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HnRNP C1/C2 May Regulate Exon 7 Splicing in the Spinal Muscular Atrophy Gene *SMN1*

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Spinal muscular atrophy (SMA) is caused by loss of *SMN1*. A nearly identical gene, *SMN2*, fails to compensate for the loss of *SMN1* because *SMN2* produces mainly an exon 7-skipped product. The +6C in *SMN1* exon 7 proceeds to include exon 7 into mRNA, while the +6U in *SMN2* causes skipping of exon 7. Here, ~45kD proteins bound to the *SMN* exon 7 RNA probe was found, and identified as hnRNP C1/C2. In gel-shift assay, hnRNP C1/C2 had a greater affinity for the RNA probe with +6C than for the RNA probe with +6U. *In vitro* splicing assay showed that anti-hnRNP C1/C2 antibody hampered splicing of *SMN1* exon 7, but did not affect splicing of *SMN2* exon 7. In conclusion, we showed the possibility that hnRNP C1/C2 enhanced *SMN1* exon 7 splicing specifically.

Spinal muscular atrophy (SMA) is a common autosomal recessive neuromuscular disorder that is characterized by degeneration of the anterior horn cells of the spinal cord, which leads to the axial and limb weakness associated with muscle atrophy. SMA is caused by the homologous deletion and/or deleterious mutation of the *SMN1* gene, which encodes the protein SMN (15). SMN is a component of nuclear structures known as gems, suggesting a role in RNA metabolism (7, 16, 20). SMN may also function in the transport of β -actin messenger RNA to the growth cones of motor neurons, which is necessary for axonal outgrowth (22).

A gene nearly identical to *SMN1*, *SMN2*, is also located on the same chromosome (15). The coding region of the *SMN2* gene differs from that of the *SMN1* gene by only a single nucleotide. As this single nucleotide change in exon 7 is synonymous, the *SMN2* gene encodes the same SMN protein as the *SMN1* gene does. However, this single nucleotide difference determines whether exon 7 is included or excluded in the mRNA: the C at position +6 of exon7 in *SMN1* allows exon 7 inclusion, leading to a full-length protein (17, 19). By contrast, the T at the same position in *SMN2* hampers the efficient inclusion of exon 7 during the splicing of *SMN2* mRNA transcripts, leading to a truncated protein (17, 19).

Two models have been presented to explain the different splicing patterns of *SMN1* and *SMN2* mRNAs, the SF2/ASF-model (4) and the hnRNP A1-model (12). Both stress the importance of the nucleotide sequences at positions +6 to +11/+12 of exon 7 of these genes. The SF2/ASF-model of Cartegni and Krainer (4) proposes that a CAGACAA sequence at this position in *SMN1*, by creating an SF2/ASF-binding exonic splicing enhancer (ESE)

motif, is essential for efficient inclusion of this exon. They explained that the C to T transition at position +6 in exon 7 of *SMN2* disrupts this exonic splicing enhancer. Kashima and Manley (12) propose otherwise: they suggest that the same single base transition, leading to a TAGACA sequence at positions +6 to +11 in *SMN2* exon 7, alters exon 7 splicing by creating an exonic splicing silencer in *SMN2*, identical to known hnRNP A1 binding sequences. They showed that depletion of hnRNP A1 significantly enhanced *SMN2* exon 7 inclusion. These two models are not necessarily incompatible because both the loss of an SF2/ASF-specific ESE and the simultaneous creation of an hnRNP A1-binding site could contribute to *SMN2* exon 7 skipping. Indeed, these proteins are known to antagonize each other. The competition between SF2/ASF and hnRNP A1 appears to be based on their relative concentrations and RNA-binding properties (3).

In this study, we focused on the upstream region of *SMN* exon 7, the nucleotide sequences at positions -12 to +6, and identified a splicing-related protein bound to this region, hnRNP C1/C2. Understanding the splicing mechanisms that differentially process these two *SMN* genes may hold the potential for therapeutic manipulation to improve SMA severity.

MATERIALS AND METHODS

Protein/RNA probe complex formation and electrophoretic mobility shift assay

In order to identify proteins that bind to SMN1 and SMN2 pre-mRNAs, we synthesized 5'-FAM-labeled 2'-O-methyl RNAs to use as RNA probes (Fig. 1). Each RNA probe (50 pmol) was added to a 20- μ l reaction mixture containing 20 mM HEPES-KOH (pH 7.9), 100 mM KCl, 1 mM DTT, 50 mM EDTA (pH 8.0), 0.45 mg/ml BSA, 2.25 mg/ml yeast tRNA, and 5 μ l of HeLa nuclear extract (CilBiotech, Mons, Belgium). To make protein-RNA probe complexes, the mixture was incubated at 30 °C for 20 min, irradiated for 10 min on ice with a UV-Stratalinker (Stratagene), and denatured at 95 °C for 5 min. The resulting complexes were analyzed by 10% SDS-PAGE. Detection of the binding complex was performed using a Fluorimager 585 (Molecular Dynamics, Inc., Sunnyvale, CA).

Mass spectrometry analysis

Sample preparation for mass spectrometry analysis was performed according to the methods of Gonnet *et al.* (8) with some modification. The bands of protein-RNA probe complexes were excised, washed with H₂O and CH₃CN, and dehydrated using a vacuum desiccator. In-gel digestion of the gel pieces was carried out: the gel pieces were rehydrated with 5 μ l of 35 mM NH₄HCO₃ (pH 8.0) containing 52.4 ng/ μ l trypsin and 20% CH₃CN, and subsequently immersed in 50 μ l of 50 mM NH₄HCO₃ (pH 8.0) overnight at 37°C. After separation of supernatants, gel pieces were extracted with 50 μ l of 5% formic acid in 50% CH₃CN for 10 minutes under stirring; this was repeated twice. The supernatants were mixed together and evaporated using a Savant Speedvac evaporator (Labquip, Ontario, Canada) to about 5 μ l. The extracted peptides were then diluted in 20 μ l of 0.2% formic acid and desalted using Zip Tip C18 pipette tips (Millipore, Billerica, MA). Elution of the peptide solution was performed with 5 μ l of 0.2% formic acid in 75% CH₃CN.

The eluted peptides were cocrystallized with equal volumes of a saturated solution of α -cyano-4-hydroxycinnamic acid in 50% CH₃CN, 0.2% formic acid, and allowed to air-dry on MALDI plates. MALDI-TOF MS and MS/MS were performed on a Voyager De-Pro (Applied Biosystems, Foster City, CA). Peptide masses were calculated by the peptide mass fingerprinting search program Mascot search (<http://www.matrixscience.com>) and ProFound (http://prowl.rockefeller.edu/profound_bin/WebProFound.exe). The masses detected by

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MS/MS were also searched using the MS-Tag program (<http://prospector.ucsf.edu/ucsfhtml4.0/mstagfd.htm>).

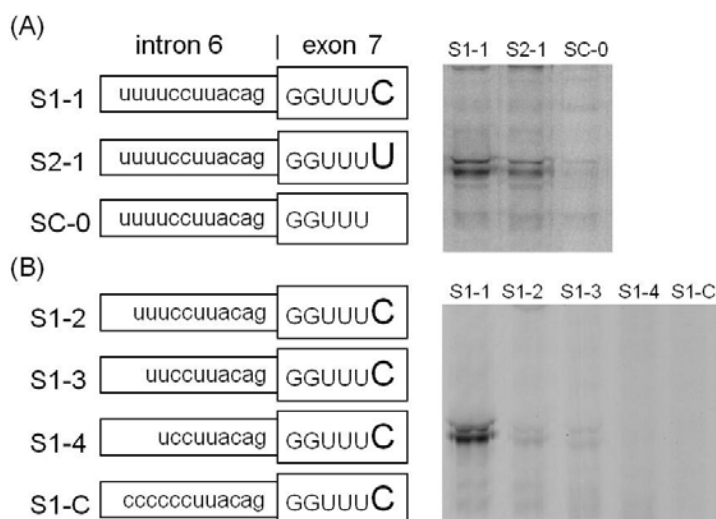


Figure1 RNA probes and electrophoretic mobility shift assay. (A) Sequences of *SMN1*- and *SMN2*-specific RNA probes and fluoro-images of protein/RNA probe complexes separated by SDS-PAGE. (B) Sequences of U-deleted *SMN1*-specific RNA probes and fluoro-images of protein/RNA probe complexes separated by SDS-PAGE.

Minigene construction

The *SMN1* minigene was constructed by overlap extension PCR, as previously described (14). To make the *SMN2* minigene, the plasmid containing the *SMN1* mini-gene was modified using the mutagenesis method of Sawano *et al.* (23): the 5'phosphorylated anti-sense primer (SMN1to2-Mut-T-R1: 5'-TTG ATT TTG TCT AAA ACC CTG TAA G-3') was used for the substitution of C-to-T at position +6 in exon 7 of the *SMN1* minigene.

Preparation of pre-mRNA and *in vitro* splicing

A total of 10 µg of each *SMN* minigene was linearized by incubation with 50 U of BamHI at 37 °C for 3 h, extracted with TE-saturated phenol:chloroform (1:1), precipitated with ethanol, washed once with 70% ethanol, and dissolved in 10 µl of TE. *In vitro* transcription was carried out using a Riboprobe *in vitro* transcription system (Promega), according to the manufacturer's instructions.

In vitro splicing reactions were carried out at 30 °C for 30 min in a total volume of 120 µl, containing 5 ng of *SMN1* (or *SMN2*) minigene pre-mRNA, 40% (v/v) HeLa nuclear extract (CilBiotech), 500 µM ATP, 20 mM creatine phosphate, 1.6 mM MgCl₂, 80 U of RNase inhibitor, and 0.8 mM DTT. To examine whether hnRNP C1/C2 plays a role in the regulation of exon 7 inclusion in the *SMN1* mRNA, anti-hnRNP C1/C2 antibody (Santa Cruz, CA, USA) was added into the HeLa cell nuclear extract.

Reverse transcription polymerase chain reaction (RT-PCR)

Following the splicing reaction, RNA, including the spliced products, was obtained from the splicing reaction mixture by phenol/chloroform (1:1, v/v) extraction and ethanol precipitation. Reverse transcription was performed in a total volume of 20 μ l, containing RNA, 50 pmol of BamEx7R primer, 1 μ l of dNTP Mix (10 mM each), 4 μ l of 5x first-strand buffer, 2 μ l of 0.1 M DTT, 40 units of RNase inhibitor, and 50 units of SuperScript II reverse transcriptase (Invitrogen, Carlsbad, CA, USA). Subsequent polymerase chain reaction (PCR) was performed in a total volume of 30 μ l, containing 1 μ l of synthesized cDNA (equivalent to 0.25 ng of pre-mRNA at the starting point of the splicing reaction), 30 pmol each of EcoRI-Ex6-FX-1 and BamEx7R primer, 250 μ M of each dNTP, and 0.7 U of Expand High Fidelity PLUS polymerase (Roche). The PCR conditions were: 94 °C for 3 min, followed by 22 cycles of 94 °C for 30 s, 60 °C for 30 s and 72 °C for 30 s, with a final extension at 72 °C for 7 min. The PCR products were electrophoresed in 2% agarose gels and stained with ethidium bromide.

HnRNP C and *SF2/ASF* siRNA transfection into HeLa cells

Duplexed siRNAs targeted against *hnRNP C* mRNA and *SF2/ASF* mRNA (Stealth Select RNAi), and an siRNA negative control (Stealth RNAi Negative Control Low GC) were purchased (Invitrogen). The sequences of 25-mer siRNAs against *hnRNP C* and *SF2/ASF* were 5'-UUC UAG AGG AUG CAU UUG ACA UGC C-3' and 5'-UUC GGA UGU CUG GAG GUA AGU UAC C-3', respectively. The siRNAs were transfected into HeLa cells grown in 6-well culture plates (30-50% confluency) using Lipofectamine 2000 (Invitrogen) according to the manufacturer's instructions. After incubating for 48 h, both native protein and RNA were extracted from the harvested cells using a protein and RNA isolation system, Ambion's PARISTM Kit (Applied Biosystems), for further analyses. The amounts of full-length (FL) and exon 7-deleted (Δ 7) transcripts in HeLa cells were determined by using RT-PCR (Fig. 4A) with primers described elsewhere (1). As a reference, glyceraldehyde-3-phosphate dehydrogenase (GAPDH) transcripts were amplified using primers against sequences in exon 8 (5'-ACC ACA GTC CAT GCC ATC AC-3') and exon 9 (5'-TCC ACC ACC CTG TTG CTG TA-3').

RESULTS AND DISCUSSION

Protein complex formation with *SMN1*- and *SMN2*-specific RNA probes

To screen for proteins bound to *SMN* exon 7 transcript, HeLa cell nuclear extracts were incubated with a FAM-labeled *SMN1*-specific RNA probe containing +6C (S1-1), a FAM-labeled *SMN2*-specific RNA probe containing +6U (S2-1), and a FAM-labeled *SMN* RNA probe with no +6 nucleotide (SC-0). After incubation, protein/RNA probe complexes were separated by SDS-PAGE (electrophoretic mobility shift assay). As shown in Figure 1A, a doublet band of protein/S1-1 and protein/S2-1 complexes was observed at ~45 kD, but the fluorescence intensity of the protein/S1-1 complex was significantly higher than that of protein/S2-1, suggesting that the protein had a greater affinity to S1-1 than to S2-1.

Identification of *hnRNP C1/C2* protein by mass spectrometry

The proteins in the ~45-kD band bound to S1-1 (45-kD complex) were enriched and purified by fractionating the HeLa cell nuclear extract by a combination of anion-exchange chromatography and reversed-phase chromatography. The purified fraction from the C18 reversed-phase HPLC separation gave only a doublet fluorescent band in an EMSA

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experiment. The doublet band was excised for identification of the proteins in the 45-kD complex.

To identify the protein, MALDI-TOF peptide-mass mapping was performed on the tryptic peptide mixture generated by in-gel digestion of the complex (Fig. 2A). Seven peptides were detected (Fig. 2C), and these were compared with the theoretical tryptic peptide masses of the protein sequences in the database. The database search indicated that the protein was hnRNP C1/C2 (NCBI accession number P07910) (Fig. 2B). The identified peptides were common to both isoforms of hnRNP C (Fig. 2B).

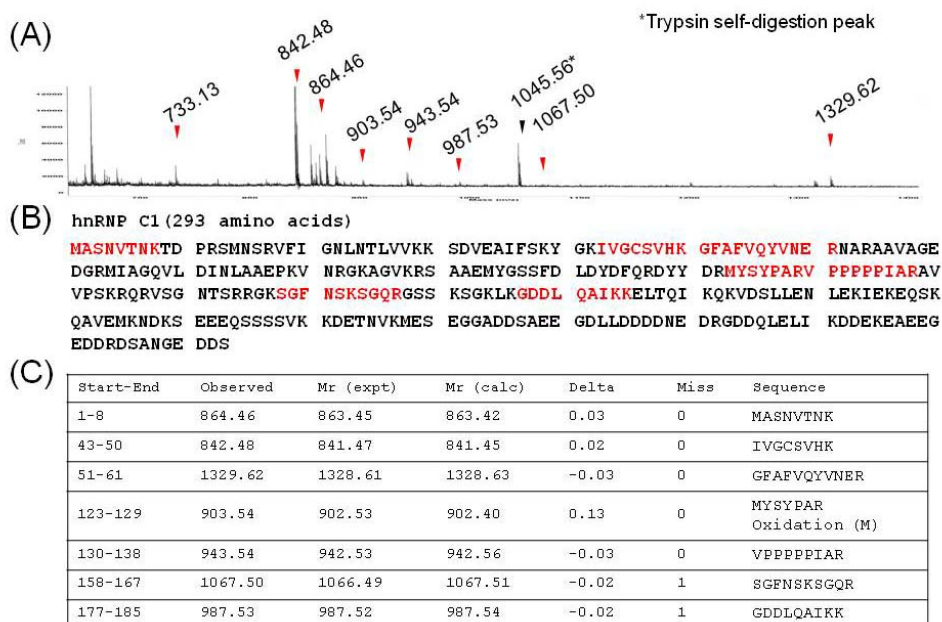


Figure2 Identification of hnRNP C1/C2 protein using MALDI-TOF peptide-mass mapping. (A) Mass spectrometry data. (B) Amino acid sequence of hnRNP C1. Matched peptides are shown in red. (C) Mascot search results. A peptide mass database, Mascot, identified seven peptides included in the protein as parts of hnRNP C1/C2.

The identity of the protein was further confirmed by post-source decay (PSD) using the MALDI-TOF MS instrument. A peptide carrying one positive charge (m/z 1329.62) was selected as a precursor ion and fragmented in the flight tube of the instrument. The NCBI database search subsequently unambiguously identified the protein as human hnRNP C1/C2 (data not shown). The fragmented peptide was found to represent the tryptic fragment GFAFVQYVNER, namely, amino acids 51-61 of human hnRNP C1/C2.

The hnRNP C1 and hnRNP C2 proteins are alternative splicing products of the same gene: the theoretical masses of C1 and C2 are 41 and 43 kD, respectively (2). Based on the molecular masses, the lower and upper fragments of the doublet band were thought to be hnRNP C1 and hnRNP C2. Western blotting analysis using anti-hnRNP C1/C2 antibody also showed a doublet band, which confirmed the results obtained using mass spectrometry (data not shown).

HnRNP C1 and C2 are among the most abundant nuclear proteins. These proteins bind to RNA as heterotetramers of C1 and C2 ((C1)₃C2) (18, 21), and are major constituents of the hnRNP complex (6). It has been previously shown that a monoclonal antibody to hnRNP C1/C2 does not inhibit spliceosome formation, but does inhibit the splicing of adenovirus 2 (Ad-2) major late transcription unit pre-mRNA *in vitro* (5). These reports indicated that hnRNP C1/C2 plays a fundamental role in RNA processing through the modulation of pre-mRNA splicing.

Protein complex formation with U-deleted *SMN1*-specific probes

To test whether the poly-U stretch is required for protein-binding, we carried out electrophoretic mobility shift assays of the protein complexes with the following FAM-labeled *SMN1*-specific RNA probes: S1-2 (in which a single U nucleotide at position -12 of *SMN1* exon 7 was deleted), S1-3 (in which a UU dinucleotide at position -11 to -12 of *SMN1* exon 7 was deleted), S1-4 (in which a UUU trinucleotide at position -10 to -12 of *SMN1* exon 7 was deleted), and S1-C (in which a UUUU tetranucleotide at position -9 to -12 of *SMN1* exon 7 was replaced by a CCCC tetranucleotide). The fluorescence of bands with these probes was null to faint (Fig. 1B).

This finding was consistent with a previous report that demonstrated that a poly-U tract may be a high-affinity binding site for hnRNP C1 (9). According to our data, the poly-U tract (UUUU tetranucleotide) at position -9 to -12 in intron 6 is essential for the binding of hnRNP C1/C2. Interestingly, our data also showed that a single nucleotide change downstream of the poly-U tract, namely, +6C to +6U in exon 7, may be critical to produce the difference in hnRNP C1 and C2 binding affinities. Koloteva-Levine *et al.* (13) also showed that for efficient binding of hnRNP C1/C2 to RNA, the context of the U-stretch is more important than the stretch itself.

***In vitro* splicing assay with an anti-hnRNP C1/C2 antibody**

To compare the splicing efficiencies of *SMN1* exon 7 and *SMN2* exon 7, we performed an *in vitro* splicing assay using *SMN1* and *SMN2* minigene constructs. The minigenes contained exon 6 (111 nt), a shortened intron 6 (289 nt), exon 7 (54 nt) and the region 5' of intron 7 (30 nt) of the *SMN1* gene (Fig. 3A). The shortened intron 6 of the minigene constructs was synthesized based on an *SMN1*-specific sequence. The splicing efficiency of *SMN1* minigene pre-mRNA was greater than that of *SMN2* minigene pre-mRNA (Fig. 3B).

To examine whether hnRNP C1/C2 plays a role in the regulation of exon 7 inclusion in the *SMN1* mRNA, an anti-hnRNP C1/C2 antibody was added to HeLa cell nuclear extract. The presence of anti-hnRNP C1/C2 antibody hampered the inclusion of exon 7 of the *SMN1* mini-gene mRNA in a dose-dependent manner (Fig. 3B). Interestingly, anti-hnRNP C1/C2 antibody did not affect the splicing efficiency of the *SMN2* mini-gene pre-mRNA (Fig. 3B).

Our *in vitro* splicing assay using minigenes showed that hnRNP C1/C2 bound the intron 6-exon 7 boundary sequence including the C at position +6 of *SMN1* exon 7, and that anti-hnRNP C1/C2 antibody decreased the splicing efficiency of *SMN1* exon 7 *in vitro*. hnRNP C1/C2 associated much less with the corresponding region including the T at position +6 of *SMN2* exon 7, and the anti-hnRNP C1/C2 antibody did not alter the splicing efficiency of *SMN2* exon 7. These results suggested that hnRNP C1/C2 binding is a determinant of exon identity and alternative splicing of *SMN1* exon 7, but not *SMN2* exon 7.

In addition, it should be noted that the shortened intron 6 sequences were common between the minigenes constructed in this study, and that several poly-U stretches exist in the intron 6 sequence. However, hnRNP C1/C2 bound to the *SMN1* minigene, but not to the

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SMN2 minigene. This finding also indicated that +6C or +6U in exon 7 may be critical to produce a difference in hnRNP C1/C2 binding affinity.

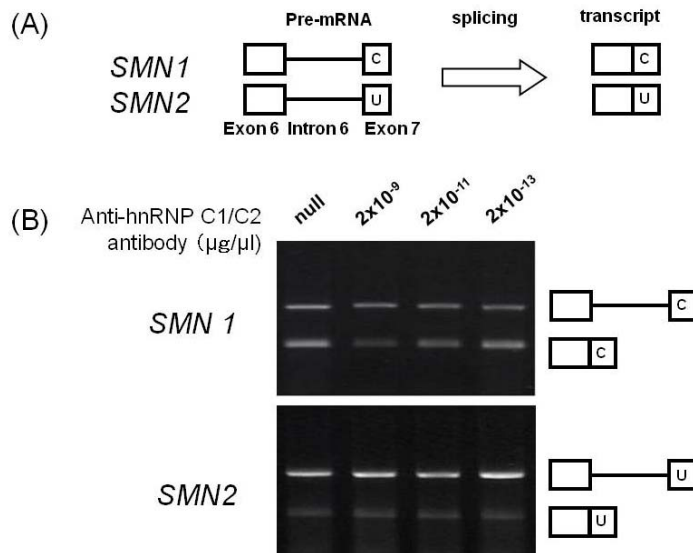


Figure3 *In vitro* splicing assay. (A) pre-mRNAs from *SMN1* and *SMN2* minigenes and a scheme of splicing. (B) *In vitro* splicing assay with anti-hnRNP C1/C2 antibody. The more anti-hnRNP C1/C2 antibody that was added, the more the exon 7 inclusion of the *SMN1* minigene mRNA was hampered. However, anti-hnRNP C1/C2 antibody did not affect the splicing efficiency of the *SMN2* minigene pre-mRNA.

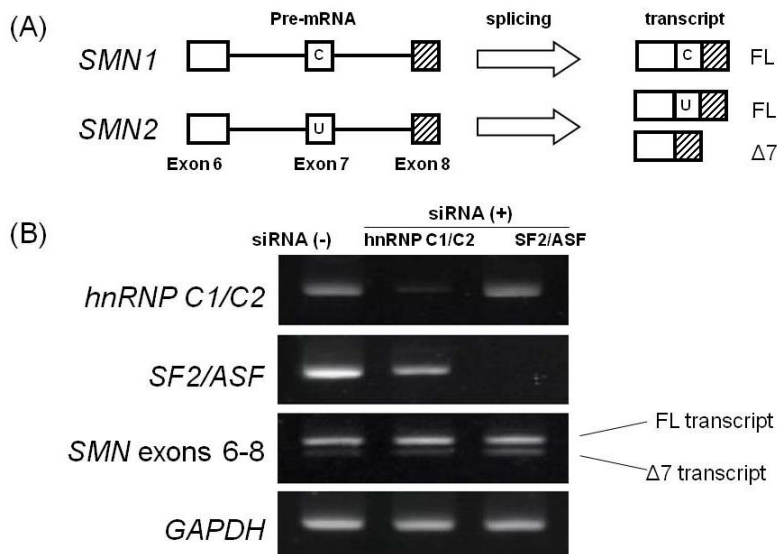


Figure4 *In vivo* splicing assay. (A) pre-mRNAs from *SMN1* and *SMN2* and a scheme of splicing. (B) *In vivo* splicing assay with siRNAs against *hnRNP C1/C2* and *SF2/ASF*. RT-PCR analysis showed that siRNAs against *hnRNP C1/C2* did not bring about significant changes in the amounts of full-length (FL) and exon 7-deletion ($\Delta 7$) transcripts, or the ratio of $\Delta 7$ /FL. Analysis with siRNAs against *SF2/ASF* showed similar results.

***In vivo* splicing assay under the condition of the *hnRNP C1/C2* gene silencing**

Then, we investigated whether *hnRNP C1/C2* is required for *SMN1* inclusion *in vivo* by using RT-PCR (Fig. 4). We transfected HeLa cells with siRNAs against *hnRNP C1/C2* or *SF2/ASF* and decreased the transcript levels by 70-80% (Fig 4B). RT-PCR analysis showed that siRNAs against *hnRNP C1/C2* did not bring about significant changes in the amounts of FL and $\Delta 7$ transcripts, or the ratio of $\Delta 7$ /FL (Fig. 4B). Analysis with siRNAs against *SF2/ASF* also showed similar findings (Fig. 4B).

In our study, neither *hnRNP C1/C2* depletion nor *SF2/ASF* depletion hampered *SMN1* exon 7 inclusion in HeLa cells. Kashima and Manley (12) showed that *SF2/ASF* depletion has no effect on *SMN* gene splicing in DT40 cells and suggested that *SF2/ASF* is not strictly required for exon 7 inclusion in *SMN1*. However, Cartegni *et al* (3) showed that mutations disrupting the *SF2/ASF*-dependent ESE motif hampered exon 7 inclusion and suggested that even if *SF2/ASF* was depleted, redundant functions of additional SR proteins or some compensatory mechanisms can maintain efficient *SMN1* exon 7 inclusion. It is more likely that *SMN* exon 7 inclusion is coordinated by more factors than are currently accounted for. Such regulation may be desirable to fulfill the requirement of differential expression of *SMN* in different cell types. In fact, a recent report demonstrated the differential regulation of *SMN* splicing in different cell-types of SMA discordant families (10, 24). For further factors affecting *SMN* exon 7 inclusion, proteins containing RNA-binding motifs should be considered, and in particular *hnRNP* proteins, because these are associated very early with nascent pre-mRNA (11). Our results do not necessarily preclude a role for *hnRNP C1/C2* as one of the determinants of *SMN1* exon 7 inclusion.

In conclusion, we identified a candidate protein, *hnRNP C1/C2*, which modulates splicing of the *SMN* exon7 by an *in vitro* splicing assay system. However, it remains unknown how *hnRNP C1/C2* cooperates or competes with splice-related proteins, such as U2AF proteins and polypyrimidine-tract binding protein, in the 3'-splice site of the alternative exon 7. In addition, *hnRNP C1/C2* depletion does not alter the splicing pattern of *SMN* genes in HeLa cells, which suggests the presence of redundant systems in the splicing machinery. Further investigation is necessary to elucidate the roles trans-acting splicing proteins including *hnRNP C1/C2* play in other cell types, notably the motor neurons affected in SMA.

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