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Evolving Concepts on Human SMN Pre-mRNA Splicing

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Abstract
SMN1 and SMN2 represent two nearly identical copies of the Survival Motor Neuron gene in humans. Deletion of SMN1 coupled with the inability of SMN2 to compensate for the loss of SMN1 leads to Spinal Muscular Atrophy (SMA), a leading genetic cause of infant mortality. SMN2 holds the promise for cure of SMA if skipping of exon 7 during pre-mRNA splicing of SMN2 could be prevented. Previous reports have shown that a C to T mutation at the 6th position of exon 7 (C6U substitution in the transcript) is the primary cause of SMN2 exon 7 skipping. Cumulative evidence suggests that C6U abrogates an enhancer associated with SF2/ASF, as well as, creates a silencer associated with hnRNP A1. There is also evidence to suggest that C6U creates an extended inhibitory context (Exinct). Recently, an intronic hnRNP A1 motif, which is not conserved between two human SMN genes, have been implicated in skipping of SMN2 exon 7. However, mechanism by which two SMN2-specific hnRNP A1 motifs interact is not known. Systematic approaches including site-specific mutations, in vivo selections, RNA structure probing and antisense oligonucleotide microwalks have revealed additional cis-elements in exon 7 as well as in flanking intronic sequences. A unique intronic splicing silencer (ISS-N1) has emerged as an effective target for correction of SMN2 exon 7 splicing by short antisense oligonucleotides (ASOs). Low nanomolar concentrations of ASOs against ISS-N1 fully restored SMN2 exon 7 inclusion and increased levels of SMN in SMA patient cells. Such a robust antisense response could be due to accessibility of the target as well as the complete nullification of a strong inhibitory impact rendered by ISS-N1. Bifunctional oligonucelotides with capability to recruit stimulatory splicing factors in the vicinity of weak splice sites of exon 7 have also shown promise for correction of SMN2 exon 7 splicing. Considering an antisense-based strategy confers a unique advantage of sequence specificity, availability of many target worthy cis-elements holds strong potential for antisense-mediated therapy of SMA.

Keywords
SMN, SMA, Alternative splicing, Antisense Oligonucleotide, RNA structure, enhancer, silencer, ISS-N1

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Point of View

Evolving Concepts on Human *SMN* Pre-mRNA Splicing

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KEY WORDS

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ABBREVIATIONS

ASO	antisense oligonucleotide
C6U	a cytosine to uridine mutation at the 6th position of SMN2 exon 7
Exinct	extended inhibitory context
ISS-N1	intronic splicing silencer N1
SMA	spinal muscular atrophy
SMN	survival motor neuron gene
TSL	terminal stem loop

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ABSTRACT

SMN1 and *SMN2* represent two nearly identical copies of the *survival motor neuron* gene in humans. Deletion of *SMN1* coupled with the inability of *SMN2* to compensate for the loss of *SMN1* leads to spinal muscular atrophy (SMA), a leading genetic cause of infant mortality. *SMN2* holds the promise for cure of SMA if skipping of exon 7 during pre-mRNA splicing of *SMN2* could be prevented. Previous reports have shown that a C to T mutation at the 6th position of exon 7 (C6U substitution in the transcript) is the primary cause of *SMN2* exon 7 skipping. Cumulative evidence suggests that C6U abrogates an enhancer associated with SF2/ASF, as well as, creates a silencer associated with hnRNP A1. There is also evidence to suggest that C6U creates an extended inhibitory context (Exinct). Recently, an intronic hnRNP A1 motif, which is not conserved between two human *SMN* genes, has been implicated in skipping of *SMN2* exon 7. However, mechanism by which two *SMN2*-specific hnRNP A1 motifs interact is not known. Systematic approaches including site-specific mutations, in vivo selections, RNA structure probing and antisense oligonucleotide microwalks have revealed additional cis-elements in exon 7 as well as in flanking intronic sequences. A unique intronic splicing silencer (ISS-N1) has emerged as an effective target for correction of *SMN2* exon 7 splicing by short antisense oligonucleotides (ASOs). Low nanomolar concentrations of ASOs against ISS-N1 fully restored *SMN2* exon 7 inclusion and increased levels of SMN in SMA patient cells. Such a robust antisense response could be due to accessibility of the target as well as the complete nullification of a strong inhibitory impact rendered by ISS-N1. Bifunctional oligonucelotides with capability to recruit stimulatory splicing factors in the vicinity of weak splice sites of exon 7 have also shown promise for correction of *SMN2* exon 7 splicing. Considering an antisense-based strategy confers a unique advantage of sequence specificity, availability of many target worthy cis-elements holds strong potential for antisense-mediated therapy of SMA.

SMN1 and *SMN2* represent two nearly identical copies of the *survival motor neuron* gene in humans.[1] *SMN1* predominantly produces full-length transcripts, whereas *SMN2* mostly produces transcripts lacking exon 7. Full-length SMN protein (the product of full-length transcript) is absolutely necessary for the survival of all higher eukaryotes.[2] Deletion of *SMN1* coupled with the inability of *SMN2* to compensate for the loss of *SMN1* leads to spinal muscular atrophy (SMA), the second most common autosomal recessive genetic disorder in humans.[1-3] It is generally believed that correction of *SMN2* exon 7 splicing holds the promise for cure of SMA. However, this requires a full understanding of *SMN2* exon 7 splicing. Comparison between *SMN1* and *SMN2* revealed a critical C to T mutation at the 6th position (C6U transition in transcript) of exon 7 of *SMN2*. C6U is sufficient to cause *SMN* exon 7 skipping.[4,5] *SMN2* exon 7 seems to have a weak 3′ splice site (3′ss). Consistently, an improved polypyrimidine tract (PPT), which defines the 3′ss, restored inclusion of *SMN2* exon 7.[6] However, an improved PPT is not sufficient to compensate for the loss of a purine-rich exonic splicing enhancer (ESE) in the middle of exon 7.[6] This ESE was identified as the binding site for splicing factor Tra2-β1.[7] Over expression of Tra2-β1 or associated factors hnRNP G and SRp30c restores inclusion of *SMN2* exon 7.[8,9] Also, STAR (Signal Transduction and Activation of RNA) family of proteins have been implicated in tissue-specific regulation of *SMN* exon 7 splicing.[10] Recent reports reveal new and rather unique cis-elements and provide an advanced understanding of *SMN* exon 7 splicing.

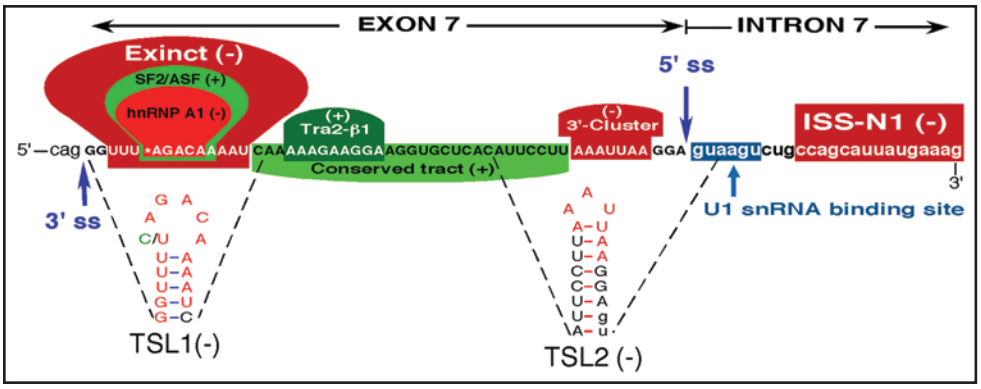

Figure 1. Diagrammatic representation of *SMN* exon 7 and adjacent intronic sequences. Sequences highlighted in red indicate negative cis-elements, whereas sequences highlighted in green indicate positive cis-elements. Capital letters represent exonic and small-case letters represent intronic nucleotides. *represents 6th exonic position that is C in *SMN1* and U in *SMN2*. U1 snRNA binding site is indicated along with positive (+) and negative (-) cis-elements, which promote and inhibit exon 7 inclusion, respectively. Exinct, Conserved tract and 3'-Cluster were discovered by in vivo selection of the entire exon 7.[17] Binding sites for splicing factors SF2/ASF, hnRNP A1 and Tra2-β1 have been described by others.[7,11-13] ISS-N1 is a novel cis-element with strong negative effect.[23] RNA structures TSL1 and TSL2 have been confirmed by enzymatic probing.[20]

COMPETING MODELS FOR SINGLE MUTATION CAUSING EXON 7 SKIPPING

Mechanism by which C6U promotes *SMN2* exon 7 skipping remains a complex issue. Theoretically, C6U could create one or a combination of situations including but not limited to abrogation of an enhancer, creation of a silencer, disruption of a stimulatory RNA structure and strengthening of an inhibitory RNA structure.[2] Based on in vitro experiments, an initial study supported an enhancer model.[11] According to this model, C6U abrogates an essential ESE associated with splicing factor SF2/ASF. Proponents of this model have recently presented additional data to support a direct role of SF2/ASF in *SMN1* exon 7 splicing.[12] However, a critical evidence against SF2/ASF model came from an in vivo experiment in which absence of SF2/ASF in a chicken cell line did not cause the anticipated skipping of *SMN1* exon 7.[13] This finding may suggest that any direct (or indirect) role of SF2/ASF is replaceable by other splicing factors in case of *SMN1* exon 7 splicing. Results obtained from chicken cell line paved the way for a competing model in which C6U creates an exonic splicing silencer (ESS) associated with splicing factor hnRNP A1.[13] According to this model, binding of hnRNP A1 to *SMN2* exon 7 causes exon 7 skipping. Strongest evidence in support of hnRNP A1 model came from RNA interference (RNAi) experiment in which depletion of hnRNP A1 promoted exon 7 inclusion in *SMN2*.[13] However, RNAi results left open the possibility of a cumulative effect of multiple hnRNP A1 targets. Indeed, a recent report has confirmed the existence of another functional hnRNP A1 motif within intron 7 of *SMN2*.[14]

Evolutionary relevant single mutations tend to have multidimensional consequences. In case of C6U, it is likely that loss of an enhancer is accompanied by the gain of a silencer. In addition, C6U may strengthen an inhibitory RNA structure, TSL1 (terminal stem loop 1), which is formed by sequences at the 5' end of exon 7 (Fig. 1).[15] Consistently, numerous mutations in the vicinity of C6U supported an Extended inhibitory context or Exinct model.[15] Additional evidence in support of Exinct model came from a recent and rather very systematic study in which several antisense oligonucleotides (ASOs) that blocked Exinct region promoted *SMN2* exon 7 inclusion.[16] In an interesting way, this ASO-based approach revisited the issue of enhancer versus silencer model associated with C6U. The fact that ASO-mediated targeting of regions encompassing C6U restores *SMN2* exon 7 inclusion supports the role of a silencer. However, due to constraints of target accessibility and some discrepancies between in vitro and in vivo results, interpretations of antisense study should be treated with caution.

MULTIPLE EXONIC CIS-ELEMENTS CONTRIBUTE TOWARD EXON 7 SPLICING

To analyze all exonic cis-elements that modulate *SMN* exon 7 splicing, a novel approach of in vivo selection was used.[17-19] This approach applied the principle of mutability to determine the relative significance of all residues within exon 7. The highly mutable positions were considered as inhibitory, whereas the conserved positions were considered as stimulatory for exon 7 inclusion. Based on mutability plot, three major regulatory elements were determined (Fig. 1). Among them are Exinct and 3'-Cluster, the negative elements located towards the 5' and 3' ends of exon 7, respectively. "Conserved tract" is the third element, which plays a stimulatory role, and is located in the middle of exon 7. Here again, independent validation of cis-elements determined by in vivo selection came from the recently reported ASO-based approach.[16] For example, ASOs that blocked inhibitory regions covering Exinct or 3'-Cluster promoted exon 7 inclusion, whereas ASOs that blocked stimulatory regions covering Conserved tract promoted exon 7 exclusion.

One of the surprising outcomes of in vivo selection of entire exon 7 was the revelation that the 5'ss of exon 7 is extremely weak.[17] This was apparent from the overwhelming selection of a nonwild type G residue at the last position (54G) of exon 7. The impact of 54G was so profound that it promoted *SMN2* exon 7 inclusion even in the absence of Tra2-β1-ESE.[17] Preference for a nonwild type G over wild type A at the last position of exon 7 could be due to increase in size of the duplex formed between the 5'ss and U1 snRNA (abbreviated as 5'ss:U1 duplex). Stimulatory impact of a long 5'ss:U1 duplex at the 5'ss of exon 7 has been recently confirmed.[20] In addition to a small size of 5'ss:U1 duplex, other factors contribute towards a weak 5'ss of exon 7. Significant among them is an inhibitory RNA structure, TSL2 (terminal stem loop 2), formed by the last 17 residues of exon 7 and the first two residues of intron 7 (Fig. 1). Breaking of TSL2 promoted *SMN2* exon 7 inclusion, whereas strengthening of TSL2 promoted skipping of exon 7 even in *SMN1*.[20] Compensatory mutations provided the ultimate proof of inhibitory role of TSL2. For example, 40G and 54C mutations separately promoted *SMN2* exon 7 inclusion by breaking TSL2, whereas together these mutations led to a complete skipping of *SMN2* exon 7 by restoring and strengthening TSL2.[20]

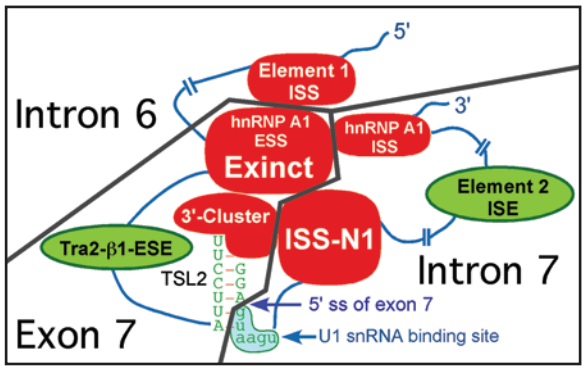

Figure 2. Diagrammatic representation of inhibitory cis-elements that promote *SMN2* exon 7 skipping. Negative cis-elements are shown in red, whereas positive cis-elements are shown in green. Thick black lines delineate exon/intron boundaries. Exinct, hnRNP A1 ESS, Tra2-ESE and 3'-Cluster are located in exon 7. Element 1 is located in intron 6, whereas ISS-N1, element 2 and hnRNP A1 ISS are located in intron 7. Partial sequence of TSL2 is shown. The 5' ss of exon 7, representing Exon 7/ intron 7 junction, and U1 snRNA-binding site are marked. As per one hypothesis, negative elements are brought together by looping out of intervening sequences.

ROLE OF INTRONIC REGULATORY ELEMENTS IN *SMN2* EXON7 SKIPPING

The 54-nucleotide long human *SMN* exon 7 is flanked by a large upstream intron 6 (~6kb) and a relatively short downstream intron 7 (~0.5kb). Exon trapping assay revealed a negative element (element 1) in intron 6 and a positive element (element 2) in intron 7 (Fig. 2).[21,22] Recently, a strong intronic splicing silencer, ISS-N1, has been identified in intron 7 (Figs. 1 and 2).[23] Inhibitory nature of ISS-N1 was confirmed by three independent approaches i.e., deletion, mutation and ASO-mediated blocking. In case of *SMN1*, deletion of ISS-N1 compensates for the loss of element 2 and Tra2-β1-ESE.[23] These results demonstrate that large intronic cis-elements, which are often impossible to predict, could have profound impact on exon definition. Also, these results suggest that unique intronic cis-elements such as ISS-N1 may have evolved to balance the stimulatory effects of positive cis-elements or vice versa. Similar to 3'-Cluster and TSL2, ISS-N1 is absent in mice. All three elements (3'-Cluster, TSL2 and ISS-N1) are in close proximity to the 5'ss (Fig. 2). One of the possible mechanisms by which ISS-N1 exerts its role could be the sequestration of the 5'ss through active collaborations with 3'-Cluster and TSL2. Presence of Exinct, which includes a putative binding site for hnRNP A1, may act as a facilitator of this process.

The finding that an additional hnRNP A1 motif is created in *SMN2* due to a single mutation in intron 7 suggests for the first time that a difference between *SMN1* and *SMN2* in the noncoding region also contributes towards *SMN2* exon 7 skipping.[14] This new hnRNP A1-ISS is located one hundred nucleotides downstream of *SMN2* exon 7. Degree of negative impact of this hnRNP A1-ISS in the context of endogenous *SMN2* has not been examined. In fact, an ASO-based approach would be the next logical experiment to evaluate and validate the negative impact of hnRNP A1-ISS. Mechanism by which two *SMN2*-specific hnRNP A1-binding sites are brought together may demand looping out of intervening sequences.[14] A similar situation could be envisioned for element 1, which is located ~100 nucleotides upstream of exon 7.[21] Looping out mechanisms, which are yet to be proved, may in fact involve complex interactions that bring distantly located negative elements in close proximity (Fig. 2). As an alternative to the looping out mechanisms, it is possible that each negative element acts independently at different stage of a rather dynamic process of exon definition.

The fact that blocking of ISS-N1 by an ASO improved inclusion of *SMN2* exon 7 suggests that inhibitory impact of C6U is subservient to other negative cis-elements that participate in making the 5'ss of exon 7 very weak. In addition, negative impact of C6U could be overcome by a variety of tailed ASOs that anneal to exon 7 in the vicinity of splice sites.[24-27] Mechanisms by which these bifunctional ASOs promote *SMN2* exon 7 inclusion involve recruitment of SF2/ASF and/or other splicing factors.[24-27] Antisense microwalks reported recently will provide ideal annealing positions for the development of future bifunctional ASOs.[16] Since *SMN* exon 7 is the last coding exon, removal of the downstream intron 7 is not essential for making full-length SMN. Consistently, an ASO that prevented intron 7 removal by blocking the 3'ss of exon 8 produced more SMN from *SMN2*.[28] This finding confirms that inhibitory effect of C6U, which weakens the 3'ss of exon 7, is realized only in the context of a strong 3'ss of the downstream exon (exon 8). To a broader significance, it has become clear that none of the negative cis-elements have irreversible impact on *SMN2* exon 7 skipping. To a direct therapeutic significance, most of the reported negative cis-elements could be targeted by ASOs. For example, low doses (in low nanomolar range) of ASOs against ISS-N1 fully restored *SMN2* exon 7 inclusion and increased levels of SMN in SMA patient cells. Such a robust antisense response could be due to accessibility of the target as well as the complete nullification of a strong inhibitory impact rendered by ISS-N1. Published reports reveal a wide variety of small compounds with potential to restore *SMN2* exon 7 inclusion in SMA.[3] But, an ASO-based strategy, which confers a unique advantage of sequence specificity, holds a strong promise for SMA therapy.

CONCLUDING REMARKS

In recent years *SMN* exon 7 splicing has become a model system to understand alternative splicing in a human disease. Initial studies began with the quest for simple mechanisms by which C6U mutation promotes *SMN2* exon 7 exclusion. Subsequent studies led to the discoveries of multiple cis-elements as potential therapeutic targets. Significantly, most of these cis-elements are context specific and are not predictable by available computational programs. Unique functional approaches and analysis tools, which helped reveal these elements, will have future implications for studies of other human diseases associated with aberrant splicing.

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