



NIH PUBLIC ACCESS

Author Manuscript

Curr Opin Mol Ther. Author manuscript; available in PMC 2011 July 20.

Published in final edited form as:

Curr Opin Mol Ther. 2009 April ; 11(2): 108–115.

Splicing therapeutics in *SMN2* and *APOB*

Bernard Khoo¹ and Adrian R Krainer²Bernard Khoo: b.khoo@ucl.ac.uk¹University College London Medical School, Department of Endocrinology, Hampstead Campus, London, NW3 2PF, UK²Cold Spring Harbor Laboratory, 1 Bungtown Road, Cold Spring Harbor, NY 11724, USA

Abstract

Splicing therapeutics are defined as the deliberate modification of RNA splicing to achieve therapeutic goals. Various techniques for splicing therapeutics have been described, and most of these involve the use of antisense oligonucleotide-based compounds that target key elements in the pre-mRNA to control splicing in the nucleus. In this review, recent developments in splicing therapeutics for the treatment of two specific diseases are described: correcting the alternative splicing of survival of motor neuron (*SMN*)² pre-mRNA to compensate for the defective *SMN1* gene in spinal muscular atrophy, and re-engineering the splicing of apolipoprotein B pre-mRNA to lower circulating cholesterol levels.

Keywords

Antisense oligonucleotide; *APOB*; apolipoprotein B; cholesterol; RNA splicing; *SMN2*; spinal muscular atrophy

Introduction

During the process of pre-mRNA splicing the spliceosome, a dynamic complex of five small nuclear ribonucleoprotein (snRNP) particles and various proteins, removes introns from the transcribed pre-mRNA and joins exons to produce a translatable mRNA [1]. Exon-intron junctions are defined by degenerate *cis*-elements: the 5' splice site, the 3' splice site and the branch-point sequence (BPS). The degenerate *cis*-elements are necessary but not sufficient for accurate splicing. Other auxiliary sequences and their cognate factors influence the selection of splice sites. 'Splicing enhancers' are sequences within the pre-mRNA that promote the use of particular splice sites. Splicing enhancers are present in exons or introns, and are recognized by activator proteins, such as serine/arginine (SR)-rich proteins [2,3], which promote spliceosomal assembly on adjacent splice sites [4]. Conversely, 'splicing silencers' are sequences that inhibit the use of particular splice sites. Elements of the splicing silencer class are recognized by various repressors, such as polypyrimidine tract binding protein (PTB) [5] and heterogeneous nuclear ribonucleoprotein A1 (hnRNP A1) [6].

In the last 15 years, increasing interest has centered on the deliberate modification of RNA splicing for therapeutic purposes. The impetus for this interest arises from the observation that various mutations that cause human genetic diseases affect splicing, with approximately 10% of mutations affecting the RNA splice sites *per se* [7]. This review describes two

therapeutic target pre-mRNAs – survival of motor neuron (SMN)2 and apolipoprotein B (APOB) – to highlight the developments in splicing therapeutics in 2007 and 2008.

SMN2: Stimulating inclusion of exon 7

Spinal muscular atrophy (SMA) is a degenerative disease of the motor neurons of the spinal cord anterior horn and lower brainstem [8]. SMA presents in childhood with muscle weakness and occasionally fatal respiratory failure, and is caused by mutations of the *SMN* locus. In humans, the *SMN* locus is part of an inverted chromosomal-segment duplication that comprises the *SMN1* gene and its paralog *SMN2*. The SMN protein is ubiquitously expressed, and the majority (60 to 90%) of the SMN protein is translated from *SMN1* mRNA. Point mutations or deletions in *SMN1* have been observed in patients with SMA [9]. The SMN protein is localized in subnuclear complexes known as Gemini or coiled bodies or 'gems', which are involved in snRNP assembly and recycling; these gems are absent or greatly reduced in the cells of patients with SMA [10].

There is only a partial deficiency of the SMN protein in SMA, as the mutated *SMN1* is partially complemented by the wild-type copy of *SMN2*. The copy number of *SMN2* is variable because of *SMN2* gene duplication or conversion. The higher the copy numbers of *SMN2*, the milder the SMA phenotype [11]. The *SMN2* gene differs from *SMN1* by five nucleotide changes, two of which occur in the exons and three of which occur in the introns [12]. One of these nucleotide changes, a translationally silent cytosine to thymine transition at position 6 in exon 7, causes skipping of exon 7 in the majority of transcripts from *SMN2* and gives rise to the SMN Δ 7 RNA isoform. The alternative splicing of *SMN2* exon 7 is caused by the disruption of an exonic splicing enhancer (ESE) sequence that is recognized by the SR protein SF2 (splicing factor 2)/ASF (alternative SF) [13] and/or by the creation of an exonic splicing silencer (ESS) recognized by hnRNP A1 and A2 [14]. Exon 7 is the last coding exon in *SMN1*, and the resulting SMN Δ 7 protein isoform lacks the C-terminal 16 amino acid residues of the SMN protein, which are replaced by 4 amino acid residues derived from exon 8 [15]. The SMN Δ 7 protein is unable to self-associate, is degraded more quickly, and is unable to effectively replace the function of the full-length SMN protein [16–18]. Approximately 20% of *SMN2* mRNA transcripts are spliced to include exon 7 and are translated to the full-length, functional SMN protein; the higher the level of SMN protein, the milder the SMA phenotype (Figure 1) [19,20].

The potential for therapy in SMA is based on correcting the aberrant splicing of the *SMN2* gene, and relies on increasing the inclusion of exon 7 in the *SMN2* pre-mRNA. The correction of the aberrant splicing of *SMN2* is in contrast to the treatment of dystrophin-related diseases, in which exon skipping is desired [21]. Progress has been made in identifying small molecules that increase *SMN2* exon 7 inclusion in the mRNA and expression of the SMN protein, including treatment with various histone deacetylase inhibitors. Examples of these and other molecules include sodium butyrate [22], phenylbutyrate [23], hydroxyurea [24], sodium valproate [25], salbutamol [26], plant-derived polyphenols (such as resveratrol and curcumin [27]), and a quinazoline compound series and an indole compound identified by HTS [28]. The anthracycline aclarubicin is also effective in increasing *SMN2* exon 7 inclusion and SMN protein expression, although the mode of action of this agent is unclear [29]. Aminoglycoside compounds that promote read-through of premature termination codons also increase SMN protein expression levels in fibroblasts derived from patients with SMA [15]. The NSAID indoprofen increases levels of SMN protein expressed from the *SMN2* gene; this agent does not have an effect on exon 7 splicing, but may instead act co-translationally to increase expression of the wild-type SMN protein [30]. Although these small molecules are promising as modulators of exon 7 splicing, they are likely to be pleiotropic in their effects. To minimize off-target effects,

methods that correct the defective splicing of SMN2 exon 7 with improved specificity have been explored. The mechanics of SMN2 exon 7 alternative splicing and the potential splicing therapies under development in 2007 and 2008 that specifically correct SMN2 exon 7 splicing to compensate for the loss of *SMN1* are described.

Enhancement of exon 7 inclusion by antisense oligonucleotides

Following on from the observation that the ESE in SMN1 exon 7 is disrupted in *SMN2* [13], a synthetic SR protein analog to foster exon 7 inclusion was designed. This comprised a chimeric molecule with a peptide-nucleic acid (PNA; a peptide-like backbone of nucleobases capable of hybridizing with RNA [31]) covalently joined to a peptide. The constructed hybrid comprised a PNA antisense oligonucleotide (ASO) moiety, which targeted nucleotide positions 7 to 18 in SMN2 exon 7, coupled to 10 serine-arginine dipeptide repeats (the RS10 domain), to form an oligopeptide that can substitute for the natural serine-arginine rich domain of SF2/ASF. This compound, SMN-RS10, was effective at inducing *SMN2* exon 7 inclusion in the mRNA transcript *in vitro* [32]. An important observation was that the PNA ASO moiety alone was partially effective in augmenting the inclusion of exon 7 in SMN2 mRNA, although the effect was strongly enhanced when the RS10 domain was attached to the ASO. The enhancement of exon 7 inclusion in SMN2 mRNA by the addition of the RS10 domain to the ASO suggests that either SMN-RS10 displaces a repressor protein from an ESS or that the formation of a secondary structure unfavorable to spliceosomal assembly is inhibited.

To investigate the mechanism of action of SMN-RS10 and optimize the placement of the ASO target (ie, to identify the SMN1 nucleotide sequence that when targeted by the ASO leads to the most improvement in exon 7 inclusion), a systematic ASO 'walk' along the entire length of SMN2 exon 7 using 2'-*O*-methoxyethyl (MOE) ASOs was undertaken [33]. The effects of the MOE ASOs on SMN2 splicing were evaluated with *in vitro* splicing assays and verified by transfection of HEK293 cells. MOE ASOs, which have a charged backbone, were transfected more efficiently than PNA ASOs. Two regions that were inhibitory to exon 7 inclusion in the SMN2 mRNA transcript were identified: one region extends from the nucleotide positions 4 to 22 (region A) and the other from 34 to 51 (region B), sandwiching a central core region from position 22 to 33 containing a 5' motif that binds the splicing activator protein transformer 2 β 1 (Tra2 β 1) [34], as well as another less well-characterized 3' ESE (Figure 2). Individual MOE ASOs targeting the nucleotide positions 7 to 21 and 34 to 48 were able to increase SMN2 exon 7 inclusion in the mRNA transcript to similar levels observed in the SMN1 mRNA. These MOE ASOs also increased the number of gems in fibroblasts derived from a patient with SMA suggesting a physiologically significant increase in the amount of functional SMN protein.

Androphy and coworkers characterized an inhibitory stem-loop structure that incorporates the 3' end of SMN2 exon 7, including two intronic residues of the 5' splice site [35]. This stem-loop structure was postulated to sequester the weak 5' splice site of exon 7 and prevent its recognition by the spliceosomal component U1 snRNP. In this regard, it is interesting that ASO 34 to 48 is complementary to the 5' strand of the stem-loop structure, and may foster exon 7 inclusion by inhibiting the formation of the stem-loop. Similarly, MOE ASO 7 to 21 and the PNA ASO 7 to 18 may encourage the inclusion of exon 7 by inhibiting the binding of splicing repressor proteins, such as hnRNP A/B proteins; these repressor proteins are thought to bind an ESS motif at positions 6 to 11 [14], or to spread from other regions when the SF2/ASF ESE motif is absent, such as in *SMN2* [36].

Weakening the 3' splice site of exon 8 of the *SMN2* gene (eg, by targeting this site with an ASO) favors exon 7 inclusion, perhaps by a competitive mechanism [37]. Dickson *et al* increased full-length SMN protein expression as well as restoration of gems in transfected

fibroblasts from patients with SMA with bifunctional 2'-*O*-methyl RNA oligonucleotides, which incorporated an antisense moiety that bound to the 3' splice site of exon 8 with an hnRNP A1-binding motif [38].

Intronic inhibitory elements within *SMN2* pre-mRNA repress the inclusion of exon 7 [39]. Targeting intronic inhibitory elements instead of exonic elements is an attractive strategy, because this is less likely to interfere with subsequent steps in mRNA processing, such as mRNA transport or translation. One such element, intronic splicing silencer element (ISS)-N1, is present within intron 7 of the *SMN2* gene and downstream of the 5' splice site of exon 7. Singh and colleagues demonstrated that binding an ASO to the ISS-N1 element restored *SMN2* exon 7 inclusion to the levels observed in *SMN1* mRNA [39]. The mapping of the *SMN2* ISS-N1 silencer motif has been refined by conducting a high-resolution ASO walk strategy in intron 7 [40]. This silencer element binds strongly and specifically to hnRNP A1/A2, and binding of hnRNP A1/A2 appears to underlie the silencing activity of the ISS-N1 element [40]. The ASOs that were most effective at increasing *SMN2* exon 7 inclusion in the mRNA transcript in HEK293 cells were infused into transgenic mice harboring the human *SMN2* gene [40]. These ASOs increased *SMN2* exon 7 inclusion in the *SMN2* mRNA in transgenic mice, and the most striking effect was observed in the liver. The inclusion of exon 7 in *SMN2* mRNA was increased from a baseline of 21 to 91% over a 4-week ASO-treatment period. Significant increases of 2- to 3-fold in exon 7 inclusion in the *SMN2* mRNA were also observed in kidney and muscle. Because these ASOs do not cross the blood-brain barrier, no increase in exon 7 inclusion was observed in the spinal cord [40].

Enhancement of exon 7 inclusion by bifunctional tailed ASOs

Bifunctional antisense oligonucleotides were generated by Skordis *et al.*, in which a 5' phosphorothioate RNA tail containing ESE motifs that bind endogenous SR proteins, or other splicing activator proteins, were combined at the 3' end of the oligonucleotide with a 2'-*O*-methyl phosphorothioate ASO moiety targeting positions 2 to 16 of *SMN2* exon 7 [41]. This strategy increased exon 7 inclusion in the *SMN2* mRNA from 57% to 84%, both *in vitro* and *in vivo* in fibroblasts isolated from patients with SMA, and partially restored the appearance of gems in the nuclei of these fibroblasts [41]. Subsequently, similar but unmodified bifunctional RNA ASOs expressed in recombinant adeno-associated virus (rAAV) vectors increased exon 7 inclusion in the *SMN2* mRNA, partially restored the appearance of gems and increased SMN protein levels [42]. In 2004, Schümperli and Pillai utilized the same bifunctional oligonucleotide design plus an additional feature wherein the oligonucleotides were coupled to a modified U7 small nuclear RNA (U7 Sm OPT, reviewed in reference [43]) at the 3' end to target the bifunctional oligonucleotide to the spliceosome and increase its efficacy in modifying splicing. Interestingly, targeting the ASO moiety to position 2 to 19 of the *SMN2* pre-mRNA (ASO 02-19-U7 Sm OPT, approximately corresponding to inhibitory region A) was only partially effective and induced exon 7 inclusion in *SMN2* mRNA only to a maximum of 41% from a baseline value of 10%. Furthermore, by removing the ESE tail, ASO 02-19-U7 Sm OPT had the reverse effect and induced exon 7 skipping in both *SMN1* and *SMN2* pre-mRNA. The increased exon skipping was attributed to steric hindrance of the 3' splice site in both *SMN1* and *SMN2* by the U7 Sm OPT stem-loop. Targeting positions 31 to 48 (ASO 31-48-U7 Sm OPT, approximately corresponding to inhibitory region B) was more successful, with the ESE-ASO 31-48-U7 Sm OPT construct able to boost exon 7 inclusion in *SMN2* mRNA to 89% [44].

A series of experiments evaluated the ability of various ESE sequences within the 5' phosphorothioate RNA tail to increase exon 7 inclusion in the *SMN2* mRNA. The first series of experiments by Skordis *et al.* utilized a purine-rich ESE sequence (GGAGGAC) to recruit SF2/ASF [41]. A bifunctional ASO incorporating a tail with a different ESE sequence (TGCGTC) optimized for the binding of the SRp55 SR protein, achieved only

partial efficacy, with an increase to 42% of exon 7 inclusion [45]. When a second ESE sequence optimized for SF2/ASF binding (CACACGA) was used, the construct had the reverse effect and caused exon 7 skipping. Similarly, ESE tails optimized for binding of the splicing enhancer proteins SRp40, SC35 and TIA-1 caused exon 7 skipping in this context [45]. This firstly demonstrates that not all splicing enhancer proteins are effective in causing exon 7 inclusion and some might even be counterproductive in causing skipping. Secondly, it is likely that the original purine-rich ESE sequence (GGAGGAC) did not recruit SF2/ASF, but actually recruited Tra2 β 1, a splicing activator protein, which naturally binds to a similar purine-rich ESE in the middle of exon 7 (Figure 2) [34].

Infection of immortalized fibroblasts from a patient with SMA with bifunctional U7 cassettes coupled to lentiviral vectors, increased SMN2 mRNA exon 7 inclusion from 54% at baseline to 75% using the ESE-ASO 02-19-U7 Sm OPT and to 97% using the ESE-ASO 31-48-U7 Sm OPT [44]. SMN protein levels were increased from the control levels of approximately 17% to approximately 50% for both constructs, and intranuclear gem numbers were also significantly increased from 17 gems/100 nuclei to 49 gems/100 nuclei [44].

Trans-splicing as a means of replacing exon 7

Lorson and colleagues developed a *trans*-splicing strategy to replace exon 7 in SMN2 mRNA [46] (reviewed in reference [47]). This approach relied on the expression of a second therapeutic *trans*-splicing RNA (tsRNA), which was engineered to base-pair to intron 6 of SMN2 [46]. The tsRNA also contained an optimized BPS and a 3' splice site, the SMN1 exon 7 sequence and a hemagglutinin tag to allow for easy monitoring of *trans*-splicing. Splicing occurred *in trans* between the SMN2 pre-mRNA and the tsRNA, such that the SMN1 exon 7 sequence was spliced into the SMN2 mRNA. In their proof-of-principle study, *trans*-splicing was demonstrated between the tsRNA and the native SMN2 pre-mRNA in HeLa cells. Furthermore, by placing the tsRNA within an rAAV vector and infecting fibroblasts from patients with SMA, *trans*-splicing was observed between the native SMN2 pre-mRNA and the tsRNA, and intranuclear gems and snRNP assembly in nuclear extracts from these cells (measured using an *in vitro* assay) were restored [46].

Re-engineering APOB mRNA to lower cholesterol

APOB is the principal structural apolipoprotein in LDL, very (V)LDL, intermediate-density lipoprotein (IDL), lipoprotein(a) (Lp(a)) and chylomicron particles [48]. The APOB pre-mRNA is composed of 29 exons, all of which are constitutively spliced into the mature APOB mRNA. There are two natural protein isoforms, the full-length APOB100 and the C-terminal truncated APOB48 forms. APOB100 is synthesized in the liver and is required for the assembly of VLDL, IDL, LDL and Lp(a) particles. Along with APOE, APOB100 is the ligand for the LDL receptor. Excess levels of the APOB100-containing particles LDL and Lp(a) have been implicated in atherogenesis [49]. The APOB48 isoform is synthesized in the intestine, is identical to the N-terminal 48% of the APOB100 protein, and is required for chylomicron assembly and intestinal fat transport. These chylomicrons are cleared from the circulation by interaction of APOE with the chylomicron remnant receptor; APOB48 cannot bind to the LDL receptor as it lacks the C-terminal domain necessary for binding. The APOB48 isoform is generated by tissue-specific RNA editing of the CAA codon to a premature UAA termination codon in the intestine. The APOB48 mRNA is edited by a protein complex known as the editosome, which consists of the catalytic subunit APOBEC-1 (APOB mRNA-editing enzyme, catalytic polypeptide-1) and accessory factors. The RNA editing site and mooring sequence necessary for editosome binding and function are within exon 26 of the APOB mRNA [50]. Because of the central role of APOB100 in atherosclerosis, this isoform has become a major therapeutic target. Downregulation of

APOB100 expression is expected to decrease total cholesterol, LDL and Lp(a) levels, and therefore prevent the development of atherosclerosis.

Modifying APOB splicing was hypothesized to cause the expression of an alternative isoform of APOB. Exon 27 was selected as the target for three reasons. First, translation of the APOB mRNA lacking exon 27 (skip 27 mRNA) would generate a C-terminally truncated isoform of APOB100, APOB87_{SKIP27}, which is similar to the C-terminal truncations observed in some patients with mutations in *APOB* causing hypobetalipoproteinemia [51]. Heterozygotes for these mutant alleles of APOB have low cholesterol and LDL levels, have lowered risks of heart attacks, and live significantly longer than normal, although these individuals may be susceptible to fat accumulation in the liver [52]. Second, the 5' splice site of exon 27, when scored for its similarity to the splice-site consensus sequence using the Shapiro and Senapathy position weight matrix, was the weakest of all the 5' splice sites of the internal exons of APOB [53]. As constitutive 5' splice sites in general demonstrate better scores than the alternatively spliced 5' splice sites [54], APOB exon 27 would be the most amenable to alternative splicing. Third, as the sequences necessary for RNA editing are present in exon 26, the skip 27 mRNA should be edited as usual in the intestine and APOB48 expression should be unaffected (Figure 3).

Therefore, 2'-*O*-methyl ASOs targeting the splice sites flanking APOB exon 27 and to the BPS of intron 26–27 were designed and transfected into HepG2 cells, which naturally express APOB100 [55]. Combination ASOs targeting two elements simultaneously in a single ASO induced exon 27 skipping, and this was most effective when both the 3' splice site and BPS were targeted. In contrast, targeting predicted ESE motifs within exon 27 to interfere with SR protein binding did not have a significant effect on exon skipping, suggesting that the constitutive incorporation of exon 27 is more dependent on the splice sites than on exonic elements, or that there is functional redundancy between the exonic elements [55].

Chabot and colleagues successfully utilized A1-tailed bifunctional oligonucleotides to induce alternative splicing in Bcl-x pre-mRNA [56]. A bifunctional oligonucleotide consisting of a combination ASO targeting two splice sites plus an RNA tail was designed to bind the hnRNP A1 protein to encourage skipping of exon 27; however, this A1 tail did not augment exon-skipping in APOB pre-mRNA, but paradoxically partially reversed the skipping of exon 27 [55]. Therefore, the splice site and BPS ASOs may not function through a simple interference with splicing-factor binding. Instead, hybridization of the combination ASOs is likely to induce a secondary structure that is unfavorable to exon 27 inclusion. It is possible that recruitment of hnRNP A1 is counterproductive, as this may cause unwinding of the secondary structure [55].

Lastly, we demonstrated that combination ASO-transfected HepG2 cells were able to translate the skip 27 mRNA to a shortened isoform of APOB100, APOB87_{SKIP27}, which included the N-terminal 87% (3929 amino acids) of APOB100, along with a divergent 37-amino acid peptide at the C-terminus and ending in a premature termination codon within the exon 28 sequence [55]. Therefore, the induction of APOB87_{SKIP27} expression *in vivo* should lead to decreased LDL and cholesterol levels, such as occurs in patients with hypobetalipoproteinemia.

As mentioned previously, because intestinal APOB mRNA editing and APOB48 expression rely on sequences within exon 26, exon 27 skipping should not affect APOB48 expression. This is in contrast to methods that rely on a generalized downregulation of APOB mRNA levels, such as chimeric ASO-mediated RNase H degradation [57], and RNAi [58]. These latter methods have been demonstrated to reduce APOB100 levels and therefore circulating

levels of cholesterol and LDL, but they also reduce the levels of APOB48 and circulating levels of chylomicrons, an unwanted side effect expected to interfere with the transport of fat from the intestine.

Conclusion

The potential treatment of SMA and high cholesterol by splicing therapeutics exemplify two key uses of this strategy: first, correcting defective splicing caused by gene mutations, such as the replacement of the defective *SMN1* gene in SMA with *SMN2*; second, to re-engineer normal pre-mRNAs to achieve the desired physiological effects, such as the re-engineering of APOB pre-mRNA to reduce cholesterol levels.

In 2008, the development of various therapeutic strategies to correct SMN2 splicing has been particularly active. The challenge now is to identify reliable and safe methods of applying these therapeutic concepts to the bedside. ASOs are known to accumulate in the liver when administered *in vivo* [59], and have been shown to switch splicing in this organ when administered to mice intraperitoneally and orally [60]. In addition, ASOs targeted against the ISS-N1 element and infused into mice are able to increase exon 7 inclusion in SMN2 mRNA in the liver, kidney, and to a lesser extent, in muscle [40]. With regards to splicing therapeutics directed against APOB, as the liver is the principal target organ, ASOs are natural candidates for such an application. The next stage in the development of these APOB ASOs will be to demonstrate their effectiveness in lowering LDL cholesterol *in vivo*, and then to demonstrate that the APOB ASOs reduce the risk of clinically relevant endpoints.

With regards to SMN2, however, there are obstacles remaining in the delivery of ASO-based therapies, particularly because the blood-brain barrier protects the CNS from these agents [61]. SMN2 ASOs administered into the systemic circulation are not able to modify the splicing of SMN2 in the spinal cord because the ASOs cannot cross the blood-brain barrier [40]. The blood-brain barrier can be bypassed by administering ASOs to the CSF, either intrathecally or intracerebroventricularly; this strategy has been shown to be effective in downregulating superoxide dismutase in spinal motor neurons and slowing disease progression in a mouse model of amyotrophic lateral sclerosis [62]. Lentiviral- or rAAV-based vectors represent an alternative technology that could allow splicing-based therapies to be administered efficiently to the CNS.

The rAAV-based vectors can be used to transduce spinal cord neurons by retrograde transport [63]. However, other concerns exist with regard to the use of viral vectors in the CNS, particularly related to the induction of an immune response in patients previously exposed to AAV [64]. Therefore, both delivery strategies for splicing therapy (viral and nonviral) will be worth pursuing.

Lastly, it is not yet known if the modification of SMN2 splicing and restoration of the expression of the SMN protein can reverse the pathophysiology of SMA and the death and degeneration of spinal motor neurons to achieve clinically relevant outcomes. This is analogous to the situation with Duchenne muscular dystrophy (DMD). The intramuscular injection of ASOs has been demonstrated to successfully restore local dystrophin expression in patients with DMD. However, in a short follow-up study of 28 days, restoration of local dystrophin expression was not accompanied by an increase in muscular strength [65]. Thus, the practical application of splicing therapeutics with regard to SMN2 is at least some distance away and awaits further development *in vivo*.

References

- of outstanding interest
 - of special interest
1. Hastings ML, Krainer AR. Pre-mRNA splicing in the new millennium. *Curr Opin Cell Biol.* 2001; 13(3):302–309. [PubMed: 11343900]
 2. Graveley BR. Sorting out the complexity of SR protein functions. *RNA.* 2000; 6(9):1197–1211. [PubMed: 10999598]
 3. Sun Q, Mayeda A, Hampson RK, Krainer AR, Rottman FM. General splicing factor SF2/ASF promotes alternative splicing by binding to an exonic splicing enhancer. *Genes Dev.* 1993; 7(12B): 2598–2608. [PubMed: 8276242]
 4. Tian M, Maniatis T. A splicing enhancer complex controls alternative splicing of doublesex pre-mRNA. *Cell.* 1993; 74(1):105–114. [PubMed: 8334698]
 5. Zheng ZM, Huynen M, Baker CC. A pyrimidine-rich exonic splicing suppressor binds multiple RNA splicing factors and inhibits spliceosome assembly. *Proc Natl Acad Sci USA.* 1998; 95(24): 14088–14093. [PubMed: 9826658]
 6. Del Gatto-Konczak F, Olive M, Gesnel M, Breathnach R. hnRNP A1 recruited to an exon *in vivo* can function as an exon splicing silencer. *Mol Cell Biol.* 1999; 19(1):251–260. [PubMed: 9858549]
 7. Stenson PD, Ball EV, Mort M, Phillips AD, Shiel JA, Thomas NS, Abeysinghe S, Krawczak M, Cooper DN. Human Gene Mutation Database (HGMD): 2003 Update. *Hum Mutat.* 2003; 21(6): 577–581. [PubMed: 12754702]
 8. Monani UR. Spinal muscular atrophy: A deficiency in a ubiquitous protein; a motor neuron-specific disease. *Neuron.* 2005; 48(6):885–896. [PubMed: 16364894]
 9. Lefebvre S, Bürglen L, Reboullet S, Clermont O, Burlet P, Viollet L, Benichou B, Cruaud C, Millasseau P, Zeviani M. Identification and characterization of a spinal muscular atrophy-determining gene. *Cell.* 1995; 80(1):155–165. [PubMed: 7813012]
 10. Paushkin S, Gubitza AK, Massenet S, Dreyfuss G. The SMN complex, an assembly of ribonucleoproteins. *Curr Opin Cell Biol.* 2002; 14(3):305–312. [PubMed: 12067652]
 11. McAndrew PE, Parsons DW, Simard LR, Rochette C, Ray PN, Mendell JR, Prior TW, Burghes AH. Identification of proximal spinal muscular atrophy carriers and patients by analysis of *SMN1* and *SMN2* gene copy number. *Am J Hum Genet.* 1997; 60(6):1411–1422. [PubMed: 9199562]
 12. Wirth B. An update of the mutation spectrum of the survival motor neuron gene (*SMN1*) in autosomal recessive spinal muscular atrophy (SMA). *Hum Mutat.* 2000; 15(3):228–237. [PubMed: 10679938]
 13. Cartegni L, Krainer AR. Disruption of an SF2/ASF-dependent exonic splicing enhancer in *SMN2* causes spinal muscular atrophy in the absence of *SMN1*. *Nat Genet.* 2002; 30(4):377–384. [PubMed: 11925564]
 14. Kashima T, Manley JL. A negative element in *SMN2* exon 7 inhibits splicing in spinal muscular atrophy. *Nat Genet.* 2003; 34(4):460–463. [PubMed: 12833158]
 15. Mattis VB, Rai R, Wang J, Chang CW, Coady T, Lorson CL. Novel aminoglycosides increase SMN levels in spinal muscular atrophy fibroblasts. *Hum Genet.* 2006; 120(4):589–601. [PubMed: 16951947]
 16. Lorson CL, Hahnen E, Androphy EJ, Wirth B. A single nucleotide in the *SMN* gene regulates splicing and is responsible for spinal muscular atrophy. *Proc Natl Acad Sci USA.* 1999; 96(11): 6307–6311. [PubMed: 10339583]
 17. Lorson CL, Strasswimmer J, Yao JM, Baleja JD, Hahnen E, Wirth B, Le T, Burghes AH, Androphy EJ. SMN oligomerization defect correlates with spinal muscular atrophy severity. *Nat Genet.* 1998; 19(1):63–66. [PubMed: 9590291]
 18. Burnett BG, Munoz E, Tandon A, Kwon DY, Sumner CJ, Fischbeck KH. Regulation of SMN protein stability. *Mol Cell Biol.* 2009; 29(5):1107–1115. [PubMed: 19103745]

19. Coovert DD, Le TT, McAndrew PE, Strasswimmer J, Crawford TO, Mendell JR, Coulson SE, Androphy EJ, Prior TW, Burghes AH. The survival motor neuron protein in spinal muscular atrophy. *Hum Mol Genet.* 1997; 6(8):1205–1214. [PubMed: 9259265]
20. Gennarelli M, Lucarelli M, Capon F, Pizzuti A, Merlini L, Angelini C, Novelli G, Dallapiccola B. Survival motor neuron gene transcript analysis in muscles from spinal muscular atrophy patients. *Biochem Biophys Res Commun.* 1995; 213(1):342–348. [PubMed: 7639755]
21. van Ommen GJ, van Deutekom J, Aartsma-Rus A. The therapeutic potential of antisense-mediated exon skipping. *Curr Opin Mol Ther.* 2008; 10(2):140–149. [PubMed: 18386226]
22. Chang JG, Hsieh-Li HM, Jong YJ, Wang NM, Tsai CH, Li H. Treatment of spinal muscular atrophy by sodium butyrate. *Proc Natl Acad Sci USA.* 2001; 98(17):9808–9813. [PubMed: 11504946]
23. Andreassi C, Angelozzi C, Tiziano FD, Vitali T, De Vincenzi E, Boninsegna A, Villanova M, Bertini E, Pini A, Neri G, Brahe C. Phenylbutyrate increases *SMN* expression *in vitro*: Relevance for treatment of spinal muscular atrophy. *Eur J Hum Genet.* 2004; 12(1):59–65. [PubMed: 14560316]
24. Grzeschik SM, Ganta M, Prior TW, Heavlin WD, Wang CH. Hydroxyurea enhances *SMN2* gene expression in spinal muscular atrophy cells. *Ann Neurol.* 2005; 58(2):194–202. [PubMed: 16049920]
25. Brichta L, Hofmann Y, Hahnen E, Siebzehnruhl FA, Raschke H, Blumcke I, Eyupoglu IY, Wirth B. Valproic acid increases the *SMN2* protein level: A well-known drug as a potential therapy for spinal muscular atrophy. *Hum Mol Genet.* 2003; 12(19):2481–2489. [PubMed: 12915451]
26. Angelozzi C, Borgo F, Tiziano FD, Martella A, Neri G, Brahe C. Salbutamol increases *SMN* mRNA and protein levels in spinal muscular atrophy cells. *J Med Genet.* 2008; 45(1):29–31. [PubMed: 17932121]
27. Sakla MS, Lorson CL. Induction of full-length survival motor neuron by polyphenol botanical compounds. *Hum Genet.* 2008; 122(6):635–643. [PubMed: 17962980]
28. Jarecki J, Chen X, Bernardino A, Coovert DD, Whitney M, Burghes A, Stack J, Pollok BA. Diverse small-molecule modulators of *SMN* expression found by high-throughput compound screening: Early leads towards a therapeutic for spinal muscular atrophy. *Hum Mol Genet.* 2005; 14(14):2003–2018. [PubMed: 15944201]
29. Andreassi C, Jarecki J, Zhou J, Coovert DD, Monani UR, Chen X, Whitney M, Pollok B, Zhang M, Androphy E, Burghes AH. Aclarubicin treatment restores *SMN* levels to cells derived from type I spinal muscular atrophy patients. *Hum Mol Genet.* 2001; 10(24):2841–2849. [PubMed: 11734549]
30. Lunn MR, Root DE, Martino AM, Flaherty SP, Kelley BP, Coovert DD, Burghes AH, Man NT, Morris GE, Zhou J, Androphy EJ, et al. Indoprofen upregulates the survival motor neuron protein through a cyclooxygenase-independent mechanism. *Chem Biol.* 2004; 11(11):1489–1493. [PubMed: 15555999]
31. Nielsen PE. Antisense properties of peptide nucleic acid. *Methods Enzymol.* 2000; 313:156–164. [PubMed: 10595354]
32. Cartegni L, Krainer AR. Correction of disease-associated exon skipping by synthetic exon-specific activators. *Nat Struct Biol.* 2003; 10(2):120–125. [PubMed: 12524529]
33. Hua Y, Vickers TA, Baker BF, Bennett CF, Krainer AR. Enhancement of *SMN2* exon 7 inclusion by antisense oligonucleotides targeting the exon. *PLoS Biol.* 2007; 5(4):e73. [PubMed: 17355180]
 - A fine-structure mapping of the splicing silencer regions flanking the Tra2 β 1 ESE in *SMN2* exon 7 was conducted using ASOs. Targeting ASOs to two key regions significantly increased *SMN2* mRNA exon 7 inclusion and partially restored the appearance of intranuclear gems.
34. Hofmann Y, Lorson CL, Stamm S, Androphy EJ, Wirth B. Htra2- β 1 stimulates an exonic splicing enhancer and can restore full-length *SMN* expression to survival motor neuron 2 (*SMN2*). *Proc Natl Acad Sci USA.* 2000; 97(17):9618–9623. [PubMed: 10931943]
35. Singh NN, Singh RN, Androphy EJ. Modulating role of RNA structure in alternative splicing of a critical exon in the spinal muscular atrophy genes. *Nucleic Acids Res.* 2007; 35(2):371–389. [PubMed: 17170000] • This study characterized ISS-N1, an intronic inhibitory element, in the

intron downstream of SMN2 exon 7. An ASO targeting ISS-N1 was shown to increase the inclusion of SMN2 exon 7 to the same level as in wild-type SMN1.

36. Cartegni L, Hastings ML, Calarco JA, de Stanchina E, Krainer AR. Determinants of exon 7 splicing in the spinal muscular atrophy genes, *SMN1* and *SMN2*. *Am J Hum Genet.* 2006; 78(1): 63–77. [PubMed: 16385450]
37. Madocsai C, Lim SR, Geib T, Lam BJ, Hertel KJ. Correction of SMN2 pre-mRNA splicing by antisense U7 small nuclear RNAs. *Mol Ther.* 2005; 12(6):1013–1022. [PubMed: 16226920]
38. Dickson A, Osman E, Lorson C. A negatively-acting bifunctional RNA increases survival motor neuron *in vitro* and *in vivo*. *Hum Gene Ther.* 2008; 19(11):1307–1316. [PubMed: 19848583]
39. Singh NK, Singh NN, Androphy EJ, Singh RN. Splicing of a critical exon of human survival motor neuron is regulated by a unique silencer element located in the last intron. *Mol Cell Biol.* 2006; 26(4):1333–1346. [PubMed: 16449646]
40. Hua Y, Vickers TA, Okunola HL, Bennett CF, Krainer AR. Antisense masking of an hnRNP A1/A2 intronic splicing silencer corrects SMN2 splicing in transgenic mice. *Am J Hum Genet.* 2008; 82(4):834–848. [PubMed: 18371932] • Using an ASO walk strategy, that is, the systematic targeting of different regions of the SMN2 pre-mRNA, the position of the ISS-N1 element was refined. The ISS-N1 element binds hnRNP A1 and A2. Mutation of the hnRNP A binding motifs within ISS-N1 weakens the inhibitory effect of ISS-N1 and overexpression of hnRNP A1. The ISS-N1 ASOs identified from the cellular assays that were most effective at inducing exon 7 inclusion in the SMN2 mRNA were tested *in vivo* in transgenic mice harboring the human SMN2 gene. The ISS-N1 ASOs were able to increase exon 7 inclusion following 4 weeks of treatment.
41. Skordis LA, Dunckley MG, Yue B, Eperon IC, Muntoni F. Bifunctional antisense oligonucleotides provide a trans-acting splicing enhancer that stimulates *SMN2* gene expression in patient fibroblasts. *Proc Natl Acad Sci USA.* 2003; 100(7):4114–4119. [PubMed: 12642665] • This was the first study to demonstrate that bifunctional ASOs targeting the defective ESE in SMN2 exon 7 were capable of correcting the defect in splicing in SMN2.
42. Baughan T, Shababi M, Coady TH, Dickson AM, Tullis GE, Lorson CL. Stimulating full-length SMN2 expression by delivering bifunctional RNAs via a viral vector. *Mol Ther.* 2006; 14(1):54–62. [PubMed: 16580882] • Bifunctional ASOs expressed by a recombinant AAV vector are able to correct SMN2 exon 7 splicing.
43. Schümperli D, Pillai RS. The special Sm core structure of the U7 snRNP: Far-reaching significance of a small nuclear ribonucleoprotein. *Cell Mol Life Sci.* 2004; 61(19–20):2560–2570. [PubMed: 15526162]
44. Marquis J, Meyer K, Angehrn L, Kämpfer SS, Rothen-Rutishauser B, Schümperli D. Spinal muscular atrophy: SMN2 pre-mRNA splicing corrected by a U7 snRNA derivative carrying a splicing enhancer sequence. *Mol Ther.* 2007; 15(8):1479–1486. [PubMed: 17505471] •• This study provided a further refinement of the bifunctional oligonucleotide concept in two key areas: using a U7 snRNA to target the oligonucleotide to the spliceosome, and coupling this to a lentiviral vector for long-term expression of the oligonucleotide.
45. Cartegni L, Wang J, Zhu Z, Zhang MQ, Krainer AR. ESEfinder: A web resource to identify exonic splicing enhancers. *Nucleic Acids Res.* 2003; 31(13):3568–3571. [PubMed: 12824367]
46. Coady TH, Shababi M, Tullis GE, Lorson CL. Restoration of SMN function: Delivery of a trans-splicing RNA re-directs *SMN2* pre-mRNA splicing. *Mol Ther.* 2007; 15(8):1471–1478. [PubMed: 17551501] •• This study demonstrated that trans-splicing can be used to repair the SMN2 mRNA by substituting in exon 7 from SMN1 from a trans-splicing RNA expressed using an rAAV vector.
47. Garcia-Blanco MA. Messenger RNA reprogramming by spliceosome-mediated RNA trans-splicing. *J Clin Invest.* 2003; 112(4):474–480. [PubMed: 12925685]
48. Rosenson, R. Lipoprotein classification; metabolism; and role in atherosclerosis. In: Rose, B., editor. *UpToDate*. Wellesley, MA, USA: UpToDate; 2005.
49. Rackley, C. Pathogenesis of atherosclerosis. In: Rose, B., editor. *UpToDate*. Wellesley, MA, USA: UpToDate; 2005.
50. Chester A, Scott J, Anant S, Navaratnam N. RNA editing: Cytidine to uridine conversion in apolipoprotein B mRNA. *Biochim Biophys Acta.* 2000; 1494(1–2):1–13. [PubMed: 11072063]

51. Whitfield AJ, Barrett PH, van Bockxmeer FM, Burnett JR. Lipid disorders and mutations in the *APOB* gene. *Clin Chem*. 2004; 50(10):1725–1732. [PubMed: 15308601]
52. Schonfeld G. Familial hypobetalipoproteinemia: A review. *J Lipid Res*. 2003; 44(5):878–883. [PubMed: 12639976]
53. Shapiro M, Senapathy P. RNA splice junctions of different classes of eukaryotes: Sequence statistics and functional implications in gene expression. *Nucleic Acids Res*. 1987; 15(17):7155–7174. [PubMed: 3658675]
54. Roca X, Sachidanandam R, Krainer A. Intrinsic differences between authentic and cryptic 5' splice sites. *Nucleic Acids Res*. 2003; 31(21):6321–6333. [PubMed: 14576320]
55. Khoo B, Roca X, Chew SL, Krainer AR. Antisense oligonucleotide-induced alternative splicing of the *APOB* mRNA generates a novel isoform of *APOB*. *BMC Mol Biol*. 2007; 8:3. [PubMed: 17233885]
56. Villemaire J, Dion I, Elela SA, Chabot B. Reprogramming alternative pre-messenger RNA splicing through the use of protein-binding antisense oligonucleotides. *J Biol Chem*. 2003; 278(50):50031–50039. [PubMed: 14522969]
57. Croke RM, Graham MJ, Lemonidis KM, Whipple CP, Koo S, Perera RJ. An apolipoprotein B antisense oligonucleotide lowers LDL cholesterol in hyperlipidemic mice without causing hepatic steatosis. *J Lipid Res*. 2005; 46(5):872–884. [PubMed: 15716585]
58. Soutschek J, Akinc A, Bramlage B, Charisse K, Constien R, Donoghue M, Elbashir S, Geick A, Hadwiger P, Harborth J, John M, et al. Therapeutic silencing of an endogenous gene by systemic administration of modified siRNAs. *Nature*. 2004; 432(7014):173–178. [PubMed: 15538359]
59. Croke ST. Progress in antisense technology. *Annu Rev Med*. 2004; 55:61–95. [PubMed: 14746510]
60. Roberts J, Palma E, Sazani P, Ørum H, Cho M, Kole R. Efficient and persistent splice switching by systemically delivered LNA oligonucleotides in mice. *Mol Ther*. 2006; 14(4):471–475. [PubMed: 16854630]
61. Khoo B, Akker SA, Chew SL. Putting some spine into alternative splicing. *Trends Biotechnol*. 2003; 21(8):328–330. [PubMed: 12902166]
62. Smith RA, Miller TM, Yamanaka K, Monia BP, Condon TP, Hung G, Lobsiger CS, Ward CM, McAlonis-Downes M, Wei H, Wancewicz EV, et al. Antisense oligonucleotide therapy for neurodegenerative disease. *J Clin Invest*. 2006; 116(8):2290–2296. [PubMed: 16878173]
63. Peel AL, Zolotukhin S, Schrimsher GW, Muzyczka N, Reier PJ. Efficient transduction of green fluorescent protein in spinal cord neurons using adeno-associated virus vectors containing cell type-specific promoters. *Gene Ther*. 1997; 4(1):16–24. [PubMed: 9068791]
64. Mandel RJ, Manfredsson FP, Foust KD, Rising A, Reimsnider S, Nash K, Burger C. Recombinant adeno-associated viral vectors as therapeutic agents to treat neurological disorders. *Mol Ther*. 2006; 13(3):463–483. [PubMed: 16412695]
65. van Deutekom JC, Janson AA, Ginjaar IB, Frankhuizen WS, Aartsma-Rus A, Bremmer-Bout M, den Dunnen JT, Koop K, van der Kooi AJ, Goemans NM, de Kimpe SJ, et al. Local dystrophin restoration with antisense oligonucleotide PRO051. *N Engl J Med*. 2007; 357(26):2677–2686. [PubMed: 18160687]

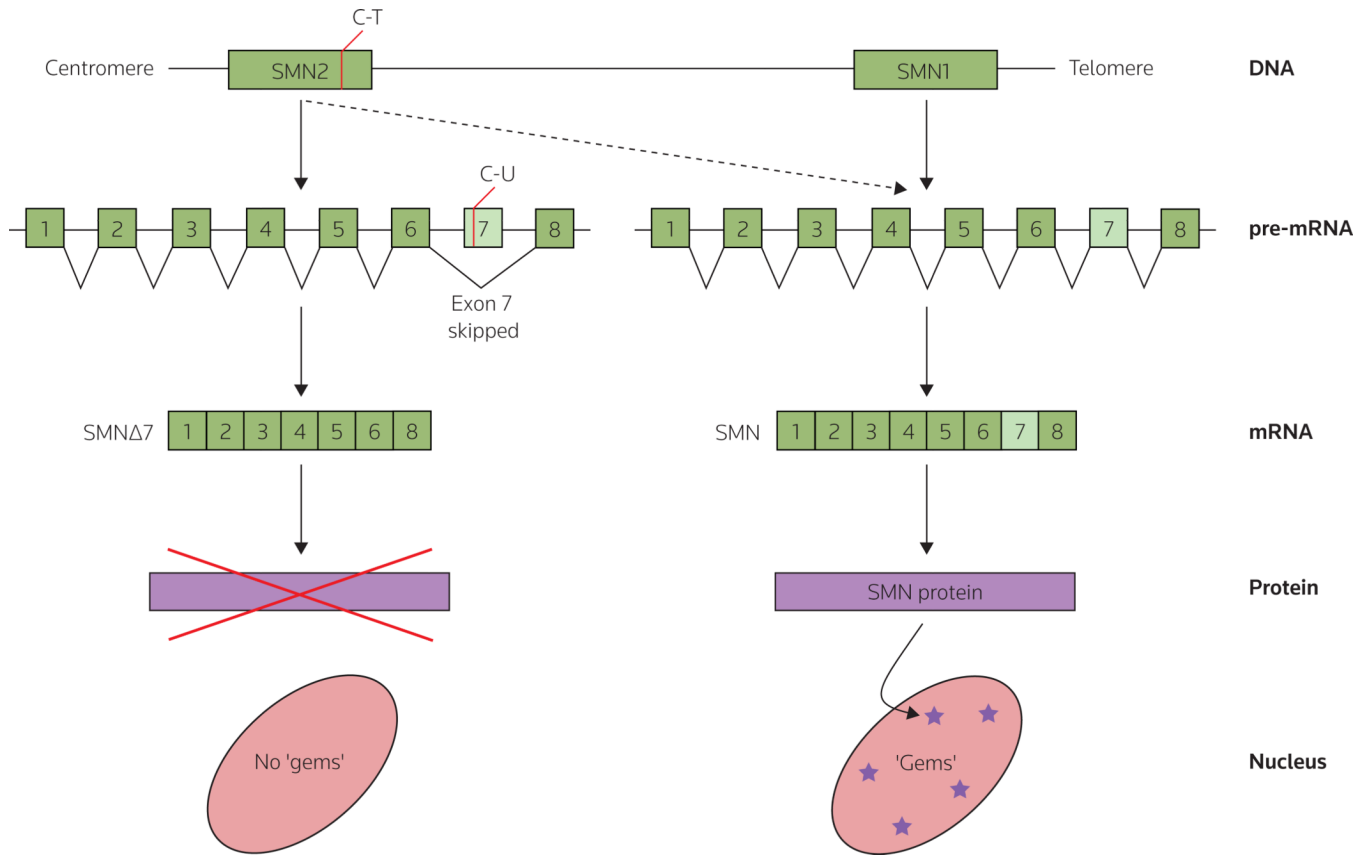


Figure 1. The genomic structure of the SMN locus

The survival of motor neuron (*SMN*)1 gene (located at the telomeric end of the chromosome) is transcribed and spliced normally to generate the SMN mRNA. The SMN mRNA is translated into the SMN protein, which is assembled into intranuclear Gemini of coiled bodies ('gems'). The effect of the cytosine (C) to thymine (T) transition (C to uridine [U] in the pre-mRNA) in the *SMN2* gene (located at the centromeric end of the chromosome) on *SMN2* exon 7 splicing and SMN protein expression is shown. A minority of the *SMN2* pre-mRNA is spliced to include exon 7 and generate the wild-type SMN mRNA (indicated by the dashed arrow). In the majority of the *SMN2* pre-mRNA exon 7 is spliced out generating the *SMNΔ7* mRNA. The *SMNΔ7* mRNA is translated to a shortened form of the SMN protein that is unstable and is unable to self-associate and assemble into intranuclear gems.

(Adapted with permission from Elsevier Ltd and Khoo B, Akker SA, Chew SL: Putting some spine into alternative splicing. Trends Biotechnol (2003) 21(8):328–330. © 2003 Elsevier Ltd)

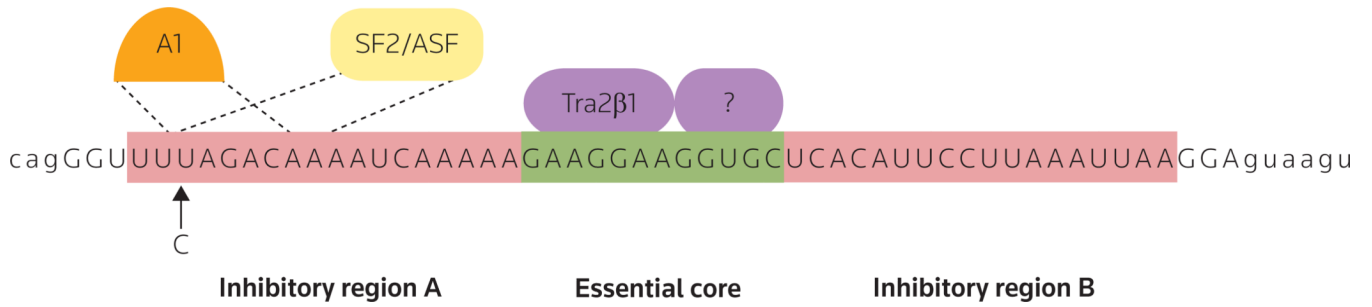


Figure 2. Regions in the SMN2 pre-mRNA that inhibit the inclusion of exon 7

The exon 7 sequence of the survival of motor neuron (SMN)2 gene is shown in capital letters, with flanking intronic sequences shown in lower case letters. The position of the cytosine (C) to uracil (U) transition in exon 7 is indicated by the arrow. Inhibitory regions A and B that inhibit the inclusion of exon 7 in the SMN2 mRNA are shown in pink, and the central core region, which appears to be essential for exon 7 inclusion, is shown in green. The splicing factors **A1** (heterogeneous nuclear ribonucleoprotein A1) [14], **SF2/ASF** (splicing factor 2/alternative splicing factor) [13], **Tra2β1** (transformer 2β1) [34], and an unknown splicing activator (?), which are thought to bind to exon 7, are shown.

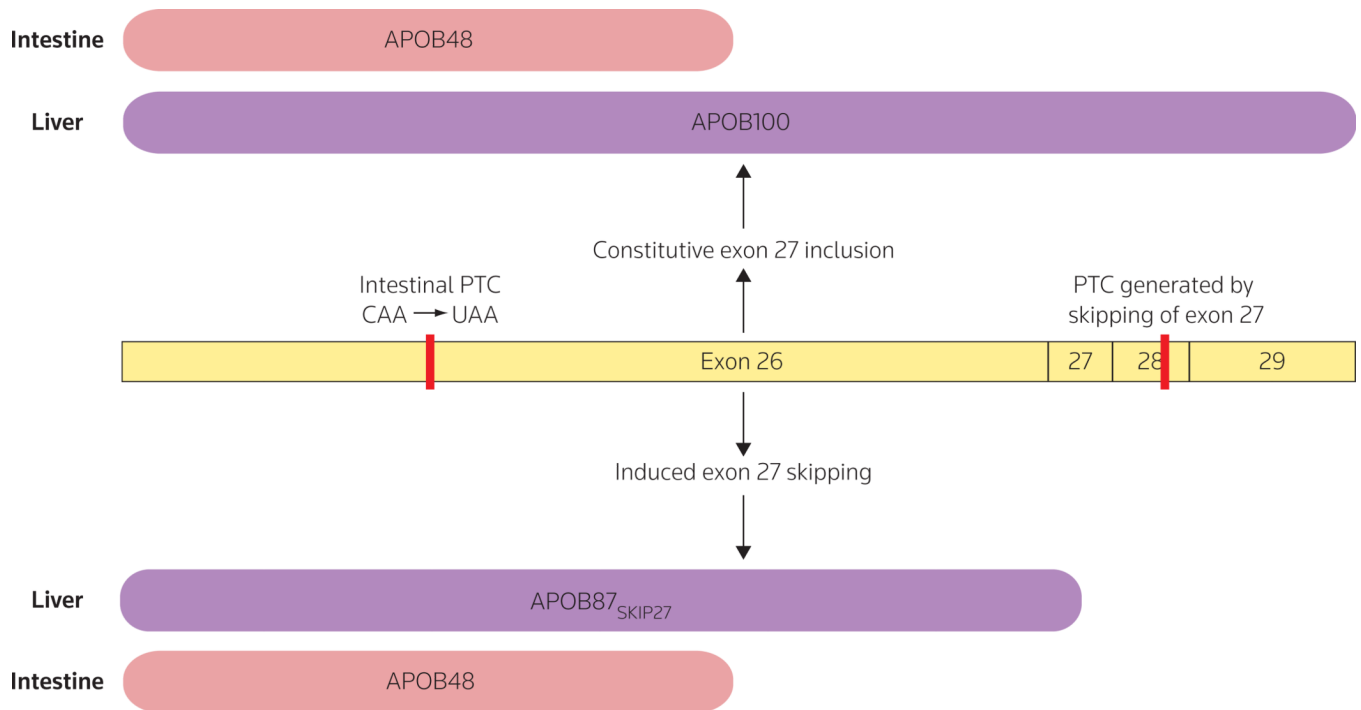


Figure 3. Skipping APOB exon 27 causes the expression of APOB87_{SKIP27}, a C-terminally truncated isoform of APOB

Constitutive exon 27 inclusion. Two isoforms of apolipoprotein B (APOB), APOB48 and APOB100, which both include exon 27 can be generated from the APOB mRNA. The APOB100 isoform is synthesized in the liver and is assembled into LDL particles, which transport cholesterol from the liver to the rest of the body and which have been implicated in atherosclerosis. APOB48 is identical to the N-terminal 48% of APOB100 and is synthesized in the intestine by tissue-specific RNA editing within exon 26, which generates a premature termination codon (PTC; indicated by the red box on the left of the Figure – sequence changed from CAA to UAA, where A = adenine, C = cytosine and U = uracil) and the C-terminally truncated protein. The APOB48 isoform is assembled into chylomicrons that transport dietary fat from the intestine. **Induced exon 27 skipping.** Modifying APOB splicing by the skipping of exon 27 can lead to the expression of two alternative isoforms of APOB, APOB87_{SKIP27} and APOB48. Because skipping exon 27 induces a frameshift, a PTC is generated within exon 28 (indicated by the red box on the right of the Figure). The skip 27 mRNA is translated in the liver to the APOB87_{SKIP27} isoform, which contains the N-terminal 87% of APOB100 plus a 37 amino acid divergent sequence derived from the frameshifted sequence of exon 28. However, as RNA editing in the intestine generates the PTC within exon 26, intestinal APOB48 expression is unaffected.