Identification of a Cis-acting Element for the Regulation of SMN Exon 7 Splicing*

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Spinal muscular atrophy results from the loss of functional survival motor neuron (SMN) alleles. Two nearly identical copies of SMN exist and differ only by a single non-polymorphic C to T transition in exon 7. This transition leads to alteration of exon 7 splicing; that is, SMN1 produces a full-length transcript, whereas SMN2 expresses a low level of full-length transcript and predominantly an isoform lacking exon 7. The truncated transcript of SMN encodes a less stable protein with reduced self-oligonucleotidization activity that fails to compensate for the loss of SMN1. In this paper, we identified a cis-acting element (element 1), which is composed of 45 bp in intron 6 responsible for the regulation of SMN exon 7 splicing. Mutations in element 1 or treatment with antisense oligonucleotides directed toward element 1 caused an increase in exon 7 inclusion. An ~33-kDa protein was demonstrated to associate with a pre-mRNA sequence containing both element 1 and the C to T transition in SMN exon 7 but not with the sequence containing mutated element 1, suggesting that the binding of the ~33-kDa protein plays crucial roles in the skipping of SMN exon 7 containing the C to T transition.

Experimental Procedures

Cell Cultures—COS-7, HEK293T, and SK-N-SH cells were used for in vitro splicing assays. COS-7 and HEK293T cells were grown in 10% fetal bovine serum/Dulbecco's modified Eagle's medium, and SK-N-SH cells were cultured in alpha-minimum essential medium with 10% fetal bovine serum. Prior to transfection, cells were plated at a density of 60–80% confluency on 3.5-cm dishes.

In Vivo Splicing—Constructs of SMN1 and SMN2 mini-genomes containing exon 6–exon 8 in a pCI mammalian expression vector were gifts from Drs. Elliot Androphy (Tufts University) and Christian Lorson (Arizona State University) (21). Mini-genomes containing SMN1 exon 6–exon 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 μl) 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 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 ACA TGC GCT AGT TC-3') and pCI reverse (5'-GTA TCT TAT CAT GTC TG-3'). PCR was performed in a total volume of 50 μl that contained 1.2 μg of first-strand cDNA, 0.4 μM each primer, 0.2 μM dNTPs supplemented with trace amounts of [α-32P]dCTP, 5 units of Taq DNA polymerase, and 10× PCR buffer (TaKaRa). Amplification conditions were as follows: an initial denaturation step (94°C/2 min), 30 cycles (94°C/30 s; 56°C/1.5 min; 72°C/1 min), and a final extension step (72°C/10 min). Reaction products were resolved by electrophoresis through a 5% acrylamide gel. PCR products were cloned into the pGEM-T vector (Promega) and sequenced. Quan-

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RESULTS

Alternative Splicing of SMN Exon 7 in Various Cells—To examine the splicing patterns of SMN exon 7, we used SMN1 and SMN2 mini-gene constructs including the genomic exon 6–exon 8 in a pCI mammalian expression vector (21) (see “Experimental Procedures”). Furthermore, we also used expression vectors containing single nucleotide conversions that were C to T or T to C substitutions in exon 7 of the SMN1 or SMN2 mini-genes, respectively. These constructs were transiently transfected into human neuroblastoma SK-N-SH cells. After collection of total RNA from the cells, RT-PCR was performed using plasmid-specific primer sets (pCI forward and pCI reverse primers) to detect RNA processing of SMN in each construct-transfected cell. The wild-type SMN1 mini-gene expressed full-length SMN transcripts with no detectable SMNΔ7, whereas the wild-type SMN2 mini-gene produced lower levels of full-length SMN and abundant SMNΔ7 (Fig. 1A). 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. These findings were consistent with previous results (21, 25) and indicated that the C to T transition in SMN exon 7 had disrupted the regulation of SMN splicing.

Identification of Cis-acting Elements Responsible for the Splicing of SMN Exon 7—To determine the cis-acting ele-
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Fig. 2. Constructions of the exon-trapping vectors and effects of deletion mutations on exon 7 splicing. A, various deletion mutants (DM1–DM6) 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 were cloned into pSPL3. BP, branching point; PPT, poly-pyrimidine tract. Element 1 (−112 to −68) and Element 2 (+59 to +124) are crucial elements for the exclusion and inclusion of SMN1 exon 7 containing the C to T transition, respectively. B and C, RT-PCR products from in vitro splicing assays. +exon 7 and −exon 7 transcripts yield 317-bp and 263-bp fragments, respectively. Each PCR product was excised from the gel, and the DNA sequence was determined. In each case, the splicing occurred at the expected sites. All deletion mutants of wild-type SMN1 (DM1–6-C) express +exon 7 transcripts (B), whereas each deletion mutant of SMN1 containing the C to T transition (DM1–6-T) produces both + and −exon 7 (C). The scores shown in B and C are percentages of exon 7 inclusion relative to the total transcripts. Values represent the means of four analyses. Note that the splicing patterns are changed in the cases where DM3-T and DM6-T were transfected compared with the full-length constructs, DM1-T or DM4-T, respectively. D, comparisons of the nucleotide sequences of element 1 and 2 between SMN1 and SMN2. *, differences in the nucleotides between SMN1 and SMN2 in these elements. Element 1 contains pyrimidine-rich sequences (underlined).
The C to T transition in the exon 7 cloned into the pCI expression vector (Fig. 3B). The maximal effects on the inclusion of exon 7 were reached at or less than 25 nM oligo-element 1. In contrast, treatment with oligo-pyr or oligo-con (data not shown) at concentrations of up to 200 nM did not lead to any changes in exon 7 inclusion. Next, we examined whether oligo-element 1 led to the inclusion of exon 7 of wild-type SMN2. As expected, treatment with the antisense oligonucleotides led to a 2.7-fold increase in the inclusion of SMN2 exon 7, similar to the case of SMN1 containing the C to T transition (Fig. 5B).

Alternatively, both oligo-pyr and oligo-con did not change the inclusion of SMN2 exon 7 (data not shown). These findings indicate that antisense oligonucleotides directed toward element 1 are an effective tool for increasing exon 7 inclusion in mutant SMN1 containing the C to T transition in exon 7 and wild-type SMN2. Moreover, taken together with the exon trapping experiments, the cis-acting element plays important roles in the regulation of the splicing of SMN exon 7 containing the C to T transition.

**A Trans-acting Protein Specifically Binds to the Pre-mRNA Sequences Containing Element 1**—To detect proteins associated with element 1, 32P-labeled RNA and SK-N-SH nuclear extracts were mixed and UV cross-linked. In this experiment, as shown in Fig. 6A, we used synthetic RNAs that were transcribed from templates of 214 bp sequences containing partial sequences of intron 6 with element 1 and exon 7 of wild-type (SMN1-C) or mutant SMN1 (C to T transition, SMN1-T). Furthermore, as a control, we synthesized RNA probes from the template, which was mutated in element 1 of SMN1-T (mSMN1-T) as described under “Experimental Procedures.”

After digestion of these complexes with RNase A, proteins cross-linked to RNA were resolved by SDS-PAGE followed by autoradiography. Several kinds of bands were detected to bind to each probe in the same pattern, but an ~33-kDa protein...
specifically bound to the SMN1-T probe but not to the SMN1-C probe (Fig. 6B). The binding of the ~33-kDa protein could not be detected in binding assays using the mSMN1-T probe, suggesting that the ~33 kDa protein specifically bound to SMN pre-mRNA containing the C to T transition, and that the element 1 was essential for this binding. We performed RNA binding experiments using shorter RNA probes containing element 1 to determine whether the ~33-kDa protein bound directly to element 1, but we could not obtain the binding activities of these shorter probes to nuclear proteins including the ~33 kDa protein. This suggests that the ~33 kDa protein...
may bind to the probes by recognizing the higher order structure of the pre-mRNA containing the C to T transition in the SMN exon 7. Therefore, it is still unclear whether or not the ~33-kDa protein binds directly to element 1.

We also observed that complexes with molecular masses about 40 kDa specifically bound to the mSMN-T probe (Fig. 6B). However, these complexes did not bind to either the SMN1-C or -T probes. The significance of this binding has yet to be elucidated.

**DISCUSSION**

SMN1 mRNA expresses a full-length transcript, whereas SMN2 produces a low level of the full-length transcript and predominantly 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 alteration of the recognition of exon 7 by components of the splicing machinery (18, 21). Cis-acting RNA elements like ESEs facilitate the splicing by recruiting general splicing factors to the adjacent exon/intron junctions (26–28). A recent study demonstrated that Tra2β-1, a member of the serine-arginine-related proteins of splicing factors, interacted with an exon 7 ESE common to both SMN1 and SMN2 pre-mRNAs (22). However, the critical C to T transition is not contained within the ESE where Tra2β-1 binds directly. Furthermore, the transition does not change the binding activity of Tra2β-1 to the ESE. Thus, it was still unclear why the C to T transition leads to the lack of exon 7 in SMN2. Therefore, in the present study, we examined the critical cis-acting elements on the SMN pre-mRNA responsible for the skipping of SMN exon 7, which contains the C to T transition.

Deletion analysis of SMN1 pre-mRNA sequences showed that the regions from −112 to −68 bp of flanking intron 6 (element 1) and from +59 to +124 of flanking intron 7 (element 2) are significant elements for the exclusion of SMN1 exon 7 containing the C to T transition and for the inclusion of SMN1 exon 7, respectively. However, deletion of these elements from wild-type SMN1 pre-mRNA did not affect the splicing of SMN exon 7. Therefore, combined conditions of deletion of these elements and the C to T transition in exon 7 are necessary for the alteration of SMN exon 7 splicing patterns. The elements that we identified in the present study have not been demonstrated to be critical for the splicing of SMN exon 7. Therefore, it is unknown why the deletion of these elements leads to changes in the processing for exon 7. However, a possible mechanism is that alteration of the splicing patterns of SMN exon 7 may result from the binding of specific regulatory factors to these elements 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 regulatory factors may sterically hinder the recognition or usage of 3′- or 5′-splice sites by splicing machineries.

Previously, heterogeneous nuclear ribonucleoprotein (hnRNP) A1 was demonstrated to inhibit splicing of HIV-tat via a hnRNP A1-responsive intron-splicing silencer (29). In this paper, hnRNP binding sites in the tat intron were shown to match the hnRNP A1 consensus binding site, UAGGG(U/A) (30). The element 1 identified in the present study is considered to act for an intron splicing silencer because of the increase in SMN splicing by deletion or mutation of the element. However, element 1 does not contain the hnRNP A1 consensus sequence, although it contains the unique pyrimidine-rich sequence. Therefore, element 1 could not be regulated by hnRNP A1, but a novel molecule(s) may associate with this element to inhibit the splicing of SMN1 containing the C to T transition or wild-type SMN2. Indeed, we found that an ~33-kDa nuclear protein could interact with RNA probes containing element 1 and the C to T transition in SMN exon 7, although it is unclear whether or not the ~33-kDa protein binds directly to element 1. The protein did not associate with the probe containing the wild-type SMN exon 7, suggesting that the ~33-kDa protein does not basically act as a regulator of the splicing of wild-type SMN exon 7, but rather it plays crucial roles in the skipping of exon 7 only in the case of SMN exon 7 containing the C to T transition. However, it remains unclear why the binding of the ~33-kDa protein to SMN pre-mRNA sequence containing element 1 inhibits the splicing and leads to the resultant exclusion of SMN exon 7. Identification and characterization of the ~33-kDa protein are needed to elucidate the mechanisms.

Only homozygous deletions or mutations of SMN1 result in the SMA phenotype (6, 9). SMA patients retain SMN2 alleles. Therefore, increasing the inclusion of SMN2 exon 7 could provide a new therapy to lower the clinical severity of SMA. We have demonstrated that treatment of cultured cells with an antisense oligonucleotide directed toward element 1, which we identified as the cis-acting element for the exclusion of SMN exon 7 containing the C to T transition, increased the inclusion of wild-type SMN2 exon 7 and SMN1 exon 7 containing the transition. The efficiencies of antisense oligonucleotides on the increase in inclusion of SMN exon 7 are dependent on the interaction between the oligonucleotides and the target sequence. The resultant binding of antisense oligonucleotides to the target sequence, such as element 1 in the pre-mRNA of SMN, may inhibit the direct interaction of splicing silencers with the element and lead to the inclusion of SMN exon 7. Under the present conditions, treatment with an antisense oligonucleotide caused a 2.5-fold increase in the inclusion of SMN exon 7, but did not induce a shift to complete inclusion of exon 7 due to the technical limits of antisense methods. These effects were equivalent to a previously reported treatment with an antisense oligonucleotide designed to hybridize to the 3′ splice junction site of exon 8 (25). In this report, the antisense oligonucleotide also increased SMN2 exon 7 inclusion 2.5-fold. Therefore, the method of antisense treatment may be limited in its effects on the increase in inclusion of SMN exon 7. It has been demonstrated that increased levels of SMN2 copy numbers correlate with decreasing severity of the SMA phenotype. Therefore, even if treatment with antisense oligonucleotides leads only to a 2.5-fold increase in full-length SMN transcripts, it may still lower the clinical severity of SMA.

In the present study, we found a pyrimidine-rich sequence in element 1 and a 66-bp sequence in element 2 that regulate the splicing of SMN exon 7 containing the C to T transition. Further experiments that clarify the functions of these elements, including the identification of their RNA-binding proteins, will address the mechanisms of splicing regulation of SMN exon 7. Moreover, based on these observations, 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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