Determinants of Exon 7 Splicing in the Spinal Muscular Atrophy Genes, *SMN1* and *SMN2*

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Spinal muscular atrophy is a neurodegenerative disorder caused by the deletion or mutation of the survival-of-motor-neuron gene, *SMN1*. An *SMN1* paralog, *SMN2*, differs by a C→T transition in exon 7 that causes substantial skipping of this exon, such that *SMN2* expresses only low levels of functional protein. A better understanding of *SMN* splicing mechanisms should facilitate the development of drugs that increase survival motor neuron (SMN) protein levels by improving *SMN2* exon 7 inclusion. In addition, exonic mutations that cause defective splicing give rise to many genetic diseases, and the *SMN1/2* system is a useful paradigm for understanding exon-identity determinants and alternative-splicing mechanisms. Skipping of *SMN2* exon 7 was previously attributed either to the loss of an SF2/ASF–dependent exonic splicing enhancer or to the creation of an hnRNP A/B–dependent exonic splicing silencer, as a result of the C→T transition. We report the extensive testing of the enhancer-loss and silencer-gain models by mutagenesis, RNA interference, overexpression, RNA splicing, and RNA-protein interaction experiments. Our results support the enhancer-loss model but also demonstrate that hnRNP A/B proteins antagonize SF2/ASF–dependent ESE activity and promote exon 7 skipping by a mechanism that is independent of the C→T transition and is, therefore, common to both *SMN1* and *SMN2*. Our findings explain the basis of defective *SMN2* splicing, illustrate the fine balance between positive and negative determinants of exon identity and alternative splicing, and underscore the importance of antagonistic splicing factors and exonic elements in a disease context.

Spinal muscular atrophy (SMA types I, II, and III [MIMs 253300, 253550, and 253400]) is an autosomal recessive neurodegenerative disease characterized by the selective destruction of spinal cord α-motor neurons. SMA is a leading genetic cause of infant mortality, with an estimated incidence of 1 in 6,000–10,000 live births, and is caused by the homozygous loss or mutation of the *SMN1* gene (Entrez Gene ID number 6606), which codes for the survival motor neuron (SMN) protein (UniProt accession number Q16637-1) (Lefebvre et al. 1995; reviewed by Frugier et al. [2002]). SMN is a ubiquitously expressed, essential protein necessary for the efficient assembly of ribonucleoprotein complexes (reviewed by Meister et al. [2002] and Gubitz et al. [2004]). *SMN2* (Entrez Gene ID number 6607), a nearly identical paralog of *SMN1,* is present in the human genome as part of an inverted duplication on 5q13. *SMN2* expresses limited amounts of functional, full-length SMN protein, which is apparently sufficient for normal activity in most cell types but not in motor neurons. *SMN2* is efficiently transcribed but cannot fully compensate for the loss of *SMN1,* because a translationally silent, single-nucleotide transition in *SMN2* at position 6 of exon 7 (c6t) causes predominant exon 7 skipping (Lefebvre et al. 1995; Lorson et al. 1999; Monani et al. 1999) and results in an unstable protein (SMNΔ7) (UniProt accession number Q16637-3) with a different C-terminus (Lorson and Androphy 2000). Increased expression from *SMN2* can reduce the severity of SMA, as seen when multiple copies of *SMN2* are present, both in patients and mouse models (reviewed by Monani et al. [2000] and Frugier et al. [2002]). Current efforts to develop therapeutics for SMA are largely directed at increasing *SMN2*-derived full-length SMN protein abundance or activity at the transcriptional and posttranscriptional levels (Jarecki et al. 2005 and references therein), including approaches aimed specifically at changing the ratio of the two main splicing isoforms in favor of the full-length active one (Lim and Hertel 2001; Miyajima et al. 2002; Cartegni and Krainer 2003; Skordis et al. 2003).

Signals located within an exon can have positive or negative effects on the recognition of that exon during splicing. Exonic splicing enhancers (ESEs) stimulate splicing and are often required for efficient intron removal, whereas exonic splicing silencers (ESSs) inhibit splicing. ESSs and ESEs frequently antagonize each

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other—for example, in the context of alternative splicing regulation (reviewed by Hastings and Krainer [2001] and Cáceres and Kornblihtt [2002]). The specific sequence and location of these *cis*-elements determine the proteins that mediate the splicing activity. Well-characterized ESEs include those recognized by SR proteins, a family of RNA-binding proteins with distinctive serine/arginine–rich domains (reviewed by Cáceres and Kornblihtt [2002] and Cartegni et al. [2002]). Some ESSs are recognized by hnRNP A1 (UniProt accession number P09651-2), an abundant RNA-binding protein that plays an important role in splicing repression (reviewed by Cáceres and Kornblihtt [2002] and Dreyfuss et al. [2002]). As exemplified by SMA, point mutations that alter splicing signals can cause a loss of normal gene expression as a result of aberrant splicing. Indeed, aberrant splicing is a prevalent cause of genetic diseases (reviewed by Cáceres and Kornblihtt [2002], Faustino and Cooper [2003], and Pagani and Baralle [2004]).

Two different models have been proposed to explain the observed difference in splicing between *SMN1* and *SMN2*: loss of an enhancer or gain of a silencer. The first model proposes the existence in *SMN1* of an ESE recognized by the SR protein SF2/ASF (UniProt accession number Q07955) (Cartegni and Krainer 2002). The C→T transition in *SMN2* disrupts this ESE and results in exon 7 skipping. This transition eliminates a high-score motif for SF2/ASF, according to ESEfinder, a program that predicts ESEs that are responsive to one of several SR proteins on the basis of experimental and statistical analyses (Cartegni and Krainer 2002; Cartegni et al. 2003). Mutational analysis, RNA-binding data, transfection experiments, and in vitro splicing assays using extracts and recombinant proteins all supported a direct role for SF2/ASF in *SMN1* exon 7 inclusion that is not maintained in *SMN2* (Cartegni and Krainer 2002), which is consistent with the notion that exon 7 skipping in *SMN2* transcripts results from the loss of the SF2/ASF–dependent ESE. Furthermore, *SMN2* splicing can be corrected by targeting exon 7 with specific synthetic effector molecules that mimic the function of SR proteins (Cartegni and Krainer 2003) or with bifunctional modified antisense oligonucleotides that comprise SF2/ASF–binding sites (Skordis et al. 2003). The second model asserts that, rather than eliminating an ESE, the presence of a T at position +6 of *SMN2* exon 7 creates an ESS that is recognized by hnRNP A1 and leads to inefficient exon 7 inclusion (Kashima and Manley 2003). Increased exon 7 inclusion following small interfering RNA (siRNA)–mediated knockdown of hnRNP A1 and the related protein hnRNP A2 (UniProt accession number P22626-2), as well as UV cross-linking assays, suggested that hnRNP A1 inhibits *SMN2* exon 7 inclusion, whereas genetic depletion of SF2/ASF in the chicken cell line DT-40 does not affect full-length *SMN1* levels (Kashima and Manley 2003). These two models are not necessarily incompatible, since both the loss of an SF2/ASF–specific ESE as well as the simultaneous creation of an hnRNP A1–binding site could, in principle, be contributing factors to *SMN2* exon 7 skipping. Indeed, these proteins are known to antagonize each other, a competition that appears to be based on their relative concentrations and RNA-binding properties (Mayeda and Krainer 1992; Cáceres et al. 1994; Eperon et al. 2000; Zhu et al. 2001; reviewed by Black [2003]).

Because of the obvious implications for SMA, a clearer understanding of the mechanism of *SMN1*/*SMN2* exon 7 splicing would significantly impact the development of therapeutic approaches. We have now rigorously tested the enhancer-loss and silencer-gain models, using extensive mutational analysis, RNA interference (RNAi)–mediated knockdown, overexpression of hnRNP A1 and SF2/ASF, in vivo and in vitro splicing assays, and RNA-protein interaction analysis. We find that *SMN2* exon 7 skipping primarily results from the loss of the SF2/ASF–dependent ESE. Although hnRNP A1 indeed has a strong inhibitory effect on exon 7 inclusion, this effect is independent of the C→T transition and, therefore, is not specific to *SMN2*. The observed antagonism between SF2/ASF and hnRNP A1 in the definition of *SMN1/2* exon 7, together with the variable expression of these splicing regulators, may cause tissue-specific differences in the extent of exon 7 inclusion and thereby contribute to the motor neuron–restricted phenotype observed in SMA.

Material and Methods

RNA Affinity Chromatography

RNA affinity chromatography was performed with the modification of a published technique (Caputi et al. 1999). RNA oligonucleotides *SMN*.WT (5′-GGUUUCAGACAAAAUCA-3′) and *SMN*.C6T (5′-GGUUUUAGACAAAAUCA-3′) were obtained from Dharmacon. One hundred micrograms of each RNA was oxidized with sodium *m*-periodate in a 24-μl reaction. One hundred microliters (1:1 slurry) of adipic acid dihydrazide agarose beads (Sigma) was mixed with the *SMN1* or *SMN2* RNA and was rotated overnight at 4°C. A 250-μl in vitro splicing reaction mix (Cartegni et al. 2002) including 100 μl of HeLa nuclear extract was added to 50 μl of the *SMN1* or *SMN2* beads that were prewashed in buffer D with 0.1 M KCl, and the mixtures were incubated for 40 min at 30°C. The beads were divided into three aliquots, which were washed three times in buffer D with either 0.1 M KCl, 0.15 M KCl, or 0.2 M KCl. After the final wash, the beads were resuspended in 40 μl of protein sample buffer and were heated at 90°C for 10 min to elute the bound proteins. Ten microliters of each protein sample was loaded on a 12% SDS polyacrylamide gel, which was then electroblotted onto a nitrocellulose membrane and was probed with monoclonal antibodies against hnRNP A1 or SF2/ASF (Hanamura et al. 1998).

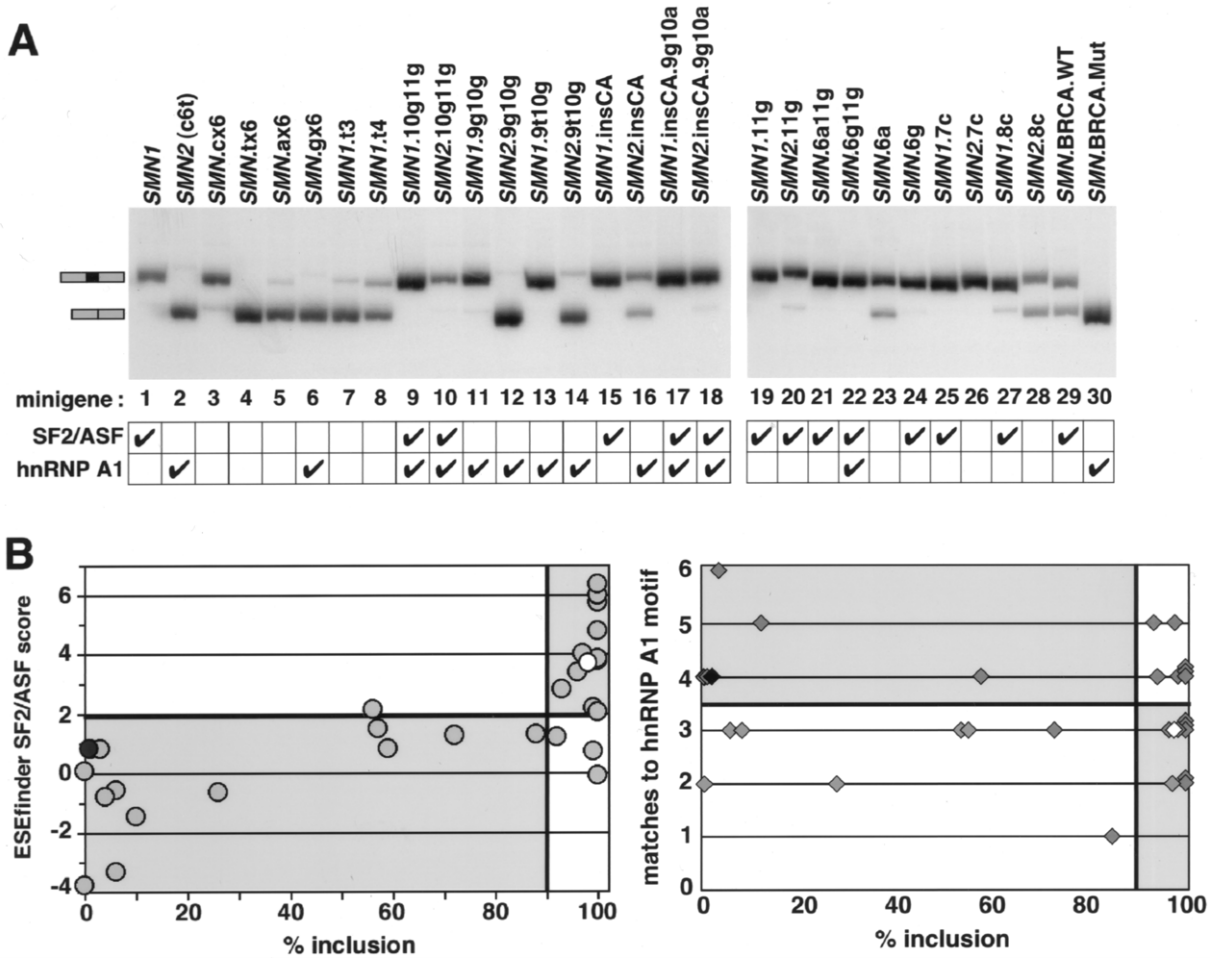


Figure 1 Mutational analysis of *SMN* exon 7 splicing. *A,* Radioactive RT-PCR analysis of RNA from *SMN* mutant minigenes expressed in 293-HEK cells. Transcripts that either include or skip exon 7 are indicated. Check marks indicate the presence of a high-score SF2/ASF motif (≥1.96), as predicted by ESEfinder 2.0 (Cartegni et al. 2003), or a ≥4 of 6 match to the hnRNP A1 consensus motif (Burd and Dreyfuss 1994). Lane numbers correspond to the 30 minigene construct numbers listed in table 1. *B,* Results of multiple experiments (table 1) quantitated by phosphorimage analysis and presented as scatterplots. The percentage of exon 7 inclusion was plotted against SF2/ASF ESEfinder 2.0 scores (*left*) or against matches to the hnRNP A1 consensus binding motif (*right*). Thresholds are set at 1.96 for SF2/ASF, at between 3 and 4 matches for hnRNP A1, and at 90% for exon inclusion. The white and black symbols represent *SMN1* and *SMN2*, respectively. Quadrants where the ESE-loss (*left*) and ESS-gain (*right*) models predict that the points should fall are shaded.

Minigene Construction, Transfections, and Expression Analysis

All *SMN* constructs were derived from pCI-*SMN*x-wt (Cartegni et al. 2002), which is a derivative of pCITel (Lorson and Androphy 2000). Mutations were introduced by site-directed mutagenesis, with the use of a QuikChange kit (Stratagene). Plasmids (1 μg) were transfected into 293-HEK cells by use of FuGENE (Roche). For SF2/ASF overexpression experiments, early-passage mouse embryo fibroblasts (MEF) cells were cotransfected with 2 μg of pCGT7-SF2/ASF and 400 ng of the *SMN* minigene plasmids. Total RNA was isolated after 48 h by use of Trizol (Life Technologies). Reverse transcription was performed using 1 μg of DNase-treated total RNA, oligo(dT), and Superscript II reverse transcriptase (Life Technologies). Semiquantitative PCR was performed with the cDNA equivalent of 10 ng of starting RNA, by use of AmpliTaq Gold (Roche) and 30 amplification cycles (94°C for 30 s, 58°C for 60 s, and 72°C for 60 s) in reactions containing [α-^{32}P]dCTP. Primers were pCIFwdB (5′-GACTCACTATAGGCTAGCCTCG-3′) and *SMN*8-300+5′R (5′-AAGTACTTACCTGAAATCTAATCCACATTCAAATTTTCTCAACTG-3′). Products were separated on 6% native polyacrylamide or 1% agarose gels. The figures show either autoradiograms or ethidium bromide–stained gels, but, in all cases, the quantitation of splicing was based on phosphorimage analysis (Fujix BAS2000). The relative abundance of each product was calculated after adjustment for differences in base composition. For the statistical analysis in figure 1 and table 1, each mutant was tested in two to four separate transfections. Correlation coefficients were calculated with the use of nonparametric Spearman rank cor-

Table 1

Summary of *SMN* Exon 7 Mutations and Levels of Exon Inclusion

Minigene Number	Minigene[a]	Sequence[b]	SF2/ASF Score[c]	hnRNP A1 Score[d]	% (±SD) Included[e]
1	*SMN1*	GGTTT***CAGACA***AATCA	***3.77***	3	98 ± 3
2	*SMN2* (c6t)	GGTTT***TAGACA***AATCA	.81	***4***	1 ± 2
3	*SMN*.cX6	GGTTT***CcccCcA***ATCA	1.29	1	88 ± 5
4	*SMN*.tX6	GGTTTTttttt AATCA	−3.77	2	0 ± 0
5	*SMN*.aX6	GGTTTaAaAa AAAATCA	−3.33	3	6 ± 7
6	*SMN*.gX6	GGTTT***ggGgggA***ATCA	.81	***4***	3 ± 3
7	*SMN1*.t3	GGTTTCAGtttAAATCA	−.56	3	6 ± 3
8	*SMN1*.t4	GGTTTCAttCttAATCA	−.65	2	26 ± 15
9	*SMN1*.10g11g	GGTTT***CAGAggA***ATCA	***5.74***	***4***	100 ± 0
10	*SMN2*.10g11g	GGTTT***TAGAggA***ATCA	***2.79***	***4***	93 ± 5
11	*SMN1*.9g10g	GGT***TTCAGgg***AAAATCA	1.19	***5***	92 ± 2
12	*SMN2*.9g10g	GGTTTTAGggAAAATCA	−.78	***6***	4 ± 5
13	*SMN1*.9t10g	GGTTT***CAGtgA***ATCA	.71	***4***	99 ± 1
14	*SMN2*.9t10g	GGTTTTAGtgAAAATCA	−1.46	***5***	10 ± 2
15	*SMN1*.insCA	GGTTTca***CAGACA***ATCA	***3.77***	3	100 ± 1
16	*SMN2*.insCA	GGTTTca***TAGACA***ATCA	.81	***4***	59 ± 5
17	*SMN1*.insCA9g10a	GGTTT***caCAGga***AAAATCA	***5.98***	***4***	100 ± 0
18	*SMN2*.insCA9g10a	GGTTT***caTAGga***AAAATCA	***3.67***	***5***	98 ± 2
19	*SMN1*.11g	GGTTT***CAGACg***AATCA	***6.34***	2	100 ± 0
20	*SMN2*.11g	GGTTT***TAGACg***AATCA	***3.39***	3	96 ± 2
21	*SMN*.6a11g	GGTTT***aAGACg***AATCA	***3.84***	3	100 ± 0
22	*SMN*.6g11g	GGTT***TgAGACg***AATCA	***4.77***	***4***	100 ± 1
23	*SMN*.6a	GGTTT***aAGACA***AATCA	1.26	3	72 ± 2
24	*SMN*.6g	GGTTT***gAGACA***AATCA	***2.19***	3	99 ± 1
25	*SMN1*.7c	GGTTT***CcGACA***AATCA	***2.04***	2	100 ± 0
26	*SMN2*.7c	GGTTTTcGACAAAATCA	−.09	3	100 ± 0
27	*SMN1*.8c	GGTTT***CAcACA***AATCA	***4.01***	2	97 ± 2
28	*SMN2*.8c	GGTTTTA***cACAAAA***TCA	1.49	3	57 ± 3
29	*SMN*.BRCA.WT	GGTTT***CtGAgtt***AATCA	***2.14***	3	56 ± 3
30	*SMN*.BRCA.Mut	GGTTT***CttAgtt***AATCA	.08	***4***	0 ± 0

[a] The 30 minigenes used in transfection experiments, in the same order as the lanes in figure 1*A*.

[b] Nucleotide sequence around the ESE/ESS motifs. The ESEfinder 2.0 SF2/ASF top-scoring motif for each sequence is shown in bold italic type (if >0). The best fit to the hnRNP A1–binding consensus (TAGGGA/T) is underlined. *SMN1* constructs have a T, and *SMN2* constructs a C, at position +6. Mutations are indicated in lowercase.

[c] Top SF2/ASF score. High-score motifs are shown in bold italic type (threshold = 1.96).

[d] Number of nucleotide matches to the hnRNP A1 hexamer consensus, shown in bold italic type when >3.

[e] Average inclusion percentages and SDs (n = 2–4) measured after radioactive RT-PCR in the linear range.

relation and a bootstrap technique to determine the CIs. Additional experiments using fewer PCR amplification cycles gave similar results.

For analysis of endogenous *SMN* splicing, 293-HEK cells were transfected with 2 μg of pCGT7-A1 expression plasmid (Cáceres et al. 1994). RT-PCR of total RNA was performed as described above, with primers *SMN*ex6FXho (5′-CGATCTCGAGATAATTCCCCCACCACCTCCC-3′) and *SMN*ex8RNot (5′-ATATGCGGCCGCCACATACGCCTCACATACA-3′). Products were purified using PCR purification columns (Qiagen), were digested with *Dde*I, and were separated on 6% native polyacrylamide gels. Protein lysates were collected with Trizol and were analyzed by western blotting with an anti-T7-Tag antibody (Novagen) and mAb 4G3 (Habets et al. 1989) against human U2B″ snRNP protein.

RNA Interference

Twenty-four hours before transfection of siRNAs with Oligofectamine (Invitrogen), 2.5×10^4 HeLa cells were seeded into 24-well plates. After 24 h, *SMN* minigenes were transfected using FuGENE, and the cells were incubated for another 24 h and then harvested for RNA isolation and western blotting. The following siRNAs were used: hnRNP A1, 5′-CAGCUGAGGAAGCUCUUCA-3′; hnRNP A2, 5′-GGAACAGUUCCGUAAGCUC-3′; SF2/ASF, 5′-ACGAUUGCCGCAUCUACGU-3′; and luciferase control, 5′-GCCAUUCUAUCCUCUAGAGGAUG-3′. RT-PCR was performed as described above. Western blotting was performed using antibodies specific for α-tubulin (Sigma B512) and for hnRNP A1 (4B10) and hnRNP A2 (DP3B3), which were kindly provided by Gideon Dreyfuss.

Nuclear Extract Depletion and in Vitro Splicing Analysis

HeLa cell nuclear extract was affinity depleted of hnRNP A/B proteins, as described elsewhere (Zhu et al. 2001). Three microliters of mock-depleted or hnRNP A/B–depleted extract was used in a standard reaction for *SMN* in vitro splicing (Cartegni and Krainer 2002). Extracts were complemented with recombinant hnRNP A1 protein, which was overexpressed in *Escherichia coli* and was purified as described elsewhere (Mayeda and Krainer 1992).

Results

SF2/ASF and hnRNP A1 Binding to SMN1 *versus* SMN2 *RNA*

We previously used site-specific labeling to show the preferential binding of SF2/ASF to exon 7 RNA from *SMN1* compared with *SMN2* (Cartegni and Krainer 2002). Preferential binding of hnRNP A1 to *SMN2* exon 7 RNA was shown by the uniform labeling of a 5′ fragment of the exon (Kashima and Manley 2003). These two observations were obtained using somewhat different methodologies and are, therefore, not directly comparable, but both results relied on immunoprecipitation after UV-induced cross-linking of radiolabeled RNAs to proteins in nuclear extract. Biases can arise because different nucleotides cross-link to proteins with significantly different efficiencies. In particular, rU generally cross-links more efficiently than does rC (Hockensmith et al. 1991). *SMN2* exon 7 RNA has a U at position +6 and would, therefore, be expected to cross-link more efficiently to a bound protein than would *SMN1,* which has a C at the same position. This difference in cross-linking efficiency could account for the apparent difference in hnRNP A1 binding to *SMN1* and *SMN2* RNAs (Kashima and Manley 2003). Conversely, this difference in cross-linking efficiency could have resulted in an underestimate of the preferential binding of SF2/ASF to the region around position +9 of *SMN1,* as noted elsewhere (Cartegni and Krainer 2002).

To obtain a more direct and unbiased assessment of proteins that bind to the RNA encompassing the C→T transition, we performed RNA affinity chromatography. The first 17 nt of *SMN1* or *SMN2* exon 7 RNA were covalently linked to agarose beads via the 3′ end and were incubated with HeLa cell nuclear extract. Proteins that remained tightly bound to each RNA after washing at different salt concentrations were analyzed using monoclonal antibodies specific for hnRNP A1 or SF2/ASF. hnRNP A1 bound efficiently to *SMN1* and *SMN2* RNA fragments with approximately equal efficiency (fig. 2, bottom). In fact, incubation of nuclear extract with either RNA resulted in the efficient depletion of hnRNP A1 from the extract, as evident in the supernatant fractions. In contrast, SF2/ASF bound very specifically to the

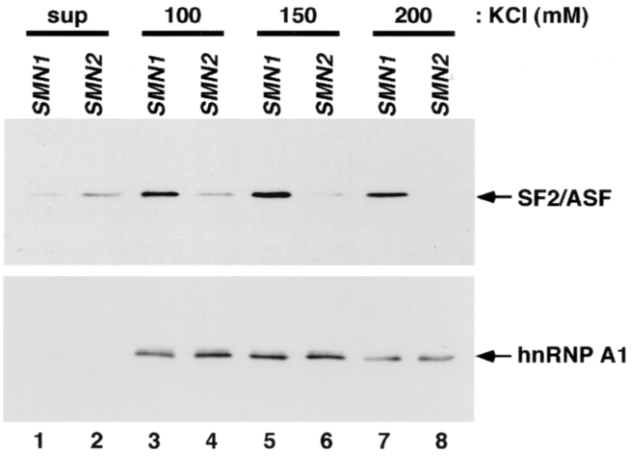


Figure 2 Binding specificity of SF2/ASF and hnRNP A1. Western blot of proteins recovered from agarose beads covalently linked to RNAs corresponding to the first 17 nt of *SMN1* or *SMN2*. The RNA beads were incubated with identical aliquots of HeLa cell nuclear extract under splicing conditions; they were then divided into three aliquots for washing at the indicated salt concentrations, and the proteins that remained bound were recovered by heating in SDS-sample buffer. Blots were probed with monoclonal antibodies against SF2/ASF (*top*) or hnRNP A1 (*bottom*). Supernatant (sup) and bound samples shown correspond to 1/100th and 1/16th of the initial binding reactions, respectively.

SMN1 RNA fragment (fig. 2, top), with minimal binding to the corresponding *SMN2* RNA, which is consistent with the notion that the C→T transition results in the loss of an SF2/ASF–dependent ESE. Although this experimental approach does not reveal the stoichiometry or the precise location of the bound proteins along the immobilized RNA probes, it is unlikely that more than one or two molecules of SF2/ASF or hnRNP A1 can bind to a 17mer RNA, given the available information about their heptamer and hexamer recognition motifs (Burd and Dreyfuss 1994; Cartegni et al. 2003), as well as previous structural data (Ding et al. 1999; Maris et al. 2005).

Extensive Mutational Analysis of SMN1 *Exon 7*

To assess the relationship between hnRNP A1 motifs and exon skipping versus SF2/ASF motifs and exon inclusion, we designed a large panel of *SMN* minigenes with different mutations surrounding the +6 position of exon 7 (table 1). We used ESEfinder 2.0 (Cartegni et al. 2003) to calculate SF2/ASF–dependent ESE motif scores and used the consensus motif derived from SELEX (Burd and Dreyfuss 1994) to assign putative hnRNP A1–binding sites. We transfected the constructs into 293-HEK cells and measured the extent of exon 7 inclusion in the spliced transcripts. The minigene constructs (minigenes

1–30) are listed in table 1 in the same order as the 30 lanes in figure 1.

In the design of the first set of mutants, we reasoned that the gain of an element should be sequence specific, requiring particular nucleotides that match the consensus binding motif. In contrast, the loss of an element should not require highly specific sequence changes as long as the element is destroyed. We initially substituted 6 nt of the presumptive splicing elements with cytosines (minigene 3: *SMN*.cX6), thymidines (minigene 4: *SMN*.tX6), adenosines (minigene 5: *SMN*.aX6), or guanosines (minigene 6: *SMN*.gX6) (table 1). Three of these four mutations caused loss of exon 7 inclusion to levels similar to *SMN2* (c6t) (fig. 1, lanes 4–6). The *SMN*.cX6 mutation was an exception (see below), since it only reduced inclusion by ~15% (fig. 1, lane 3). Substitution with a more limited number of thymidines (minigene 7: *SMN1*.t3 and minigene 8: *SMN1*.t4) in the putative enhancer element, while maintaining the C at position +6, also led to very significant exon skipping (table 1 and fig. 1, lanes 7 and 8). These two mutants are equivalent to ones first used to identify the sequences surrounding position +6 as being important for efficient *SMN1* exon 7 inclusion (Lorson and Androphy 2000). The ability to cause exon 7 skipping in such a nonspecific manner strongly suggests that the mutations disrupt a positively acting splicing element (i.e., an ESE) required for *SMN1* exon 7 inclusion.

To assess the involvement of hnRNP A1 in exon 7 splicing, we next tested whether mutations that strengthen or weaken hnRNP A1–binding motifs correlate with the extent of exon 7 skipping. *SMN1* and *SMN2* have three and four nucleotide matches, respectively, to the optimal hnRNP A1–binding hexamer, TAGGGA/T (Burd and Dreyfuss 1994). We tested three pairs of *SMN1*/*SMN2* minigenes with double mutations at positions +9 to +11 (10g11g, 9g10g, and 9t10g) combined with C or T at position +6 (minigenes 9–14, table 1 and fig. 1). Despite having at least the same number of matches to the consensus hnRNP A1 motif as *SMN2,* all but two of the mutations resulted in increased exon 7 inclusion, showing that the introduction of an hnRNP A1–binding motif does not consistently result in exon 7 skipping. In the remaining two cases, a perfect or near-perfect match at that site (i.e., minigene 12: *SMN2*.9g10g and minigene 14: *SMN2*.9t10g) was indeed associated with exon skipping, but because these mutations also simultaneously eliminate the SF2/ASF high-score motif, the cause of exon skipping cannot be unequivocally assigned (table 1 and fig. 1, lanes 12 and 14).

The presence of UAG at the 5′ end of the hnRNP A1 consensus motif appears to be important for hnRNP A1 binding and silencing activity in other contexts (Burd and Dreyfuss 1994; Caputi et al. 1999). To further evaluate whether the presence of an hnRNP A1–binding site correlates with *SMN2* exon 7 skipping, we tested two sets of mutations (minigenes 25–28), 7c and 8c, that target the UAG/CAG motif in *SMN1* and *SMN2* and are equivalent to previously tested mutations (Kashima and Manley 2003). Both *SMN1*.7c and *SMN1*.8c maintain a high-score SF2/ASF motif and, as predicted by the ESE-loss model, gave high levels of exon 7 inclusion (fig. 1, lanes 25 and 27). *SMN2*.7c lacks both a high-score SF2/ASF motif and an hnRNP A1–binding motif but maintained a high level of exon 7 inclusion (lane 26). In *SMN2*.8c, the SF2/ASF motif was also eliminated, correlating with the ~50% exon 7 skipping despite the absence of the UAG motif (lane 28). Indeed, when a UAG motif was present without the concomitant disruption of the ESE (lanes 10, 18, and 20), exon 7 inclusion was close to 100%, pointing to a marginal role of the UAG motif in this context.

Several mutants have good matches to both SF2/ASF and hnRNP A1 motifs (table 1, minigenes 9, 10, 17, 18, and 22) and gave high levels of exon 7 inclusion (fig. 1), suggesting that the SF2/ASF high-score motif is the functional or dominant element in determining exon 7 splicing. Two of these mutants are part of a set specifically designed to test this hypothesis, by inserting a CA dinucleotide upstream of the +6 C or T in *SMN1* or *SMN2,* respectively (table 1, minigenes 15 and 16). The insertion, which leaves the preexisting SF2/ASF and hnRNP A1 putative sites unaltered, maintained the high level of exon 7 inclusion for *SMN1* and partially improved it for *SMN2* (fig. 1*A*, lanes 15 and 16). When combined with a 9g10a mutation in the context of *SMN2* (table 1, minigene 18), the CA dinucleotide insertion restores a high-score SF2/ASF motif and simultaneously strengthens the hnRNP A1–binding motif. Despite the strengthened hnRNP A1 motif, exon 7 inclusion increased to nearly 100% (fig. 1, lane 18), indicating that the presence of an hnRNP A1 motif at that position contributes minimally to the levels of exon 7 skipping, whereas an SF2/ASF motif appears to be a strong determinant of inclusion.

Eight mutations had neither SF2/ASF motifs nor hnRNP A1 motifs (table 1, minigenes 3–5, 7, 8, 23, 26, and 28). Six of these eight mutations gave significant exon 7 skipping, consistent with the lack of the ESE motif (fig. 1, lanes 4, 5, 7, 8, 23, and 28). The other two mutations, *SMN2*.7c and, to a lesser degree, *SMN*.cX6, gave levels of exon 7 inclusion comparable to *SMN1* (lanes 3 and 26). Given that exon 7 inclusion for the other mutations does not correlate with the lack of an hnRNP A1–binding site, we infer that 7c and cX6 may fortuitously introduce an unrelated protein-binding site and/or affect RNA secondary structure in a way that facilitates splicing.

We also tested an SF2/ASF–dependent ESE found in

BRCA1 exon 18, which is abrogated by a point mutation that causes skipping of the exon (Liu et al. 2001). In the context of the *SMN* minigene, the heterologous *BRCA1* ESE stimulated exon 7 inclusion above *SMN2* levels, whereas complete exon skipping was observed with the mutant version (table 1, minigenes 29 and 30, and fig. 1). Together with the reciprocal finding that the *SMN1* SF2/ASF ESE, but not the *SMN2* version, can replace the *BRCA1* ESE (Cartegni and Krainer 2002), this result shows that different SF2/ASF ESE heptamers are functionally interchangeable.

Our ESE-loss model takes into account mutations that were tested in previous studies (Lorson and Androphy 2000; Cartegni and Krainer 2002; Kashima and Manley 2003) and that were also included in the present study to confirm previous results and to comprehensively reassess the roles of SF2/ASF and hnRNP A1. For example, one set of mutants (table 1, minigenes 19–24) was originally designed to eliminate the SF2/ASF–dependent ESE by introducing changes at position +6 and, then, to restore it by means of a compensatory mutation, a11g (Cartegni and Krainer 2002). In agreement with our previous observations, exon 7 inclusion was rescued (fig. 1, lanes 19–24). Two constructs, however, gave significantly different results compared with a previous report (Kashima and Manley 2003). In that study, *SMN2*-TG and *SMN2*-8C rescued *SMN2* exon 7 inclusion to levels similar to *SMN1*. Our equivalent constructs, *SMN2*.9t10g and *SMN2*.8c, gave only 13% and 56% exon inclusion, respectively (table 1, minigenes 14 and 28, and fig. 1*A*), consistent with the reduction in the SF2/ASF motif scores (see the "Discussion" section).

It is apparent from the above results that not every mutant analyzed is consistent with the presence or absence of either an SF2/ASF or an hnRNP A1 motif. This is to be expected, because any point mutation can potentially create or destroy additional regulatory elements. However, the contribution of a particular motif to the recognition of exon 7 can be assessed by the extent of correlation between splicing and motif scores when the entire panel of mutants is analyzed. We, therefore, compiled the results from all of the mutations (table 1) into a data set presented as scatterplots (fig. 1*B*). The graphs show the relationship between exon 7 inclusion and either ESEfinder SF2/ASF motif scores (Cartegni et al. 2003) or matches to the hnRNP A1–binding site consensus (Burd and Dreyfuss 1994). The threshold for the hnRNP A1–binding motifs is set at more than three matches, because the transition from three matches in *SMN1* to four matches in *SMN2* was proposed to be the determinant of *SMN2* exon 7 skipping (Kashima and Manley 2003). To set a high threshold for ESE activity, the extent of exon 7 inclusion considered indicative of high inclusion was set at 90%. According to the ESE-loss model, the data points should be distributed in the lower left and upper right quadrants, which was indeed the case for 26 of the 30 experimental points (fig. 1*B*). Moreover, there is a strong correlation between SF2/ASF scores and exon 7 inclusion (Spearman rank correlation coefficient 0.75; 95% CI 0.53–0.88; perfect correlation = 1) but not between hnRNP A1–binding motifs and exon 7 skipping (correlation coefficient −0.14; 95% CI −0.48 to 0.25; perfect correlation = −1). Indeed, the ESS-gain model predicts that the data points should be distributed in the lower right and upper left quadrants, but, instead, the distribution appears to be random (fig. 1*B*).

One caveat of this statistical analysis is that the SF2/ASF and hnRNP A1 motifs were originally derived by different methods, and they have different extents of degeneracy. To allow an unbiased comparison of the correlation between exon 7 inclusion and the two motifs, we derived a position weight matrix for hnRNP A1 from the original SELEX data (Burd and Dreyfuss 1994), using the same methodology as originally employed for the derivation of the SF2/ASF motif (Liu et al. 1998). The new hnRNP A1 matrix and the statistical analysis of the data are presented in appendix A (online only) and gave results consistent with the match/mismatch approach. Once again, there was no correlation between hnRNP A1 motif scores and the extent of exon 7 skipping (figs. A1 and A2 [online only]). Taken together, these results strongly suggest that the presence of an SF2/ASF motif is important for exon 7 inclusion, whereas an hnRNP A1 motif is neither necessary nor sufficient to promote exon 7 skipping.

Effects of Depletion and Overexpression on SMN Splicing in Vivo

We have confirmed and extended previous work that demonstrated that simultaneous siRNA-mediated knockdown of hnRNP A1 and a closely related hnRNP A/B family member, hnRNP A2, significantly increases *SMN2* exon 7 inclusion in HeLa cells (Kashima and Manley 2003) (fig. 3*A* and 3*C*). The original interpretation of these results was that hnRNP A1/A2 inhibits splicing of *SMN2* exon 7 via an ESS introduced into *SMN2* by the C→T transition. Although this is a formal possibility, we wished to address the specificity of the hnRNP A1/A2 effect and an alternative interpretation—namely, that nonspecific binding by hnRNP A1/A2 (or specific binding elsewhere) becomes inhibitory in the absence of the SF2/ASF–dependent ESE.

If the observed hnRNP A1/A2 inhibitory activity is general, rather than specific, it should repress exon 7 inclusion irrespective of the C→T transition. We, therefore, designed a mutation that weakens exon 7 inclusion in *SMN1* without affecting the region of the SF2/ASF–dependent ESE. The mutation is a C→G transversion

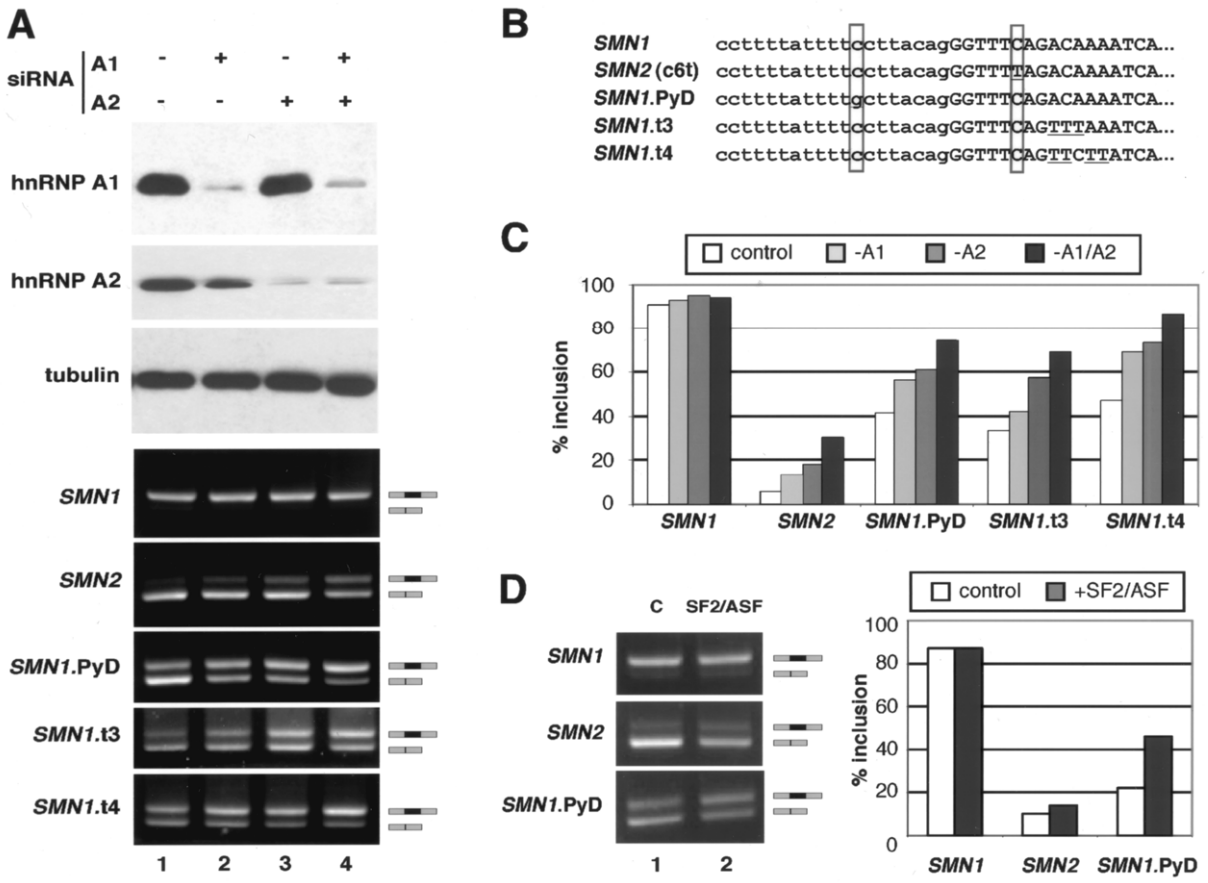


Figure 3 Analysis showing that depletion of hnRNP A1/A2 in vivo relieves exon 7 skipping, irrespective of the C→T transition. *A,* HeLa cells were treated with siRNAs against hnRNP A1, A2, or both and then were transfected with *SMN* minigene plasmids. Endogenous levels of hnRNP A1 and A2 and tubulin control were measured by western blotting 24 h after transfection (*upper images*). Radioactive RT-PCR analysis was performed, and spliced products were visualized by ethidium-bromide staining (*lower images*). *B,* Sequences of the relevant portions of intron 6 (*lowercase*) and exon 7 (*uppercase*) in the minigenes. Mutations are underlined, and the −8 and +6 positions are boxed. *C,* RT-PCR products shown in panel A were quantitated on a phosphorimager. Note that the *SMN1*.t3 and *SMN1*.t4 mutants gave higher levels of inclusion in HeLa cells than in 293-HEK cells (compare with fig. 1*A* and table 1). *D,* Overexpression of SF2/ASF promotes exon 7 inclusion in *SMN1*.PyD. MEF cells were cotransfected with an SF2/ASF expression plasmid or vector alone (C, lane 1) and the indicated *SMN* minigenes. RT-PCR analysis and quantitation were performed as in panels A and C.

within the polypyrimidine tract of intron 6 (*SMN1*.PyD) (fig. 3*B*). This mutation weakens the 3′ splice site and decreases exon 7 inclusion by >50% (fig. 3*A*). If hnRNP A1–mediated repression is indeed dependent on the T at position +6, there should be no effect of hnRNP A1/A2 depletion on splicing of this minigene, because the C was retained and no ESS was introduced. However, hnRNP A1/A2 depletion resulted in an approximately twofold increase in exon 7 splicing with this mutant (*SMN1*.PyD) (fig. 3*A*, lanes 1–4, and fig. 3*C*).

We next analyzed two additional mutations, *SMN1*.t3 and *SMN1*.t4, which have a C at position +6 of exon 7 but have neither SF2/ASF nor hnRNP A1 motifs (table 1). As predicted by the ESE-loss model, exon 7 inclusion levels were low with both mutants. Upon hnRNP A1 and A2 depletion, however, inclusion increased approximately twofold (fig. 3*A* and 3*C*), supporting the conclusion that the hnRNP A/B–mediated inhibition of exon 7 inclusion is an indirect effect, independent of the C→T transition in *SMN2* and consistent with the general inhibitory properties of hnRNP A/B proteins (Mayeda et al. 1993; Cartegni et al. 2002; Black 2003).

The *SMN1*.PyD mutant splices at suboptimal levels but, at the same time, maintains an intact, responsive ESE and, therefore, can be used to test whether SF2/ASF can promote exon 7 inclusion in cells. We overexpressed SF2/ASF in MEF cells, which have low basal levels of endogenous SF2/ASF, compared with transformed cell lines. As expected, there was no significant improvement in splicing with the *SMN1* and *SMN2* minigenes, since *SMN1* exon 7 inclusion is already very efficient and *SMN2* lacks the SF2/ASF motif (fig. 3*D*). In contrast,

the level of exon 7 inclusion from the *SMN1*.PyD mutant increased about twofold, indicating that the ESE is responsive to varying levels of SF2/ASF in vivo.

If hnRNP A1 specifically inhibits exon 7 inclusion in *SMN2,* then hnRNP A1 overexpression would be expected to inhibit inclusion even further, whereas *SMN1* should not be affected. In contrast, if hnRNP A1–mediated inhibition is a global and/or nonspecific effect, its overexpression should also cause an increase in *SMN1* exon 7 skipping. To determine which prediction is correct, we transiently overexpressed hnRNP A1 in 293-HEK cells and analyzed the splicing of RNA transcripts from the endogenous *SMN1* and *SMN2* genes. The endogenous *SMN2* gene can be distinguished from *SMN1* by a G→A transition in the 3′ UTR in exon 8 that creates a *Dde*I site (Gennarelli et al. 1995; Parsons et al. 1996). Analysis of *Dde*I-digested RT-PCR reactions showed that the *SMN1* cDNAs from control cells correspond to essentially 100% inclusion of exon 7 (fig. 4, lane 1). In contrast, overexpression of hnRNP A1 resulted in the appearance of *SMN1* transcripts lacking exon 7 (lane 2), indicating that hnRNP A1 can inhibit endogenous *SMN1* exon 7 splicing. An increase in exon 7 skipping was also observed for the *SMN2* transcripts.

Inhibition of SMN1 *and* SMN2 *Exon 7 Splicing by hnRNP A1 in Vitro*

The role of hnRNP A1 in *SMN1* and *SMN2* exon 7 splicing can be tested directly with the use of an in vitro splicing assay (Cartegni and Krainer 2002). *SMN1* exon 7 inclusion is less efficient in vitro than in vivo, facilitating the detection of changes that lead to increased exon inclusion. Indeed, depletion of hnRNP A/B proteins from nuclear extracts (fig. 5*A*) resulted in a significant increase in *SMN1* exon 7 inclusion, relative to mock-depleted extracts (fig. 5*B,* lanes 1 and 4). This effect was even greater than that on *SMN2* (fig. 5*B,* lanes 7 and 10). The addition of recombinant hnRNP A1 resulted in dose-dependent inhibition of both *SMN1* and *SMN2* exon 7 inclusion (fig. 5*B,* lanes 4–6 and 10–12). Because *SMN1* exon 7 inclusion was also inhibited, these results further demonstrate that the hnRNP A1–mediated effect is independent of the C→T transition in *SMN2*.

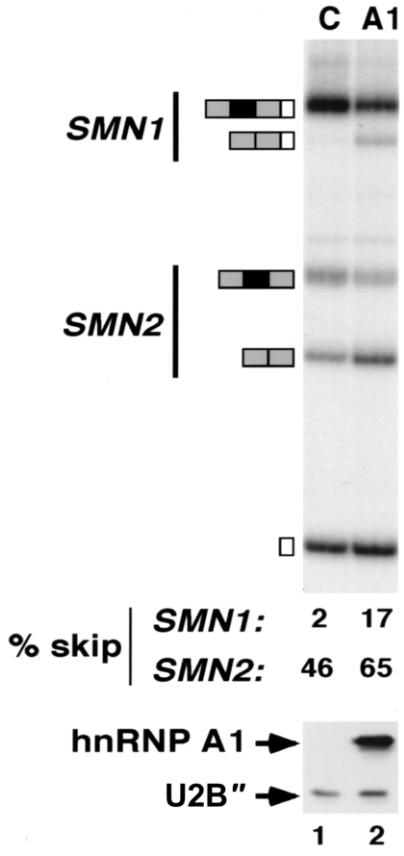


Figure 4 Analysis showing that overexpression of hnRNP A1 in vivo inhibits *SMN1* and *SMN2* exon 7 inclusion. *Top,* Radioactive RT-PCR analysis was performed with RNA from control mock-transfected 293-HEK cells (C, lane 1) or cells expressing T7-tagged hnRNP A1 (lane 2). To distinguish between *SMN1* and *SMN2* endogenous mRNAs, the cDNAs were digested with *Dde*I to specifically cleave *SMN2* in exon 8. Products were separated on a 6% native polyacrylamide gel. The white box represents the 3′ end of exon 8 that was cleaved from *SMN2* spliced products. The proportion of *SMN1* and *SMN2* transcripts lacking exon 7 (% skip) is shown below the autoradiogram. *Bottom,* Western blot of lysates from mock-transfected cells or cells expressing T7-tagged hnRNP A1. Blots were probed with anti-T7 antibody and an antibody against U2B″ as a control.

Discussion

The *SMN2* gene can potentially express sufficient functional SMN protein to fully compensate for the loss of *SMN1* in all patients with SMA, which makes it an attractive target for drug therapy. The development of therapeutic approaches would be greatly facilitated by precise knowledge of the mechanism by which *SMN2* exon 7 fails to be efficiently incorporated into mature transcripts. The identification of the *cis*-elements and *trans*-acting factors involved is essential for the optimization of current approaches and/or for the identification of new drug targets. Two different models, which are not necessarily incompatible, were previously proposed to explain the different splicing patterns of *SMN1* and *SMN2*: loss of an SF2/ASF–dependent enhancer in *SMN1* (Cartegni and Krainer 2002) or gain of an hnRNP A1–dependent silencer in *SMN2* (Kashima and Manley 2003). We have now systematically assessed the roles of hnRNP A1 and SF2/ASF in *SMN1/2* exon 7 splicing and conclude that the primary determinant of the extent of exon 7 inclusion is the SF2/ASF–dependent ESE. Interestingly, hnRNP A1 can antagonize SF2/ASF

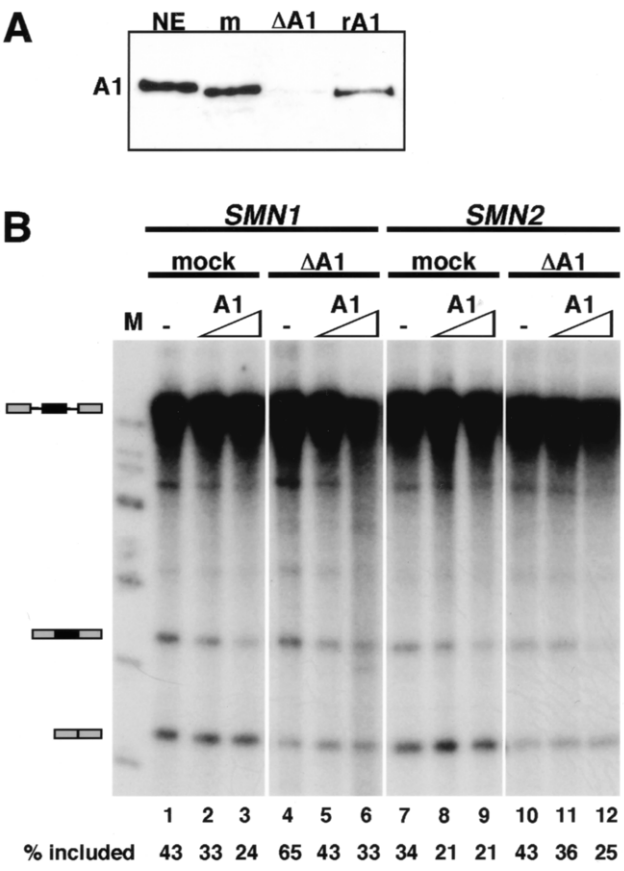


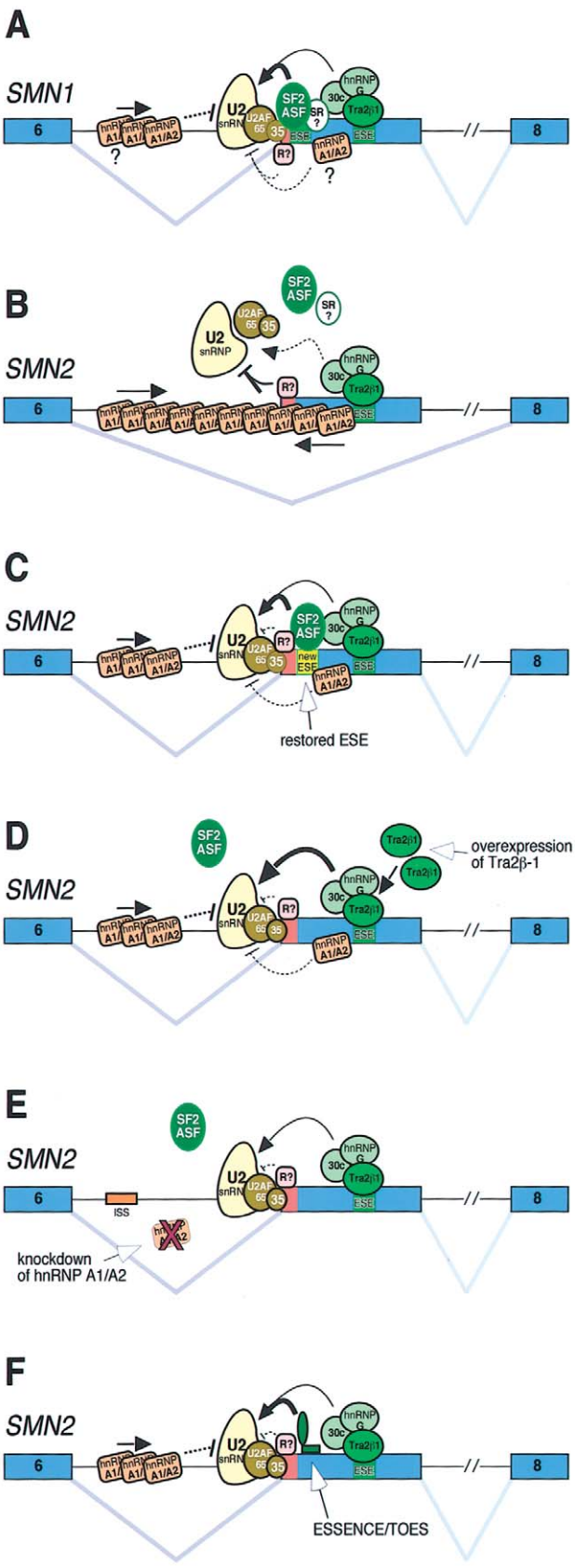
Figure 5 Analysis showing that hnRNP A1 inhibits *SMN1* and *SMN2* exon 7 splicing in vitro. *A,* Western blot of hnRNP A1 in 1 μl of HeLa nuclear extract (NE), mock-depleted extract (m), extract depleted of hnRNP A/B proteins (ΔA1), and 50 ng of recombinant hnRNP A1 (rA1). *B,* In vitro splicing of *SMN1* and *SMN2* transcripts in 3 μl of mock- or hnRNP A/B–depleted extract supplemented with 0 (−), 170, or 340 ng of recombinant hnRNP A1. Marker lane is indicated (M).

and, indeed, promote exon 7 skipping. However, this activity of hnRNP A1 is not specific to *SMN2* but rather reflects the general inhibitory effect of hnRNP A1 on many alternatively spliced exons (reviewed in Cartegni et al. 2002), illustrating the antagonism between these two splicing effectors in a physiological context.

We have confirmed the loss of a putative ESE motif in *SMN2* predicted by ESEfinder (Cartegni et al. 2003), using a very different ESE-prediction program, RESCUE-ESE (Fairbrother et al. 2004). However, the presence of a bona fide hnRNP A1 ESS motif in *SMN2* remains uncertain. Two methods to identify ESS elements (Wang et al. 2004; Zhang and Chasin 2004) do not predict the creation of a silencer by the c6t transition (data not shown). Kashima and Manley (2003) compared the sequence of *SMN2* with known or putative hnRNP A1–dependent ESSs from four genes. They noted that the sequence of *SMN2* exon 7 at positions 6–11 is identical to *CD44* (Entrez Gene accession number 960) exon v5 at positions 4–9 and that both have some resemblance to the hnRNP A1 SELEX motif (Burd and Dreyfuss 1994). In *CD44*, several linker-scan mutants throughout the entire exon v5, including one that overlaps the UAGACA sequence, result in higher levels of exon v5 inclusion (König et al. 1998). Later, it was shown that the activity of a dispersed silencer within exon v5 is controlled by hnRNP A1, but this ESS appears to be a composite of multiple elements located in subdomains throughout the exon, and no specific high-affinity binding site could be identified (Matter et al. 2000). Therefore, we cannot infer that positions 4–9 in *CD44* exon v5 and, by analogy, positions 6–11 in *SMN2* define an hnRNP A1–binding site, especially considering that this particular sequence fails to inhibit splicing when analyzed in a heterologous context (Kashima and Manley 2003). Similar to the situation in *CD44* exon v5, we found that hnRNP A1 plays a role in *SMN2* exon 7 skipping but does so in a general manner, perhaps dependent on other exon features or on the surrounding intronic regions rather than on the proposed hnRNP A1 motif.

In contrast to the general inhibitory effect of hnRNP A1 on *CD44* and *SMN2* splicing, bona fide examples of hnRNP A1–specific ESS elements have been identified (Cartegni et al. 2002). One such element, ESS3, is found in HIV-1 *tat* exon 3 (Amendt et al. 1995; Staffa and Cochrane 1995; Si et al. 1998; Tange et al. 2001; Zhu et al. 2001; Damgaard et al. 2002; Marchand et al. 2002). ESS3 comprises the sequence UAGUGA, which matches the high-affinity hnRNP A1–binding hexamer at five positions. This motif is required for direct interaction with hnRNP A1 (Zhu et al. 2001) and for hnRNP A1–mediated inhibition of splicing in vivo (Staffa and Cochrane 1995) and in vitro (Si et al. 1998; Zhu et al. 2001; Damgaard et al. 2002). One of the *SMN2* exon 7 mutants tested by Kashima and Manley (2003), *SMN2*-TG, introduces a sequence identical to the *tat* ESS3 motif. Despite the fact that this mutation creates a nearly perfect match to an hnRNP A1–binding site that is a proven hnRNP A1–dependent ESS in its original context, those authors reported that this mutation rescues exon 7 inclusion. When we analyzed an equivalent mutant, *SMN2*.9t10g (fig. 1), we found that this mutation resulted in exon 7 skipping, a finding consistent with the introduction of a genuine hnRNP A1–mediated silencer element in this case and also with the concomitant disruption of the SF2/ASF motif.

The most compelling previous evidence supporting a direct role for hnRNP A1 in *SMN2* exon 7 skipping was the activation of exon 7 inclusion upon transient knockdown of hnRNP A1/A2 proteins by siRNAs (Kashima and Manley 2003). We confirmed this result, but when



we included controls for specificity, using three different mutants (*SMN1*.PyD, *SMN*.t3, and *SMN*.t4) that result in exon 7 skipping without introducing an hnRNP A1 motif, we found that knocking down hnRNP A1/A2 led to a comparable increase in exon 7 inclusion in all cases (fig. 3). We conclude that repression of exon 7 inclusion by hnRNP A1/A2 is a generalized effect, independent of the C→T transition. Thus, most of the results in the Kashima and Manley (2003) report can be explained by the general property of hnRNP A1 to promote skipping of alternative exons (Mayeda et al. 1993), rather than by an *SMN2*-specific effect.

On the basis of our present results and of previous work, we propose a new model for *SMN1* and *SMN2* exon 7 splicing, which is depicted in figure 6. According to this model, hnRNP A1 acts in a general manner to block splicing of exon 7 in the absence of the dominant SF2/ASF–dependent ESE (fig. 6*B*). Consistent with the known RNA-binding and repressor properties of hnRNP A1 (Zhu et al. 2001; reviewed by Cartegni et al. [2002]), its binding to *SMN1* and *SMN2* RNAs may nucleate from as-yet-uncharacterized high-affinity sites at some distance from the ESE. The presence of the SF2/ASF–dependent ESE in *SMN1* supersedes this inhibition (fig. 6*A*). The same is true when exon 7 definition in the *SMN2* context is strengthened by various means, such as the reconstitution of a new ESE at a similar position (fig. 6*C*), the overexpression of proteins that bind to other enhancer elements in exon 7 (fig. 6*D*), the targeting of synthetic molecules that mimic SR protein functions—for example, ESSENCE (Cartegni and Krainer 2003) and TOES (Skordis et al. 2003) (fig. 6*F*)—or the reduction

Figure 6 Models for the effects of the *SMN2* C→T transition on exon 7 splicing. Multiple positive and negative determinants influence *SMN1* and *SMN2* splicing. In *SMN1* (*A*), efficient splicing is driven by the interactions of SF2/ASF with the ESE in the +6c region and of other factors with a separate enhancer downstream. There may also be an interplay between the various elements and additional, perhaps redundant, factors ("SR?") might be involved. In this strong exon definition context, a possible hnRNP A1/A2 role and/or the action of a putative repressor ("R?"), are not sufficient to inhibit splicing. The loss of the SF2/ASF–dependent ESE in *SMN2* (*B*) allows a secondary, non–*SMN2*-specific inhibitory action of hnRNP A1/A2, possibly in collaboration with "R?". Positive interactions downstream cannot counteract this inhibitory activity. Exon 7 inclusion in *SMN2* can be rescued to *SMN1*-like levels by other means (*white arrows*), such as reconstitution of the SF2/ASF–dependent ESE (or other ESEs) (*C*), overexpression of some splicing activators (e.g., Tra2β1) (*D*), down-regulation of hnRNP A1/A2 by RNAi (*E*), or the presence of compounds that functionally replace SR proteins or ESEs (e.g., ESSENCE or TOES) (*F*). Fluctuations in different cells and tissues in the relative levels of SF2/ASF, hnRNP A1/A2, and/or the additional splicing factors involved would therefore determine the precise pattern of *SMN2* exon 7 splicing. Green boxes represent known ESEs. Red and orange boxes represent putative ESS and ISS elements, respectively.

in the levels of inhibitory factors by siRNA treatment (fig. 6*E*).

Other splicing factors, such as Tra2β1, SRp30c, and hnRNP G (UniProt accession numbers P62995-1, Q13242, and P38159, respectively), have also been shown to influence *SMN1* and *SMN2* exon 7 inclusion via elements in the central portion of exon 7 (Hofmann et al. 2000; Hofmann and Wirth 2002; Young et al. 2002), and additional regulatory elements, both in exon 7 (Singh et al. 2004*a*, 2004*b*) and in the flanking introns (Miyajima et al. 2002; Miyaso et al. 2003), appear to be important for exon 7 recognition (fig. 6). Because *SMN1* and *SMN2* exon 7 sequences are identical, with the exception of the C→T transition at position +6, regulatory sequences that do not encompass this position are unlikely to be directly involved in the *SMN2* exon 7 splicing defect. However, they clearly play an important role in exon 7 splicing efficiency and may influence the overall effect of the C→T transition.

It is possible that another repressor ("R?," fig. 6) different from hnRNP A1/A2 recognizes *SMN2* sequences with some degree of specificity. In support of this notion, a recent study showed that, in the absence of the ESE, several mutations in the first 5 nt (GGTTT) of *SMN2* exon 7 partially rescue splicing (Singh et al. 2004*a*), and this motif is also present in 11 of 141 recently described ESS decamer elements (Wang et al. 2004). However, this putative ESS at the beginning of the exon is not sufficient to overcome the effect of the SF2/ASF ESE (fig. 1 and table 1). Consistent with this finding, our reanalysis of the extensive mutational and iterative selection data of Singh and colleagues (2004*a*, 2004*b*) shows a good correlation between exon 7 inclusion and the presence of an SF2/ASF ESE motif (fig. A3 [online only]). In particular, in the functional SELEX data obtained by randomization of the first 6 nt of exon 7 (Singh et al. 2004*a*), SF2/ASF–dependent ESE motifs are present in 33 of the 42 selected clones (fig. A3 [online only]). Likewise, partial randomization of the entire exon (Singh et al. 2004*b*) resulted in high-score SF2/ASF motifs at the original position (6–12) in 25 of the 59 selected exons and in 40 of the 59 exons when partial overlaps with the original position were considered (data not shown). Because only ~4% of all possible 16,384 heptamers represent high-score SF2/ASF motifs (Wang et al. 2005), it is clear that these motifs are greatly enriched among sequences selected for their ability to promote exon 7 inclusion, thus strongly reinforcing the notion that the SF2/ASF–dependent ESE is the primary splicing determinant in this region and that the activity from any putative inhibitory sites is secondary to the loss of the ESE.

Previous negative results from in vivo SF2/ASF–depletion experiments suggested that SF2/ASF is not strictly required for exon 7 inclusion in *SMN1*. The depletion of SF2/ASF from cells, either genetically in the chicken cell line DT-40 (Kashima and Manley 2003) or by use of siRNA-mediated knockdown in HeLa cells (L. Cartegni, M. L. Hastings, J. A. Calarco, E. de Stanchino, and A. R. Krainer, unpublished data), has little effect on *SMN1* splicing. Although a decrease in *SMN1* exon 7 inclusion in the absence of SF2/ASF is a logical expectation, there are several possible alternative explanations for the lack of such an effect. First, SF2/ASF, which is a general splicing factor, may be important for both exon 8 and exon 7 splicing. There are six high-score SF2/ASF motifs in exon 8 (data not shown) and, if any of them represent genuine ESEs, the depletion of SF2/ASF may equalize the competition between the 3′ splice sites preceding these two exons, thereby resulting in no effect (or even in a positive effect) on overall exon 7 inclusion. Indeed, blocking the 3′ splice site of exon 8 with antisense oligonucleotides increases the extent of exon 7 inclusion (Lim and Hertel 2001). Second, because the depletion of SF2/ASF compromises DT-40 cell viability (Wang et al. 1996), this approach may not provide a direct indication of normal SF2/ASF activity, since secondary effects associated with cellular stress and apoptosis are likely in play. Third, SR proteins are well known to have partially redundant functions (reviewed by Cartegni et al. [2002]). As previously suggested (Cartegni and Krainer 2002), additional SR protein(s) may recognize the ESE in the absence of SF2/ASF and substitute for it in ESE-dependent exon 7 splicing, so that a double depletion might be required to observe an effect. By analogy, in the case of hnRNP A1, the simultaneous depletion of the related protein hnRNP A2 is required to observe a strong effect on *SMN2* exon 7 inclusion (fig. 3*a*) (Kashima and Manley 2003). Fourth, compensatory mechanisms might also be triggered or enhanced through auto- and cross-regulatory loops involving different splicing factors and their respective alternative splicing isoforms; many examples of such regulatory loops have been described elsewhere (reviewed by Bourgeois et al. [2004]). Thus, a reduction in the levels of SF2/ASF could directly or indirectly lead to changes in the levels of other splicing factors, which would, in turn, maintain efficient *SMN1* exon 7 inclusion.

Any of these scenarios, or a combination of them, could account for the absence of an apparent effect of SF2/ASF depletion on *SMN2* splicing. On the other hand, there is substantial evidence supporting a direct role for SF2/ASF in *SMN1* exon 7 splicing. Mutational analysis shows a strong correlation between exon 7 inclusion and the presence of an ESE-dependent SF2/ASF motif (fig. 1), which also enhances splicing in a heterologous context (Cartegni and Krainer 2002). SF2/ASF preferentially interacts with *SMN1* compared with

SMN2 transcripts (fig. 2) and makes direct contact with the ESE site (Cartegni and Krainer 2002). SF2/ASF overexpression can promote *SMN1* exon 7 inclusion in a weakened context that preserves the ESE (fig. 3*D*), and SF2/ASF, but not another SR protein, SC35, specifically activates *SMN1* exon 7 splicing in an in vitro S100-complementation assay (Cartegni and Krainer 2002). As is the case for many genes (reviewed by Cartegni et al. [2002] and Black [2003]), SF2/ASF–dependent splicing is counteracted by hnRNPA1/A2 in an antagonistic relationship that is likely physiologically relevant for the modulation of *SMN* splicing as well.

Our results from overexpression of hnRNP A1 (fig. 4) indicate that changes in the cellular levels of hnRNP A1 can modulate the extent of exon 7 inclusion in both *SMN1* and *SMN2*. Likewise, SF2/ASF overexpression experiments (fig. 3*D*) showed that exon 7 inclusion is sensitive to the cellular levels of SF2/ASF. These results suggest that exon 7 inclusion is determined in part by the cellular levels of these two proteins, which have opposite effects on splicing. The balance between SF2/ASF and hnRNP A1 activity in exon 7 inclusion appears to hinge upon the recognition of the ESE in exon 7 by SF2/ASF, as inactivation of this ESE in *SMN2* results in the substantial loss of exon 7 inclusion. This hypothesis predicts that, in the absence of hnRNP A1, the ESE should become dispensable for the splicing of exon 7, which is, indeed, the case (figs. 3 and 5). Because we find no evidence that hnRNP A1 is acting via a specific binding site created in *SMN2*, we presume that hnRNP A1 inhibition of exon 7 splicing is mediated by as-yet-undetermined splicing silencer sequences. Putative high-affinity binding sites in and around exon 7, calculated with a newly derived hnRNP A1 matrix, are shown in appendix A (figs. A1 and A5 [online only]).

The reason why *SMN1* gene disruption causes the selective degeneration of spinal cord motor neurons without affecting other tissues is not known. A threshold level of SMN protein is essential for cell viability (reviewed by Frugier et al. [2002]) and, clearly, the low level of full-length transcripts produced from *SMN2* is sufficient for the survival of most cell types in patients with SMA and mouse models. It is possible that motor neurons have a greater need for the SMN protein or that SMN performs additional, neuron-specific functions (reviewed by Sendtner [2001] and Gubitz et al. [2004]). Alternatively, *SMN2* may produce less functional SMN protein in spinal motor neurons compared with other cells, which would only be detrimental in affected SMA individuals (Coovert et al. 1997). One intriguing possibility is that α-motor neurons express high levels of hnRNP A/B proteins and/or low levels of SF2/ASF or other relevant splicing factors, resulting in increased skipping of *SMN2* exon 7. Although there is no direct evidence that this is, in fact, the case for motor neurons and SMA, the relative levels of hnRNP A1/A2, SF2/ASF, and other SR proteins do vary with cell type and developmental stage (Hanamura et al. 1998; reviewed by Dreyfuss et al. [2002]), and our results demonstrate that SF2/ASF and hnRNP A1 levels can determine the extent of exon 7 splicing.

Any treatment that improves the ratio of *SMN* full-length to SMNΔ7 from *SMN2* might have therapeutic value, but targeting splicing factors could be problematic. For example, hnRNP A1 could be viewed as a possible therapeutic target, given its inhibitory effect on *SMN2* exon 7 splicing (Kashima and Manley 2003). However, in light of its properties as a global splicing inhibitor and its involvement in many other cellular processes (reviewed by Dreyfuss et al. [2002]), targeting hnRNP A1 could lead to undesirable effects. Likewise, most SR proteins probably regulate the alternative splicing of numerous genes, and it may be difficult to identify small molecules that modulate their effects with a high degree of specificity and/or without associated toxicity. Nevertheless, drugs with even limited specificity could provide a favorable risk/benefit ratio, especially considering the severity of SMA and some of the other disorders associated with defective splicing. The recent identification of small-molecule inhibitors specific for individual SR proteins provided the first indication that this family of proteins can be targeted pharmacologically (Soret et al. 2005). Gene-specific strategies that rely on antisense base pairing to increase *SMN2* exon 7 inclusion provide an intrinsically higher degree of specificity, although they face their own unique obstacles.

Mutations in *cis*-elements that result in splicing alterations are commonly observed (reviewed by Cáceres and Kornblihtt [2002], Faustino and Cooper [2003], and Pagani and Baralle [2004]). However, how such mutations exert their effects is not always obvious. Because *cis*-acting mutations that affect splicing may result in gain of function (e.g., creation of an ESE or ESS element), loss of function (e.g., destruction of an ESE or ESS element), or even a combination of both, in addition to potential alterations in RNA secondary structure (reviewed by Buratti and Baralle [2004]), a wide variety of approaches should be employed, whenever possible, to elucidate the underlying mechanisms.

Our findings demonstrate the complexity of the relationship between positive and negative splicing elements and the factors that recognize them, and they underscore the importance of exon identity determinants for both constitutive and alternative splicing. The results also illustrate the need for a thorough understanding of splicing mechanisms, to facilitate accurate phenotypic risk assessment, and for the development of effective therapeutic treatments for human genetic diseases.

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Web Resources

Accession numbers and URLs for data presented herein are as follows:

Entrez Gene, http://www.ncbi.nlm.nih.gov/entrez/query.fcgi?db=gene/ (for *SMN1, SMN2,* and *CD44*)
ESEfinder 2.0, http://rulai.cshl.edu/tools/ESE/ (for identifying putative SR protein–dependent ESEs and calculating motif scores)
Online Mendelian Inheritance in Man (OMIM), http://www.ncbi.nlm.nih.gov/Omim/ (for SMA types I, II, and III)
Pictogram, http://genes.mit.edu/pictogram.html/ (for pictogram representations of position weight matrices)
RESCUE-ESE, http://genes.mit.edu/burgelab/rescue-ese/ (for identifying candidate ESEs)
UniProt, http://pir.uniprot.org/index.shtml/ (for SMN, SMNΔ7, hnRNP A1, hnRNP A2, SF2/ASF, Tra2β1, SRp30c, and hnRNP G)

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