RNA Biology 7:4, 430-440; July/August 2010; © 2010 Landes Bioscience

Repair of pre-mRNA splicing
Prospects for a therapy for spinal muscular atrophy

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Key words: antisense oligonucleotides, bifunctional RNA, exon definition, gene therapy, motoneurons, splicing enhancer, U7snRNA

Recent analyses of complete genomes have revealed that alternative splicing became more prevalent and important during eukaryotic evolution. Alternative splicing augments the protein repertoire—particularly that of the human genome—and plays an important role in the development and function of differentiated cell types. However, splicing is also extremely vulnerable, and defects in the proper recognition of splicing signals can give rise to a variety of diseases. In this review, we discuss splicing correction therapies, by using the inherited disease Spinal Muscular Atrophy (SMA) as an example. This lethal early childhood disorder is caused by deletions or other severe mutations of *SMN1*, a gene coding for the essential survival of motoneurons protein. A second gene copy present in humans and few non-human primates, *SMN2*, can only partly compensate for the defect because of a single nucleotide change in exon 7 that causes this exon to be skipped in the majority of mRNAs. Thus *SMN2* is a prime therapeutic target for SMA. In recent years, several strategies based on small molecule drugs, antisense oligonucleotides or in vivo expressed RNAs have been developed that allow a correction of *SMN2* splicing. For some of these, a therapeutic benefit has been demonstrated in mouse models for SMA. This means that clinical trials of such splicing therapies for SMA may become possible in the near future.

Introduction

In higher eukaryotes, most transcripts of protein coding-genes are subject to splicing. Moreover, alternative splicing provides variant transcripts that may encode different proteins or that may be alternatively productive or non-productive with regard to a given protein product. The human genome is estimated to contain ~25,000 protein-coding transcription units, whereas the number of proteins could be considerably higher.[1,2] Although other mechanisms (such as RNA editing, post-translational modifications) contribute to this increase in protein diversity, alternative splicing is clearly its main source. Alternative splicing is particularly important for the development and differentiation of complex tissues such as the nervous system. Large scale studies reveal that ~95% of human genes undergo alternative splicing.[3-5] Considering the importance of constitutive and alternative splicing, it is not surprising that defects in these processes can generate aberrant transcripts with diverse pathologic consequences. In this review, we will briefly describe causes and effects of errors in constitutive and alternative splicing. Then, we will describe the therapeutic strategies currently being developed to circumvent these effects, with a special focus on the human inherited disease Spinal Muscular Atrophy (SMA).

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Submitted: 04/18/10; Accepted: 04/30/10
Previously published online:
www.landesbioscience.com/journals/rnabiology/article/12206

The Importance of Exon Definition

In higher eukaryotes and especially in humans, introns are much longer than exons (~3,500 bp compared to ~150 bp, respectively).[6] Moreover, the signals recognized by the splicing machinery, the 5' and 3' splice sites (SS) and the branch points (BP), are highly degenerate (**Fig. 1A**).[7] Many sequences within a transcription unit have the potential to act as ss, but only a minority of them are true ones in the sense that they are used in constitutive or alternative splicing. All sequences that resemble the 5' or 3' SS consensus but that are not used in splicing are defined as cryptic SS.

This degenerate nature of SS and BP sequences and the frequent occurrence of cryptic signals raises the question what distinguishes true signals from cryptic ones. The answer is that additional informations encoded in the sequence of a primary transcript help to define the exons of a transcription unit.[7] These informations can be found in exons or in introns, and are correspondingly called exonic splicing enhancers (ESEs), exonic splicing silencers (ESSs), intronic splicing enhancers (ISEs) and intronic splicing silencers (ISSs). The enhancers promote exon inclusion, whereas the silencers repress it. They do so by recruiting RNA-binding proteins that act as splicing regulators (**Fig. 1B**).

The predominant proteins acting as promoters by binding to ESEs, the so-called SR and SR-related proteins (SRp), contain one or more RNA recognition motifs (RRM) and a region of alternating serines and arginines. The RRM allows them to bind to target sequences in pre-mRNAs, whereas the SR repeats act as protein interaction domains with which these proteins can help to recruit parts of the spliceosomal machinery to the nearest intron-exon boundary, thereby improving exon definition.[8-10] In contrast, the hnRNPs (heterogenous nuclear ribonucleoproteins) rather act as silencers by antagonising the recognition of splice sites directly or by disturbing the link between enhancers and

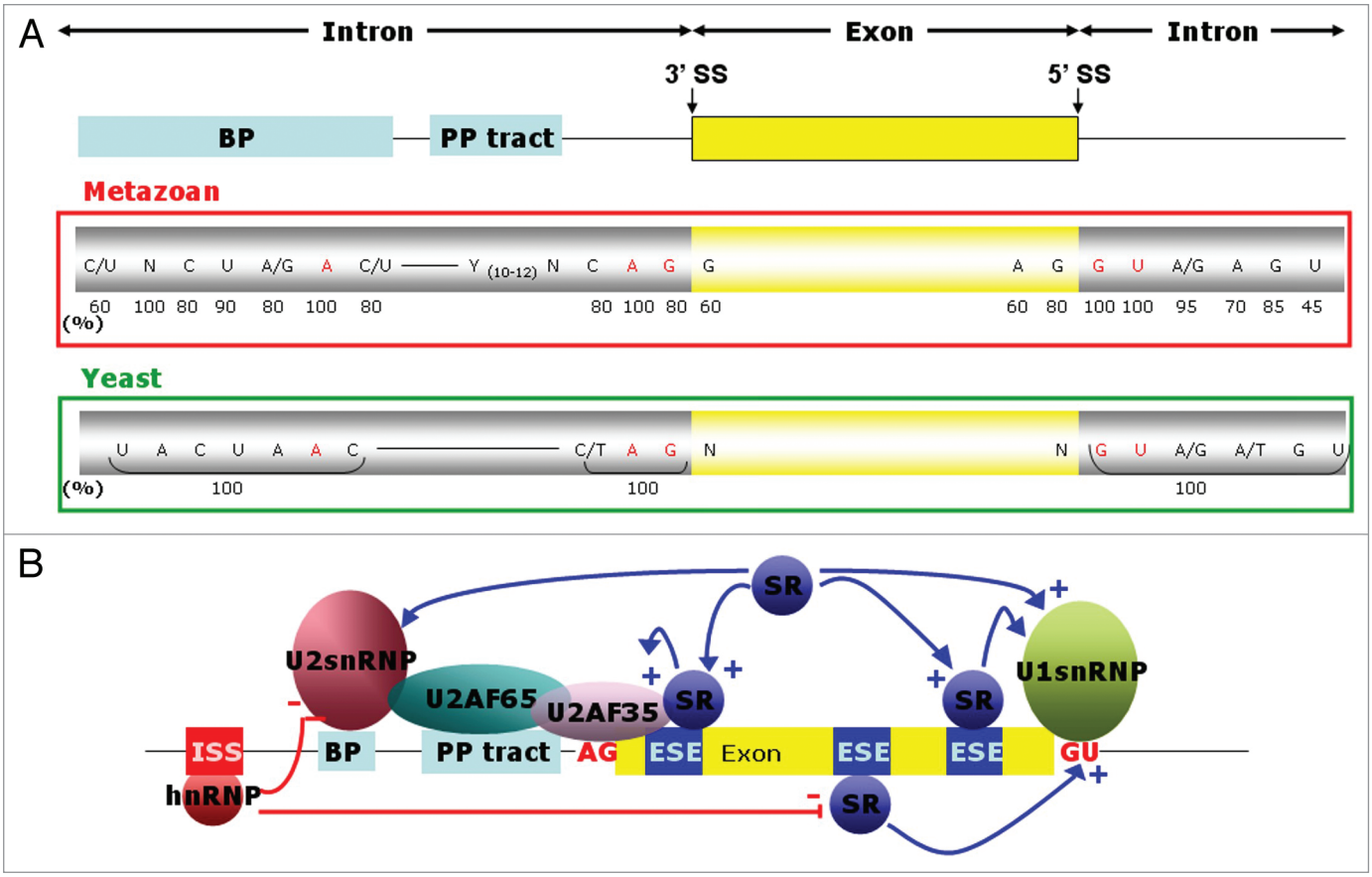

Figure 1. Cis acting sequence elements and trans-acting factors determining exon definition. (A) Conserved sequences flanking metazoan and yeast exons. The exon is symbolized by a yellow rectangle. The flanking introns are symbolized by lines in which the branch point (BP) sequence and polypyrimidine tract (PP tract) are highlighted as light blue boxes. The conserved nucleotides of the BP, PP tract, 3' splice site (3' SS) and 5' splice site (5' SS) are shown below with numbers indicating the percent prevalence of the most frequent nucleotides at each position. Shown in red are the branch point adenosine as well as the virtually invariant last two and first two nucleotides of the introns. (B) Role of splicing activators and repressors in splicing modulation. SR proteins bind to exonic splicing enhancers (ESE; dark blue) via their RNA recognition motifs and favour the recruitment of the splicing machinery (+) at the 5' and 3' SS, mainly by stabilizing the interaction between snRNPs and the pre-mRNA. SR proteins also act by direct protein-protein interactions with U1 snRNPs at the 5' SS, and with the U2 snRNP and U2AF co-factors at the 3' SS. In contrast, hnRNP proteins most frequently bind to intronic splicing silencers (ISS; red) and are involved in repression (-) of splicing, by exerting negative effects on U snRNPs or SR proteins. These different interactions are represented by arrows.

their interactors.[11,12] In general, binding sites for SR proteins are more frequent in true exons, and hnRNP binding sites are more often found in introns.

Nevertheless, the positive or negative roles of these factors in splicing are not so simply defined, since in certain exons, they may also act in an opposite way. Moreover additional proteins not belonging to these two groups, such as proteins of the CELF/Bruno-like family,[11,13] can act both as enhancers or modulators of splicing. In the end, the definition of an exon and hence its inclusion in the mature mRNA is the composite result of the sequences and relative positions of splicing signals (SS and BP), enhancers and silencers, the amounts of enhancing and repressing trans-acting factors and their interaction with each other as well as with components of the basic splicing machinery. Even the secondary structure of the RNA to be spliced may sometimes influence exon definition.[14]

Alternative splicing results when the interplay of all these sequences, splicing regulators and the general splicing factors that are directly involved in the spliceosome cycle can be interpreted in different ways. For many transcritpion units, multiple splicing isoforms can persist as mature mRNAs in a given cell. However, SS and exon choices can also vary within a given cell under different physiological conditions. Most important are changes in splicing patterns that occur during development while cells differentiate. Some of these splicing differences may persist and establish long-lasting tissue- or cell type-specific patterns of splicing.

The differential expression of mRNA isoforms by alternative splicing can depend on several parameters. Splicing regulatory proteins can be controlled in their abundance or activity. Of particular importance is the tissue-specific distribution of RNA-binding proteins acting as splicing regulators. In the central nervous system (CNS), alternative splicing is particularly important, as it allows for the differentiation of neurons in several ways.[15-18] An interesting switch in the abundance of the splicing regulator polypyrimidine tract binding protein (PTB) to its neuronal counterpart nPTB represents an important trigger in neuronal

differentiation. The downregulation of PTB is initiated by a micro-RNA, miR-124 and is then maintained by nPTB which negatively affects the inclusion of exon 11 in the PTB mRNA.[19-21] This PTB/nPTB switch leads to differential splicing of a few hundred mRNAs and thus plays a major role in reprogramming the cells that will become neurons. Another set of splicing regulators, the Nova proteins, are specifically expressed in neurons and appear to induce a similar switch which leads to synaptic maturation.[22-24] These are only a few examples which demonstrate the importance of alternative splicing in developmental processes, as a single splicing regulatory protein can affect the expression of dozens or hundreds of other genes.

Splicing and Disease

Viewing the importance and complexity of splicing, the fact that it plays an important role in many diseases is not surprising. Early studies indicating that ~15% of point mutations leading to genetic disease were due to RNA splicing defects[25] were probably underestimates for several reasons. The early genetic analyses relied exclusively on DNA samples and concentrated on the sequences of exons and their flanking SS. Only with recent progress in DNA sequencing, intronic sequences have really come under scrutiny. Moreover, some missense mutations may act through their effects on splicing rather than on the function of the protein.[26] Additionally, silent mutations which may have been classified as polymorphisms may in fact alter binding sites for splicing regulators.[27,28] Bioinformatic programs to predict regulatory binding sites (ESE, ESS, ISE, ISS) are still not very reliable. Finally, the organization of human genetic sampling is not geared to analyze RNA; this is even more of a problem when it is difficult or even impossible to obtain biopsies from the affected tissues.[29]

This slow development of splicing analyses as part of the genetic testing process is reflected by the fact that most splicing mutations reported so far are ones that destroy a SS, or create one by mutating a related sequence.[27,28] However, it is clear that splicing defects may also arise by mutations that cause steric block of SS,[30] modulate SS communication[31,32] or change RNA folding.[33,34] It is interesting that only very few mutations have been found in the core spliceosomal components and the splicing regulatory proteins, certainly due to lethal effects during early development.[35] An increasing number of mutations that is being recognized are those that affect regulatory sites in RNA (binding sites for SR and hnRNP proteins and other splicing regulators).[27,28] A particularly interesting example is Myotonic Dystrophy which is caused by a CUG trinucleotide expansion. The expanded repeats sequester the muscleblind protein that acts as a splicing regulator.[36] The resulting pleiotropic splicing defects are the immediate cause of the Myotonic Dystrophy.

SMA, Not a Splicing Disease by Itself, Calls for a Splicing Therapy

The neuromuscular disorder Spinal Muscular Atrophy (SMA) occurs with a carrier frequency between 1/35 and 1/80 leading to an affected individual every 1 in 6,000 to 1 in 10,000 live births.[37-39] It is the second most common autosomal recessive disease in Caucasians after Cystic Fibrosis and a frequent cause of early infant mortality. Ailment progression is characterized by a degeneration of α-motoneurons in the anterior horn of the spinal cord resulting in symmetrical muscle weakness and paralysis of both, proximal and distal muscles. Depending on the time of onset, severity of phenotypes and age at death, SMA has been divided into several types:

- Type I (Werdnig-Hoffmann disease), the most severe form, characterized by appearance of muscular weakness during the first months of life and an average lifespan of 8 months.
- Type II (intermediate form), with clinical manifestation starting 6–18 months after birth and life expectancy of 2–30 years.
- Type III (Kugelberg-Welander disease); first impacts are typically observed after the second year of life; patients often get wheelchair-bound within or after adolescence.

Two additional types are described in the literature, namely the very severe type 0, with prenatal onset and early neonatal death and type VI, a genetically heterogeneous appearance with comparatively mild consequences emerging only during adulthood.[40]

More than 95% of all SMA cases are due to mutations or deletions in the gene *SMN1 Survival of Motor Neuron 1,* located in chromosome region 5q12-5q13.[38] This region contains a 500-kb inverted duplication. The telomeric *SMN1* and centromeric *SMN2* genes differ in only a few nucleotides (none of which affect the encoded protein sequence). The most relevant of these is a C to T transition in exon 7 (see below). Due to gene conversion and duplication events, the number of *SMN2* copies in patients can vary, and this was shown to modulate the severity of the disease phenotype.[41] The SMN protein is essential for normal development and cell survival, as homozygous deletions of the single *Smn* gene in mice have been shown to be lethal in early embryonic stages.[42,43] Human survival is only possible due to the second gene copy, and all SMA patients therefore carry at least one *SMN2* gene.

The SMN protein is composed of 249 amino acids and migrates at 38 kDa on SDS gels.[44] Although it is ubiquitously expressed, its levels vary between different tissues. In healthy adults, particularly high amounts of the protein are found in spinal cord, kidney, liver and brain tissue, whereas skeletal and cardiac muscle show intermediate amounts, and even lower quantities occur in fibroblasts and lymphocytes.[45] Its expression is also developmentally regulated, displaying the highest abundance in prenatal phases.[46]

That SMN is an essential protein for all cells can be explained by its well characterized biochemical function. Together with a small number of other proteins, called Gemins 2–8, it forms the SMN complex which mediates the cytoplasmic assembly of the spliceosomal small nuclear ribonucleoproteins (snRNPs) U1, U2, U4 and U5, their counterparts from the minor spliceosome (U11, U12, U4atac) and the U7 snRNP involved in histone RNA 3' end processing.[47-52] Besides this cytoplasmic function, the SMN complex is also found in nuclear foci, called gems (for gemini of Cajal bodies), which often colocalize with Cajal bodies. In these locations, the SMN complex is proposed to play a role in snRNP

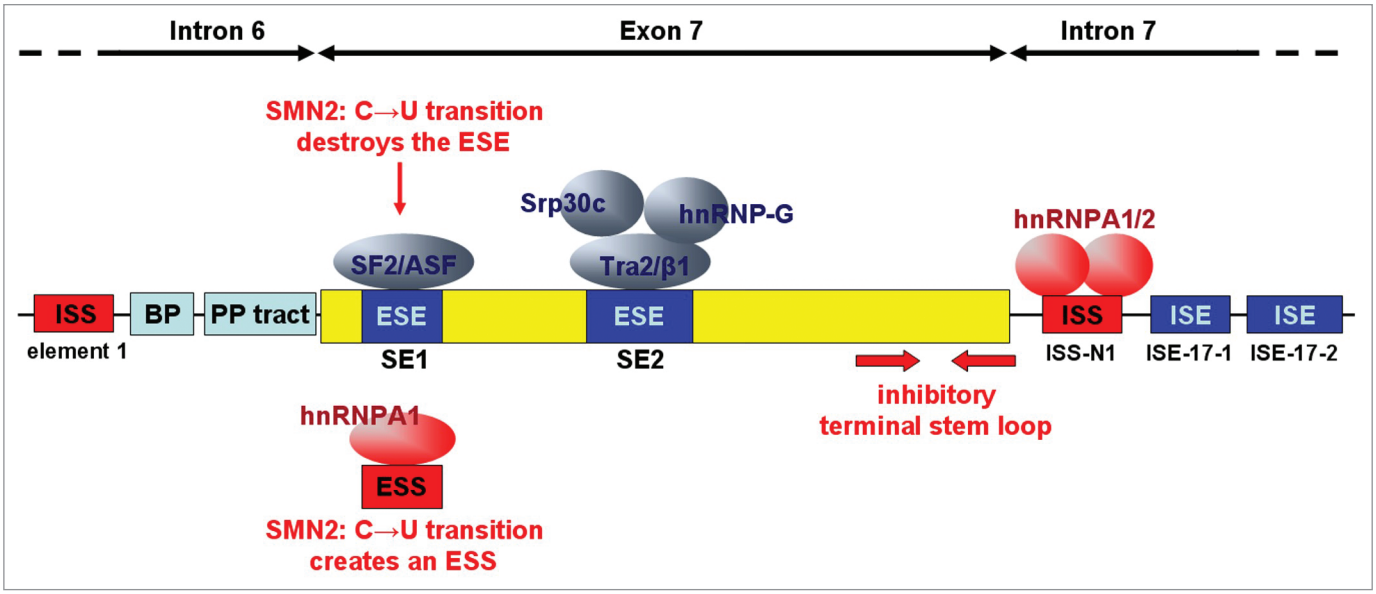

Figure 2. Splicing architecture of exon 7 of the human *SMN1* and *SMN2* genes. The diagram represents exon 7 (yellow box) and its flanking intronic regions (lines). Elements inhibiting exon 7 inclusion are shown in red, whereas the positive elements are represented in dark blue. The suboptimal branch point (BP) and polypyrimidine tract (PP tract) are indicated in light blue. SF2/ASF and Tra2/β1 bind to the exonic splicing enhancers SE1 and SE2, respectively. The recognition of SE1 by SF2/ASF is prevented in *SMN2*, due to the C → U transition. This sequence alteration also creates a hnRNP A1-dependent splicing silencer (see main text for refs.).

maturation, recycling or turnover.[53,54] Moreover, SMN has been proposed to play a role in nuclear import of snRNPs,[55] together with snurportin which is an import adaptor recognizing the trimethyl guanosine cap structure of snRNAs that is generated after SMN-mediated assembly has taken place.[56]

Besides this well-characterized function in snRNP maturation, SMN has also been proposed to play a direct role in splicing[48] or to participate in the assembly of other ribonucleoprotein particles.[57]

It is presently still not clear if the motoneuron defects seen in SMA patients are related to SMN's role in snRNP metabolism. Although partial and unequal deficiencies in snRNP concentrations have been observed,[58,59] it is still unclear whether the primary defect is a disturbance of splicing patterns that would be most pronounced or whose effects would be most damaging for motoneurons.[60] An alternative explanation is that some additional but less well characterized interactions[61] and functions of SMN could be vital to the maintenance of motoneuron functionality and viability.[62,63] This latter view is supported by studies showing that the SMN protein is present in transport granules of cultured motoneurons travelling down the axon.[64,65] Some of SMN's interaction partners such as hnRNP R and Q,[66] profilins (proteins associated with microfilaments)[65,67] or the Fragile X Mental Retardation protein FMRP[68] support such a peripheral function. Thus it has been proposed that SMN could be involved in the transport or translational regulation of actin mRNA or other so far not characterized mRNAs and thereby control the organisation of the axon terminal.[62,65] Interestingly, the phenotypes observed in SMA mouse models all show first perturbations of the neuromuscular junctions, before an actual cell death can be confirmed in the spinal cord.[69-76] This strongly favours a dying back mechanism whereby the functionality of the synapse in the periphery gets primarily disturbed, suggesting initial mechanisms being disrupted at this place.[77] It has to be mentioned that the the two hypotheses—splicing defect versus motoneuron-specific role—do not exclude each other and can actually be combined.

The Architecture of *SMN* Genes with Regard to Exon 7 Definition

Although the two *SMN* gene copies are nearly identical, *SMN2* is not able to completely substitute for the loss of function due to destroyed *SMN1*. The problem is that one of the previously mentioned nucleotide differences, the C → U transition at position 6 of exon 7, changes the splicing pattern such that this exon is mostly excluded from the native mRNA, thereby leading to the predominant production of a truncated protein during translation.[78] This truncated protein (SMNΔ7) retains some functionality, as evidenced by slightly longer survival times when it is expressed in SMA mice (on a background of deleted mouse *Smn* genes and two copies of human *SMN2*).[79] However, SMNΔ7 is unable to oligomerize[80] and therefore very unstable.[81]

The question how an exchange of a single nucleotide can have such a detrimental effect on pre-mRNA splicing can partly be explained by the structure and sequence of this exon and its environment. Of three initially characterized ESEs,[81] two, SE1 and SE2, seem to be functional (**Fig. 2**). SE2 can be bound by the SR-like splicing factor hTra2-β1, which increases the inclusion of this exon in a dose-dependent manner[82] by forming stable complexes with two other splicing factors, namely SRp30c[83] and hnRNP G.[84] The SE1 element acts as an SF2/ASF-dependent splicing enhancer which is destroyed by the critical C6U

mutation.[85,86] However, this mutation additionally appears to create a new exonic splicing silencer signal (ESS) that is bound by hnRNP A1.[87,88]

Obviously, the exon 7 seems to be heavily dependent on regulatory splicing signals, all the more as it is flanked by two intronic splicing silencer sequences (ISSs), one lying within intron 6 (termed element 1; **Fig. 2**)[89] and the other in intron 7 (termed ISS-N1).[90] The latter was recently shown to contain two binding sites for hnRNP A1/A2.[91] Finally, two additional intronic splicing enhancer sequences were discovered in intron 7 that strongly influence the exon 7 inclusion ratio.[92]

Compared to the average length of human exons, which lies around 129 base pairs,[93] this crucial exon 7 appears to be extremely short, consisting of only 54 bp. An in vivo SELEX study performed with a randomized *SMN1* exon 7 sequence came to the conclusion that both, the 5' and the 3' endings seem to predispose it for exclusion.[94] On the 5' end, positions 5–15 create an overall negative surface for splicing. The 3' end shows an element with negative splicing impacts as well, namely at position 45–52. This region also seems to be involved in a secondary structure that affects exon 7 inclusion.[95] Finally, the last exonic nucleotide represents an A instead of the canonical G found in more than 80% of all human exons.[7] As a consequence, the binding affinity of the U1 snRNP is reduced.[94,96] In addition to these exonic features, the polypyrimidine tract at the 3' SS[81] and the BP[97] preceding exon 7 are suboptimal.

In summary, the SMN exon 7 is rather poorly defined, and positive and negative elements create a very sensitive equilibrium towards exon inclusion. The vast majority of transcripts from the *SMN1* gene (approximately 90%) contains all 9 exons (full-length transcripts) and the remaining 10% are mostly missing exon 5.[45] In *SMN2*, however, this balance is disturbed by converting an enhancer motif into a silencer, and the vantage therefore tips to predominant exclusion. The *SMN2* gene in fact produces different mRNA isoforms, some lacking exon 5, exon 7, or even both exons as well as full-length mRNA. The full-length mRNA accounts for only 20–30% of the total,[98,99] even though this percentage strongly varies between different patients and tissues. Thus, the production of full-length SMN protein is severely reduced in SMA patients, since a functional *SMN1* gene copy is absent.

Promising Approaches to Improve *SMN2* Exon 7 Inclusion

Strategies aiming to repair splicing defects are being developed for several diseases and by many different laboratories. SMA is only one example, albeit an important one for which multiple strategies have been tested.

However, before we delve into a discussion of these approaches, it should be mentioned that strategies which are not directed at correcting *SMN2* splicing are also being tried with some success. For example, it is possible to enhance the promoter activity of the *SMN2* gene. Valproate, a histone deacetylase (HDAC) inhibitor has been shown to increase the production of full-length SMN and to extend the survival of transgenic SMA mice deleted for mouse *Smn* and expressing human *SMN2*.[79,100,101] However, a study performed with SMA patients showed no increase of SMN.[102] Another HDAC inhibitor, phenylbutyrate, yielded promising results in vitro,[103] but a test performed on a large cohort of SMA type II patients did not show an improvement.[104] Hydroxyurea (HU), a ribonucleotide reductase inhibitor, is also able to increase the expression of *SMN* in SMA cells in vitro,[105,106] and a test performed on 33 patients of SMA types II and III, showed a slight increase in muscle strength and full-length SMN mRNA in some patients.[106] A recent drug screen revealed that certain 2,4-diaminoquinazoline derivatives also stimulate the SMN promoter and can increase the lifespan of SMNΔ7 SMA mice by approximately 21–30% when given prior to motoneuron loss.[107] Overall, these studies need further investigation and cohort selection to really evaluate the gain obtained with these treatments. In addition to such efforts to increase *SMN2* transcription, a forced translational read-through of the premature termination codon of SMNΔ7 transcripts by aminoglycoside compounds was also shown to increase functional SMN protein levels in fibroblasts derived from SMA patients.[108] Finally, it should be mentioned that a gene replacement therapy is also a valid option, despite the size of the SMN gene. Two recent reports have demonstrated that different adeno-associated virus (AAV) vectors expressing SMN cDNA can reach motoneurons and partly rescue newborn mice that would otherwise show a severe SMA phenotype.[109,110] Moreover, neural stem cell therapy may also be an option for treating SMA.[111]

Small molecule drugs improving *SMN2* exon 7 inclusion. Focusing on splicing correction strategies for SMA, certain drugs have been shown to modulate alternative splicing decisions,[112,113] but in general their specificity is low and side effects are therefore a major concern. Concerning SMA, beneficial splicing effects were reported for aclarubicine,[114] but the drug proved to be quite toxic. Another drug found to improve *SMN2* exon 7 inclusion is a tetracycline-like compound, termed PTK-SMA1.[115] However, this compound does not appear to cross the blood-brain barrier, so that further chemical derivatives or sophisticated modes of application such as cerebrospinal fluid instillation may be required.

Oligonucleotide-based approaches. Very specific splicing correction can be obtained by the use of antisense oligonucleotides (AONs). AONs are ideally suited to hybridize to specific pre-mRNA sequences involved in the splicing of a particular gene.[116] They can contain different types of modifications that stabilize them and/or allow for more efficient target sequence hybridisation.[117] Most often this approach is used to block a particular splice site or splicing enhancer and thereby to induce the skipping of target exon.[116,118] However, in the case of SMA the problem is not to skip an exon but to enhance its utilisation by the splicing machinery which asks for more sophisticated approaches. Another problem specific for using AONs to treat SMA may again be how to cross the blood-brain barrier and to reach the spinal cord motoneurons.

One of the AON strategies used in the SMA context (**Fig. 3A**) is based on the idea that exon 7 skipping at least partly results from a competition between the 3' SS of exons 7 and 8. Thus, to

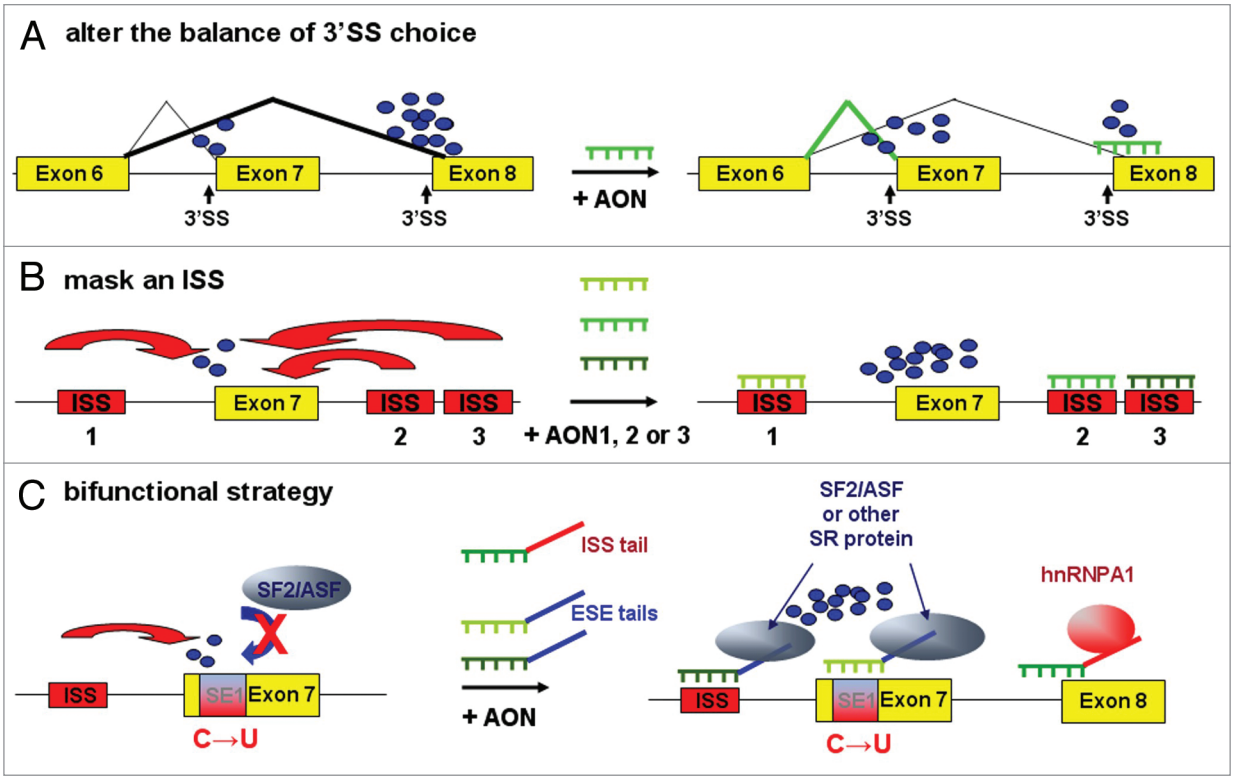

Figure 3. Schematic representation of the different AON-based splicing correction strategies for SMA. Red and blue arrows indicate negative and positive splicing effects, respectively. Small blue dots signify unspecified splicing factors necessary for exon definition and splicing. (A) In a concept of 3' SS competition where exon 8 prevails over exon 7, an AON masking the 3' SS of exon 8 will partly shift splicing factor recruitment to exon 7. (B) Masking any of the flanking ISS by an AON can stimulate exon 7 inclusion in the mRNA. (C) The bifunctional strategy allows to tether binding sequences for different SR proteins to either ISS element 1 or the altered SE1 sequence and thereby to enhance the recruitment of the splicing machinery to exon 7. As the mutated SE1 element is also a splicing silencer, both approaches have dual effects by masking a negatively acting element and by recruiting positively acting SR proteins. An AON targeting the exon 8 3' SS that bears a tail with a binding sequence for hnRNP A1 will also more efficiently shift splicing factor recruitment to exon 7 than the corresponding tail-less AON (see main text for refs.).

change the balance of 3' SS use in favour of exon 7, AONs targeting the 3' SS and BP region of exon 8 were tested. Indeed, several of these AONs increased the proportion of final transcripts with inclusion of exon 7 in tissue culture experiments.[119] Other AON strategies aimed at masking inhibitory sequences that limit exon 7 inclusion (**Fig. 3B**). In fact AONs have been instrumental at defining several of these inhibitory elements. In particular, AONs masking sequences just downstream of the exon 7 5' SS contributed to the discovery of the potent ISS-N1 and caused reinclusion of exon 7 in SMA patient fibroblasts.[90] In a recent study, even shorter AONs were tested, and an 8-mer was shown to induce specific exon 7 inclusion and to restore SMN protein levels in SMA patient cells even when applied in nanomolar concentrations.[120] Finally, the periodic intracerebroventricular instillation into SMA mice of a longer AON targeting this ISS-N1 restored SMN expression in brain and spinal cord up to 50% of the level found in control mice and led to a slight amelioration of disease symptoms.[121]

A systematic screen of a large number of AONs hybridizing to different positions of exon 7 revealed several AONs that led to efficient exon 7 reinclusion.[122] These oligonucleotides may either have interfered with crucial binding sites for splicing regulatory proteins or have disrupted inhibitory RNA secondary structures. The same principle of oligo walk was also performed in the intronic regions flanking exon 7, leading to the discovery of a weak ISS in intron 6 and of two tandem hnRNP A1/A2 motifs[91] in the previously described strong intron 7 ISS-N1.[90] Certain AONs targeting this region also increased *hSMN2* exon 7 inclusion in the liver and kidney of SMA mice.

An interesting expansion of the antisense approach was introduced almost simultaneously by two groups in 2003. In this so-called bifunctional strategy, a functional moiety is added to an AON (**Fig. 3C**). Both groups targeted the region of *SMN2* exon 7 that carries the C → T transition and used a functional moiety that should replace the missing splicing enhancer protein. In one case, the functional moiety used was a repeating SR peptide coupled to the AON.[123] In the other study an RNA sequence containing a repeated binding sequence for a SR protein (ASF/SF2) was added.[124] Both of these approaches indeed led to considerable exon 7 reinclusion and a partial restoration of SMN protein levels in SMA patient fibroblasts. Two further developments of this bifunctional concept were recently published by Lorson and colleagues. In one case, an AON targeting the 3' SS region of exon 8 was combined with a functional sequence able to attract hnRNP A1 and thereby to increase the inhibitory effect on exon 8.[125] In the other case, the antisense moiety targeted the element 1 ISS

located in intron 6 of *SMN2*, whereas the functional moiety was an ESE tail recruiting positive splicing factors.[126] Both of these approaches led to improved exon 7 reinclusion compared to the corresponding simple AONs lacking the functional moiety. They were also tested by intraventricular injections into the brains of SMA mice. In both studies, increases in brain SMN protein levels were observed. In the second study, SMN levels were also increased in various sections of the spinal cord and a small positive effect on weight development and survival of the mice could be demonstrated.[126] This, so far, has been the only study that has demonstrated a therapeutic effect of an oligonucleotide in a mouse SMA model.

Based on all these studies, multiple oligonucleotides—simple AONs or bifunctional ones—are now available that potently improve *SMN2* exon 7 inclusion. The main problem currently preventing their use in clinical studies is the fact that they do not cross the blood-brain barrier. It is not clear if cerebrospinal fluid instillations or other types of introduction into the central nervous system will be a valid option for human patient therapy, especially if the treatments have to be applied on a life-long basis. Another point to consider is whether a repeated deposition into the central nervous system of oligonucleotides that are chemically designed to be undegradable or slowly degraded will not have unexpected long-term toxic consequences.

Approaches based on in vivo expressed RNAs. To circumvent the problems of oligonucleotide delivery and to allow for a more permanent therapy, antisense sequences designed for splicing modulation can also be introduced into RNA expression cassettes designed to be transcribed in vivo within the target cells. As splicing occurs in the nucleus, expression cassettes based on small nuclear RNAs are particularly suited for this purpose, and the most frequently used expression system is based on a modified gene for U7 snRNA.[127,128] The U7 snRNP is an essential factor involved in 3' end processing of animal replication-dependent histone mRNAs, that lack the typical poly(A) tail present on all other eukaryotic mRNAs.[129,130] A set of three point mutations introduced in the non-canonical Sm binding site of U7 snRNA (AAU UUG UCU AG) that render it similar to the consensus Sm binding site of the spliceosomal snRNAs (AAU UUU UGG AG) leads to an increased nuclear accumulation of the snRNP particle (termed U7 Sm OPT), which however is non-functional in histone RNA processing.[131,132] The reason for this lack of function is the replacement of the U7-specific Sm core structure, composed of Lsm10 and Lsm11 and five Sm proteins, by a standard Sm core composed of all seven Sm proteins found in spliceosomal snRNPs.[52,130,133] These observations fostered the idea that this "generic," but function-less U7 Sm OPT snRNP could serve as a platform for antisense sequences designed to modulate specific splicing events (**Fig. 4A** and inset). As was the case for AONs, such modified U7 snRNAs were initially almost exclusively designed to induce exon skipping in genes involved in human diseases such as β-thalassemia,[134-137] Duchenne muscular dystrophy,[138-140] or HIV/AIDS.[141-143]

Focusing on SMA, a number of different U snRNA-based approaches have been used with variable success (**Fig. 4A**). Several U7 Sm OPT derivatives targeting the 3' SS and BP region of *SMN2* exon 8 were found to induce exon 7 reinclusion,[144] similar to what had been observed with AONs targeting the same sequences.[119] In our laboratory, we have been able to reproduce these findings, but have found this strategy to be less efficient than the bifunctional approach (see below).[96] Such U7 constructs were also stably introduced into SMA patient fibroblasts by means of lentiviral[96] or adenoviral[145] vectors leading to a stimulation of full length *SMN2* mRNA and protein.

In our laboratory, we have tested multiple U snRNA-based approaches to enhance *SMN2* exon 7 inclusion. Based on the observation that the BP preceding exon 7 is suboptimal, we tried to express a U2 snRNA derivative with increased complementarity to this BP region.[97] However, no improvement of *SMN2* splicing could be observed, despite the fact that a BP mutation increasing the complementarity to U2 snRNA stimulated exon 7 inclusion. It is possible that the U2 modification interfered with U2 snRNP assembly or that the limiting step in *SMN2* exon 7 definition is not the binding of the U2 snRNP but the prior recognition of the BP region by the splicing factor SF1.[146,147] Another strategy was to design a modified U1 snRNA that would bind more efficiently to the suboptimal exon 7 5' SS that features an adenosine in the last exon position. Although this approach led to a strong exon 7 inclusion, the stable expression of this U1 snRNA was toxic to cells, most likely because splicing efficiencies of other exons containing an A in this position were altered or because of the activation of cryptic splice sites (Marquis J, Meyer K and Schümperli D, unpublished observations). We also tried to target the intron 7 ISS-N1 with U7 Sm OPT derivatives, but this caused an even stronger skipping of exon 7, possibly due to steric interference of the U7 snRNP moiety with spliceosome assembly at the 5' SS.[96]

The most efficient and viable approach in our hand, however, has been an adaptation of the bifunctional AON approach to a U7 Sm OPT construct.[96] Two different regions in exon 7 were targeted, and the U7 snRNA derivatives additionally contained different sequence repeats able to bind to various SR proteins. One of these RNAs targeting the 3' region of exon 7 with an ESE tail induced 90% of full-length *SMN2* mRNA both in a HeLa cell minigene test system and in SMA patient fibroblasts. In the latter cells, the level of SMN protein was increased to about 50% of the level seen in wild-type human fibroblasts which contain two copies each of *SMN1* and *SMN2*. Considering that human SMA carriers with a single *SMN1* copy are fully viable, this increase was highly significant. More important, however, transgenic SMA mice carrying this U7 construct showed an extended lifespan (median of 124 days, compared to 5–7 days for untreated SMA animals), a greatly improved muscle performance and normal motoneuron counts at 1 month of age.[148] Early ultrastructural alterations in neuromuscular junctions of the diaphragm of SMA mice were also prevented.[76] These studies proved for the first time that the idea of improving *SMN2* splicing to cure SMA is not a mere hypothesis but, in fact a realistic strategy. It should also be mentioned that some success in cell culture models was reported for a similar bifunctional RNA driven by the U6 promoter, but lacking a snRNA backbone.[149] However, using an almost identical construct, we have not been able to reproduce this result in our hands.[96] The bifunctional RNA was almost undetectable and

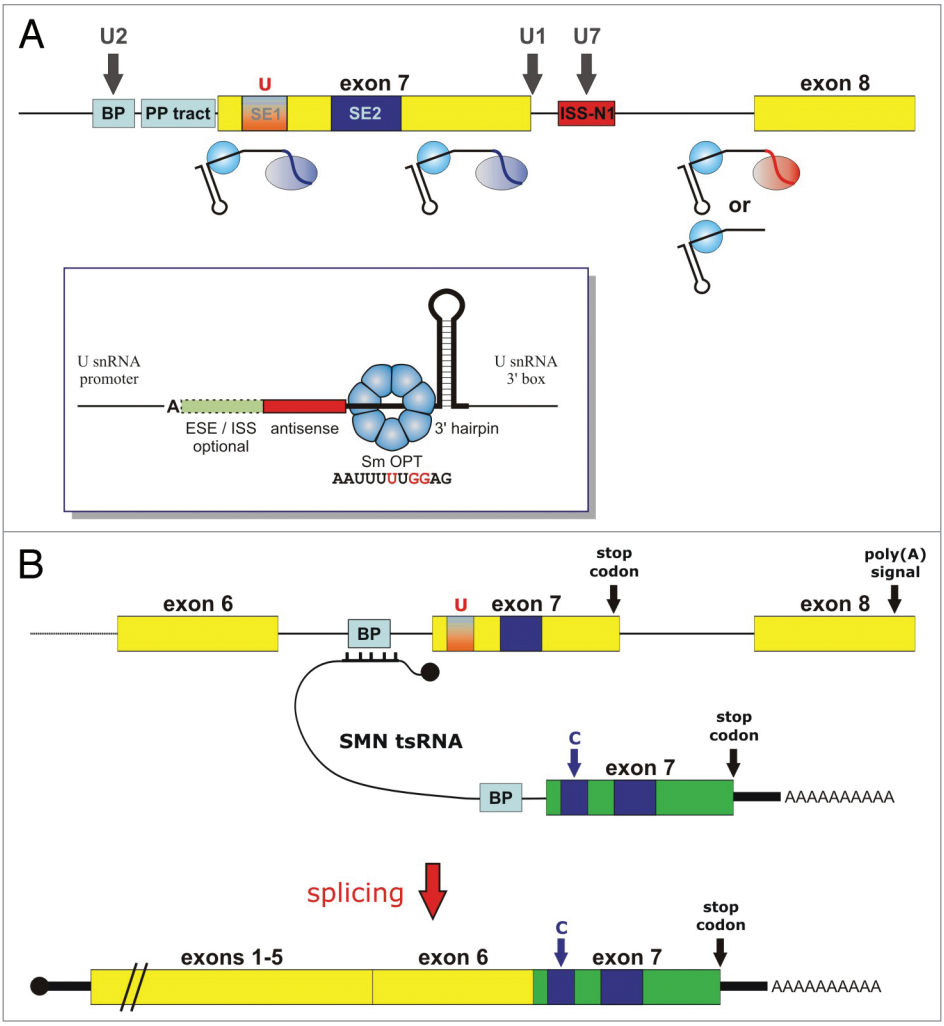

Figure 4. Scheme depicting splicing correction strategies for SMA based on in vivo expressed RNAs (A) SnRNA-based strategies. Bifunctional U7 Sm OPT derivatives have been designed to tether SR proteins to various positions in exon 7. Alternatively, U7 Sm OPT derivatives can mask the BP and 3' SS of exon 8, either with or without tethering hnRNP A1. Shown with dark grey vertical arrows are snRNA-based strategies that were either not successful (U2 snRNA fully complementary to BP upstream of exon 7), inhibitory (U7 Sm OPT targeting ISS-N1) or toxic to cells (U1 snRNA fully complementary to the exon 7 5' SS). Inset: Basic structure of a U7 Sm OPT derivative. The important elements are (from 5' to 3'): the antisense sequence, the Sm OPT site capable of assembling with a heptameric Sm core of the standard Sm proteins (nucleotide changes respective to wild-type U7 snRNA are shown in red), and a 3'-terminal hairpin which stabilises the RNA. Splicing enhancer or silencer sequences can be added at the 5' end to generate bifunctional U7 snRNAs. Note that transcription from a U snRNA promoter is important to allow efficient 3' end formation at the U snRNA 3' box and assembly into a snRNP particle. Moreover, mammalian U7 snRNAs start with an adenosine residue. (B) Trans-splicing strategy. A SMN-specific trans-splicing RNA (tsRNA) will bind to the BP/3' SS region upstream of *SMN2* exon 7 (for simplicity only the BP is shown) and will contain a strong BP/3' SS leading into a *SMN1*-specific version of exon 7 (shown in green) and ending in a poly(A) tail downstream of the stop codon. After splicing, this *SMN1*-specific exon 7 will be fused to the body of the endogenous *SMN2* mRNAs containing exons 1–6. Black knobs indicate the cap structures at the 5' ends of the RNAs involved (see main text for refs.).

presumably also not concentrated in the nucleus, underlining the importance of a snRNA backbone for RNA stability and nucleoplasmic accumulation.

An entirely different approach to repair the splicing defect in *SMN2* is the use of a method known as spliceosome-mediated RNA trans-splicing (SMaRT).[150] It consists of repressing the 3' SS of an exon by an antisense RNA sequence that is followed by an exon to be trans-spliced to the body of the message (**Fig. 4B**). In particular, Coady and coworkers have developed a therapeutic trans-splicing RNA base-pairing with the intron 6 of *SMN2* and containing the *SMN1* exon 7 sequence.[151] The final product after splicing is a chimeric mRNA containing *SMN2*-exons 1–6, followed by *SMN1* exon 7. The transduction of SMA fibroblast cells with an AAV vector carrying this construct increased SMN protein levels.

For a somatic gene therapy in experimental animals or in human patients, these therapeutic RNA genes will have to be introduced into spinal cord motoneurons. This will require appropriate transfer vectors. Some initial success was obtained by Azzouz, Mazarakis and coworkers by using lentiviral vectors,[152,153] but recent results indicate that self-complementary AAV9 vectors can efficiently transduce motoneurons, when injected into

newborn mice, cats or non-human primates.[109,154,155] In particular, two groups have recently reported a significant clinical amelioration in SMA mice after scAAV9- or scAAV8-mediated introduction of a SMN cDNA.[109,110] Future work will have to reveal which type of gene therapy is most efficient in a human clinical setting, a gene replacement approach as used in these two studies or one of the splicing correction strategies described above.

Acknowledgements

We are thankful for financial support by the Kanton Bern as well as by grants of the AFM (Association Française contre les Myopathies), EURASNET (European Network of Excellence on Alternative Splicing), Swiss Foundation for Research on Muscle Diseases (to K.M.) and Swiss National Science Foundation (grant 3100A0-120064).

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