Modulation of SMN pre-mRNA splicing by inhibition of alternative 3’ splice site pairing

Sharlene R. Lim and Klemens J. Hertel*

Department of Microbiology and Molecular Genetics,
College of Medicine,
University of California, Irvine,
Irvine, CA 92697-4025,
USA

*Corresponding author:
Department of Microbiology and Molecular Genetics,
College of Medicine,
University of California, Irvine,
Irvine, CA 92697-4025,
USA

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Summary

Spinal muscular atrophy is caused by the loss of functional survival motor neuron (SMN1) alleles. A translationally silent nucleotide transition in the duplicated copy of the gene (SMN2) leads to exon 7 skipping and expression of a non-functional gene product. It has been suggested that differential SMN2 splicing is caused by the disruption of an exonic splicing enhancer. Here we show that the single nucleotide difference reduces the intrinsic strength of the 3’ splice site of exon 7 two-fold while the strength of the 5’ splice site of the exon 7 is not affected. Thus, a decrease in splice site strength is magnified in the context of competing exons. These data suggest that lower levels of exon 7 definition not only reduce intron 6 removal but more importantly, increase the efficiency of the competing exon 7 skipping pathway. Antisense oligonucleotides were tested to modulate exon 7 inclusion which contains the authentic translation stop codon. Oligonucleotides directed towards the 3’ splice site of exon 8 were shown to alter SMN2 splicing in favor of exon 7 inclusion. These results suggest that antisense oligonucleotides could be used as a therapeutic strategy to counteract the progression of SMA.
Introduction

Proximal spinal muscular atrophy (SMA)\(^1\) is a common human genetic disease that is the leading cause of hereditary infant mortality (1-3). It is characterized by the progressive degeneration of the anterior horn stem cells of the spinal cord with consequent paralysis of the trunk and limbs. Three clinical groups of the disease (I - III) have been described based on the decreasing severity of the symptoms (4). SMA has been linked to deletions or mutations of the Survival of Motor Neuron (SMN) gene, which has been mapped as an inverted repeat to chromosome 5 at 5q13 (4). Homozygous absence of the telomeric copy (SMN1) correlates with development of SMA (5,6). By contrast, alterations within the centromeric SMN gene (SMN2) do not produce any known phenotype (4). It has been demonstrated that SMN levels produced from the SMN2 locus modify the severity of the disease in a dose-dependent manner (7,8). However, SMN2 alone cannot provide protection from SMA (9-11).

The majority of the SMN transcript consists of nine exons encoding for a 294 aa (38 kDa) gene product. The ubiquitously expressed protein is detected at especially high levels in neuronal cells (7,8). Until recently, it was unclear why only mutations in the telomeric SMN1 mutations result in SMA. A genomic sequence comparison of the two genes revealed that SMN1 and SMN2 encode for the identical protein (12). However, three alternatively spliced transcripts generated with different efficiencies have been described for each locus (4). SMN1 primarily produces the full-length form of SMN, whereas differential splicing of the SMN2 pre-mRNA predominantly produces an isoform lacking exon 7 (SMN\(\Delta7\)). Comparison of SMN transcripts

\(^1\) The abbreviations used are: SMA, Spinal Muscular Atrophy; SMN, Survival of Motor Neuron; SMN1, telomeric copy of SMN; SMN2, centromeric copy of SMN; ESE, exonic splicing enhancer; py, polypyrimidine tract; 3'ss, 3' splice site; 5'ss, 5' splice site; HPRT, hypoxanthine phosphoribosyltransferase; RT-PCR, reverse transcription-polymerase chain reaction.
revealed a direct relationship between SMA and exon 7 skipping. Furthermore, it was demonstrated that SMN\(\Delta 7\) is unable to efficiently self-associate, a property that correlates with disease severity (13). Thus, SMN\(\Delta 7\) is not functionally equivalent to full length SMN and the failure of SMN2 to fully compensate for mutations in SMN1 is due to a differential pre-mRNA splicing event.

The only critical nucleotide change between SMN1 and SMN2 affecting the inclusion of exon 7 has been pinpointed to a single C to T base difference located six nucleotides inside exon 7 (12,14) (Fig. 1). This transition may disrupt a putative exon splicing enhancer (ESE) (14), a cis-acting element that generally serves as binding sites for members of the serine-arginine (SR)-rich family of splicing factors (15). An assembled splicing enhancer complex in turn promotes splice site and exon recognition by assisting in the recruitment of the splicing machinery to the adjacent intron through protein-protein interactions (16,17). Previous work demonstrated that the recognition of weak splice site signals could be aided significantly by the presence of ESEs. By contrast, ESEs have little or no detectable effect on the activation of strong splice sites that conform to the consensus sequence (15-18). Most ESEs studied in mechanistic detail activate 3 splice sites that contain sub-optimal polypyrimidine tracts (py), a stretch of nucleotides immediately upstream of the exon/intron junction. Because py tracts and the 3 splice site serve as binding sites for the U2 snRNP auxiliary factor (U2AF) (19-23), a model has emerged suggesting that enhancer complexes assembled on ESEs recruit U2AF to a weak 3 splice site (24-26). In the case of SMN2, it was suggested that the C to T nucleotide difference in exon 7 might disrupt the integrity of a putative ESE to a degree that renders spliceosomal assembly and exon 7 recognition insignificant (12,14). As a consequence, exon 7 is skipped in SMN2 and SMN\(\Delta 7\) is the product of the alternative processing event. A recent
report has tested this hypothesis and demonstrated the presence of an ESE within exon 7 (27). However, the critical C to T transition observed between SMN1 and SMN2 was not contained within this region. In fact, within the nucleotide sequences around the C to T transition, no detectable ESE activities were observed. Thus, it is still unclear why the C to T transition leads to exon 7 skipping in SMN2.

Because SMN2 cannot compensate for mutations within SMN1, SMN1 is the SMA determining gene. More importantly, however, because all individuals with SMA have retained their SMN2 allele, therapy directed towards increasing SMN2 exon 7 inclusion could provide a promising tool to lower the clinical severity of SMA. Recent studies have demonstrated that overexpression of hTra2, a splicing factor that was shown to interact with exon 7 of SMN, was able to tilt the balance of exon 7 inclusion in the SMN2 splicing pathway such that the majority of SMN2 mRNAs were full length (28).

In order to gain insights into the mechanism that leads to SMN2 exon 7 skipping, we have evaluated and compared the relative strength of exon 7 splice sites. Here we provide evidence that the C to T transition within exon 7 of SMN2 decreases the intrinsic strength of the upstream 3’ splice site (3’ss) by two-fold. By contrast, the 5’ splice site (5’ss) of exon 7 is not affected by the nucleotide transition. We conclude that the competition between the 3’ss of exons 7 and 8 to pair with the 5’ss of exon 6 amplifies the relatively minor differences in exon 7 splice site strength. These observations suggested that modulation of the 3’ss competition could provide the means to facilitate the inclusion of exon 7 which contains the authentic translation stop codon into the SMN2 splicing pathway. We find that the application of antisense oligonucleotides that reduce the recognition of the 3’ splice site of exon 8 increase the incorporation of exon 7 into the fully processed transcript.
**Materials and Methods:**

**Plasmids**

SMN1 and SMN2 mini-gene constructs containing exon 6, intron 6, exon 7, intron 7, and exon 8 in a pCI mammalian expression vector background were a gift from Elliot Androphy. **SMNΔ8** constructs were generated by deleting a NotI and XcmI fragment from the SMN mini-gene. **SMNΔ6** was generated through the deletion of a PstI and NheI fragment of the SMN mini-gene. **hetSMN** constructs were generated through XhoI and BclI digestion of the SMN mini-gene. The deleted region contained exon 6 and most of intron 6. However, in contrast to the **SMNΔ6** constructs, the **hetSMN** constructs retain a pseudointron inherent to the pCI backbone. The pseudointron is located upstream of the SMN sequences and contains a 5’ splice site derived from exon 1 of β-globin and a 3’ splice site derived from IgM.

**Transfections and RT-PCR analysis**

Human cervical carcinoma (C33a) cells were grown in minimal essential media (MEM) (Bio-Whittaker) supplemented with 2 mM glutamine and 10% fetal bovine serum (FBS). Transfections were performed using the lipofectamine2000 transfection kit (Gibco-BRL). 1 µg of DNA was used to transfect approximately 3x10^5 cells plated 24 h prior to transfection. At 24 h post-transfection, cells were harvested and total RNA was isolated using TRIzol reagent (Gibco-BRL). Total RNA was reverse transcribed (RT) with Moloney murine leukemia virus reverse transcriptase for 1 hour at 37°C using poly-dT priming. Radioactive PCR analysis was then performed with different primer sets to identify spliced products. PCR was done for 20 cycles in the presence of 0.2 mM dNTP supplemented with trace amounts of [³²P]α-dATP.
Each cycle consisted of a 45 sec denaturation step at 94 °C, a 45 sec annealing step at 56 °C, and a 90 sec extension step at 72 °C. For analysis of transfections with SMNΔ8, primers used selectively amplified both unspliced and spliced products. Primers used to amplify unspliced products were pCI-for (5-GCTAACGCAGTCAGTGCTTC-3) and pCI-rev (5-GTATCTTATCATGTCTGCTCG-3). To amplify spliced products, primers used were Tin6-for (5-TAATACGACTCATAAGCCATATAAAGCTATCTATATATAGC-3) and pCI-rev.

For analysis of transfections with SMNΔ6 and hetSMN, pCI-for and pCI-rev primers were used. As a normalization control, the hypoxanthine phosphoribosyltransferase (HPRT) gene was simultaneously amplified by using the primers HPRT-Fw 5’AAG GAG ATG GGA GGC CAT and HPTR-Rev 5’GTT GAG AGA TCA TCT CCA CCA AT. Reaction products were boiled and fractionated on a 6% polyacrylamide gel. The resulting bands were visualized and quantitated using a PhosphorImager. The splicing efficiency was calculated after normalization to the internal control HPTR by computing the fraction of spliced product counts over the sum of spliced and unspliced counts. Transfection experiments were repeated at least three times.

Antisense oligonucleotide application

Stable cell lines expressing either the wt SMN1 or SMN2 mini-gene were generated by G418 selection. Briefly, C33a cells were co-transfected with the plasmid of interest and pSV_neo, a neo selection plasmid (a gift from Dr. Marian Waterman), using the lipofectamine2000 reagent (Gibco-BRL). After 12 hrs, G418 was added to the medium to a final concentration of 2 mg/ml. Selected clones were sub-cultured and amplified. All antisense oligonucleotides containing 2’-O-methyl and phosphorothioate backbone modifications were obtained from Dharmaco
Research, Inc., Boulder. Antisense oligonucleotides were added to stable cell lines in exponential growth phase using lipofectamine 2000. The final concentration of antisense oligonucleotides in the culture medium was 100 nM unless stated otherwise. Cells were harvested and total RNA was isolated using TRIzol reagent (Gibco-BRL) 24 hrs after antisense oligonucleotide addition unless stated otherwise. As a loading control, the hypoxanthine phosphoribosyltransferase (HPRT) gene was simultaneously amplified by using the primers HPRT-Fw  5’ AAG GAG ATG GGA GGC CAT, and HPTR-Rev  5’ GTT GAG AGA TCA TCT CCA CCA AT. The sequence of the antisense oligonucleotides directed towards the branch point (oligo_{bp}) is, 5’-CUCAUAUGUCAGAUGUACAG-3’, towards the polypyrimidine tract (oligo_{py}) is CCUGCAAAUGAGAAAUUAG, and towards the splice site junction (oligo_{jnct}) is CUAGUAUUUCCUGCAAAUGAG.

**Results**

It has been suggested that differential processing of the SMN1 and SMN2 pre-mRNAs is caused by the disruption of an ESE located within exon 7 of the transcript (12,14). The single nucleotide difference within exon 7 of SMN1 and SMN2 is located six nucleotides downstream of the 3’ splice site junction. While the C to T transition does not change the coding sequence of the transcript, the recognition of exon 7 by components of the splicing machinery is severely altered. Less than 20% of SMN2 pre-mRNAs are spliced to include exon 7, while more than 90% of SMN1 pre-mRNAs are processed to include exon 7. Generally, exon skipping is a consequence of the inability of the splicing machinery to recognize and define the exon (29). Exon definition in turn predominantly relies on the strength of 3’ and 5’ splice site signals.
flanking the exon. Cis-acting RNA elements like ESEs or exonic splicing silencer elements facilitate this process by recruiting general splicing factors to the adjacent exon/intron junctions, thus augmenting the intrinsic strength of 3’ or 5’ splice site (16,30). Because the C to T transition in SMN2 is proposed to disrupt a putative ESE element it is expected that the efficiency at which the splicing machinery recognizes the 5’ or 3’ splice sites will be reduced. To test this hypothesis, we generated a set of SMN mini-genes that were assayed in cell transfection splicing assays.

*The C to T transition in SMN2 decreases the utilization of the 3’ splice site of exon 7.*

To test the effects the C to T transition has on the strength of the 3’ss or 5’ss of exon 7, we designed two sets of identical mini-genes for SMN1 and SMN2 (Fig. 2A). SMN8 lacks the majority of intron 7 and all of exon 8 and thus tests the activity of the 3’ss of exon 7. SMN6 lacks exon 6 and the vast majority of intron 6 and thus tests the activity of the 5’ss of exon 7. Compared to the wild-type substrate, these pre-mRNAs do not contain competing exons. Therefore, splice site activation leads to a single intron removal event. Processed as well as unprocessed RNA were amplified from total RNA preparations by PCR using intron specific or exon specific primer sets (see Materials and Methods). In the native context of competing splice sites, processing of the *SMN1* and *SMN2* wild-type mini-genes lead to the characteristically strong difference in exon 7 inclusion (Fig. 2B, C). However, as demonstrated in Fig. 2B, the difference of intron 6 removal was observed to be less pronounced for the *SMN18* and *SMN28* mini-genes (lanes 5 and 6). Prior to calculating processing efficiencies, band intensities were normalized to the intensity of an internal control, the amplification product of the hypoxanthine phosphoribosyltransferase (HPRT) gene (see Material and Methods). Within a data set of four
independent transfection experiments, a reproducible difference in splice site strength was determined to be approximately two-fold (compare splicing efficiencies of 20±3% for SMN18 with 9±2% for SMN28) (Fig. 2B, Table 1). Thus, the C to T transition in SMN2 decreases the intrinsic strength of the 3’ss strength of exon 7 by two-fold.

To investigate if the C to T transition in SMN2 leads to a decrease in the strength of the 5’ss of exon 7, we compared the SMN6 substrates. As demonstrated in Fig. 2C, the steady state levels of intron 7 removal were observed to be surprisingly low (0.5±0.3% for SMN16 and 0.8±0.4% for SMN26, Table 1). Because we did not detect a statistically significant difference between the efficiencies of SMN16 and SMN26 splicing, we conclude that the C to T transition in SMN2 does not affect the 5’ss strength of exon 7 within the context of the single intron pre-mRNA.

The experiments described above indicate that the 3’ss of exons 7 and 8 compete to pair with the 5’ss of exon 6. To evaluate the importance of exon 6 nucleotides in this competition we exchanged exon 6 of SMN with exon 1 derived from β-globin to generate a heterologous substrate, hetSMN (Fig 2A). As summarized in Table 1, quantitative analysis of the transfection assays show that the ratio of exon 7 inclusion observed for the hetSMN pre-mRNAs are very similar to the levels observed with the wild-type SMN mini-genes (Table 1). We conclude that the decision to include or exclude exon 7 into the splicing pathway is independent of the nature of the upstream exon and its 5’ splice site.

Taken together, these results demonstrate that the nucleotide difference in SMN2 decreases the utilization of the 3’ss but not of the 5’ss of exon 7. Interestingly, the data also suggest that the relatively minor differences in the strength of the SMN1 and SMN2 exon 7 splice sites are drastically amplified in the native cis-competition context.
Antisense oligonucleotide application to modulate the competition of exon 7 and exon 8 increase
the ratio of exon 7 inclusion in SMN2.

Because a greater number of SMN2 gene copies in SMA patients correlates with a milder
phenotype of SMA, it has been postulated that the low amount of full length SMN generated
from SMN2 modulates disease severity (7,8). Alteration of the SMN2 splicing pattern to
generate higher fractions of full length SMN gene product may therefore be a promising
therapeutic approach to treat SMA. Our mutational analysis suggested that the splicing
machinery recognizes and processes exon 7 efficiently in the absence of a splice site choice.
Therefore we reasoned that an antisense oligonucleotide directed towards the 3’ splice site of
SMN2 exon 8 could potentially tilt the balance in favor of exon 7 inclusion. As illustrated in
Fig. 3A, this antisense oligonucleotide strategy may also lead to intron 7 retention. However,
intron 7 retention does not change the coding region for SMN because the translation stop codon
is located within the last nucleotides of exon 7. Stable cell lines expressing the SMN1 and
SMN2 wt mini-genes were created by G418 selection. The cells were then incubated in the
presence of various antisense oligonucleotides designed to hybridize to components of the 3’ss of
exon 8, the proposed branch point sequence (oligo_{bp}), the polypyrimidine tract (oligo_{py}), or the
splice site junction (oligo_{junct}). As illustrated in Fig. 3B, treatment of the cell lines with
oligo_{junct}, but not with oligo_{bp} (or oligo_{py}, data not shown) led to a reproducible increase in the generation
of exon 7 containing mRNA. As expected, we also detected a significant fraction of stable SMN
RNA that retained intron 7. Quantitative analysis indicates that the antisense oligonucleotide
application leads to a five-fold increase in the ratio of exon 7 inclusion over exon 7 skipping.
The absolute levels of exon 7 inclusion increased from 20% in the absence of antisense
oligonucleotides up to 50% in the presence of the junction antisense oligonucleotide. Thus, treatment of the stable cell lines expressing the SMN2 mini-gene with the junction antisense oligonucleotide modulates splice site pairing to include exon 7. As illustrated in Fig. 4A, the application of the antisense oligonucleotide is concentration dependent with an apparent $K_D$ of $20 \pm 10$ nM. Maximal splice site modulation is reached at approximately 100 nM oligo\text{jnct}. In comparison, incubation of the bp or py antisense oligonucleotides at concentrations of up to 500 nM did not lead to any measurable increase in the fraction of exon 7 inclusion (data not shown). Fig. 4B shows a time course of the antisense oligonucleotide treatment. Modulation of SMN2 splicing is observed within the first 7 hours of oligo\text{jnct} transfection and is maintained throughout the length of the experiment (72 hours). Maximal efficiency was observed after 24 hours of treatment and the steady decrease in antisense activity thereafter is most likely due to the degradation of the 2 O-methyl phosphorothioate oligonucleotide. While oligo\text{jnct} manipulated the ratio of exon 7 inclusion in SMN2 pre-mRNA splicing, its effect on SMN1 processing was manifested predominantly by the generation of intron 7 retaining SMN1 RNAs (data not shown). We conclude that an antisense oligonucleotide treatment directed towards the intron 7/exon 8 junction efficiently and specifically modulates the splicing pattern of SMN2.

**Discussion**

SMA is caused by gross mutations in the telomeric copy of the duplicated SMN genes. The centromeric copy of SMN, SMN2, cannot compensate for the loss of SMN1 because the majority of SMN2 pre-mRNAs undergo differential RNA splicing. A single C to T nucleotide difference that lies within exon 7 of the SMN transcript leads to preferential exon 7 exclusion in SMN2,
ultimately leading to production of a truncated and unstable peptide. Thus, the C to T transition drastically affects the efficiency and kinetics at which the splicing machinery recognizes and defines exon 7. In order to gain insights into the mechanisms by which the C to T transition alters exon 7 definition, we have compared the strength of the SMN1 and SMN2 exon 7 splice sites. Our data demonstrate that in the absence of competing splice sites, i.e. in the context of a single intron removal event, a two-fold difference was observed between the 3 splice sites of SMN1 and SMN2. In contrast, we did not detect a difference between the 5’ splice sites. Thus, the C to T transition does not appear to drastically decrease the intrinsic strength of the exon 7 splice sites. The two-fold variance clearly cannot account for the greater difference observed in the native context of competing exons. While 90% of SMN1 mRNAs include exon 7, only 20% of exon 7 containing mRNAs are observed for SMN2. Assuming similar transcriptional activities, the difference in the mole number of full length SMN mRNAs produced from each SMN locus is therefore approximately five-fold. In order to evaluate these differences, it is useful to express the percentage of exon 7 inclusion by the fraction of the apparent rate constant of exon 7 inclusion ($k_{\text{inc}}$) over the apparent rate constant of exon exclusion ($k_{\text{ex}}$). Thus, the fraction of exon 7 inclusion equals to $k_{\text{inc}}/k_{\text{ex}}$. Because the ratio of exon 7 inclusion is 90% for SMN1 pre-mRNA processing, the rate of exon inclusion is 9 times faster than the rate of exon skipping. In case of SMN2, $k_{\text{ex}}$ is 5 times faster than $k_{\text{inc}}$ leading to a 20% inclusion efficiency.

In the context of competing exons, $k_{\text{inc}}/k_{\text{ex}}$ therefore changes from 9 in SMN1 to 0.2 in SMN2, a 45-fold difference. Removal of either intron 6 or intron 7 most likely commits the splicing pattern to exon 7 inclusion. Thus, changes in the rate of exon inclusion depend on the efficiency of either intron 6 or intron 7 splicing. Because our comparison of intron 6 and intron 7 removal
indicates that neither process is altered by more than two-fold, it is tempting to argue that the
SMN2 characteristic exon 7 skipping phenomena arises mainly by increasing the efficiency of
the competing exon exclusion reaction. Based on the above comparisons, a two-fold decrease in
the efficiency of intron 6 or intron 7 removal is expected to be accompanied by a 20-fold
increase in the rate of \( k_{\text{ex}} \). These considerations suggest that a more efficient recognition of
exonic sequences, by the action of exonic splicing enhancers for example, affect not only the rate
of intron removal but also the competing rate of exon skipping. Therefore, exon definition may
incorporate a mechanism that also influences rates of splice site juxtapositioning.

Alternative splicing of the CD45 pre-mRNA has recently been attributed to the
combined actions of exonic enhancer and silencer elements (31). It was shown that a single
nucleotide polymorphism, like SMN located within exonic sequences, changed the ratio of
\( k_{\text{inc}}/k_{\text{ex}} \) by approximately 30-fold. Using single intron substrates it was further demonstrated that
the efficiency of only one of the two intron removal events was altered by approximately four
fold. By analogy to the above considerations, these results are in agreement with the notion that
an increase in the level of exon definition divergently alters the rates of exon inclusion and exon
exclusion. A more efficient recognition and assembly of the exonic region by the splicing
machinery leads to a less efficient splice site pairing of the upstream splice site with the
competing downstream splice site. As demonstrated recently, recruitment of spliceosomal
factors to non-splicing competent regions caused inhibition of intron removal\(^2\). In this case, it
was argued that the non-productive splicing complex efficiently competes for splice site pairing
across the intron, thereby reducing the probability of a productive juxtapositioning event with a

\(^2\) Schaal, T.D., Hertel K.J., Reed R., Maniatis T., in preparation
downstream splice site. We would like to suggest that the C to T transition in SMN2 leads to an overall decrease in the ability of exon 7 to be recognized as exonic. As the affinity of splicing factors to interact with exon 7 decreases, so does the activity of flanking intron removal. In addition, a less defined exon 7 region reduces its ability to prevent exon 7 skipping, and consequently the competing splice site pairing event between the 5 splice site of exon 6 and the 3 splice site of exon 8 becomes the preferred splicing pathway. However, we cannot exclude the possibility that the design of our single intron substrates deleted cis-acting elements required for the full manifestation of the splicing effect caused by the C to T transition.

Our experiments indicate that the C to T transition in SMN2 does not drastically alter the kinetics of intron 6 or intron 7 removal. If unprocessed and processed SMN8 and SMN6 transcripts are assumed to have similar half-lives, a comparison of their steady state levels strongly suggests that intron 6 removal occurs much faster than intron 7 removal. These conclusions imply that intron 6 removal precedes processing of the terminal intron. Consistent with this interpretation, we reproducibly detect low steady state levels of the SMN1 intron 7 retaining splicing intermediate (Fig. 3B).

Because all individuals with SMA have retained their SMN2 allele, therapy directed towards increasing SMN2 exon 7 inclusion could provide a very promising tool to lower the clinical severity of SMA. Our comparison of SMN18 and SMN28 processing demonstrated a low but measurable difference in the efficiency of intron 6 removal. These data suggested that the 3’ss of exon 7 of SMN1 and SMN2 have similar affinities for components of the splicing machinery and that the C to T transition in SMN2 does not abolish the recognition of the 3’ss of SMN2 exon 7. These observations prompted us to test an antisense oligonucleotide based approach to modulate the frequency of exon 7 inclusion in SMN2. By blocking the 3 splice site
of exon 8 with an antisense probe, we hoped to manipulate the apparent competition between the 3 splice sites of exon 7 and exon 8. In designing the antisense oligonucleotide, we took advantage of our observation that the 3 splice site of SMN2 exon 7 is quite competent to pair with the 5 splice site of SMN2 exon 6 and the fact that the SMN translation termination codon resides within exon 7. Of the three tested oligonucleotides only one, oligojunct, showed any significant effect in modulating SMN2 exon 7 inclusion. As expected from the experimental design, oligojunct caused an accumulation of RNA species that retained intron 7. In addition, oligojunct marginally increased the fraction of fully spliced product. The failure of oligopy and oligobp to induce any splicing alterations may either be caused by accessibility complications, or by their inability to reduce spliceosomal assembly, even when associated with their target sequence. The concentration dependence of oligojunct illustrates the efficiency of an interaction between oligojunct and the target sequence. The concentration of oligojunct required for half maximal modulation (20±10 nM) favorably compares with other reports utilizing antisense oligonucleotide approaches to alter pre-mRNA splicing patterns (32-35). As expected from half-life considerations, the oligojunct splicing effect diminishes over time.

It is unclear at the present time what minimal levels of SMN expression are required to protect against SMA or to prevent the progression of the neuro-degenerate disease. The overall efficiency of the oligojunct application is illustrated in Fig. 3B. At the conditions tested, the presence of the oligojunct leads to a 2.5 fold increase in the concentrations of RNAs containing exon 7. This numerical increase appears modest in magnitude. However, because it has been demonstrated that increased levels of SMN2 copy numbers correlate with decreasing severity of
SMA, a 2.5-fold increase in the levels of exon 7 containing RNA may be very significant in raising SMN levels to concentrations that allows some level of protection against disease progression. While the application of oligo_jnct demonstrates the feasibility of the antisense approach, it also serves as a stepping stone for the search of related oligonucleotides with higher efficacy.

A recent study demonstrated that the over-expression of hTra2, a human splicing factor that has been shown to interact with an exon 7 ESE common to both SMN1 and SMN2 pre-mRNAs, is able to efficiently alter the fraction of exon 7 inclusion in SMN2 (28). The strategy used in this approach was to increase the probability of an interaction between hTra2 and the pre-mRNA. Activating exon 7 definition in SMN2 therefore committed the splicing machinery to include exon 7 more frequently into the splicing pattern. While the results of the exon 7 activation studies were very encouraging, the overexpression of a splicing factor with general functions in pre-mRNA splicing could lead to undesired alterations of other important pre-mRNAs. Here we have tested a splicing modulation approach that by design incorporates a high degree of specificity. The mechanism of oligo_jnct action relies on its specific interactions with the 3 splice site of exon 8. Association of oligo_jnct with its target renders it inaccessible for recognition by the splicing machinery. Oligo_jnct therefore competes with the splicing machinery for interactions with the 3’ss of exon 8. While antisense oligonucleotide applications are highly specific in nature, their efficacy depends largely on target accessibility, as demonstrated by the failure of oligo_bp and oligo_py to affect SMN2 pre-mRNA splicing. These oligonucleotides in essence served as controls to demonstrate that the processing modulation caused by oligo_jnct is a
consequence of a specific and productive interaction with the target RNA and not by a non-specific antisense oligonucleotide effect.

While the antisense approach appears very promising in principle, the ultimate test of the application will be to evaluate if the presence of oligo_jnct or a related oligonucleotide leads to an increase in the levels of full-length SMN protein. Although oligo_jnct directed intron 7 retention will generate a mRNA that, when translated, will produce functional SMN protein, it is unknown if SMN intron 7 retention alters the stability of the RNA or its ability to cross the nuclear membrane. Further experiments will address the outcome of these important issues.

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References


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Figure Legends

Fig. 1  Comparison of the SMN1 and SMN2 genes.

(A) Schematic of SMN mini-gene constructs containing the C to T transition within exon 7 and their preferred splicing pathways. Solid lines represent major pathways, dotted lines represent minor pathways. The thick bars within exon 7 highlight the position of the C to T transition and the UAA translation stop codon. (B) SMN RNA sequences of the region including exon 7. Exon 7 sequences are in uppercase. Intron sequences are in lowercase. The C to T transition is highlighted by the large font, the UAA stop codon at the 3’ end of exon 7 is represented in both.

Fig. 2  Comparison of SMN1 and SMN2 exon 7 splice site strength.

(A) SMN substrates tested. SMN8 and SMN6 constructs were created by deletion of exon 8 or exon 6, respectively. For hetSMN exon 6 was deleted and replaced with a heterologous beta-globin sequence containing a functional 5 splice site. Grey boxes show the relative positions of the primer annealing sites. Arrows above the boxes indicate the directionality of the primer. The thick bars within exon 7 highlight the position of the C to T transition and the UAA translation stop codon. (B) Representative autoradiogram of three independent transfection experiments. [32P]α-dATP-labeled RT-PCR of total RNA isolated from C33a cells 24 h post-transfection with 1 µg of SMN or SMN8. Different primer sets were used to selectively amplify spliced or unspliced products. Lanes 1 and 2 are SMN1 and SMN2 wt, lanes 3-6 are SMN1Δ8 and SMN2Δ8. Splicing efficiencies for SMN1Δ8 and SMN2Δ8 are indicated below lanes 5 and 6 and represent average values from three independent experiments (20±3% for SMN1Δ8 with 9±2% for SMN2Δ8). (C) RT-PCR of total RNA isolated from transfection experiments with 1 µg of SMN
or SMN6. Lanes 1 and 2 are SMN1 and SMN2 wt, lanes 3 and 4 are SMN1Δ6 and SMN2Δ6. Splicing efficiencies for SMN1Δ6 and SMN2Δ6 are indicated below lanes 3 and 4 and represent average values from three independent experiments (0.5±0.3% for SMN1Δ6 and 0.8±0.4% for SMN2Δ6).

Fig. 3 Antisense oligonucleotides modulate SMN2 splicing.

(A) Strategy used to modulate SMN2 alternative splicing. An antisense oligonucleotide directed towards the intron 7/exon 8 junction reduces the recognition of the exon 8 3’ splice site. As a consequence, the splice site pairing competition between exons 7 and 8 may be tilted to favor exon 7 inclusion. The thick bars within exon 7 highlight the position of the C to T transition and the UAA translation stop codon. Grey boxes indicate the positions of the primer annealing sites.

(B) Addition of oligo_junct (lanes 3 and 4) but not oligo_bp (lanes 5 and 6) modulates the efficiency of SMN2 exon 7 inclusion. The percent exon 7 inclusion and intron 7 retention are shown below each lane. Exon 7 inclusion is defined as the sum of fully spliced and intron 7 retained mRNAs.

Fig. 4 Time and concentration dependence of oligo_junct antisense application.

(A) Autoradiogram showing the effects increasing concentrations of oligo_junct has on the splicing pattern of the SMN2 mini-gene. The quantitation for each concentration tested is shown below.

(D) Graph representing the time course of the antisense oligonucleotide application. Oligo_junct was added at a final concentration of 100 nM to SMN2 expressing stable cell lines.
Figure 1

A

\[ \text{SMN1} \]

\[
\begin{array}{c}
\text{6} \\
\text{7} \\
\text{8}
\end{array}
\]

\[
\begin{array}{c}
\text{UAA} \\
\text{C}
\end{array}
\]

\[ \text{SMN2} \]

\[
\begin{array}{c}
\text{6} \\
\text{7} \\
\text{8}
\end{array}
\]

\[
\begin{array}{c}
\text{UAA} \\
\text{T}
\end{array}
\]

B

\begin{align*}
\text{SMN1} & : & \text{Intron 6} & : & \text{Exon 7} & : & \text{Intron 7} \\
\text{acuuccuuauuuuccuuacagGGUUUCAGACA...UAAGGAuagucugccagc} & & & & \text{3' Splice Site} & & \text{5' Splice Site} \\
\text{SMN2} & : & \text{acuuccuuauuuuccuuacagGGUUUTAGACA...UAAGGAuagucugccagc}
\end{align*}
Figure 3

A

\[ \text{SMN2} \text{ 6} \quad \text{7} \quad \text{8} \]

\[ 80\% \quad 20\% \]

\[ + \text{antisense to exon8 3'ss} \]

\[ \text{SMN2} \text{ 6} \quad \text{7} \quad \text{8} \]

B

<table>
<thead>
<tr>
<th></th>
<th>SMN2</th>
<th></th>
<th>SMN1</th>
</tr>
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<tbody>
<tr>
<td></td>
<td>none</td>
<td>junc</td>
<td>bp</td>
</tr>
<tr>
<td>24 hrs</td>
<td>16</td>
<td>15</td>
<td>53</td>
</tr>
<tr>
<td>48 hrs</td>
<td>38</td>
<td>53</td>
<td>13</td>
</tr>
</tbody>
</table>

% exon 7 inclusion: 16, 15, 53, 38, 17, 13, 95
% intron 7 retention: 0.5, 0.4, 22, 16, 0.8, 0.6, 3
Figure 4

A

SMN2

M 0 25 50 75 100 150 200 300 nM

1600

500

1 2 3 4 5 6 7 8

B

fraction exon7 inclusion

0 0.1 0.2 0.3 0.4 0.5 0.6

junction antisense [nM]

0 50 100 150 200 250 300 350

fraction exon7 inclusion

0 0.1 0.2 0.3 0.4 0.5

hrs

0 10 20 30 40 50 60 70 80
Table 1

Splicing efficiency of SMN mini-genes

<table>
<thead>
<tr>
<th>substrates</th>
<th>SMN1 allele</th>
<th>SMN2 allele</th>
<th>SMN1 allele</th>
<th>SMN2 allele</th>
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</thead>
<tbody>
<tr>
<td>SMN</td>
<td>22±3</td>
<td>22±2</td>
<td>92±3</td>
<td>16±2</td>
</tr>
<tr>
<td>SMN8</td>
<td>20±3</td>
<td>9±2</td>
<td>n.a.</td>
<td>n.a</td>
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<tr>
<td>SMN6</td>
<td>0.5±0.3</td>
<td>0.8±0.4</td>
<td>n.a.</td>
<td>n.a</td>
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<tr>
<td>hetSMN</td>
<td>12±1</td>
<td>10±2</td>
<td>86±4</td>
<td>14±2</td>
</tr>
</tbody>
</table>

n.a., not applicable