Heterogeneous Sp1 mRNAs in Human HepG2 Cells Include a Product of Homotypic trans-Splicing*

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Sp1 is one of the well documented transcription factors, but the whole structure of human Sp1 has not been determined yet. In the present study, we isolated several cDNAs representing two forms of human Sp1 mRNA with different 5′-terminal structures in HepG2 cells. Isolation of a genomic clone established that one of the cDNAs represents the mRNA having consecutive alignment of exons, which allowed deducing the complete amino acid sequence for human Sp1. Another cDNA clone had a surprising structure that possessed an alignment of exons 3-2-3. Both reverse transcriptase-polymerase chain reaction and RNase protection assays confirmed accumulation of the two forms of Sp1 mRNA in HepG2 cells. Because Southern blot analysis suggested that exon 3 is of a single copy in the genome, the cDNA clone having the duplicated sequences for exon 3 appeared to reflect the trans-splicing between pre-mRNAs of human Sp1.

Transcription factor Sp1 was initially identified as a protein that bound to multiple GGGCGG sequences (GC boxes) in the SV40 early promoter (1). Subsequent studies have shown that Sp1 also interacts with GC boxes in the promoters of cellular and other viral genes and activates expression of those genes (2, 3). Although Sp1 has been regarded as a ubiquitous transcription factor that regulates transcription from TATA-less promoters of housekeeping genes, recent studies have suggested that Sp1 may be also involved in specific gene activation through modulation of its abundance and phosphorylation and glycosylation states in response to a variety of signals (4–8). Likewise, our preliminary studies concerning gene expression responsive to insulin suggested that synthesis and/or degradation of Sp1 protein might be regulated by insulin. Accordingly, we started structural study of human Sp1 mRNA to enquire any account for that apparent insulin effect in its mRNA structure. Despite a large number of reports concerning the function of Sp1, the complete structure of human Sp1 protein has not been established yet. The reported cDNA clones for human Sp1 still lack the N-terminal and the upstream noncoding regions (9, 10), although a DNA-binding domain having three zinc finger motifs and four transcriptional activation domains, termed domains A, B, C, and D (11), have been identified in the partial Sp1 structure. We report here accumulation of two forms of human Sp1 mRNA in HepG2 cells and the evidence that one form of the products was generated by homotypic trans-splicing. The complete structure of human Sp1 protein was also deduced from the cDNA sequence.

EXPERIMENTAL PROCEDURES

5′ Rapid Amplification of cDNA Ends and Reverse Transcriptase-Polymerase Chain Reaction—Total RNA was extracted from HepG2 cells using ISOGEN (Nippongene, Toyama, Japan) according to instruction of the manufacturer. The single strand cDNA for 5′ RACE1 was prepared by in vitro synthesis of cDNA with avian myeloblastosis virus reverse transcriptase XL (Takara Shuzo, Tokyo, Japan) using total RNA (5 μg) and the primer RT (5′-TCTGTCTTTTG-3′) and digestion of the template RNA with RNase H. When nucleotide positions were numbered relative to the transcription start site that was identified in this study, the primer RT corresponded to the positions from 2197 to 2186. The same nucleotide numbering was adopted throughout this paper. 5′ RACE was carried out using a 5′ Full RACE Core Set (Takara Shuzo). The first PCR was performed using the single strand cDNAs concatenated by T4 RNA ligase and primers S1 (5′-GGTCCGAGATCCTCTCTCC-3′, positions 2144–2163) and A1 (5′-ACCTGTGAAAGTTGTTGG-3′, positions 2136–2117) through a 25-cycle-amplification (94 °C for 30 s, 52 °C for 30 s, and 72 °C for 4 min). Then, a nested PCR was applied to the first PCR products under the same condition using primers S2 (5′-GATCCCCATGTTGGCTACCC-3′, positions 2164–2183) and A2 (5′-GAATTCTCAAAGGTCACTCCAT-3′, positions 2116–2096). Each primer contained both the sequence for a proper segment in Sp1 gene and the sequence (underlined) for creation of a restriction site. Each product of the nested PCR was cloned into pUC vector for DNA sequencing.

For detection of the Sp1 mRNA with the exon 3-2-3 alignment by RT-PCR, a 25-cycle amplification (94 °C for 30 s, 52 °C for 30 s, and 72 °C for 50 s) was applied to the single strand cDNA that was used for 5′ RACE and appropriate pairs of the following primers; primer T1 (5′-CAACACTACTCTTTATATCCCCC-3′, positions 3265–3284), primer T2 (5′-AATGACGCTGTCTTCTTGG-3′, positions 3285–3304), primer T3 (5′-CCACCATGTTGGAAATGACAGCT-3′, positions 1541–1560), primer T4 (5′-ATGAAATGAAGTTGGTGG-3′, positions 1557–1576), primer T5 (5′-CTGGATGTTGCAACCGTACCT-3′, positions 2116–2097), and primer T6 (5′-ATGACTTGGCAGCATTGG-3′, positions 2156–2137). For detection of the Sp1 mRNA with the exon 2-3-2 alignment by RT-PCR, a single strand cDNA was prepared by reverse transcription from poly(A)+ RNA (1 μg) of HepG2 cells using the primer R8 (5′-TGCCGAGTCAGAGACGTGTTG-3′); this primer was synthesized referring to the cDNA sequence in the downstream of exon 3. The first PCR was carried out using the primer X2 (5′-GTTGGCTGTCTTCTTGG-3′), primer T3 and primer 2R-1 (5′-AGGCCACACGTACCT-3′, positions 1635–1616). The nested PCR was prepared by 25-cycle amplification (94 °C for 30 s and 72 °C for 2 min) using the following primers; primer R14 (5′-TCAATGTTGGATGGCTTCTG-3′, positions 1561–1539 and positions 3502–3499), primer R15 (5′-TTGGACGTGTCAGCTGTCACCA-3′, positions 147–170), primer R16 (5′-TCCATGGATGAACTAGCTACCA-3′, positions 1550–1576), and primer R17 (5′-CGAGCCGGAATCTGGAATCTG-3′, positions 2017–2041).

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The abbreviations used are: RACE, rapid amplification of cDNA ends; RT, reverse transcriptase; PCR, polymerase chain reaction; bp, base pair(s); kb, kilobase pair(s); nt, nucleotide(s); ESE, exonic splicing enhancer.
Isolation of a Genomic Clone—Human genomic DNA was prepared from HepG2 cells according to a standard protocol (12), and completely digested with XbaI. Then a size-fractionated pool of the DNA fragments was ligated with the plaque vector ADASH II (Stratagene, La Jolla, CA) to construct a human genomic library. This library was screened by a plaque hybridization technique using the 120-kb DNA fragment of human Sp1 cDNA (positions 56–175) as a probe.

**Primer Extension Analysis**—The primer PE1 (5′-ATGGTGGCAGCT-GAGGGACAAG-3′, positions 173–152) was end-labeled with [γ-32P]ATP using T4 polynucleotide kinase (Nippongene). The 32P-labeled primer (1.2 × 10^6 cpm) was incubated with 16 μg of poly(A) + RNA from HepG2 cells at 30 °C for 16 h. The primer-RNA hybrid was precipitated with ethanol and subjected to reverse transcription with avian myeloblastosis virus reverse transcriptase at 42 °C for 60 min. The extended product was analyzed on a 6% polyacrylamide gel containing 8 M urea.

**RNase Protection Assay**—To construct template plasmids for in vitro synthesis of riboprobes, DNA fragments were amplified by PCR with two sets of primers. The primers XA1 (5′-AATAAGCTTGTTCGGTGTCGC-TCTGTCAGC-3′, positions 81–100) and XA2 (5′-TTATCATAGAG-GCACCACCCACCATTACAC-3′, positions 1636–1616) were used with the template of the 0.41-kb 5′ RACE product, and primers BA1 (5′-AATA-AGCTTCAACCCACCATTACAC-3′, 3332–3349) and BA2 (5′-TA-ATCTGAGATGCCCCATATTGCC-3′, 1615–1598) were used with the 1.6-kb 5′ RACE product. These primers contained additional sequences (underlined) for creation of XbaI or HindIII site at each end. Each amplified DNA fragment wasinserted between XbaI and HindIII sites of pBluescript KS. The resulting plasmids were linearized by digestion with HindIII, and antisense riboprobes were synthesized from these T7 promoter-containing plasmids in the presence of [α-32P]CTP using T7 RNA Synthesis Kit (Nippongene). The riboprobe for detection of the β-actin mRNA was also synthesized using a β-actin human antisense control template (Nippongene). RNase protection assays were performed with an RPA II kit (Ambion, Inc., Austin, TX) according to the manufacturer’s instructions. In brief, riboprobes (each 5 × 10^6 cpm) were incubated at 42 °C for 16 h with RNA samples as indicated in the figure legends. Then they were digested for 30 min at 37 °C with 200 μl of a mixture of RNase A (0.5 unit/ml) and RNase T1 (100 unit/ml). The protected products were analyzed on a 6% polyacrylamide gel containing 8 M urea.

**Genomic Southern Blot Analysis**—Genomic DNA (2 μg) from HepG2 cells was digested completely with a restriction enzyme (BamHI, EcoRI, PstI, or XbaI), electrophoresed on a 7% agarose gel, and transferred onto a Hybond-N membrane (Amersham Pharmacia Biotech). The DNA on the membrane was allowed to hybridize with the 32P-labeled DNA fragment (positions 3225–3502) that corresponded to exon 3 of the human Sp1 gene and washed under stringent conditions (0.2 × SSPE plus 0.1% SDS, 15 min at 65 °C, two times).

**RESULTS**

**Two Forms of Human Sp1 cDNA with Different 5′-Terminal Regions**—To obtain a human Sp1 cDNA clone containing the 5′-terminal region, we employed a 5′ RACE method using total RNA from HepG2 cells. The primers were designed to anneal specifically to the sequence in a 5′-terminal region of human Sp1 on the basis of the data registered in GenBank™ (accession number J03133). As shown in Fig. 1A, three kinds of DNA fragments with respective sizes of 0.34, 0.41, and 1.6 kb were mainly amplified. The sequence analysis revealed that all the products indeed possessed in common a known sequence in Sp1 gene; however, these products were classified into two types based on the sequence upstream of this sequence. The products of 0.41 and 0.34 kb had a new and identical sequence in the immediate upstream of the known sequence, although the 0.41-kb product contained the further upstream sequence of 71 bp long (Fig. 1B). By contrast, the 1.6-kb product had an unexpected structure, in which another established sequence in the downstream region of Sp1 gene was also linked upstream of the common sequence (Fig. 1B).

Because we obtained different cDNA clones for the 5′-terminal region of Sp1 mRNA, we analyzed the Sp1 gene in the human genome to elucidate the mechanism of the generation of these differences. In genomic Southern analysis with XbaI digestion, a single band of 14 kb was detected using the probe obtained from the 0.41-kb product (data not shown); thus we constructed a genomic library from the XbaI digest to obtain the genomic clone containing the 5′ region of human Sp1 gene. The screening of this library with the same probe yielded a single positive clone, which was named Sp1E1 (Fig. 1C). Characterization and sequence analysis revealed that this genomic clone contained both the common Sp1 sequence and the new sequence that was found in the present 5′ RACE products (accession number AB039286). This result indicated that the 0.41- and 0.34-kb products contained an upstream exon of human Sp1 gene.

To confirm whether the 5′-terminal end of the 0.41-kb product represented the 5′-terminus of Sp1 mRNA, we next performed primer extension analysis with poly(A) + RNA isolated from HepG2 cells. This experiment showed only a single band representing the product extended 56-bp from the 5′ end of the 0.41-kb product (Fig. 2B). There is no consensus sequence for the splice acceptor site in the genomic sequence preceding the one corresponding to the 5′ end region of 0.41-kb product. We assume, therefore, the position 56 bp upstream from the 5′ end of the 0.41-kb product as the transcription start site of human Sp1 gene (Fig. 2A). The genomic structure up to 266 bp upstream of this putative transcription start site did not contain any TATA box-like sequence but four possible GC boxes at positions starting from -231, -192, -139, and -9, respectively, thus suggesting the possible auto-regulation in Sp1 function (Fig. 2A). Although the 0.41- and 0.34-kb products did not contain the expected 5′ terminus of Sp1 mRNA, the newly determined sequence in these products had a stop codon in the frame for Sp1 protein (Fig. 2A). Therefore, the first methionine codon in this open reading frame appeared to be the initiation codon and the complete amino acid sequence of human Sp1 protein was thus deduced from the DNA sequences of the 0.34- and 0.41-kb products. The deduced amino acid sequence of human Sp1 is composed of 785 amino acid residues.
calculated molecular mass is 80,691 Da. The resulting amino acid sequence of the N-terminal region showed a high homology with those of mouse and rat Sp1 proteins (Fig. 3).

Comparison of sequences of the genomic and cDNA clones revealed exon-intron boundaries in the 5′-terminal region of Sp1 gene (Fig. 1, B and C). The ASp1E1 clone contained first three exons of the Sp1 gene; the sizes of these exons were 178, 155, or 1513 bp, respectively. It was also shown that the 1.6-kb product had a 3′-terminal region of 97 nt in exon 2 in the mRNA related to the 1.6-kb product. Together, the fragment with 97 nt corresponded to the expected part of alignment by RT-PCR. The 5′ end positions of 5′ RACE products of 0.41 and 0.34 kb are also shown by small bent arrows. The position of the extended product is indicated by an arrowhead. The transcription start site, which is shown by a bent arrow, is the position of the extended product indicated by an arrowhead. The upstream exon 3 encoded exactly the same amino acid encoded Asp and Gly, respectively. The codon between exons 3 and 4 was GGT, which was the identical amino acid residues to those of human Sp1. The amino acid sequence of the N-terminal region showed a high homology with those of mouse and rat Sp1 proteins (Fig. 3). The amino acid sequence of the N-terminal region of human Sp1 protein that is deduced from the DNA sequences of the 0.41- and 0.34-kb products is compared with those of mouse and rat Sp1 proteins. Asterisks indicate the identical amino acid residues to those of human Sp1. Gaps are inserted for the maximum homology. The boxed amino acid residue is the first one encoded by the DNA sequence previously deposited in GenBank (accession number J03133).

Fig. 3. Comparison of amino acid sequence of Sp1. The amino acid sequence of the N-terminal region of human Sp1 protein that is deduced from the DNA sequences of the 0.41- and 0.34-kb products is compared with those of mouse and rat Sp1 proteins. Asterisks indicate the identical amino acid residues to those of human Sp1. Gaps are inserted for the maximum homology. The boxed amino acid residue is the first one encoded by the DNA sequence previously deposited in GenBank (accession number J03133).

Fig. 4. Detection of the Sp1 transcript with the exon 3-2-3 alignment by RT-PCR. A, the positions and directions of primers T1 through T6 in the cDNA structure are indicated by arrows. The sizes of the expected fragments are also shown. B, the amplified products were analyzed on a 1.5% agarose gel, and their sizes are shown at the side of arrowheads. Lane P, the product of the nested PCR with primers T2 and T5 following the PCR with primers T1 and T6; lane 2, the product of nested PCR with primers T4 and T5 following the PCR with primers T3 and T6; lane 3, the product of PCR with primers T6 and T5; lane M, 50-bp ladders as size markers.
these results directly demonstrate the presence of two forms of Sp1 mRNAs with different 5′-terminal structures in HepG2 cells. Furthermore, the signal intensity of each protected fragment also suggested a significant level of accumulation of either form of Sp1 mRNA in HepG2 cells.

The Sp1 mRNA with the Exon 3-2-3 Alignment Is Produced by trans-Splicing.—Because genomic rearrangement can cause exon duplication in mRNA (13), we examined whether or not exon 3 of Sp1 gene is duplicated in the genome of HepG2 cells. Genomic Southern blot analysis was performed with genomic DNA digests with various restriction enzymes using a DNA fragment from exon 3 as a probe. As shown in Fig. 6, a single band was detected in each lane (lanes 4–7). In addition, the signal intensities of these bands were almost the same as that of the control band for a single copy (lane 3). This estimation was further validated by a parallel Southern analysis for an established single copy gene, p53 gene, applied to the same DNA digests (data not shown). These results suggested that exon 3 exists as a single copy in the genome. Thus, not the genomic duplication but an RNA editing mechanism, i.e. formation of circular RNA or trans-splicing, appeared to give rise to the Sp1 mRNA with the exon 3-2-3 alignment. Because circular RNAs lack poly(A) tails per se, we next performed RNase protection assay with poly(A)⁺-rich RNA and poly(A)⁻-rich RNA (Fig. 7). The distribution of the Sp1 mRNA with the exon 3-2-3 alignment to these two fractions was similar to that of the cis-splitted Sp1 mRNA. In addition, the β-actin mRNA that was used as a marker of fractionation, was distributed similarly. Therefore, we concluded that the Sp1 mRNA with the exon 3-2-3 alignment was produced by trans-splicing between two Sp1 pre-mRNAs.

We also investigated the structure upstream of the 3-2-3 alignment of the trans-spliced Sp1 mRNA by RT-PCR. To examine whether exons 1 and 2 are located in the upstream of the 3-2-3 alignment, first PCRs were carried out with primers X2 and 2R-1 primers which were designed to anneal specifically to exon 1, exon 2, or exon 3, and the exon 3-2 junction sequence, respectively (Fig. 8A). Then the nested PCRs were done using the primers R15, R16 or R17, and R14, which were designed to anneal specifically to exon 1, exon 2, or exon 3, and the exon 3-2 junction sequence, respectively (Fig. 8A). When the first PCR product with T3 and 2R-1 primers was used as a template, the amplified products were observed by DNA sequencing of these products established their expected structure. In contrast, no product was observed by the nested PCR when the first PCR product with X2 and 2R-1 primers was used as a template (lanes 1). The specificity of the primers used were verified in the negative control PCRs using an EcoRI-XhoI fragment (Fig. 1C) of a genomic DNA clone (lanes G) or the cDNA clone containing the exon 1-2-3-4 alignment (lanes C) as a template, and the positive control PCR using the recombinant clone having the exon 1-2-3-2-3 alignment as a template (lanes R). Taken together, the trans-spliced mRNA appeared to have exon 2 but not exon 1 in its 5′ region.
DISCUSSION

Here, we cloned human Sp1 cDNAs that represent two forms of mRNAs with different structures in the 5’-terminal region. The results of RT-PCR and RNase protection assay confirmed that two Sp1 mRNAs are really present and accumulated in HepG2 cells. One of them is generated through a well studied cis-splicing process, and the other with the exon 2-3-2-3-alignment is by trans-splicing. Consistently, we detected heterogeneous RNA species in Northern analysis of RNA from HepG2 cells using a Sp1 cDNA fragment (positions 2765–3295) as a probe; the main band was approximately 8.2 kb, whereas the other two were minor but still marked representing smaller mRNAs (data not shown). The main band probably corresponds to the main bands previously reported (4, 9, 14) for the human Sp1. Other distinct bands we observed correspond to smaller RNAs whose occurrences may depend on cell lines and/or tissues, because the smaller species also seem to be detected in MKN-28 (4) but not in HeLa cells (9). Although we determined the structure of the 5’-terminal region of human Sp1 mRNA in this study, the sequence for the 3’ noncoding region remains undetermined. We currently suspend, therefore, the identification of those multiple bands in the Northern analysis until accomplishment of the whole structure for Sp1 mRNA.

trans-Splicing is an RNA editing mechanism that produces mature mRNA from separate pre-mRNAs. In trypanosomes, nematodes and some other lower organisms, spliced leader RNA, which is similar to spliceosomal U small nuclear RNAs, was ligated at the 5’ ends of diverse nuclear mRNAs (15–17). Another type of trans-splicing has been discovered in plant mitochondria and chloroplasts. In this trans-splicing, formation of group II intron-like structures by base pairing between complementary segments of introns in separate pre-mRNAs seems to be essential (17–19). In mammalian cells, trans-splicing was first demonstrated in vivo and in vitro using artificial RNA substrates (20, 21), and spliced leader RNA and actin-1 pre-mRNA from Caenorhabditis elegans (22). Subsequently, a few examples of trans-splicing as a natural event have also been found in mammalian cells (23–25). These trans-splicings occur between different pre-mRNAs. On the other hand, very recent studies unveiled trans-splicing between identical pre-mRNAs, namely homotypic trans-splicing, in mammalian cells in the expressions of the rat carnitine octanoyltransferase gene (26), the rat SA gene (27), and the rat voltage-gated sodium channel gene (28). Our present finding with the human Sp1 gene adds another distinct example to the homotypic trans-splicing in mammalian cells, suggesting this type of trans-splicing might be rather a general mechanism for regulation of phenotype expression in mammalian cells.

Based on our findings in this study, we propose the model of the homotypic trans-splicing (Fig. 9). In this model, we present the trans-spliced Sp1 mRNA that lacks exon 1. The reason that we did not detect the trans-spliced Sp1 mRNA having exon 1 is obscure. However, the presence of alternative transcription start sites in intron 1 can be a candidate account, because we observed multiple products in the primer extension analysis with the exon 2-specific primer (data not shown). It has been proposed that trans-splicing process in mammals proceeds in spliceosome complexes and through partial base pairing between two precursor mRNAs (29). By the survey of complementarity between a segment in the upstream of exon 2 and one in the downstream of exon 3, we found two sets of complementary sequences (Fig. 9). We also found an exonic splicing enhancer (ESE)-like sequence (GAGGAGGAGGG, positions 1680–1690) in exon 2 of the Sp1 gene (Fig. 9). The ESEs, which are known to be involved in a weak splice site selection in alternative splicing with cooperation of serine/arginine-rich splicing factors (SR proteins), are usually purine-rich sequences in the exons downstream of a regulated 3’ splice site (30–32). Recently, it has been also demonstrated that ESE and SR proteins are important for trans-splicing by an in vitro assay system (33, 34). Furthermore, two putative ESE elements were also reported in the carnitine octanoyltransferase gene (26). Thus, the above-mentioned complementary sequences and the ESE-like sequence are possibly involved in the case of Sp1 mRNA maturation as well.

The biological significance of trans-splicing for Sp1 remains
elusive. Because exon 3 in Sp1 mRNA mainly encodes the transcrip
tional activation domains A and B (11), the product of the Sp1 mRNA with duplicated exon 3, if translated, has dou-
bled transcrip
tional activation domains. Although we sug
gested that the trans-splicing form of human Sp1 mRNA lacked the first exon, this possibility still remains because of the presence of the second methionine in exon 2 (Fig. 3), which might serve as a translational start site. Such a product may show stronger ability for transactivation, because synergistic activation among activation domains, named superactivation, was demonstrated; an added transactivation domain elevated the ability of a truncated Sp1 having domains for DNA binding and transactivation (35, 36). In this case, the trans-splicing will result in a positive regulation. In other cases, this trans-splicing might contribute to a negative regulation by producing nonfunctional mRNA and reduces the functional Sp1 mRNA. Although the result of RNase protection assays suggests that the Sp1 mRNA with the exon 2-3-2-3 alignment has a poly(A) tail, this possibility also remains. It is already proposed that trans-splicing is a novel mechanism for regulating the cellular events because the trans-spliced rat SA mRNA is tissue-spe
cific, and trans-splicing of pre-mRNA of rat voltage-gated sodi
dium channel is regulated by a nerve growth factor (27, 28).

Finally, our study also established the complete amino acid se
quence of human Sp1. A partial DNA sequence of 313 bp that covers only the N-terminal coding region of Sp1 recently ap
appeared in the data base (accession number AJ272134), and this sequence perfectly matched to our sequence. Although the newly identified amino acid sequence is not so large, this infor
mation is valuable because a region close to the N terminus of human Sp1 is critical for susceptibility to proteasome-depen
dent degradation (37). In addition, the three different sizes of mRNAs of mouse Sp1 were observed during spermatogene
sis, one of which encoded a Sp1 lacking the 7 amino acid resi
dues of N terminus (38). Therefore, the complete structure of human Sp1 identified in this study will be also useful for further investigation concerning the regulation of Sp1 function.

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