Spinal muscular atrophy is caused by the homozygous loss of survival motor neuron 1 (SMN1). SMN2, a nearly identical copy gene, differs from SMN1 only by a single nonpolyomorphic C to T transition in exon 7, which leads to alteration of exon 7 splicing; SMN2 leads to exon 7 skipping and expression of a nonfunctional gene product and fails to compensate for the loss of SMN1. The exclusion of SMN exon 7 is critical for the onset of this disease. Regulation of SMN exon 7 splicing was determined by analyzing the roles of the cis-acting element in intron 7 (element 2), which we previously identified as a splicing enhancer element of SMN exon 7 containing the C to T transition. The minimum sequence essential for activation of the splicing was determined to be 24 nucleotides, and RNA structural analyses showed a stem-loop structure. Deletion of this element or disruption of the stem-loop structure resulted in a decrease in exon 7 inclusion. A gel shift assay using element 2 revealed formation of RNA-protein complexes, suggesting that the binding of the trans-acting proteins to element 2 plays a crucial role in the splicing of SMN exon 7 containing the C to T transition.

Spinal muscular atrophy (SMA) is a common autosomal recessive disorder characterized by the loss of motor neurons in the spinal cord, which presents as proximal, symmetrical limb, and trunk muscle weakness that ultimately leads to death (1). The survival of the motor neuron (SMN) gene has been identified as the disease-causing gene of SMA and is present on chromosome 5 at 5q13 (2, 3). Humans contain two nearly identical copies of the SMN gene, SMN1 and SMN2. These genes encode an identical protein, a 294-amino acid RNA-binding protein. Only homozygous deletions or mutations of SMN1 result in the SMA phenotype (4–15).

SMN1 mRNA expresses a full-length transcript, whereas SMN2 produces low levels of the full-length transcript and high levels of an isoform lacking exon 7 (SMNΔ7) (2, 16, 17). The SMNΔ7 protein is presumed to be less stable (18) and has a reduced ability to oligomerize, explaining why SMN2 cannot prevent SMA (2, 19, 20). The critical difference between SMN1 and SMN2 is a silent nucleotide transition in SMN exon 7. SMN1 contains a C located six nucleotides inside exon 7, whereas SMN2 contains a T at this position. This transition is believed to inhibit one of the splicing regulatory elements, called exonic splicing enhancer (ESE), within exon 7 (21). A previous report demonstrated the presence of an ESE within exon 7 and that human Tra2-β1, a member of the serine-arginine-related proteins of splicing factors, binds to the elements and stimulates an ESE (22). Recently, it was discovered that a single nucleotide change occurs within a heptamer motif of the ESE, which in SMN1 is recognized directly by SF2/ASF (23). The abrogation of the SF2/ASF-dependent ESE is considered to be the basis for the inefficient inclusion of exon 7 in SMN2. However, it is unclear whether Tra2-β1 and SF2/ASF functionally cooperate to promote the inclusion of the exon and whether other factors are involved in the regulation of the splicing of SMN exon 7.

Previously, we tried to determine the critical cis-acting elements on the SMN pre-mRNA responsible for the aberrant splicing of the SMN exon 7 containing the C to T transition (24). We identified two cis-acting elements (elements 1 and 2) responsible for the regulation of SMN exon 7 splicing. The mutation in element 1, which is composed of 45 bp in intron 6, or treatment with antisense oligonucleotides directed toward element 1 caused an increase in exon 7 inclusion. The ~33-kDa protein was demonstrated to associate with element 1 in the SMN exon 7 containing the C to T transition, suggesting that the binding of the ~33-kDa protein to element 1 plays crucial roles in the skipping of the SMN exon 7 containing the C to T transition. Element 2 was composed of 66 bp in intron 7 and plays roles in the inclusion of exon 7, but the detailed mechanisms responsible for the splicing regulation of this element were unclear. In this report, we investigate the functions of element 2, which we identified previously as being involved in the regulation of SMN exon 7 splicing.

EXPERIMENTAL PROCEDURES

Cell Cultures—COS-7 and SK-N-SH cells were used for in vivo splicing assays. COS-7 cells were grown in 10% fetal bovine serum/Dulbecco’s modified Eagle’s medium, and SK-N-SH cells were cultured in α-minimum essential medium with 10% fetal bovine serum. Prior to transfection, the cells were plated at a density of 60–80% confluency on 3.5-cm dishes.

In Vivo Splicing—Constructs of SMN1 and SMN2 mini-genes containing exons 6–8 in a pCI mammalian expression vector were gifts from Drs. Elliot Androphy (Tufts University) and Christopher Lorson (Arizona State University) (21). Mini-genes containing SMN 1 exons 6–8 and the C to T transition in exon 7 cloned into the pCI vector were mutated in element 1 by site-directed mutagenesis. The constructs (1.0 μg) were transfected into cells using LipofectAMINE reagent or LipofectAMINE ACE reagent (Invitrogen) according to the manufacturer’s protocol. Transfected cells were lysed in buffer RLT (Qiagen), and the
total cellular RNA was purified using the RNeasy mini kit (Qiagen). First strand cDNA was synthesized in a 20 µl reaction volume using a random primer (TaKaRa) and Moloney murine leukemia virus reverse transcriptase (Invitrogen). PCR amplification analysis of the plasmid-derived cDNAs was performed using the primer set, pCI forward (5'-GCT AAG AAC GTC ATG CCT 3' and pCI reverse (5'-GTA TCT TAT CAT GTC TGC TCG 3'). PCR was performed in a total volume of 50 µl that contained 1.2 µg of first strand cDNA, 0.4 µM of each primer, 0.2 µM dNTPs supplemented with trace amounts of [γ-32P]dCTP, 5 units of rTaq DNA polymerase, and 10X PCR buffer (TaKaRa). The amplification conditions were as follows: an initial denaturation step (94°C for 2 min), 30 cycles (94°C for 30 s, 56°C for 1.5 min, and 72°C for 1 min), and a final extension step (72°C for 10 min). The reaction products were separated by electrophoresis through a 5% acrylamide gel using a 12 bp DNA ladder (Pharmacia) as a molecular weight marker.

PCR products were cloned into the pGEM-T vector (Promega) and sequenced. Quantification of the density of each band was carried out using a densitography program (ATTO). The ratios of inclusion of exon 7 were quantitated and expressed as percentages of inclusion relative to the total intensities.

**Exon Trapping Systems**—Various deletion mutants of SMN1 containing intron 6, exon 7, and intron 7 were generated by PCR using the following primer sets: 600Fwd (5'-AAG CGT GCC ATG AGC CAC TGC AAG AAA AC-3') and OR (5'-GGA TCC GAG AAT TCT AGT AGG GAT GTA G-3') for I7DM3, and 600Fwd and OR-IR (5'-GGA TCC GAG AAT TCT AGT AGG GAT GTA G-3'). These PCR products were digested with BSA2 and TAA CCT TTC AAC T-3' and sequenced. PCR was performed in a total volume of 40 µl using a pSPL3 vector-specific primer set, SD6 (5'-AAG AAA AC-3'), and SA2. PCR was performed in a total volume of 40 µl. Amplification conditions were as follows: an initial denaturation step (94°C for 2 min), 30 cycles (94°C for 30 s, 56°C for 1.5 min, and 72°C for 1 min), and a final extension step (72°C for 10 min). The reaction products were separated by electrophoresis through a 5% acrylamide gel using a 12 bp DNA ladder (Pharmacia) as a molecular weight marker.

**Gel Mobility Shift Assay**—For the collection of nuclear extracts, SK-N-SH cells were homogenized in 50 volumes of 10 mM HEPES (pH 7.9) containing 10 mM KCl, 1 mM EDTA, 1 mM EGTA, 5 mM dithiothreitol, and 1 mM phenylmethylsulfonyl fluoride at 4°C. Buffers and any other solutions used in this study were sterilized before each use by filtration through a Steritop (Millipore Corp.) with a pore size of 0.2 µm. All cell cultures were stored at ~8°C as nuclear extracts for gel mobility shift assay.

For gel mobility shift assays, the reactions were carried out in a final volume of 20 µl (binding buffer: 20 mM HEPES, pH 7.9, 72 mM KCl, 1.5 mM MgCl2, 0.78 mM magnesium acetate, 0.52 mM dithiothreitol, 3.8% glycerol, 0.75 mM ATP, and 1 mM GTP) by mixing various concentrations of nuclear extracts with radioisotope-labeled RNA oligonucleotide (present at a final concentration of 6 nM). The oligonucleotide sequences were 5'-GUG GAA AAC AAA UGU UUU UGA ACA-3' (oligo-element 1), and 5'-GUG GAA AAC AAA UGC CCC UGA ACA-3' (oligo-mutant element 2). The samples were incubated at room temperature for 25 min and then were loaded on a 4% native acrylamide gel in a loading buffer containing Tris-HCl, 15% glycerol, and 1.5 mM EDTA. The gel was run at a constant 100 V for 2 h at 4°C. The gel was then dried and exposed to autoradiographic film.

**Identification of the Unique Stem-Loop Structure in the SMN Intron 7, Which Enhances the Exon 7 Splicing**—It is well known that SMN1 mRNA expresses a full-length transcript, whereas SMN2 produces low levels of the full-length transcript and high levels of an isoform lacking exon 7 (SMN2). Indeed, the phenomena were confirmed by the transient transfection of SMN1 and SMN2 mini-gene constructs, including the genomic exons 6–8 in a pCI mammalian expression vector (Fig. 1A). Furthermore, the substitution of C to T located six nucleotides inside exon 7 of SMN1 led to the exclusion of exon 7, and the splicing patterns of exon 7 were similar to those of wild type SMN2. In contrast, the mutant SMN2 (substitution of T to C in SMN2) mini-gene produced full-length SMN transcripts (Fig. 1A). To determine the cis-acting elements responsible for the skipping of SMN exon 7, we constructed various deletion mutants of both wild type SMN1 and mutant SMN1 (C to T transition in exon 7) mini-genes that contain exon 7 with flanking introns 6 and 7, and they were cloned into the exon trapping vector, pSPL3 (Fig. 1B). Previously, we identified a cis-acting element for the regulation of SMN exon 7 splicing in the intron 6 (element 1) (24). Element 1 has been demonstrated to be composed of 45 bp, and the core was a pyrimidine-rich sequence responsible for the negative regulation of SMN exon 7 splicing. Further, we also found element 2, which was composed of 66 bp in the intron 7 and plays roles in the inclusion of exon 7, but the detailed mechanisms responsible for the splicing regulation of this element remain unclear. In the present study, we first tried to identify the minimum sequence of element 2 essential for the activation of the splicing of SMN exon 7.

When the various deletion mutants of wild type SMN1 (I7DM1-4-C) were transfected into COS-7 cells, all of the constructs expressed the full-length type of mRNA including SMN exon 7 (Fig. 1C, + Exon7) but never expressed isoforms lacking exon 7 (Fig. 1C, − Exon7). In contrast, the longest construct of mutant SMN1 (I7DM1-T), which contained a C to T transition in the SMN1 exon 7 with 235 bp of the flanking intron 6 and 124 bp of the flanking intron 7, expressed mRNAs with both the inclusion and exclusion of exon 7 (51% inclusion) (Fig. 1C). The ratios of exon inclusion using mini-genes containing the C to T transition in exon 7 cloned into exon trap vectors were higher than those using the SMN mini-genes containing exons 6–8 cloned into the pCI vector. The differences indicate that there may be some cis-acting elements that regulate the splicing of SMN exon 7 in regions that are different from the mini-gene sequences (about 400 bp) cloned into the exon trap vector. However, because the mini-genes containing the C to T transition in SMN1 exon 7 cloned into the exon trap vector (pSPL3) showed a significant increase in the exclusion of exon 7, these constructs are thought to be useful for determining the cis-elements responsible for the exclusion of exon 7 in the 400-bp mini-gene.

For the deletion mutants in the flanking intron 7, the deletion mutant I7DM4-T, which contained a deletion of 66 bp at the 3'-flanking regions from I7DM1-T, increased the exclusion of SMN exon 7 (23% inclusion), whereas the splicing pattern of deletion of the mutant I7DM3-T, which contained a deletion of...
52 bp from I7DM1-T, was similar to that of I7DM1-T (Fig. 1C). Therefore, the 14-bp region from +59 to +72 of the flanking intron 7 may be a critical element for the inclusion of SMN exon 7 containing the C to T transition. The position of this element within intron 7 is presented in Fig. 1D. Analysis of the higher order structure by the MFOLD program (26) showed that element 2, composed of 24 nucleotides, possessed a unique stem-loop structure (Fig. 1D). The sequence showed a complete matching between SMN1 and SMN2, suggesting that element 2 may be important for the regulation of the splicing of the SMN 1 exon 7 containing the C to T transition and wild type SMN2.

Functional Analyses of the Intronic Splicing Enhancer Element—To determine whether the stem-loop structure in element 2 is important for regulation of the splicing of the SMN exon 7 containing C to T transition, we variously mutated the element 2, composed of 24 nucleotides, possessed a unique stem-loop structure (Fig. 1D). The sequence showed a complete matching between SMN1 and SMN2, suggesting that element 2 may be important for the regulation of the splicing of the SMN 1 exon 7 containing the C to T transition and wild type SMN2.

When A-U base pairs in the stem were substituted to G-C base pairs, inclusion of exon 7 was slightly decreased (construct F in Fig. 2A). This finding suggests that the higher ordered stem structure in element 2 is important, but the sequences of the A-U base pairs are not very important.

To test whether element 2 can activate the splicing of the SMN exon 7 containing the C to T transition, we constructed exon trap vectors containing three or four repeats of element 2 that were tandemly inserted into the I7DM1-T vector, as shown in Fig. 3A. The insertion of multiple element 2 in the intron 7 of SMN increased the inclusion of SMN exon 7 in an inserted number-dependent manner (Fig. 3B). Altogether, it is demonstrated that element 2 is an intronic splicing enhancer of SMN exon 7 containing the C to T transition.

Data base analysis showed there were several genes containing similar sequences to element 2 in its intron. Some genes that reveal complete matching to the stem-loop structure within element 2 are listed in the Table I. As shown in the table, these elements listed are positioned relatively close to the 5’ splice sites of each intron similar to element 2 of the SMN gene. Furthermore, we found expressed sequence tag clones of MRPS35 (mitochondrial ribosomal protein S35) and RDGGBB (retinal degeneration Bβ) that have alternative splicing variants both including and excluding its exon close to the stem-loop structure, suggesting that the stem-loop structure within element 2 could play a role in the alternative splicings of several genes.

A trans-Acting Factor Specifically Binds to Element 2—Because a disruption of element 2 revealed dynamic changes in
the splicing patterns of SMN exon 7, it is possible that trans-acting factors specifically bind to element 2 and regulate its splicing through direct binding. Therefore, we performed gel shift assays using 32P-labeled oligo-element 2 and nuclear extracts of neuroblastoma SK-N-SH cells. Efficient binding was observed, and the binding activity was increased corresponding to the amounts of nuclear extracts (Fig. 4A). To confirm that the binding was specific, competition studies with unlabeled oligo-element 2 were performed. Preincubation of nuclear extracts with 25-fold molar excesses of unlabeled oligo-RNAs resulted in a nearly complete elimination of the activity, whereas preincubation of the extracts with equivalent amounts of unlabeled mutated oligonucleotides of element 2 (oligo-mutant element 2), which were mutated by substitution of T to C (construct C), as shown in Fig. 2A, did not inhibit the binding of the RNA-nuclear proteins (Fig. 4B). These data demonstrate that a trans-acting factor specifically binds to element 2 in the intron 7 of SMN pre-mRNA and that binding to the cis-acting element 2 may enhance the splicing of the SMN exon 7 containing the C to T transition.

We performed UV cross-linking experiments using SMN pre-mRNA, but we could not detect any difference in proteins binding to SMN pre-mRNA between wild type and mutant SMN pre-mRNA, which deleted element 2. Because many bands were detected in both binding assays and bands of interest may be hidden in the many bands, we could not obtain further information concerning putative trans-acting factor(s) bound to this enhancer element, such as the molecular weight(s) of the factor(s).

**Effects of Treatment with Antisense Oligonucleotides**—Having demonstrated that element 2 in intron 7 of the SMN gene plays a crucial role in the regulation of exon 7 splicing and that trans-acting proteins could directly bind to this element, it was of interest to examine whether the treatment of cells transfected with I7DM1-T exon trapping vectors with antisense oligonucleotides was sufficient to lead to a decrease in the inclusion of SMN exon 7 containing the C to T transition.

We performed UV cross-linking experiments using SMN pre-mRNA, but we could not detect any difference in proteins binding to SMN pre-mRNA between wild type and mutant SMN pre-mRNA, which deleted element 2. Because many bands were detected in both binding assays and bands of interest may be hidden in the many bands, we could not obtain further information concerning putative trans-acting factor(s) bound to this enhancer element, such as the molecular weight(s) of the factor(s).
As-element2 led to a decrease in the inclusion of SMN exon 7, which was dependent on the amount of antisense oligonucleotides (Fig. 5B). Treatment with the As-con showed no change in the splicing of SMN exon 7 (Fig. 5C). In contrast, treatment with As-pyr showed a slight decrease in the inclusion (data not shown). The polypyrimidine tract is essential for the pre-mRNA splicing. Therefore, the As-pyr was expected to work effectively as the antisense oligonucleotides that blocked its cis-acting element. Similarly, the As-element 2 caused an inhibition of the splicing of SMN exon 7, suggesting that the results supported the above findings that element 2 is an important cis-acting for the splicing of SMN exon 7 containing the C to T transition.

**DISCUSSION**

SMN1 mRNA expresses a full-length transcript, whereas SMN2 produces a low level of full-length transcript predominantly as an isoform lacking exon 7 (2, 16, 17). The critical difference between SMN1 and SMN2 is a silent nucleotide transition in SMN exon 7. That is, SMN1 contains a C located six nucleotides inside exon 7, whereas SMN2 contains a T at this position. This transition leads to an alteration in the rec-
An Intrinsic cis-Element for SMN Exon 7 Splicing

Fig. 5. Treatment with antisense oligonucleotides directed toward element 2 decreases the expression of transcripts containing SMN exon 7. A, the regions where the three types of antisense oligonucleotides (As-element2, As-con, and As-pyr) can hybridize. B, RT-PCR of in vivo splicing using the exon trapping system. Treatment with As-element2 led to a decrease in the ratio of exon 7 inclusion relative to total transcripts. The effects observed were dependent on increasing concentrations of As-element2 (lower panel). C, treatment with As-con did not affect the splicing of SMN exon 7. The percentages of exon 7 inclusions are shown below each lane, and represent the means for the four experiments.

Omission of exon 7 by components of the splicing machinery (20, 21). A previous report demonstrated the presence of an ESE within exon 7 and that human Tra2-β1, a member of the serine-arginine-related proteins of splicing factors, binds to the elements and stimulates an ESE (22). It was recently reported that a single nucleotide change occurs within a heptamer motif of the ESE, which in SMN1 is recognized directly by SF2/ASF (23). The abrogation of the SF2/ASF-dependent ESE is considered to be the basis for the inefficient inclusion of exon 7 in SMN2. However, it is unclear whether Tra2-β1 and SF2/ASF functionally cooperate to promote the inclusion of the exon and whether other factors are involved in the regulation of the splicing of SMN exon 7. Therefore, we have examined one of the critical cis-acting elements that we identified in a previous study of the SMN pre-mRNA responsible for the skipping of the SMN exon 7 containing the C to T transition.

Deletion analysis of SMN1 pre-mRNA sequences showed that the regions from +59 to +72 of the flanking intron 7 are significant elements for the inclusion of the SMN1 exon 7 containing the C to T transition. However, deletion of these elements from wild type SMN1 pre-mRNA did not affect the splicing of SMN exon 7. Therefore, although element 2 does not play a role in exon 7 splicing of the wild type SMN, it is necessary for enhancing the exon 7 splicing of the mutant type SMN. It is not known why element 2 affects only the splicing of SMN exon7 containing the mutation. A possible mechanism is that the alteration of the splicing patterns of the SMN exon 7 may result from the binding of a specific regulatory factor, as shown in Fig. 4A, to element 2 caused by changes in the higher order structure of the pre-mRNA containing the C to T transition in exon 7, and the binding of the regulatory factor may activate the recognition or usage of 3’ or 5’ splice sites by splicing machineries such as small nuclear ribonucleoproteins or serine-arginine-related proteins (27, 28).

It has previously been reported that the binding of the polypyrimidine tract-binding protein to the enhancer pyrimidine tract is functional in that the exon inclusion increases when in vivo levels of polypyrimidine tract-binding protein increase (29). Members of the CELF family of RNA-binding proteins, including CUG-BP, have also been known to bind to a conserved intronic splicing element (MSE containing CUG motif) and positively regulate alternative splicing (30, 31). Element 2, identified in the present study, is considered to act for an intronic splicing enhancer because of the decrease in SMN splicing by deletion or mutation of this element. However, element 2 does not contain the consensus sequences to which the polypyrimidine tract-binding protein or the CELF family bind, although it contains the unique stem-loop structure. Therefore, element 2 could not be regulated by these proteins. However, a novel molecule may associate with the element to enhance the splicing of the SMN exon 7 containing the C to T transition. Indeed, we found specific interactions of element 2 oligonucleotide and nuclear extracts. The element that we identified in the present study has not been shown to be critical for splicing or for the specific binding site of splicing factors. Therefore, the mechanisms responsible for the regulated splicing of SMN exon 7 by the cis-acting element remain unknown.

Identification and characterization of the trans-acting factors that bind to the element are needed to elucidate the mechanisms. Data base analysis showed a complete matching of the stem-loop structure of the element 2 nucleotide sequence to the intron sequences of several genes. Although a detailed analysis of the splicing of these genes is needed, it raises the possibility that these genes may also be regulated by an alternative splicing by this cis-acting element and its trans-acting proteins.

In summary, we found a stem-loop structure within element 2 in the intron 7 of SMN that enhances the splicing of the SMN exon 7 containing the C to T transition. In addition to element 1, which has been demonstrated to be a negative regulator of the splicing, it is important for an understanding of the mechanisms of splicing of SMN exon 7 containing the C to T transition to identify the trans-acting RNA-binding proteins that specifically interact with these elements. Experimental manipulation to modify the function of the cis-acting elements or the trans-acting factors might allow the development of therapeutic strategies for SMA.

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An Intronic Splicing Enhancer Element in Survival Motor Neuron (SMN) Pre-mRNA
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