alignments in poly(A)$^+$ RNA-rich preparation showed that both alignments arose from *trans*-splicing rather than circularization of a single strand. The identification of the *trans*-spliced products in both rat and human raises the possibility that *trans*-splicing on Sp1 pre-mRNA is rather common to mammals. It was observed that the level of the *trans*-spliced variants varies in different rat organs.

Keywords: Sp1; Spliced variant; Tissue-specificity; Transcription factor; *trans*-Splicing

trans-Splicing is an inter-molecular reaction between a splice donor and a splice acceptor in two separate pre-mRNAs. Although this reaction was initially shown to occur in vitro [1,2], the *trans*-splicing between different pre-mRNAs has been shown to be a natural biological event in trypanosoma, nematodes, plant chloroplasts, and mammalian cells [3–7]. Recently, *trans*-splicing between pre-mRNAs of a kind, namely homotypic *trans*-splicing, has also been discovered in mammalian cells. Homotypic *trans*-splicing produces heterogeneous mRNAs for rat carnitine octanoyltransferase, rat SA, and rat voltage-gated sodium channel [8–10]. Although the biological significance of homotypic *trans*-splicing has not been established yet, the *trans*-spliced rat SA mRNA is produced in a tissue-specific manner and nerve growth factor enhances the level of the *trans*-spliced mRNA for voltage-gated sodium channel [9,10]. Thus, homotypic *trans*-splicing in mammals can be a regulatory mechanism that generates diverse gene products.

Previously, we demonstrated a *trans*-spliced product of Sp1 mRNA having the exon 3–2–3 alignment in human HepG2 cells [11]. Sp1 is a mammalian transcription factor that binds to the GGGCGG sequence in the promoters of various genes and activates transcription. Although Sp1 had been regarded as a ubiquitous transcription factor for constitutive expression of housekeeping genes, recent studies suggested that Sp1 was also involved in specific gene activation in response to a variety of signals [12–16]. It is known that alternative splicing that generates isoforms of transcription factors is frequently associated with tissue-specific and/or signal-responsive cellular regulatory mechanisms [17]. Accordingly, we investigated tissue-specific expression of the *trans*-spliced Sp1 mRNA variants. For this purpose, we first confirmed that the *trans*-spliced Sp1 mRNA is also present in rat cells as in human cells. Then, tissue-specificity in terms of such splice variation was analyzed using RNA from different tissues of the rat.

Materials and methods

RNA preparation and cDNA synthesis. Total RNA was isolated from rat primary culture cells derived from adipose tissues using TRIzol Reagent (Gibco-BRL) according to manufacturer's instruc-

☆ The nucleotide sequence determined in this paper has been deposited in DDBJ/GenBank/EMBL database (Accession No. AB077988).

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tion. Total RNA was also prepared from various tissues of the rat (7 week male Wistar, Cler, Japan). Poly(A)$^+$ RNA-rich and poly(A)$^-$ RNA-rich fractions were prepared from total RNA of the cultured cells as described previously [11]. The first strand cDNA was synthesized with the total RNA and avian myeloblastosis virus reverse transcriptase XL (Takara Shuzo) or Superscript II (Invitrogen), using the primer RT (5′-TCTGTTCCTTTG-3′) or the primer Rev (5′-TTGCTGCCATTGGTACTGT-3′), which was complementary to the sequence from +379 to +368 or from +404 to +386 of the rat Sp1 mRNA, respectively. The first strand cDNA was also synthesized with the oligo$(dT)_{18}$ primer. Numbering of positions relative to the translational start site is adopted throughout this paper, based on the sequence information on the rat Sp1 mRNA in the database (GenBank Accession No. D12768).

PCR primers used in this study. The DNA sequences and the positions of PCR primers used in this study are listed below: rT1, 5′-CAAGCCCAGACAATCACCTTG-3′ (positions +1441 to +1461); rT2, 5′-TATGCAGGGTGTTTCCTTGG-3′ (positions +1467 to +1486); rT3, 5′-ACCAAGATCACTCAATGGATGAAG-3′ (positions +8 to +31); rT4, 5′-GAAGTGACAGCTGTGAAGATTG-3′ (positions +28 to +49); rT5, 5′-CTGTGAGGTCAAGTTCACCT-3′ (positions +298 to +279); rT6, 5′-ATGATCTGCCAGCCATTGGC-3′ (positions +338 to +319); rRPA2 (5′-CGTCTAGACCGTTATTG CCACCAACACCTT-3′, positions +74 to +53); rRPA3 (5′-CG TCTAGACTCATTGGGTGACTCAATTCTGC-3′, positions +231 to +209); rRPA4, 5′-TCGAAGCTTTTACACCCATTGCCTCAGC TG-3′ (positions +1514 to +1534); rRPA5 (5′-GGCAAGCTTGCTCG CCCCTCAGCTGCCAC-3′, positions −31 to −12); rRPA6 (5′-GGC TCTAGAGGGAGTTGTTGCTGTTCTCATT-3′ (positions +253 to +232); P1, 5′-TGCAGCAGAATTGAGTCAC-3′ (positions +205 to +223); P2, 5′-GTGGCAGCAAACTGCAGCTG-3′ (positions +584 to +565); DK1, 5′-CCTCAGCTGCCACCATGAGC-3′ (positions −24 to −5); DK4, 5′-TGCTGCTACTGCCTGTGCTGC-3′ (positions +142 to +122); DK5, 5′-CAGGCCTGCCTTTGGCTATAGC-3′ (positions +1652 to +1673). Several of these PCR primers had a sequence (underlined) for creation of a restriction site in addition to the Sp1 sequence.

Isolation of a genomic clone and DNA sequencing. A DNA fragment corresponding to the region (positions −31 to +142) of the rat Sp1 mRNA was amplified by PCR with rat cDNA, using PCR primers rRPA5 and DK4. Using this PCR product as the probe, a rat genomic library (Clontech) was screened by a plaque hybridization technique according to the standard protocol [18]. The nucleotide sequence of the genomic clone was determined by the dideoxy chain termination method using a Thermo sequenase II dye terminator cycle sequencing kit (Amersham Pharmacia Biotech) and an automated DNA sequencer (ABI 373S DNA Sequencing System).

Southern blot analysis. Rat genomic DNA (Clontech) was completely digested with appropriate restriction enzymes, electrophoresed on a 0.7% agarose gel, and transferred onto a nylon membrane (Hybond-N, Amersham Pharmacia Biotech). The DNA on the membrane was hybridized with the ^{32}P-labeled DNA fragment (positions +205 to +584), whose region was amplified by PCR using primers P1 and P2, at 65 °C for 16 h. Then, the membrane was washed in 2× SSC for 15 min at 65 °C two times, in 2× SSC plus 0.1% SDS for 30 min at 65 °C, and in 0.1× SSC for 10 min at 65 °C and subjected to autoradiography.

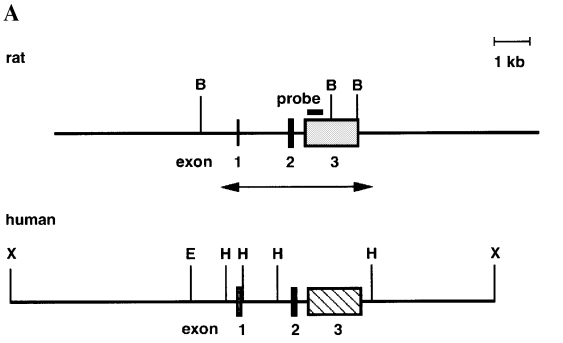
RNase protection assays. To create the template plasmids for in vitro synthesis of the riboprobe 3–2 and the riboprobe 3–3, two DNA fragments were amplified by PCR using respective pairs of primers rRPA2 and rRPA4, and rRPA3 and rRPA4. The templates used for this amplification were the DNA fragments obtained in the experiment presented in Fig. 3. Each amplified fragment was digested with *Hin*dIII and *Xba*I, and then inserted between *Hin*dIII and *Xba*I sites of pGEM-3Zf(+) (Promega). Antisense riboprobes were synthesized from T7 promoter within the vector in the presence of [α-^{32}P]UTP using a T7 RNA synthesis Kit (Nippongene). RNase protection assays were performed as described previously [11].

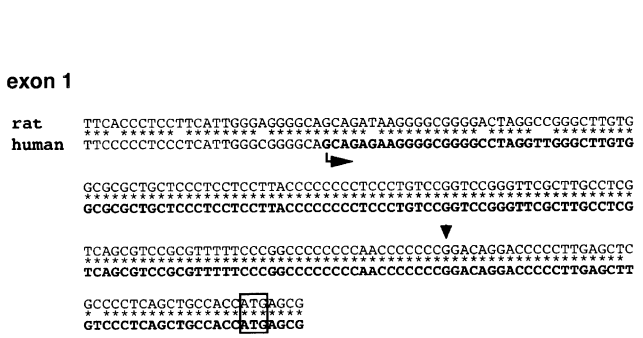
Results and discussion

Structure of rat Sp1 gene

Some *cis*-splicing events have been shown to be species-specific [19,20]. In addition, the *trans*-splicing on carnitine octanoyltransferase gene was observed in rat but not in other mammals [21]. To evaluate the possibility that the *trans*-splicing on Sp1 gene also occurs in rat cells as in human cells, we first need to determine the intron–exon structure of the rat Sp1 gene. In human cells, the *trans*-spliced mRNA of Sp1 is produced by attack of the donor site within the intron 3 on a pre-mRNA of Sp1 to the acceptor site within the intron 1 on another [11]. On the other hand, the intron 3 in the human Sp1 gene was judged to be very large (>40 kb) on the basis of the draft database on human genome (Accession No. NT_009563). Therefore, we screened the rat genomic clone for this study with a DNA probe encoding only the 5′ terminal region of the rat Sp1 cDNA (−31 to +142). The Southern blot hybridization analysis indicated that the sequence of the probe DNA was located on a 3.5 kb *Bam*HI fragment from the genomic DNA clone (Fig. 1A). The fragment showed a striking homology with the human gene in its sequence around the transcription start site (Fig. 1B). We therefore assumed that transcription of the rat Sp1 gene also starts from the position corresponding to the transcription start site of the human gene. We could uniquely assign the exons 1 through 3 of the rat Sp1 gene on the basis of this striking homology and by comparison with a partial rat Sp1 cDNA in the database (Fig. 1B). The position and size of the introns 1 and 2 in the rat Sp1 gene were closely similar to those in the human Sp1 gene. The intron 3 of the rat gene might be very large as that of the human gene, because Southern blot hybridization analysis suggested that our clone did not cover the exon 4 (data not shown). In addition, sequence analysis revealed the following discrepancy between our sequence and the sequence of rat Sp1 cDNA that was already registered in the database: two short sequences (positions −13 to −4 and positions +232 to +237) were missing in our genomic clone. These sequences were also missing in our cDNA clone that was subsequently isolated by reverse transcriptase-polymerase chain reaction (RT-PCR, data not shown). In spite of these discrepancies, we adopted the numbering of positions according to the sequence of rat Sp1 mRNA in the database, so that one can easily specify the positions of the primers we used (see Materials and methods).

Since it is known that the DNA sequences in introns affect splice site selection and efficiency of splicing [22–24], we compared the DNA sequences of introns of the rat Sp1 gene with those of the human gene (Fig. 1C). The two genes have diverse DNA sequences in introns whereas they showed very high homology in exons 1, 2,





C

intron 1

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rat      1 GTAAGGATGAGTCCACTCCAAGCCTAGGGGTGGGAGGGGAGTGAGGGGGCGCGCGCGAGG
human    1 GTAAGGATGAGTCCACTCCAAGCTTAGGGGTGGGAGGCGAGTGAGGGGGCGCGCGCGAGG
         61 GCCGACCGGGCCGGTCCCCGCTGTG-AGGGGGGGCGGGCGGGA---AGCGGCGGCGGCGGC
         61 GCCGACCGGGCGATCCCCGCCGTGAAGCGGGGGCGGGCGGGAGGCGGCGGCGGCGGCGGC
        117 CTTGGTCCCGCCCGGGGCGGAGGGAAGGGAGGGAGAAGGGGCAGTGGCGGGGCCTCGGAG
        121 CTAGGTCCCGCCCGGGGCGGAGGGAAGGGAGGGAGACGGGGCAGTGGCGGGGCCTCCGAG
        177 GAGGAGGGGGATGGGCTACCCGCCCCGGGGGAGGGGGCAGCCTGGCCTCGCCCGCCCCCT
        181 GAGGAGGGGGATGGGCCGCCCGCCCCGGGGGAGGGGGCAGCGTGGCCTCGCCCGCCCCCT
        237 GCCCGCCCCGGCCACGGGGGACGGGCCTTACCCCCCACTACCCGGCCGGCCGCCTGAGGC
        241 GCCCGCCCCGGCCACGGGGGACGGGCCTTACCCCCCACTACTCGGCCGGCCGCCTGAGGC
        297 TCCTCCTGTCGGGGGGCTGGCGCCGCGGGGGCGGCCCGACCAGTGCAGGCCCCGCCTGGGC
        301 TCCTCCCGCCGGGGGCTGGAGCCGCGGGGGCGGCCCGAGCAGCGAAGGCCCCGCCCGGGC
        357 CAACCGCCTCCCCGGTCCGCCCTCCGGTCCCAGCCTGCTCC-AGGCCCTCC--CCCGCAC
        361 CAACCGCCTGCCTGGTCCGCCCTCTGGTCGCCGCCTGCTCCAAGGCCCTCCTCCCCCCAC
        414 -CCCCGTAAATTTCCCTT---------CCCCCATCCGACCCGCCCTTGCCACGCCCCTAAC
        421 TTTCCGTAGATTTCCCTTCCCCCCGCCCCCCACCGTCCCGCCCTTTCCACGCCCCCTCAC
        465 CCCGAAACCGCCCCTTCCTTCTATGGCCCGGCCCCTCAGCCCCAAACC---TGGGTTCTA
        481 CCCGAAACCGCCCTTTCCCTC-AGGGCCC-GCCCTCCGCCCCGAAACCTTCTGACTACTA
        522 AGCCCTAAACCATTTTAGCCTCTTACCTGCCCTCTTCACATGTTTCCTCACCTCCACTTA
        539 ACCTGTAGATCCCTTTAGTTCCTTAGCAGTATTC-ACAAATGTTT------TTCTACTCA
        582 CAATTTACCCTTTCTCGGCCCCTTCCTGTTTTTGTTTACACTTAATCTGTCTCCATCTCT
        592 CACTTTTTAC-TTCTTTTTCCCCTCTCGTTACTTTTCACAC--------T-TGGATTTTT
        642 TTTAGTCCTAATTTTAGCCCACTTTCCATCCACCTATGTGTTGGGTCTCCTTTTCCTTCC
        642 GTTTGTCTTTTTTTTTTTTTTTTTTTTTTGAGACGGA-GTTTTCGCTCTTGTTACCCAGAC
        702 AGGATGCTTTTTTGTTTTTCTTTTTGAGATAGGGTCTATGTGGTACTCGTAGTAACTCGA
        701 TGGA--ATGCAATGGTGCGATCTCGGCTCACTGCAACCTCTGCCTCCCGGATTCAGGCGA
        762 AGTTACTATGCAGACCTCAAACTTTTAGCTACCTAGTTGCCTGCCTCTTCCTCCTGAGTG
        759 TTCTCCTGCCTCAGCCTC-CGGGAGTAGCT--GGAATTACAGGCATGCGCCACC---ACA
        822 CTGGGATTAGAGTGTGCCATCACTAGGCCCCACTTTTTTTTTTTTTTTTTTTAATTTCTG
        813 CCCGGCTAATTTTGT---ATTTTTAGTAGAGAC---------AGGGTTTCTCCATGTTGG
        882 AGACACTGCTCTCAAATTCCCTCAAAAGTGCTGTTTGCATTGAGATCAGTTCTTACTCTG
        861 TCAGGCTGGTCTCAAAACTCCCGACCCCAGGTGATCTGCCTGCCTCCGCCTCCCAAAGTA
        942 TAGGTCCGGCAGACCTAAGAACCACCTGGCTCATCCT-TCCAAGTCTTGGGATTATAGGG
        921 ATGGGATTACAGGCGT---GACCACCGCGCCTGGCCTCTCCATCTCTTTTAAACTTA--A
       1001 TGTGAGTCACCATGCCTGGCTTCCAAAGCAATTTTTGAGTCATTTCTG----GGGTTAAT
        976 TTTTAGCAAGCTTTCCATGCACCCACATGTTGTTTTATGCCTTACCTGTCCTTTTCCTTT
       1057 TTATGCCTGGGAAAATAGTGACCTGTCTTG-GACAC-CATTAAGCC-ATGTGCCTGCTC-
       1036 TTTTCCCTTAGGATACTTTTTCTTATTTAGAAACTCTCACCATGCCTACCGTCCTTCACA
       1113 --TGC-ATGCCTATGGC----TTTG----GCATCTCAGTTTAA---TGTGTAACTGAGAA
       1096 TTTCCTATCCCCAAAGCACTTTTTGAATGTTTTCTTAGTTTAATTTTGTACAAGGGAGAA
       1159 TTTCTTTGAACTCTTGTCTCCACTTCCAAAATACTGGGATTTTAGGCCTACCACCATAAC
       1156 TTAAGGCCCACTTTTGGCACATTGAACCTAGTTCATGTCCTGCATGCCCTGCAGCTCCCC
       1219 T-AGCTGT--------ATCTGACTAATACCTTTAAAAAAAAACATTTTATTAGTCATTGCT
       1216 TCTGCCCTCCAATCCATTTTCTTTATACTGGTGGCTTTCGCCTTCCTGTTAGTCATTGCT
       1271 -GGTGGTCAAAACGT-----TAATTTTAAATCTTAATGT-TTTTC-CCCCTCAA------
       1276 ATTTCATCATAGCCTCCTTTTATCCTTCCTACTTCATTTCTTTTCTTCCCTCAAGTTTAC
       1317 CTTATTTGTTTTGTAATTATTTTTAG
       1336 GTTGTTTGTTTTTTAATTATTTT-AG
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intron 2

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rat      1 GTGAGTG---ATCAAAGA-TGGGGAAGGTATTGAAAGGGTATAAGTATTCTTAGA-AACC
human    1 GTAAGTGATAATCATAGAGTGGGGAAGGTGTTGAGAAGATGTAAATATTCTTAGATAATT
         56 TCCTTA-----ACCAAAAACTCTGGGAGAAAGTACAGTAGGGAAGTACACACAGAAAGAT
         61 GCCTTACTCTTCACAGAAAGCGTTTTAGGGAG-AGACTAAACCATTTTATAGATAAGGAA
        111 AGGTGATCTAGAGGGTCCCCAAATAGGAA---AGTTGGAACAGTACACAGAAGTTAGCATT
        120 GTAAGAGAATGGAGATCCCCAAAGACAAAGGAGTTTCAGCAATACAGATAGGTCAGCTTT
        169 TTGTTTCTGGTTTTTGCTCTTTATATGCCTGCATTGTTGCTGTTTGTCTGTTGAATACTG
        180 TTGTTTCTGTTTTTTGCTCCTTGTCTG--CACTACGTTGCTGTTTATTTGTTGTATACTG
        229 CCCCCTAGGCCAGCTATTGGGTGCCACTAACTCCTTTCCTCTCCCTTATTTTCGGCCAG
        238 CCCCCTAGGCTGGCAGCTGGGTGTCACTAACTCCTTTCCTCTCCCTTATTTTCGGCCAG
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intron 3

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rat      1 GTAAGACATCCATTCTT-TGCACTAATCCCAGACTAATGCTTCCCTGATA-CT---AC-T
human    1 GTAAGATTTCCAATCTTGTGCATTTATTGGGAACCAACTCTAGCATCGTAGCTGAAACTT
         55 GAGTGTAAAG-GGGGAAGTG-GAACTCTGAGAT----GTTTCCTTAAGGCTAAACA----
         61 GAGTCTAAAGAAAGGAATAGAGCCTTTTGAGATAACACTTTCTTGAGGGTTAGTCATTTA
        105 CCCAAAGCAA---GTGTAAAATA-TTTCAGTCAGGATAGTTGGGAGCTGAATTAATGTCT
        121 GGCAAATAAATTTGAGAAATAGAGATTCTGTTTAAGTAATTTGGAACTGAAGTAAAGGAA
        161 TCTTAGTTGGATCTGATGAGAAAGAAGCACTCCTGAGTAATGCCCTGTGGACTCTACTTT
        181 ACTTCTCCAGTTAGTTTGAGGAAGGAGCACTTCTGGGCAATATTCTGGAGTCTGTAATAC
        221 CCTGTTGTCTTGG-ACCACAAACTAAAGCTTG-GTTGCTT
        241 TCAGTGTTTTTGGTGCTGCAGAATAATGGTTGACAAGCTT
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Fig. 1. Structure of the rat Sp1 gene in comparison with the human counterpart. (A) Schematic structures of intron–exon organization of rat and human Sp1 genes are shown. Exons are shown with shadowed boxes and the 5′ flanking regions and introns are shown with lines. The region where the DNA sequence was determined in this paper is indicated with a double-headed arrow. The region used as the probe for Southern blot hybridization was indicated with the bold line above the schematic structure of rat Sp1 gene. Single letter abbreviations stand for respective restriction sites: B, *Bam*HI; E, *Eco*RI; H, *Hin*dIII; and X, *Xba*I. (B) Comparison of the DNA sequence around the transcription start site of the human Sp1 gene and the corresponding sequence of the rat Sp1 gene. The transcription start site of the human Sp1 gene is marked with a bent arrow and the 5′ end of rat Sp1 cDNA registered in the database is marked with an arrowhead. The translational initiation codons in the rat and the human Sp1 genes are boxed. The exon 1 sequence of the human Sp1 gene is shown with bold letters. (C) Comparison of intronic sequences (introns 1, 2, and 3) of the rat (upper) and human (lower) Sp1s. Asterisks indicate the identical bases in the two sequences. Gaps are inserted for the maximum sequence homology. Since we determined only the 258-bp sequence starting from the 5′ splice site for the intron 3, the two sequences are compared within that length.

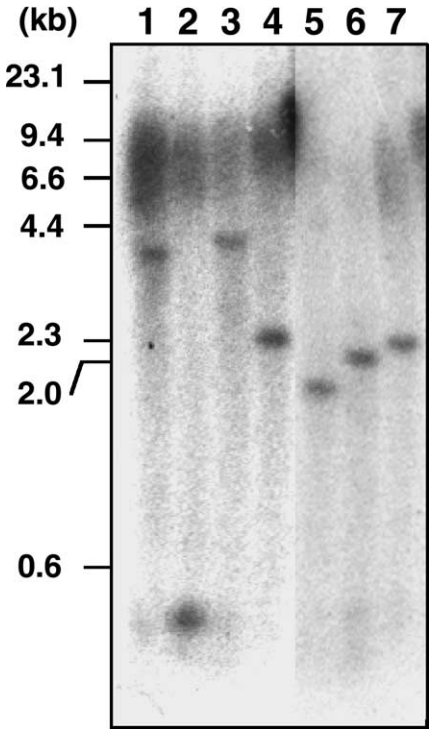

Fig. 2. Genomic Southern blot analysis. Rat genomic DNA (4 μg) was completely digested with *Hin*dIII (lane 1), *Pst*I (lane 2), *Eco*RI (lane 3), *Xba*I (lane 4), *Xba*I and *Hin*dIII (lane 5), *Xba*I and *Eco*RI (lane 6), or *Xba*I and *Sal*I (lane 7), respectively. The DNA fragment derived from a near 5′-terminal region of the exon 3 was used as a probe (see Fig. 1A).

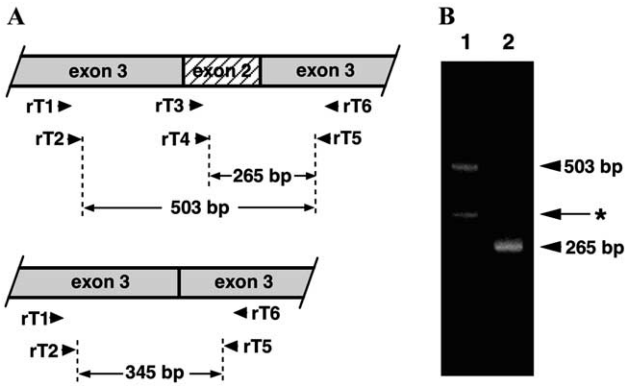

Fig. 3. Detection of Sp1 mRNAs with exon 3–2–3 and exon 3–3 alignment in RT-PCR. (A) Positions and directions of primers used for RT-PCRs were shown by arrowheads in the postulated cDNA structures. For detection of *trans*-spliced Sp1 mRNA, the first PCR with primers rT1 and rT6 was followed by the second PCR with primers rT2 and rT5. Detection of *cis*-spliced mRNA was performed through the first PCR with primers rT3 and rT6 and the second PCR with primers rT4 and rT5. (B) The products of the PCRs for *trans*-spliced mRNA (lane 1) and the product of the PCR for *cis*-spliced mRNA (lane 2). PCRs were performed for 30 cycles in the first and second amplifications. Because different amounts of the template cDNA were used for each PCR, the intensity of each band does not accurately reflect the levels of *cis*- and *trans*-spliced mRNAs. The sizes of the amplified products are shown at the side of arrowheads. The arrow marked with an asterisk indicates an additional PCR product (see text).

and 3 (96%, 85%, and 94%, respectively, data not shown). However, the intronic sequences extending for approximately 500 bp from the 5′ splice site of the intron 1 were highly conserved in the rat and human. In addition to this homologous sequence, some conserved sequences including splice consensus sequences at the donor and acceptor sites were found. These sequences may be critical for precise splicing.

To determine the copy number of the Sp1 gene in the rat genome, genomic Southern blot analysis was performed using genomic DNA digested with various restriction enzymes (Fig. 2). Only a single band of a comparable intensity was detected in each lane by using the partial sequence of the exon 3 as a probe (see Fig. 1A), suggesting that, at least, the probed sequence of the exon 3 of the rat Sp1 gene is a single copy in the genome.

trans-Spliced Sp1 mRNA in rat cells

To examine whether the Sp1 mRNA with the exon 3–2–3 alignment is present in rat cells as in human cells, RT-PCR analysis was carried out with total RNA from cultured rat cells. The PCR primers were designed to amplify a 503-bp DNA fragment, if such a type of Sp1 mRNA was present in rat cells (Fig. 3A). A positive control RT-PCR that intended to amplify a 265-bp cDNA fragment from *cis*-spliced Sp1 mRNA was also performed at the same time (Fig. 3A). As shown in Fig. 3B, the DNA fragments with expected sizes were amplified through PCRs and DNA sequencing confirmed their identities. In addition to the 503-bp DNA fragment, an unexpected PCR product of 345-bp was also detected in this PCR analysis (Fig. 3B, lane 1). DNA sequence analysis revealed that this product had the alignment of exon 3–3 (see Fig. 3A). This tandem alignment had not been detected in the human Sp1 mRNA. We also carried out RNase protection assays to detect Sp1 mRNA with the exon 3–2–3 or the exon 3–3 alignment in the rat cells, using two antisense riboprobes (Fig. 4A). It was expected that hybridization of the riboprobe 3–2 to the junction of the exon 3–2 alignment would produce a protected fragment of 238 nt. In addition, hybridization of this riboprobe to the exon 1–2 junction region and to the exon 3–4 or exon 3–3 junction should produce the 67 nt fragment and the 171 nt fragment, respectively. Such bands were clearly observed with the rat total RNA, but not with the yeast RNA used as a negative control. Similarly, all the expected bands were observed with the rat RNA using the riboprobe 3–3. However, the intensity of the band that appeared to reflect either the exon 3–2–3 or the exon 3–3 alignment was stronger than we had expected on the basis of the results of PCR and Northern

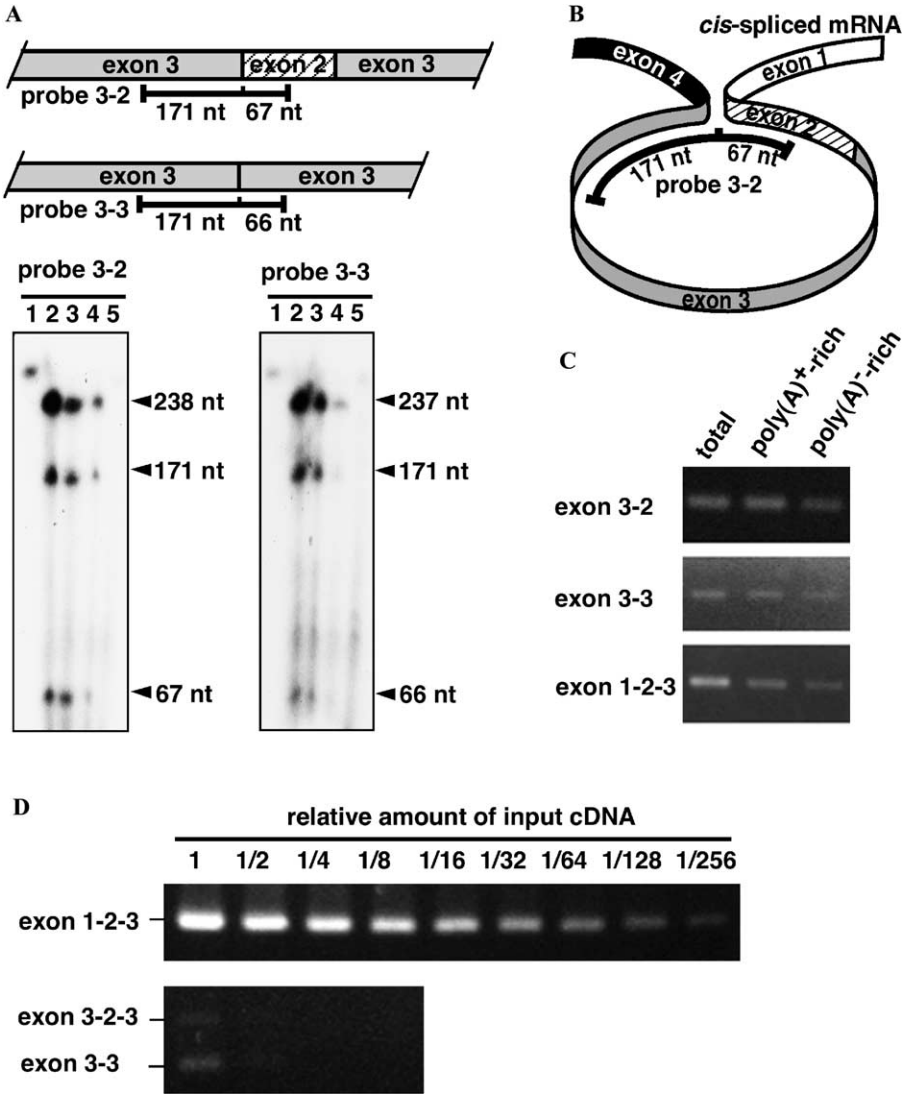

Fig. 4. Analyses of the Sp1 mRNAs with exon 3–2–3 and exon 3–3. (A) RNase protection assay. The targeting positions of antisense riboprobes were shown in the schematic structure of Sp1 mRNA. The sizes of the segments that hybridize to each exon are also shown. The riboprobes were incubated with 40 μg of the total RNA from cultured rat cells (lane 2), poly(A)$^+$ RNA-rich fraction (lane 3), poly(A)$^-$ RNA-rich fraction (lane 4) or 40 μg yeast RNA (lane 5). Poly(A)$^+$ RNA-rich and poly(A)$^-$ RNA-rich fractions were prepared from 40 μg of the total RNA. The undigested riboprobe was also loaded (lane 1). Protected fragments were indicated with arrowheads. (B) A possible hybridization of the probe 3–2 to the *cis*-spliced Sp1 mRNA. (C) RT-PCR analysis. First strand cDNA was synthesized from the total RNA (total), poly(A)$^+$ RNA-rich or poly(A)$^-$ RNA-rich fractions using the oligo(dT)$_{18}$ primer. PCR amplification was performed with primers DK1 and rRPA6 for the detection of the exon 1–2–3 alignment and with primers rRPA4 and rRPA6 for the detection of the exon 3–3 alignment. The detection of the exon 3–2 alignment was performed through the first PCR amplification with primers rRPA4 and rRPA6 and the nested PCR amplification with primers DK4 and DK5. (D) Quantitative RT-PCR for estimation of the ratio of Sp1 mRNAs with the exon 3–2–3 or exon 3–3 alignment to *cis*-spliced Sp1 mRNA. PCR amplification (38 cycles) was performed with primers rRPA4 and rRPA6 for the detection of the exon 3–2–3 and the exon 3–3 alignments, or primers DK1 and rRPA6 for the detection of the exon 1–2–3 alignment, using different dilutions of the first strand cDNA shown at the tops of respective lanes. The first strand cDNA obtained with total RNA from thymus was used, because the level of Sp1 mRNA was highest in thymus.

blot analysis (data not shown). In many studies, RNase protection assays were employed for detection of specific RNA molecules and analyses of their levels. However, as discussed recently in a study on *trans*-splicing using RNase protection assay [9], the protected bands apparently reflecting the *trans*-spliced mRNA may be also caused by hybrid formation of a riboprobe with such a looped *cis*-spliced mRNA as illustrated in Fig. 4B. In the present case, the 5′-side of the riboprobe 3–2 can form a base-pairing with the 5′-side of the exon 2 and the 3′-side of the same riboprobe with the 3′-side of the exon 3. A similar hybridization of the riboprobe 3–3 to the *cis*-spliced mRNA is also possible. RNase digestion of such hybrid molecules is expected to pro-

duce the protected fragments which are as large as the ones produced from the hybrid between the riboprobe and the mRNA with the exon 3–2 or exon 3–3 alignment. Accordingly, the protected bands with the sizes of 238 nt and 237 nt in our RNase protection assay may not be exclusively correlated to the exon 3–2 or exon 3–3 alignment. For this reason, we estimated the ratio of Sp1 mRNA with the exon 3–2 or the exon 3–3 alignment to *cis*-spliced Sp1 mRNA by quantitative RT-PCR rather than the RNase protection assay. Based on comparison of the intensities of the bands for the amplified Sp1 cDNAs that were made from various dilutions of the whole cDNA, the ratio of each Sp1 mRNA with the exon 3–2 or the exon 3–3 alignment to *cis*-spliced mRNA was estimated to be approximately 1% (Fig. 4D).

Circularization can also attach the tail of an exon to the head of either itself or a preceding exon in the pre-mRNA [25–27]. Therefore, we examined whether the exon 3–2 or the exon 3–3 alignment can be detected in the mRNA having the poly(A) tail, because circularization deletes the poly(A) tail. In RT-PCR with poly(A)$^+$ RNA-rich fraction or poly(A)$^-$ RNA-rich fraction, both the products from the exon 3–2 and exon 3–3 alignments were more abundantly amplified from poly(A)$^+$ RNA-rich fraction than from the poly(A)$^-$ RNA-rich one. The distribution ratio of either product between poly(A)$^+$ RNA-rich and poly(A)$^-$ RNA-rich fractions was also similar to that of *cis*-spliced Sp1 mRNA which is polyadenylated (Fig. 4C). These results suggest that both variants with the anomalous exon alignments contained poly(A) tails. Since we have already shown that the exon 3 is a single copy in the rat genome (Fig. 2), a possibility that a *cis*-splicing involving duplicated exons in a single gene caused the mRNA with the exon 3–2–3 alignment and the exon 3–3 alignment is ruled out. These results suggest that both variants of Sp1 mRNA with the exon 3–2–3 or the exon 3–3 alignment are produced by homotypic *trans*-splicing in rat cells.

Distribution of trans-spliced variants of Sp1 mRNA among rat organs

To investigate whether the level of the *trans*-spliced Sp1 mRNA varies in different organs of rat, we performed semi-quantitative RT-PCR using the primers that could amplify both the exon 3–2–3 and 3–3 alignments at the same time. In agreement with the previous study reporting that the level of Sp1 mRNA varies depending on both cell type and developmental stage [28], we observed different levels of the total Sp1 mRNA among various tissues of a rat (data not shown). To compare relative levels of the *trans*-spliced variants among organs, we estimated abundances of the two *trans*-spliced mRNAs relative to those of the total Sp1 mRNAs in each organ. As shown in Fig. 5, relative intensities for both *trans*-spliced bands were markedly lower in spleen than in other organs.

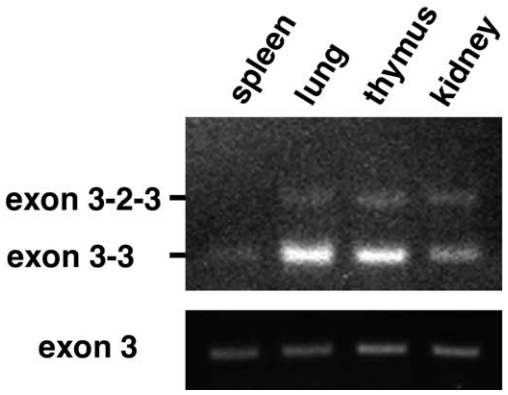

Fig. 5. Tissue dependent *trans*-splicing. The RT-PCR analysis of *trans*-spliced variants with RNAs from spleen, lung, thymus, and kidney was shown (the upper panel). A pair of primers (rRPA4 and rRPA6) that allowed both amplifications of the exon 3–2–3 alignment and the exon 3–3 alignment at the same time was used. The result is shown after normalization on the basis of the result of RT-PCR for the amplification of the exon 3 (the lower panel), because the Sp1 mRNA level is different in these tissues.

Here, we showed that *trans*-splicing produces heterogeneous Sp1 mRNAs not only in human cells but also in rat cells. This fact prompts the idea that *trans*-splicing on Sp1 pre-mRNA can be a universal process in mammals. On the other hand, the exon 3–3 alignment was detected only in the rat and the relative levels of the two *trans*-spliced variants seemed tissue-dependent. These variations may reflect any species- and tissue-dependent controls of gene expressions. As shown in Fig. 1B, both homologous and non-homologous sequences were found in the introns of rat and human Sp1 genes. The differences of intronic sequences might be responsible for the differences in the *trans*-splice site selection and production of the variant with the exon 3–3 alignment in the rat, though the relevant element cannot be specified at this stage. The Sp1 mRNA with the exon 3–2–3 alignment can encode Sp1 protein with repeated transcriptional activation domains [29], whereas the Sp1 mRNA with the exon 3–3 alignment can encode only a truncated form of Sp1 protein because this duplication brought about frame shift and created a stop codon in the second exon 3. One of the possible reasons why mammalian cells produce the spliced variants of Sp1 mRNA is that these spliced variants encode altered functional proteins. Otherwise, these spliced variants might be abortive or formed as a result of post-transcriptional regulation for decrease of the level of productive mRNA. The key question in this context is therefore whether the *trans*-spliced mRNAs are translated into functional proteins or not, which is still left to be clarified.

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