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The role of RNA structure in regulating pre-mRNA splicing

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Abstract

Pre-mRNA splicing is the process of removing non-coding introns from RNA transcripts and is carried out by the spliceosome, along with other auxiliary factors. In general, research in splicing has focused on the sequences within the pre-mRNA, without taking into account the structures that these sequences might form. However, we propose that the role of RNA structure deserves more consideration when thinking about splicing mechanisms. RNA structures can inhibit or aid binding of spliceosomal components to the pre-mRNA, or can increase splicing efficiency by bringing important sequences into close proximity. Recent reports have also identified proteins and small molecules that can regulate splicing by modulating RNA structures, thus expanding our knowledge of the mechanisms used to regulate splicing.

An Introduction to Splicing and its Importance

For many years the size of an organism's genome was thought to dictate its complexity. However, when it was discovered that humans have a similar number of genes compared to less complex organisms, it became clear that other cellular processes were playing a role in our complexity. The variety of ways in which pre-mRNAs can be processed provides a number of mechanisms through which complexity can arise from genomes. In particular, alternative pre-mRNA splicing allows for a large number of mRNAs to arise from a single pre-mRNA. Generally, any sequence that is excised is defined as an intron, and the coding regions that are retained are exons. However, there are many ways in which a single transcript can lead to various mRNAs; this is known as alternative splicing. For example, coding exons can be removed, or portions of an intron (or an entire intron) retained. A recent study estimated that up to 95% of genes can be alternatively spliced to some degree, indicating that splicing is a common way to generate vast diversity within cells [1].

The process of splicing is carried out by the spliceosome, a large protein–RNA complex. It is arguably one of the most complex machines in the cell, as it is composed of more than a hundred protein factors and five small nuclear ribonuclear proteins, snRNPs (for review see [2], and Figure 1 for a diagram of splicing). An open question in the field of splicing is how the spliceosome identifies exons, as they are generally quite small compared to the intervening intronic sequences. In less complex organisms, sequences that aid in splicing tend to be more conserved, such as the 5' splice site, the branch-point, poly-pyrimidine tract (py-tract) and the 3' splice site (Figure 2). In higher eukaryotes, however, these sequences can be less conserved,

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although most introns are still spliced appropriately. How does the spliceosome accomplish this task? Auxiliary sequences are known to aid in splicing. For instance, a sequence within an exon is called an Exonic Splicing Enhancer (ESE), or an Intronic Splicing Enhancer (ISE) if it lies within an intron. Conversely, there are silencing signals that repress splicing; these are referred to as Exonic Splicing Silencers (ESS) or Intronic Splicing Silencers (ISS), depending on their location. Structures within pre-mRNAs also play a role, by displaying splicing signals, by placing certain sequences in proximity to each other, or by masking other signals.

This review covers recent developments in the field of RNA structure and the role it can play in splicing regulation. A focus will be placed on RNA structures that directly regulate splicing, and on two recent discoveries in the splicing field. The first is that proteins can regulate splicing by modulating the secondary structure of the pre-mRNA. The second is that small molecules and metabolites have been shown to regulate alternative splicing by binding and affecting the structure of the pre-mRNA.

RNA Structures that Directly Regulate Splicing

It is well documented that RNA structures can directly regulate splicing. Here we will discuss two basic classes: RNA structures that inhibit splicing and RNA structures that aid splicing. When studying RNA structure and splicing, most structures are initially predicted using various computer modeling programs, such as mFold, and verified *in vitro* using biochemical methods. Mutations that disrupt the structure and compensatory mutations that restore the proposed structure are commonly used *in vivo* to validate proposed structures. These methods are not always conclusive, and in some cases NMR or crystallography have been used to verify structures. Conservation of structures can also be studied across species to determine the importance of any given RNA structure [3]. Co-variation of base-pairs (e.g. G-C change to A-U) in RNA structures between species provides further evidence that the RNA structure forms and has an important function, although lack of sequence conservation suggests that part of the RNA might only be important structurally [4].

An additional issue to keep in mind when thinking about structures within pre-mRNAs is the fact that splicing can occur co-transcriptionally [5]. Many older and simplified models suggest that pre-mRNAs are fully transcribed, then fold, and are spliced. Instead, current data shows that the pre-mRNA can be actively spliced as it is being transcribed [5]. This means that slow folding RNA structures (even if quite stable) might not have time to form in the pre-mRNA before splicing occurs. Therefore, the rate of transcription (which varies) might have a significant role in determining if RNA structures affect splicing. The competition between folding of RNA structures and protein binding single-stranded conformations of the RNA also should be considered.

Structures that inhibit splicing

The RNA portion of the snRNPs involved in the initial interaction with the pre-mRNA are known to base-pair to sequences within the pre-mRNA. It is therefore reasonable to hypothesize that RNA structures can inhibit splicing by preventing access to these sequences. However, these internal structures might not always be extremely stable, and a competition would exist between the internal structure and the base-pairing of the snRNP to the pre-mRNA. The equilibrium between these two interactions (intramolecular versus intermolecular) can vary drastically, and therefore the subsequent splicing efficiency will be directly correlated to the stability of the internal structure. Structures that block snRNP binding appear to be a common mechanism through which RNA structure can inhibit splicing. Many examples of this type of inhibition are listed in Table 1, and the general themes for this type of inhibition will be discussed.

In several cases, native RNA structures have been identified that sequester important sequences (such as the 5' or 3' splice sites, the branch-point or the py-tract) in base-paired structures [6–15] (Figure 2B). In other examples, artificially inserted structures have been used by researchers to sequester these signals to assess their role in splicing [16–21]. When the 5' splice site or branch-point are structured, recruitment of the U1 or U2 snRNP is inhibited [8,9,15, 22,23]. In other cases, structures might also inhibit proteins from binding the pre-mRNA. The U2AF heterodimer consists of two proteins, U2AF65 and U2AF35, which bind the py-tract and 3' splice site, respectively, and help recruit the U2 snRNP to the branch-point [2]. Biochemical and structural data indicate that U2AF65 recognizes the py-tract as a single-stranded site [24], and not surprisingly, when the py-tract is structured U2AF65 binding is inhibited and splicing is blocked [23]. Structures at the 3' splice site can strongly inhibit splicing (Table 1), but it is unclear if the structures repress splicing by reducing U2AF35 binding, or if a later step in splicing is affected, such as the 2nd chemical step of splicing, where the upstream exon is ligated to the downstream exon. *In vitro* splicing assays show little free upstream exon or lariat for pre-mRNAs that have structured 3' splice sites [10,11,13,16], suggesting that an earlier step in splicing is affected.

A variety of examples (Table 1) have been identified where RNA structures cause skipping of exons, but it is unclear if this is a common regulatory mechanism. A recent bioinformatics study found that introns that are predicted to contain more stable RNA structures are less likely to be spliced efficiently, when compared to introns with less predicted structure [25]. This finding suggests that RNA structure might be a common method used to regulate alternative splicing by repressing usage of exons.

Structures that aid splicing

Surprisingly, there are just as many examples of RNA structure aiding splicing as there are of structures inhibiting splicing (Table 2; Figure 2). One general theme seen is that structures in the pre-mRNA can bring important splicing signals into closer proximity. For instance, the 3' splice site and branch-point are generally in close proximity. However, in some introns they can be hundreds of nucleotides away, but the introns still splice efficiently [26,27]. In some of these introns, structured regions between the branch-point and 3' splice site have been found that make the effective distance shorter, allowing distal branch-points to be used. In other examples, structures have been found in short introns that bring the 5' and 3' splice sites into closer proximity, and are hypothesized to increase splicing due to the effects on distance between the splice sites [28–31].

In other introns, some structures consist of very long-range interactions, which are also proposed to bring the 5' and 3' splice sites closer [32–35]. In many examples, proposed stems have loops that are up to a kilobase or larger in size, and disruption of the proposed stems reduces splicing efficiency. This finding suggests that long-range interactions are real; however, it is technically challenging to verify if these long-range interactions occur *in vivo*, or if their function is to bring splicing signals into closer proximity.

Another interesting example of a long-range interaction is observed in *Drosophila melanogaster* Down syndrome cell adhesion molecule (*Dscam*). In the *Dscam* pre-mRNA, exon 6 has 48 possible exons, and only one is chosen in any given mRNA. Long-range RNA structures are proposed to determine the selection of exon 6, where a selector sequence near the 5' splice site of the intron base-pairs with a docking sequence near a 3' splice site directly upstream of a potential exon [36]. A selector sequence exists upstream of every possible exon, but there is only one docking sequence at the 5' end of the intron. If this structure does promote inclusion of the exon directly following the “chosen” selector sequence, this could explain how only a single exon is chosen when multiple exons 6 might be included in the mRNA.

Long-range interactions are also an appealing model for bringing distant splice sites into closer proximity, as there are many substantially large introns that are multiple kilobases in size found in higher eukaryotes. A recent study provides evidence that this might indeed be a common regulatory mechanism. Raker and colleagues identified 202 phylogenetically conserved potential structures in the twelve *Drosophila* genomes, with the majority of proposed stems having extensive loops spanning over 1,000 bases [37]. The proposed stems were enriched within introns surrounding alternatively spliced exons, and modifications to the structures altered splicing patterns [37]. However, more research is needed to determine how common the mechanism of long-range interactions is for splicing of large introns.

A final example of RNA structure aiding splicing is the use of structures to display splicing signals. In some examples, cryptic splice sites are sequestered in structures [27,38]. When the structures are disrupted, the splicing machinery uses these incorrect splice sites. As many introns have multiple cryptic splice sites, structures that mask these signals could be a common mechanism for selection of proper splice sites over cryptic ones. In another example, an important ESE in the EDA exon of the fibronectin pre-mRNA was displayed in a loop of a stem [39,40]. If the structure was altered and the ESE was no longer single-stranded, SR proteins (a family of RNA binding proteins that contain serine and arginine repeats that generally bind ESEs and function as splicing enhancers) no longer bound the ESE and the exon was skipped. A bioinformatics approach with human sequences found that enhancer or silencer signals are strongly predicted to lie in unstructured regions (presumably so these signals can be bound by proteins) [41]. The signals were no longer effective in regulating splicing when known or predicted signals were placed into structured elements [41]. This finding argues that splicing signals must not only be present in the primary RNA sequence, but also displayed properly.

Proteins that Regulate Splicing by Modulating RNA Structure

Recently, a number of proteins have been shown to regulate splicing by modulating RNA structures, or binding structural elements. In some examples, the role of the protein and its effect on the RNA structure and splicing are thought to be understood, whereas the mechanisms are less clear in other examples.

Proteins that stabilize/destabilize regulatory RNA structures

MBNL1—An example of a protein regulating RNA splicing by modulating RNA structure is muscleblind-like 1 (MBNL1). Identified through its role in the disease myotonic dystrophy, MBNL1 is an alternative splicing factor that binds structured RNA and can function as either a repressor or as an enhancer of various alternatively spliced cassette exons [42,43]. The best articulated binding site for MBNL1 in a cognate pre-mRNA is within the human cardiac troponin T (*TNNT2*, also known as *cTNT* in many publications) pre-mRNA. MBNL1 represses inclusion of *TNNT2* exon 5 [23,44], and binds a stem-loop directly upstream of the exon [23, 44]. Binding by MBNL1 at this stem-loop blocks the splicing factor U2AF65 from binding the py-tract [23]. U2AF65 must bind the py-tract in a single-stranded structure, and MBNL1 binds the sequence in an alternative conformation, with at least the lower region of the stem-loop remaining intact [23] (Figure 3A). By blocking U2AF65 binding, subsequent U2 snRNP recruitment is inhibited, causing exon 5 to be skipped [23]. Stabilization of the stem-loop also represses exon 5 independently of MBNL1, implying that the role of MBNL1 is to regulate access of U2AF65 to the py-tract [23,44].

MBNL1 also recognizes a stem-loop directly upstream of exon F in the mouse *Tnnt3* pre-mRNA, suggesting that MBNL1 might commonly bind structured elements within pre-mRNAs [45]. However, a recent crystal structures of two zinc fingers from MBNL1 in complex with

a short RNA indicates that MBNL1 binds at least a few of the RNA bases in a single-stranded conformation and might create loops in the pre-mRNA when it binds [46].

DEAD-box RNA helicase p72—RNA helicases are important for splicing as they help to recycle snRNPs [2]. However, a recent example indicates that RNA helicases might also affect splicing by destabilizing inhibitory structures in a pre-mRNA. The DEAD-Box RNA helicase p72 increases levels of exon 4 inclusion in a *CD44* minigene [47]. A stem-loop sequesters the 5' splice site directly following exon 4, and p72 is hypothesized to increase exon 4 inclusion by destabilizing this stem (Figure 3B). Although it was not directly shown that p72 destabilizes the structure, mutations that disrupt the stem also increased exon 4 inclusion [47]. The role of RNA helicases has generally been restricted to their role in snRNP recycling, but it is possible that many inhibitory RNA structures could be regulated by other RNA helicases, pointing to a more direct role for the helicase protein family in regulating pre-mRNA splicing.

Binding of regulatory proteins to structured RNA elements

L30—Many years ago, the ribosomal protein L30 (originally named L32) from *Saccharomyces cerevisiae* was shown to cause retention of an intron in its own transcript by binding a stem-loop that encompasses the 5' splice site of the intron [48–52]. When bound to the 5' splice site, L30 surprisingly still allowed for the recruitment of the U1 snRNP to this splice site, despite the structure surrounding the 5' splice site [50]. However, recent work showed that subsequent recruitment of the U2 snRNP at the downstream branch-point is inhibited by L30 [52]. It remains unclear why L30 binding has no effect on U1 binding at the 5' splice site, but only on subsequent recruitment of the U2snRNP at a distal location. One possible model is that L30 interacts with U1 in a way that inhibits U1 from interacting and with and recruiting U2 [52].

Rev / hnRNP A1 and the HIV-1 pre-mRNA—In a few cases, proteins that were hypothesized to regulate splicing via RNA structure were subsequently found to have no effect on RNA structure. The HIV-1 pre-mRNA is highly structured in some of its intronic regions. At the 5' major splice donor, a structure inhibits viral infectivity [7], whereas structures at the 3' end of the intron affect 3' splice site choice [6]. It was proposed that hnRNP A1 altered the secondary structure of the pre-mRNA in a way that also affects splicing [53], but recent reports did not find this to be the case [53,54]. Instead, it appears that hnRNP A1 may sterically inhibit U2AF binding and usage of the A3 splice site [55]. In addition, the HIV-encoded Rev protein was hypothesized to alter the HIV-1 pre-mRNA transcript upon binding. But it had no effect on the structure or splicing of the HIV-1 pre-mRNA, only on the efficiency of its transport to the cytoplasm [56].

“Looping out” of exons to repress their inclusion

hnRNP A1—The regulation of exon 7B of the *Hnrnpa1* pre-mRNA is quite complex and multiple levels of regulation have been identified. On one level, the hnRNP A1 protein represses inclusion of the exon [57,58]. Structures have also been shown to directly regulate 5' splice site usage (Table 1) [15]. Binding sites have been identified both upstream and downstream of the exon, and hnRNP A1 has been hypothesized, via homo-dimerization, to “loop out” the exon and cause its repression [57,59] (Figure 3C). One report observed that hnRNP A1 causes skipping of the exon by reducing U1 recruitment to the 5' splice site after the exon [59]. Another study found that hnRNP A1 helps recruit an unidentified inhibitory factor to the 3' splice site directly upstream of the exon, and that this factor inhibits binding of U2AF65 and U2AF35 to the 3' splice site [57]. The two models are compatible, although it remains unclear if both are required for repression of the “looped out” exon, or if they are redundant mechanisms.

PTB—PTB represses at least 6 exons, and has binding sites both upstream and downstream of these repressed exons, leading to a similar “looping out” model [60] (Figure 3C). It appears that PTB blocks a later step in splicing compared to hnRNP A1. Specifically, PTB blocks the definition of the intron following the “looped out” exon, after the exon has been defined by snRNPs. For example, when PTB “loops out” the *Src* N1 exon, it blocks the interaction between U1 bound at the 5’ splice site adjacent to the N1 exon and U2 at the downstream exon 4 [61]. Although current examples (hnRNP A1 and PTB) only include protein factors, we also speculate that RNA–RNA interactions could “loop out” exons to regulate splicing.

Regulation of Splicing by Small Molecules

Another recent addition to the regulatory mechanisms surrounding pre-mRNA splicing has been that metabolites and small molecules can bind RNA structures and directly regulate splicing. Although only a few examples have been described, this is a burgeoning field and more examples will likely be found in the future.

The metabolite thiamine pyrophosphate (TPP) is a coenzyme derived from vitamin B1. It is made in bacteria, fungi, plants and is essential in humans. TPP-binding riboswitches were first identified in bacteria, but recent work identified TPP riboswitch-like sequences within eukaryotes [62–66]. In many cases, the potential riboswitch was predicted to lie within intronic sequences, and increased levels of TPP were seen to affect the alternative splicing of these introns [62–66]. TPP was observed to tightly bind the riboswitch sequences *in vitro* and induce structural changes [62], indicating that the metabolite directly regulates splicing by modulating RNA structure. In one case, the intron affected by TPP was within the 3’ untranslated region (UTR) of a gene involved in TPP synthesis [64] (Figure 4). TPP inhibited splicing of the intron and led to reduced stability of the pre-mRNA, providing a negative feedback loop for TPP synthesis [64].

RNA aptamers recognized by small molecules have also been inserted into pre-mRNAs to regulate splicing. The theophylline aptamer was inserted into an adenovirus *in vitro* splicing construct to encompass the 3’ splice site and splicing was inhibited by theophylline [67]. The tetracycline aptamer was inserted to overlap with the 5’ splice site of a reporter gene containing introns from either actin or the U3 snRNP [68]. Aptamers placed near the 5’ or 3’ splice site reduced splicing efficiency by more than 50% upon the addition of tetracycline, especially when multiple aptamers were inserted into the intron [68].

Pentamidine is a small molecule that was recently discovered as a potential therapeutic for myotonic dystrophy, as it was able to target the toxic CUG RNA repeats that cause this disease and release the protein MBNL1, which is sequestered to the repeats [69]. Coincidentally, this study also showed that pentamidine can directly affect the alternative splicing of exon 5 of the *TNNT2* pre-mRNA, whose splicing is mis-regulated in myotonic dystrophy [23]. Pentamidine appears to inhibit the inclusion of exon 5 by binding the same-stem loop recognized by MBNL1 to inhibit U2AF65 binding, leading to exon 5 skipping [69]. These examples suggest that targeting RNA structures in pre-mRNAs is a potentially powerful approach for the design of therapeutics to correct the many pre-mRNA processing errors that cause human disease.

Concluding Remarks

The historic view of pre-mRNA processing was that the pre-mRNA was a passive molecule who was acted upon by proteins to produce a mature message; coating of the RNA by hnRNPs kept it mainly unstructured, allowing other proteins and snRNPs to scan the pre-mRNA for regulatory sequences. However, this view has been largely discarded, as examples have shown that the pre-mRNA itself is an active participant in its regulation and processing (Box1). In the variety of structures that it can take, pre-mRNA structures are key for appropriately displaying

splicing sequences and recruiting splicing proteins. All the major splicing signals can be affected by structures. Furthermore, a burgeoning field is providing new examples of proteins and small molecules that modulate regulatory RNA structures. Investigating the role of RNA structure in pre-mRNA splicing is sure to produce new mechanisms and new factors (both protein and small molecules) that can regulate pre-mRNA splicing.

BOX 1

Outstanding questions in the field

1. *How does the spliceosome accurately determine splice sites in pre-mRNAs that are many kilobases in size?* The lack of strong consensus splicing signals in many introns makes it unclear how the spliceosome finds the correct splice sites. RNA structure within the pre-mRNA may provide an important mechanism for the spliceosome to find the splice sites. Studies suggest that RNA structure in the pre-mRNA can be used to display sub-optimal signals, or to mask sites that might be used incorrectly as splice sites.
2. *Are long-range RNA interactions commonly used to regulate splicing?* A few examples have demonstrated that long-range RNA interactions are important for splicing, but it remains unclear how common these interactions are for splicing in general. Furthermore, how do these long-range RNA interactions occur when RNA binding proteins are coating the RNA and splicing can occur rapidly?
3. *Is modulating the structure of the pre-mRNA by proteins and small molecules a common mechanism to regulate alternative splicing?* A few examples in specific pre-mRNAs have been identified where the binding of proteins or small molecules stabilizes one RNA structure over another structure; the different RNA structures favor different splicing outcomes. However, it will be interesting to see if this is a common mechanism used by many different proteins and small molecules to regulate splicing by directly affecting RNA structures within the pre-mRNA.
4. *How important are RNA tertiary structures for regulation of splicing?* Although there is one example of the TPP riboswitch that relies on tertiary structure for its ability to regulate splicing, it is unclear if RNA tertiary structures will be found to commonly regulate splicing.

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Figure 1. Model of spliceosomal assembly

Exons are represented by blue boxes and introns are depicted as a black line. The branch-point adenosine and 5' and 3' splice sites are noted. Spliceosomal assembly is seen to be a sequential process, at least *in vitro*, as different proteins and protein–RNA complexes (known as snRNPs, green) load onto the pre-mRNA. Note that, in E complex, the proteins SF1, U2AF65 and U2AF35 (orange) recognize sequences in the 3' end of the intron and help recruit the U2 snRNP to the intron in A complex

Figure 2. Structures that directly regulate splicing

The primary mechanisms through which RNA structures can either repress or aid in splice site selection are described in this figure. **A)** Diagram of the canonical consensus sequences within a pre-mRNA which are important for splicing. **B)** Representative diagram of structures that inhibit splicing. Shown are stem-loops that repress binding of the U1 snRNP (green) to the 5' splice site, the U2 snRNP to the branch-point, U2AF65 and U2AF35 (orange) to the 3' splice site and an SR protein (pink) to a sequence within the exon. **C)** Representative diagram of structures that aid splicing. Depicted is a structure that brings the 5' and 3' splice sites into closer proximity, a stem that brings the 3' splice site and branch-point into closer proximity,

a stem that masks a cryptic 3' splice site (denoted as YAG*) and a stem that properly displays an enhancer sequence in the exon that an SR protein binds.

Figure 3. Proteins that regulate splicing by modulating RNA structure

Three mechanisms that proteins use to regulate splicing by modulating RNA structure are described in this figure. **A)** MBNL1 (turquoise) inhibits U2AF65 (orange) binding and U2 (green) recruitment to the 3' splice site of intron 4 in the cardiac troponin T pre-mRNA. MBNL1 and U2AF65 compete by binding mutually exclusive RNA structures. **B)** The DEAD-box RNA helicase p72 (purple) is hypothesized to increase exon 4 inclusion in the *CD44* pre-mRNA by destabilizing a stem that encompasses the 5' splice site following exon 4. **C)** hnRNP A1 (light blue) and PTB (lime green) are hypothesized to cause skipping of exons by "looping out" of the exon. hnRNP A1 causes skipping of exon 7B of its own pre-mRNA by both directly inhibiting binding of the U1 snRNP to the 5' splice site following the exon, and by helping to

recruit a protein whose identity is unknown (?; gold) to the 3' splice site directly upstream of the exon. This unidentified factor inhibits U2AF65/U2AF35 binding to the 3' splice site. PTB represses inclusion of exon N1 in the *Src* pre-mRNA. PTB did not affect binding of the U1 snRNP to the 5' splice site, but did inhibit U2AF65/U2AF35 binding to the downstream 3' splice site.

Figure 4. Thiamine pyrophosphate (TPP) binds a riboswitch that affects splicing for a negative feedback loop in its own biosynthesis pathway

Thiamine C synthase (THIC; blue) is a protein in the biosynthesis pathway for TPP (turquoise), which is a vitamin B1 coenzyme. High levels of TPP lead to inhibition of splicing of an intron in the 3' UTR of the *THIC* pre-mRNA. Splicing stabilizes the pre-mRNA, whereas the unspliced pre-mRNA is degraded more quickly. TPP binds a riboswitch that encompasses the 3' splice site of the intron, and represses splicing. This action provides a negative feedback loop to inhibit THIC protein production when adequate levels of TPP have been produced.

Table 1

Structures that inhibit splicing

Organism	Gene	Effect on Splicing	Ref.
Naturally occurring structures			
<i>Homo sapiens</i>	<i>SMN1, SMN2</i>	Exon 7 is alternatively spliced, and multiple structures can affect splicing. A structure 55 nt ¹ after exon 7, and a stem that sequesters the 5' ss ² after the exon can both repress exon 7 inclusion. A stem within the exon itself does not affect its repression.	[8,70,71]
<i>Homo sapiens</i>	<i>HGH</i>	A stem before exon 3 was predicted to encompass the upstream of the two possible 3' ss. Mutations that destabilize the stem increase usage of the upstream splice site, whereas mutations that further stabilize the stem repress usage of the upstream 3' ss <i>in vitro</i> .	[12]
<i>Homo sapiens</i>	<i>PS2</i>	A stem-loop is predicted within exon 5. When deleted or destabilized, exon 5 levels increase <i>in vivo</i> .	[72]
<i>Homo sapiens, Mus musculus</i>	<i>Tau</i>	Exon 10 is encompassed in a stem-loop. Destabilizing the stem-loop increases exon 10 inclusion by increasing U1 snRNP binding, whereas stabilization decreased exon 10 inclusion <i>in vivo</i> . However, one report [73] claims the stem-loop does not affect exon 10 inclusion <i>in vivo</i> .	[14,22,73,74]
<i>Mus Musculus</i>	<i>Hnrnpa1</i>	A stem-loop sequesters the 5' ss directly following exon 7B. Destabilizing the stem-loop increases exon 7B inclusion	[15]
<i>Mus musculus</i>	<i>Ncam</i>	A structure in intron 18 reduces <i>in vitro</i> splicing efficiency of the intron by inhibiting U2AF65 binding.	[75]
<i>Mus musculus</i>	<i>IgM</i>	A stem-loop sequesters the 3' ss of the C4-M1 intron. Destabilizing the stem increases splicing <i>in vitro</i> .	[13]
<i>Gallus gallus</i>	β -TM	Extensive structure is predicted to encompass exons 6A and 6B. The structures surrounding exon 6B inhibit recruitment of the U1 snRNP <i>in vitro</i> , and repress exon 6B inclusion both <i>in vitro</i> and <i>in vivo</i> .	[9,76,77]
Duck hepatitis B virus	pre-genomic RNA	A proposed structure encompasses both the 5' and 3' ss of an inefficiently spliced intron.	[10]
Rous sarcoma virus	n/a	A 23 nucleotide stem-loop interacts aberrantly with the U1 or U11 snRNPs to inappropriately recruit them to the 3' ss and poison splicing.	[78]
HIV-1	n/a	Stabilization of a stem-loop around the 5' major splice donor strongly reduces viral infectivity, whereas compensatory mutations restore infectivity. Another structure is found around the py-tract for the A3 splice site and destabilization of the stem increases usage of the A3 site.	[6,7]
Artificially inserted structures			

Organism	Gene	Effect on Splicing	Ref.
Adenovirus	ADML tripartite leader	Artificial stem-loops can loop out exon 2 and reduce exon 2 inclusion <i>in vitro</i> and <i>in vivo</i> . A less robust effect is observed when the 5' or 3' ss are placed within a structure.	[20]
<i>Saccharomyces cerevisiae</i>	RP51A	Inserted stem-loops can sequester the 5' ss or branch-point and reduce splicing efficiency <i>in vitro</i> and <i>in vivo</i> .	[18]
<i>Saccharomyces cerevisiae</i>	ACT	A stem-loop was inserted directly after the branch-point strongly inhibits the second step in splicing <i>in vitro</i> . A stem-loop 23 nt further downstream does not affect splicing.	[19]
<i>Mus musculus</i>	α -TM	Stem-loops inserted in intron 2 and reduce splicing efficiency <i>in vitro</i> . The structures encompass either the 3' ss or lie between the py-tract and the 3' ss.	[16,17]
<i>Nicotiana plumbaginifolia</i>	Syn7 model intron	Inserted stem-loops can sequester the 5' or 3' ss and reduce splicing efficiency <i>in vitro</i> .	[11]
<i>Oryctolagus cuniculus</i> (rabbit)	β -globin	An inserted stem which sequesters the 5' ss also reduces splicing efficiency <i>in vitro</i> . When the 5' ss is placed in the loop >54 nt, the site is used <i>in vivo</i> , but usage is never observed <i>in vitro</i> .	[21]

¹ nt- nucleotide

² ss- splice site

Table 2

Structures that aid splicing

Organism	Gene	Effect on Splicing	Ref.
Structures that bring important sequences into closer proximity to each other			
Adenovirus	ADML tripartite leader	The 3' ss ^I region is heavily structured, providing a reason for why a variety of 3' ss are chosen. A stem-loop in the E1A intron is thought to bring the 3' ss closer to the 3 possible branch-points. Destabilizing the stem represses splicing, whereas compensatory mutations restore splicing <i>in vitro</i> . Greater stability increases splicing efficiency.	[26,79]
<i>Drosophila melanogaster</i>	<i>Dscam</i>	A long-range "iStem" is important for selection of a single exon 4 from many possible exons. For exon 6, "docking site" and "acceptor site" sequences are proposed to be involved in the choice of a single exon.	[36,80]
<i>Homo sapiens</i>	<i>FGFR2</i>	Structure is predicted after exon IIIb. Mutations that destabilize the structure decrease usage of the exon, whereas compensatory mutations restore usage <i>in vivo</i> . There are conflicting reports of whether a stem with a specific sequence is required, or if a stem with any sequence is sufficient.	[32–34]
<i>Saccharomyces cerevisiae</i>	<i>RP51B</i>	A predicted stem-loop brings the 5' and 3' ss into closer proximity. Splicing efficiency is reduced <i>in vivo</i> and splicing complex formation <i>in vitro</i> when the stem is mutated. Compensatory mutations to restore the stem restore normal splicing efficiency.	[28,30]
<i>Saccharomyces cerevisiae</i>	<i>ACT</i>	A structure is essential for usage of the correct 3' ss <i>in vivo</i> , and not a cryptic ss. This structure also brings the 3' ss closer to the branch-point, which is 125 nt ² upstream.	[27]
<i>Saccharomyces cerevisiae</i>	<i>YRA1</i>	A very long intron, for yeast, is made effectively shorter by a stem with a large loop. Mutations to the stem decrease splicing efficiency and compensatory mutations restore splicing efficiency <i>in vivo</i> .	[35]
<i>Saccharomyces cerevisiae</i>	U3 snoRNA precursor	A stem-loop in the U3 snoRNA precursor brings the 5' and 3' ss into close proximity.	[31]
Structures that display important splicing sequences, or mask inappropriate splicing signals			
<i>Homo sapiens</i> , <i>Mus musculus</i>	<i>FN</i>	A stem-loop displays an important ESE within the EDA exon. Mutations to the structure affect ESE display.	[39,40]
Adenovirus	E3 transcription unit	A stem-loop structure is required for correct usage of a 5' ss <i>in vitro</i> . The stem-loop sequesters another cryptic 5' ss.	[38]
<i>Rattus norvegicus</i>	calcitonin (<i>CGRP</i>)	A stem-loop encompasses the branch-point, py-tract and a suboptimal 3' ss. Mutations to the stem decrease usage of the 3' ss, whereas compensatory mutations restore usage <i>in vitro</i> .	[81]
Structures with unknown functions			
<i>Homo sapiens</i>	<i>COL2A1</i>	A biochemically verified stem-loop following a weak 5' ss enhances usage of the ss <i>in vivo</i> .	[82]
<i>Drosophila melanogaster</i>	<i>Adh</i>	A structure is predicted in intron 1 upstream of the branchpoint. Mutations which destabilized the structure decreased splicing efficiency <i>in vivo</i> , whereas compensatory	[83]

Organism	Gene	Effect on Splicing	Ref.
		mutations that restored the structure also restored splicing efficiency.	
<i>Saccharomyces cerevisiae</i>	YL8A	<i>In vivo</i> splicing efficiency of a short exon is found to be increased by flanking stem-loops in the adjacent introns.	[84]
<i>Saccharomyces cerevisiae</i>	tRNA ^{Leu} precursor	Destabilization of the tRNA's D stem reduces splicing of the precursor <i>in vitro</i> . Compensatory mutations restored splicing.	[85]
<i>Nicotiana plumbaginifolia</i>	Syn7 model intron	Stem-loops were inserted directly upstream of the 3' ss and were observed to increase splicing efficiency <i>in vitro</i> .	[11]

² nt- nucleotide,

¹ ss- splice site