Alternative splicing and its regulation under normal and abnormal conditions

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Examensarbetet utfört vid IFM Biologi
2010-05-31

LITH-IFM-G-EX--10/ 2350—SE
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During the maturation of pre-mRNA introns are removed and exons are spliced together, to form a primary transcript, a reaction that is catalyzed by the spliceosome. Alternative splicing is a complex reaction that mainly utilizes one of four mechanisms; exon skipping, 5' splice site choice, 3' splice site choice and intron retention. To achieve accurate splicing four sequence elements are essential, two of which are located in the splice sites themselves; 5' splice sites and 3' splice sites, but also the polypyrimidine tract and the branch point sequence. Alternative splicing can be regulated by histone or chromatin modulations, siRNA, transcription efficiency and various proteins, many of which belong to either the SR protein family or the hnRNP family of proteins. SR proteins usually promote exon inclusion, while hnRNP proteins usually promote exon skipping. There are also regulatory elements that are called exonic splicing enhancers or silencers depending on if they promote or inhibit the inclusion of the exon they reside in. These elements also exist in introns and are then called intronic splicing enhancers or silencers. The enhancer elements are most commonly targeted by SR proteins and the silencer elements are usually targeted by hnRNP proteins. This paper will mainly focus on the regulation of alternative splicing and the role of alternative splicing under abnormal conditions, such as when mutations cause disease.
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Abstract
During the maturation of pre-mRNA introns are removed and exons are spliced together, to form a primary transcript, a reaction that is catalyzed by the spliceosome. Alternative splicing is a complex reaction that mainly utilizes one of four mechanisms; exon skipping, 5' splice site choice, 3' splice site choice and intron retention. To achieve accurate splicing four sequence elements are essential, two of which are located in the splice sites themselves; 5’ splice sites and 3’ splice sites, but also the polypyrimidine tract and the branch point sequence. Alternative splicing can be regulated by histone or chromatin modulations, siRNA, transcription efficiency and various proteins, many of which belong to either the SR protein family or the hnRNP family of proteins. SR proteins usually promote exon inclusion, while hnRNP proteins usually promote exon skipping. There are also regulatory elements that are called exonic splicing enhancers or silencers depending on if they promote or inhibit the inclusion of the exon they reside in. These elements also exist in introns and are then called intronic splicing enhancers or silencers. The enhancer elements are most commonly targeted by SR proteins and the silencer elements are usually targeted by hnRNP proteins. This paper will mainly focus on the regulation of alternative splicing and the role of alternative splicing under abnormal conditions, such as when mutations cause disease.

Keywords
Alternative splicing, ESE, ESS, hnRNP proteins, ISE, ISS, Regulation, Spliceosome, SR proteins.
List of abbreviations

ASF/SF2 – Alternative splicing factor/splicing factor 2
ATM - Ataxia telangiectasia gene
BR genes – Balbiani ring genes
CFTR gene – Cystic fibrosis transmembrane conductance regulator
Clk/Sty – Family of protein kinases
ESE – Exonic splicing enhancers
ESR - Exonic splicing regulatory sequences
ESS – Exonic splicing silencers
dsDNA – Double stranded DNA
GRD – Glycine rich domain
GST-MS2 protein – Coat protein
hnRNP – Heterogeneous nuclear RNA
Htra2-β1 – A serine-protease
IRS – Intronic splicing regulatory sequences
ISPE – Intronic splicing processing element
ISE – Intronic splicing enhancers
ISS – Intronic splicing silencers
nPTB – Neuronal PTB
nSR100 – neural-specific SR related protein, 100 kDa
PDH – Pyruvate dehydrogenase
Pol II – Polymerase II
PSF – PTB-associated splicing factor
PTB – Polypyrimidine tract binding protein
RRM – RNA recognition motifs
RS domains – Arginine-serine rich domain
siRNA – Small interfering RNA
SMA – Spinal muscular atrophy
SMN – Survival of motor neuron gene
sp1 – Protein that promotes initiation of transcription
snRNP - Small nuclear ribonucleoproteins
SR proteins – Serine/arginine-rich proteins
SRrp37 – SR related protein 37
SRPK – Protein kinase family
ssDNA-protein – Single stranded DNA
SW6 – VP16 mutant
TGS - Transcriptional gene silencing
U2AF – Non-snRNP protein required for U2snRNP binding
VP16 –Protein that promotes transcription
Introduction

During the maturation of pre-mRNA the non-coding introns are removed and the coding exons are spliced together, to form a primary transcript. This process is essential for the construction of proteins in higher eukaryotes (Xiang et al., 2005). The size of the human genome, 25,000 genes, can not singlehandedly be accounted for the human complexity. In alternative splicing several isoforms of mRNA are constructed from a single gene or locus, in some cases thousands of transcripts are created, which gives rise to numerous different functional proteins (Zhou et al., 2009; Koren et al., 2007). Extensive alterations of transcripts lead to different products which affect the function of genes in biology, disease and evolution (Zhou et al., 2009). A quantitative study performed by Mironov et al., 1999, has revealed that at least one third of human genes are alternatively spliced and more recent studies predict that up to 90% of human genes are alternatively spliced (Koren et al., 2007).

The splicing reactions are catalyzed by the spliceosome. The spliceosome serves as the site for the two transesterification reactions that remove an intron and connect the two nearby exons (Xiang et al., 2005). The spliceosome consists of numerous, at least a hundred, proteins and five small nuclear ribonucleoproteins (snRNP), U1, U2, U5 and U4/U6. The U4/U6 and U5 snRNPs are also referred to as tri-snRNP. The formation of the spliceosome is initiated by the ATP-independent interaction of the U1snRNP with the 5′ splice site which forms the E (early) complex. This complex also contains the U2snRNP that is loosely associated with the pre-mRNA. The following step is an ATP-dependent step where the U2snRNP base-pairs with the branch site which stabilizes the complex, now called the A complex or the prespliceosome. These steps in the assembly are important for the recognition of splice sites and the regulation of alternative splicing is often accomplished by modifying the organization of the E and A complex formation. To form the spliceosome from the prespliceosome the tri-snRNP binds to the pre-spliceosome to form the spliceosomal B complex. After the first transesterification reaction of splicing, complex C is formed. After the second transesterification the mRNA and the excised intron are released and the spliceosome dissociates (Figure 1) (Hartmuth et al., 2002).

![Figure 1. The stepwise assembly of the spliceosome. From Stark and Lührmann (2006).](image-url)
There are two models for the mechanism of exon and intron recognition and selection by the splicing machinery, intron definition and exon definition. In the intron definition the splicing machinery recognizes an intronic unit and is placed across the intron. This mechanism is thought to be old in an evolutionary perspective and the introns are selected to remain short. Exon definition takes place when the machinery instead is placed over exons, which limits the length of the exon. It is believed that the higher amount of GC in exons allows the exon to be defined. Exon definition has been evolved later in evolution and is the main mechanism in higher eukaryotes (Keren et al., 2010).

There are mainly four pathways of alternative splicing; exon skipping (or cassette exons), 5’ splice site choice, 3’ splice site choice and intron retention (Figure 2). In cassette exons a whole exon is either skipped or included in the mature mRNA transcript. In the 5’ – and 3’ splice site choices the exons are flanked by a constitutive splice site on one side and by two or more competing alternative splice sites on the other side. This results in an alternative region that is either included or excluded in the mRNA transcript (Koren et al., 2007). The alternative splicing is frequent in the 5’UTR region and is linked to different starting points of transcription which enables the cell to use different starting points for the translation. This gives rise to proteins with additional domains, instead of variations of the same domain (Mironov et al., 1999). Intron retention is the least studied pathway for alternative splicing, mainly due to the fact that these variants are thought to be largely derived from un-spliced or partially spliced pre-mRNAs (Galante et al., 2004). This is believed to be the result of failure of the splicing machinery to recognize weak splice signals flanking the introns or other sequences. In intron retention a sequence, which might be a real intron, can be spliced out as an intron or an intron can be retained which generates different mRNA transcripts. The retained introns exhibit weaker splice signals and are associated with genes that have shorter introns. They also exhibit higher levels of expression and have a lower density of ESSs and ISSs than traditionally spliced genes (Ding et al., 2009).

Figure 2. The main mechanisms of alternative splicing. The red dotted lines and the red arrows depict the alternative splicing and the green lines and arrows depict constitutional splicing. In the first three illustrations the yellow boxes show the exons, or part of exons that is either included or excluded and the intron retention shows a part of an exon that is excised as an intron. From Wang and Burge (2008).

To achieve accurate splicing four splice sequences, located in the introns are essential, two of which are located in the splice sites themselves; 5’ splice sites (GT), 3’ splice sites (AG). The other two splicing signals are the polypyrimidine tract and the branch point sequence.
(Tsai et al., 2007). These four signals are called the core splicing signals (Wang and Burge, 2008). The 5\’ splice site contains a 9-base consensus sequence that includes a universally conserved GU. This is surrounded by additional nucleotides that are less conserved. The 3\’ splice site includes a conserved AG which is surrounded by less conserved C and G. Upstream of the AG region there is a 10-base sequence that is rich in pyrimidines and this region is known as the polypyrimidine tract. The fourth element that is required for correct splicing is the branch point. In the first step of splicing the 5\’ exon-intron boundary is attacked by a nucleotide located near the 3\’ end of the intron. This result in cleavage and the formation of a lariat structure where a new 5\’-2\’ phosphodiester bond is formed. This takes place at the so called branch point, upstream of the 3\’ end of the intron. The branch point usually consists of an A with a loose consensus of YNYURAY surrounding it. This region is not very conserved and the position varies which can result in so called cryptic branch points which can be found in most pseudo exons. There is evidence that the branch point is recognized early in splicing by splicing factors which indicates that the location of the branch point is important in the recognition of the 3\’ splice site (Zhang et al., 2005).

There is a certain type of exons called composite exons. There are two classes of composite exons. The exons of the first class are followed by a 3\’splice site and a polyA site. The competition for inclusion between two such exons results in the production of a mature mRNA that contains two possible 3\’-end termini. This class is termed as skipped exons because the nearest exon in the pair is used as a terminal exon, skipping the last one, or they are both skipped entirely. The second class consists of exons with 3\’ and 5\’ splice sites which are immediately followed by a polyA site. They are characterized by a suboptimal 5\’ splice site and a weak polyA site. This second class of competing composite exons can be used as terminal or internal exons depending on the cell, which means that the first one could terminate the processing of the mRNA, or be included which gives the second exon the role of terminal exon. The alternative splicing of IgM is an example of composite exons (Anquetil et al., 2009).

**Regulation of alternative splicing**

The regulation of alternative splicing depends on the interaction of a large number of splicing factors and regulatory elements in the pre-mRNA. One group of such factors consists of widely expressed proteins that seem to have wide-ranging roles in mRNA biogenesis. This group can be divided into two sub groups; the SR proteins and the hnRNP proteins, where the former tends to promote exon inclusion and the latter usually has the opposite effect (Ouyang, 2009). The regulation also depends on regulatory elements that are called exonic splicing regulatory sequences, ESRs, which are divided into exonic splicing enhancers (ESE) or exonic splicing silencers (ESS). There are also intronic splicing regulatory sequences (IRS) that are divided into intronic splicing enhancers (ISE) or intronic splicing silencer (ISS) (Goren et al., 2006). In addition to these ways of regulation the splicing can also be affected by the rate and amount of pausing of transcriptional elongation (Alló et al., 2009).

**Exonic/intronic splicing enhancer/silencer**

There are cis acting regulatory elements that are called exonic splicing regulatory sequences, or ESRs which are usually very short motifs, 4-18 nucleotides. These can either be ESEs or ESSs depending on if they promote or inhibit the inclusion of the exon they reside in. The alternative spliced exons that are either short in length or contain weak 5\’ splice sites are those who show the highest conservation of the ESRs, which suggest that, the splicing of
these exons depend on the presence of ESRs (Goren et al., 2006). There are also intronic splicing regulatory elements, ISRs, which are either ISEs or ISSs depending on if they enhance or inhibit the usage of adjacent splice sites from an intronic location. Both the exonic and intronic splicing regulatory elements often function by recruiting trans-acting splicing factors that modulate exon selection and regulate alternative splicing by activating or suppressing splice site recognition or the spliceosome assembly. ESEs are the most understood element and it often bind to the positive regulators of the SR family, so called because of it contains arginine-serine repeats (Xiang et al., 2005; Wang and Burge, 2008). ESEs often contain repeats of the motif GARGAR where R is a purine, so called purine-rich domains (Gallego et al., 1997).

SR proteins participate in splicing by enhancing the recruitment of the spliceosome components U1 and U2. In the absence of the ESE the binding of the spliceosome components to the 5’ splice site and branch point is less stable and the rate of the spliceosome formation is slowed down. The splicing event can instead be inhibited by splicing silencers, located either in exons or introns. They act by binding to a group of proteins that include members of the heterogeneous nuclear ribonucleoprotein, hnRNP, family. If a silencer element is present in an exon (ESS) it suppresses the exon definition, a mechanism that involves binding of a splicing co-activator. When a silencer element is located in the intron (ISS) it suppresses the recognition of the splice site, which is important for alternative splicing (Szeszel-Fedorowicz et al., 2006).

However a recent study (Goren et al., 2006) suggests that the same ESR sequence can function as an enhancer in one exon and as a silencer in another. This was demonstrated by mutating four ESRs located in three different exons and observing their effect on splicing, but also by sliding two known binding sites for two SR proteins, ASF/SF2 and SRp40, along an alternatively spliced exon. The different positions of these two SR binding sites along the exon had different effects of the alternative splicing pattern. SRp40 is known to be a strong silencer but in several of the cases it was found to be a strong or moderate enhancer. Similarly, ASF/SF2 is known to be an enhancer it exerted silencing effects in several positions. There were also significant variations in the levels of enhancing or silencing effects depending on the locations along the exon. This suggests that the distinction between the ESE and ESS elements is not only dependent on the sequence, but also on the location (Goren et al., 2006).

**Intronic regulatory elements/pseudo exons**

Human introns generally consist of thousands of bases and contain both cryptic, or pseudo, splice sites and cis-acting regulatory elements. The potentially cryptic sequences must be distinguished from real exons and skipped in the mature mRNA. These sequences can be the cause of non-functional pre-mRNA isoforms and normally, inclusion of pseudo exons is actively inhibited by the formation of RNA secondary structures that prevent recognition of cryptic 5’ and 3’ splice sites (Pastor et al., 2009; Dhir et al., 2010). Genomic variants that change the splicing regulatory elements can affect the normal splicing, which in turn can cause a range of diseases. These mutations can be located either in near proximity to the exon or in distant regions of the intron. The mutations that lead to intronic alterations far away from the exon are often considered as neutral or non-important when it comes to pre-mRNA processing. However, there is increasing evidence that these intronic mutations play an important role and are associated with human disease. They act by creating new splice sites or strengthen the pre-existing cryptic splice sites, thus creating alternative splice sites (Pastor et al., 2009).
One example of this is the \textit{ATM} gene and mutations in this gene can cause ataxia telangiectasia. Ataxia telangiectasia is a rare autosomal recessive genetic disorder that is characterized by progressive neurodegeneration, immunodeficiency and a high risk of cancer. There is also telangiectasia, dilation of small blood vessels, in eyes and skin (Lavin \textit{et al.}, 2007). The \textit{ATM} gene contains a pseudo exon which is included in the mature mRNA through a complex mechanism. The inclusion of the pseudo exon is inhibited by the binding of the U1snRNP at an intronic splicing processing element, or ISPE. When this ISPE is intact the U1snRNP binds to it, but the spliceosomal assembly and the formation of the A complex is ineffective. This suggests that the U1snRNP mediated recruitment of U2snRNP is unsuccessful, which would make the U1snRNP a splicing silencer in this situation. This leads to the repression of the \textit{ATM} upstream cryptic 3’ splice site and repression of the pseudo exon. The binding of U1snRNP to the ISPE element also reduces the binding affinity for ASF/SF2 to an enhancer sequence that is located in the pseudo exon, which also leads to repression of the pseudo exon. (Dhir \textit{et al.}, 2010). A mutation in the ISPE element in \textit{ATM} leads to activation of two nearby pre-existing cryptic splice sites, which leads to intron removal around the pseudo exon. The ISPE deletion removes the static interference of U1snRNP on the cryptic splice site and results in alternative splicing in the upstream section in the intron and activates excision of the upstream portion of the intron. This also generates a splicing precursor. Further processing of the precursor to a cryptic exon depends on the presence of a downstream ISE located in an antisense Alu. Alu repeats are located in the human genome in gene-rich regions and are specially imbedded within introns and Alu sequences containing splicing regulatory elements and can change regular splicing into alternative splicing. The ISE embedded in the antisense Alu facilitates recognition of the weak 5’ splice site. This leads to an incorporation of the cryptic exon into the mature mRNA and it is responsible for ataxia telangiectasia (Figure 3 and 4) (Pastor \textit{et al.}, 2009).

\textbf{Figure 3.} The cryptic exon inclusion when the cryptic splice site is activated due to the mutation. From Pastor \textit{et al.} (2009).

\textbf{Figure 4.} In the WT the binding of U1snRNP to the ISPE interferes with the recognition of the nearby cryptic 3’ splice site and no precursor is constructed, which leads to normal processing. The mutant ISPE (triangle) removes the interference of the U1snRNP, which activates the cryptic 3’ splice site and the intermediate precursor is formed. In the presence of the ISE element (yellow circle) this intermediate is processed to mature mRNA that contains the cryptic exon. From Pastor \textit{et al.} (2009).
Transcription efficiency
In mammalian cells the methylation of intragenic DNA initiates the formation of a closed chromatin structure which reduces the efficiency of RNA polymerase II (Pol II). The reduced efficiency of Pol II induces an alternative exon inclusion. The rate of transcription also affects the outcome when two splicing reactions occur simultaneously during the transcription. Fast and highly efficient transcription favors exon skipping whilst slow and not as efficient transcription favors the inclusion of an exon (Alló et al., 2009).

Histone modifications and Chromatin modifications
Nucleosomes are depleted in introns that are neighboring an acceptor splice site and at the polyA site and abundant in exons. The position of the nucleosomes seems to be independent of transcription but highly expressed genes have reduced levels of nucleosomes on the exons, compared with genes with low expression rates. This suggests that there might be modifications during transcription and the different levels of nucleosomes in exons and introns might facilitate exon definition. The higher levels of nucleosomes increase the strength of splice sites. The nucleosomes will slow Pol II down which increases the possibility that the splicing factors recognize a weak splice site. The histone molecules may contribute to the recognition of regulatory elements on the mRNA by splicing regulators and hence affect the splice site selection (Fox-Walsh and Fu, 2010).

siRNA and gene silencing
Small interfering RNAs, siRNAs trigger transcriptional gene silencing (TGS) in human cells. This is achieved through heterochromatin formation at target DNA sequences and the process includes recruitment of chromatin-modifying enzymes which results in methylation of histones and DNA. siRNAs can also regulate gene expression in mammalian cells through the histone modification and DNA methylation. Recent findings by Alló and colleagues, 2009, indicate that siRNA targeting gene sequences surrounding an alternative exon can regulate its alternative pre-mRNA splicing. It is the siRNA that target the intron downstream of the alternative exon that promotes inclusion of the alternative exon. The siRNA surrounding the alternative exon forms a closed chromatin structure that prevents efficient Pol II elongation. This delay in elongation gives more time to the splicing machinery for the recognition and inclusion of an alternative exon. The interactions between siRNA and Pol II have been experimentally validated by Alló and colleagues (2009), with the use of a promoter which could be activated by Sp1 (promotes initiation) and VP16 (promotes initiation and elongation). When the transcription of the genes was transactivated by Sp1 the siRNA increased the exon inclusion but when the VP16 transactivator was used this effect vanished. However when a VP16 mutant, SW6 which does not support elongation, was used the effect of the siRNA was not removed (Alló et al., 2009).

SR proteins
Members of the serine/arginine-rich (SR) protein family are important components in constitutive and alternative splicing, as well as in many other aspects of mRNA metabolism such as mRNA transport, translation and stability in eukaryotic cells (Saiprasad and Anireddy, 2010). It is not yet concluded whether all individual SR proteins can perform all of the different SR protein functions or if several independent SR proteins take part in excision of each intron. It is possible that alternative splice site choices are decided by which combination of splicing factors, including SR proteins, which associate with a pre-mRNA. SR proteins typically contain one or two N-terminal RNA recognition motifs (RRM) RNA binding
domains (RBD) and also a C-terminal arginine/serine rich (RS) domain that is variable in length (Gravely and Maniatis, 1998; Björk et al., 2009). The SR protein specificity for substrates is defined by their RNA recognition motif domain. There are also some RNA sequences that have specificity for SR proteins. Natural pre-mRNA probably contains a combination of these motifs and it is likely that these sequences on the pre-mRNAs determine the gene-specific SR protein association patterns. However, it is also possible that other proteins, such as hnRNP proteins, influence the binding of SR proteins and one such protein, hrp59, is believed to bind to a specific pre-mRNA at an exon site that overlaps with the SR protein exon site, thus inhibiting the binding of the SR protein (Björk et al. 2009). The SR proteins are phosphorylated at the serine residues in the RS domain and activated by two families of protein kinases, SRPK and Clk/Sty. SRPK1 binds to a motif in ASF/SF2 that restricts phosphorylation to the N-terminal of the RS domain whilst Clk/Sty can phosphorylate the whole of the RS domain of other SR proteins, which results in a hyperphosphorylated state (Long and Caceres, 2009). This posttranslational modification is required for the SR protein to be able to interact with a carrier protein, SR-transportin, that translocates the SR protein from the cytoplasm to the nucleus and the amount of phosphorylation is essential for the SR protein activity in alternative splicing (Aubol et al. 2003; Ma et al., 2009; Ma et al., 2009).

SR proteins have been shown to interact with each other and RNA molecules, but also with proteins in the splicing machinery, such as the U1snRNP (Gravely and Maniatis, 1998; Björk et al., 2009). It is also possible that the SR proteins facilitate the recruitment of the tri-snRNP (Long and Caceres, 2009). All of these protein-protein interactions require the RS domain which, when phosphorylated is thought to function in the formation of protein-protein and protein-RNA interactions required for spliceosome assembly (Calarco et al., 2009). The phosphorylated RS domains bind splicing signals that are partially base paired with U1snRNPs and promote this base pairing (Björk et al., 2009). The RS domains can also contact the mRNA directly via the branch point and 5’ splice site, which suggest an alternative mechanism for spliceosome assembly (Long and Caceres, 2009). About 40 RS domains have been related to splicing in mammalian cells and it is suggested that the levels of these proteins may contribute to cell- and tissue-dependent alternative splicing (Calarco et al., 2009). One function of the SR proteins, such as ASF/SF2, is to promote the binding of U1snRNP to 5’ splice sites (Li and Manley, 2005). SR proteins may also function by ensuring correct pairing of 5’ and 3’ splice sites by simultaneous interaction with U1snRNP and U2AF, which interacts with the 5’ and 3’ splice site respectively. U2AF is a non-snRNP protein which consists of two subunits that is required for the binding of U2snRNP to the pre-mRNA branch site and it binds specifically to the polypyrimidine tract at 3’ splice sites (Figure 5).

![Figure 5. Simultaneous binding of SR proteins to U1snRNP and U2AF. From Long and Caceres (2009).](image-url)
In addition to these constitutional functions the SR proteins also play an important role in regulating alternative splicing (Graveley and Maniatis, 1998). Binding of SR proteins to ESEs prevents exon skipping, which ensures correct 5’ to 3’ linear order of exons in spliced mRNA. There are two models to which this could be explained. The first is relying on the ability of the ESE-bound SR proteins to recruit and stabilize interactions between the U1 snRNP at the 5’ splice site and U2AF at the 3’ splice site. This process is called exon definition. The other model is based on the capability of ESE bound proteins to antagonize the effects of hnRNP proteins, which recognize ESSs. The SR proteins may also form protein-protein interactions across introns to juxtapose the 5’ and 3’ splice sites early in the spliceosomal assembly in an RS dependent manner. The SR protein ASF/SF2 is also able to interact directly with the RNA at the branch point to promote spliceosomal assembly (Long and Caceres, 2009). The SR proteins that are attached to a splicing enhancer can also recruit components of the splicing machinery to nearby enhancer-dependent introns with weak splice sites. Introns often contain non-consensus pyrimidine tracts to which U2AF binds weakly. It has been shown that the small subunit of the U2AF protein can simultaneously interact with the large subunit as well as the SR proteins within the enhancer complex. This stabilizes the U2AF protein binding to the weak 3’ splice site (Graveley and Maniatis, 1998).

Not all SR proteins contain an RS domain and those who lack that domain are mostly the ones that have been shown to have tissue-specific expression patterns. Several of these proteins play an important role in cell- and tissue-specific alternative splicing. They include the neuronal-specific Nova-1/2 and HuB proteins, the neuronal- and muscle-expressed proteins Fox-1/2 and MBNL family proteins and the neuronal-, myoblast- and testis-expressed nPTB protein. nPTB is a paralog of the widely expressed PTB splicing repressor protein. The switch from expression of PTB to nPTB has been connected to regulation of neuronal alternative splicing and results in the formation of less repressive nPTB-bound splicing complexes that are responsive to positive-acting factors in neurons. The mechanism of action is however not well understood (Calarco et al., 2009). One tissue- and vertebrate-restricted RS domain protein, neural-specific SR related protein of 100 kDa (nSR100) has been identified. The lack of this protein results in exclusion of a large number of alternative exons in the nervous system. These exons are mainly located in genes with important roles in neural cell differentiation. nSR100 creates neuronal specificity in alternative splicing by activating nPTB expression and by binding directly to its regulated target transcripts (Calarco et al., 2009).

SC35

Experiments with the SR protein SC35 in the Balbiani ring (BR) genes performed by Björk and colleagues, 2009, have shown that individual SR proteins have several different functions in the cell nucleus during processing and transport of a single pre-mRNP, so called when a eukaryotic mRNA particle is transported from the nucleus to the cell cytoplasm. On the BR genes the interference with SC35 does not block initiation or elongation but the distribution of nearby BR pre-mRNPs changed. Is has been shown that SC35, in mammalian cells, recruits the kinase positive transcription elongation factor b to the elongation complex. This kinase is important to RNA polymerase II and elongation. The interference with SC35 leads to an increased number of transcripts in the distal region, which is consistent with the fact that the elongation process will be slowed down with the impairment of SC35 function (Björk et al., 2009).

Pyruvate dehydrogenase (PDH) complex deficiency is a common genetic disorder of mitochondrial energy metabolism. It is a major cause of lactic acidosis and most of the cases are sporadic and result from a mutation arising within the germ cells of one of the parents. At least 75 mutations have been identified in the E1α subunit-encoding gene (E1α is a subunit of
the PDH complex) and two exonic mutations have been associated with a skipping of exon 6. A shift from G to A in the intron downstream of exon 7 5’ splice site causes alternative splicing which involves the use of a cryptic 5’ splice site instead of the normal 5’ splice site. This mutation creates, or strengthens a SC35 binding enhancer element and also strengthens a pre-existing ASF/SF2 binding motif. SC35 activates the cryptic 5’ splice site which leads to the retention of intronic sequences. Increased levels of siRNA have been shown to reduce the levels of SC35 and thereby reversing the positive regulation of SC35 and increasing the amount of normal mRNA (Gabut et al., 2005).

**SRrp37**
SRrp37, SR related protein 37, is a recently found splicing regulator protein and its expression shows tissue specific expression patterns and it has also been shown that SRrp37 is involved in alternative splicing initiation. SRrp37 has been found to co-localize with SC35 in nuclear specks, but it is also found in nucleoli. This might be due to maturation or a traffic pathway for this protein. Newly synthesized endogenous SRrp37 is localized in speckles, whereas the mature protein is present in nucleoli. This subcellular localization of SRrp37 in both nucleoli and nuclear speckles suggest a role in both rRNA and mRNA processing. A large amount of a protein in the nucleoli is common with proteins that are either ribosomal proteins or proteins involved in the maturation of rRNA, whereas location in the speckles suggests that the protein is involved in pre-mRNA splicing. Experiments performed by Ouyang, 2009, have indicated that SRrp37 prefers not to use 5’ splice sites but instead promotes the use of a 3’ splice site. This stands in contrast to other SR proteins, such as ASF/SF2, which tends to promote both 3’ and 5’ splice site choices (Ouyang, 2009).

**ASF/SF2**
ASF/SF2, or Alternative splicing factor/splicing factor 2, is a sequence specific RNA binding protein with two RNA binding domains and a C-terminal RS domain for protein-protein interaction (Aubol et al., 2003). It promotes the binding of U1snRNP and recognition of 5’ splice sites. This increase in affinity for the 5’ splice site is the result of ASF/SF2 promoting of the annealing of complementary RNA (Eperon, et al., 1993). Furthermore, it interacts with the C-terminal domain (CTD) of RNA polymerase II which is necessary for its recruitment to the transcription sites (Li and Manley, 2005). It is foremost a nuclear protein, but it is known to shuttle between the cytoplasm and the nucleus. Phosphorylation and dephosphorylation are crucial in the splicing function of ASF/SF2 and affect its cellular localization (Mouton and Tsokos, 2010). The phosphorylation of the RS domain in ASF/SF2 acts to enhance protein–protein interactions with other RS domain-containing splicing factors, such as one subunit of the U1snRNP. The phosphorylation that is necessary for the activation of ASF/SF2 only takes place at one or more of the 20 serine residues in the RS domain (Aubol et al., 2003). ASF/SF2 tends to favor the downstream 5’ splice site; however, at higher concentrations additional 5’ splice sites could be recognized and thus activated. This would probably be those 5’ splice sites that are closest to the 3’ splice site due to proximal localization, or even cryptic 5’ splice sites (Zuo and Manley, 1994; Wang and Manley, 1995). ASF/SF2 can also promote a proximal choice between two competing 3’ splice sites. In this case, the 5’ splice site is fixed and ASF/SF2 promotes the use of a weak 3’ splice site. ASF/SF2 can interact with U2AF which binds to the branch point near the 3’ splice site. To evaluate the ability of ASF/SF2 to sense the proximity or distance, the effect of hnRNP A1 must be taken into consideration. hnRNP A1 promotes distal splicing and antagonizes the proximal splicing that is favored by ASF/SF2, which indicates that both ASF/SF2 and hnRNP A1 play important roles in splice site choices (Bai et al., 1999).
The β-tropomyosin gene generates a number of different protein products due to tissue-specific alternative splicing. It contains three pairs of exons that are mutually exclusive and in two of these the exon choice is related to the use of different promoters or polyadenylation signals. The third pair of exons, 6A and 6B, are selected in a tissue-specific manner and are independent of promoter or polyadenylation site choice. Exon 6A is included and expressed in fibroblasts and smooth muscle cells and exon 6B is exclusively included in skeletal muscle cells. Sequences at the 3’ end of the intron between the two exons and within exon 6B have been shown to repress the use of the 3’ splice site choice in exon 6B in non-muscle cells. A pyrimidine-rich region in the intron downstream of exon 6A is a splicing enhancer essential for recognition of the 5’-splice site of exon 6A. The SR protein ASF/SF2 is able to activate the use of exon 6A through specific recognition of the S4 enhancer sequence. That is somewhat unexpected, since ASF/SF2 often is involved in the recognition of exonic enhancer sequences rich in purines. The splicing factor SC35 on the other hand behaves as an inhibitor of the 6A exon and directly antagonizes ASF/SF2. It has been shown that abundance of these proteins differs between skeletal muscle cells and non-skeletal muscle cells. The shift towards SC35 in skeletal muscle cells contributes to the exclusion of exon 6A in these types of cells (Gallego et al., 1997).

**hnRNP proteins**

hnRNPs, or heterogeneous nuclear ribonucleoproteins, are a family of proteins that have many functions. They are involved in telomere biogenesis, mRNA stability and turnover, cytoplasmic trafficking of mRNA and in splicing control (Moran-Jones et al., 2009). In splicing, they are trans-acting factors that bind to either ESSs or ISSs. This functions to inhibit the use of certain splice sites during splicing events. However, there are some cases where hnRNPs promote splicing instead of inhibit it. The usual structure of hnRNP proteins consist of two or more RNA binding domains and a secondary domain. The secondary domain is thought to facilitate protein-protein interactions and ssDNA-protein interactions. But it is also involved in RNA-protein interactions, just as the RNA binding domains. Furthermore, most hnRNPs can interact with other hnRNPs (Clower et al., 2010). There are about 20 classes of hnRNP proteins and the hnRNP A/B family includes the paralogue A0-A3 and the last three proteins have multiple isoforms that is the result of alternative splicing (Zhu et al., 2002; Landsberg et al., 2006). When they are bound to ESSs or ISSs, they can bind along the RNA through cooperative binding, which can interfere with the binding of spliceosomal components or activating proteins, such as the SR proteins (Clower et al., 2010). It has also been suggested that several proteins of the hnRNP family can multimerize, thus creating regions of silencing across exons or modulate the conformation of the pre-mRNA and thereby influence exon recognition (D’Ambrogio et al., 2009).

**hnRNP A1/A2**

hnRNP A1 is one of the most common hnRNP proteins and is involved in many alternative splicing events. In humans it is a 320 amino acid protein with two N-terminal RNA recognition domains and the secondary domain is rich in glycine (GRD, glycine rich domain), a structure that it shares with hnRNP A2 (Clower et al., 2010). hnRNP A2 is a nuclear protein that is involved in RNA packaging and splicing, but also telomere biogenesis, cytoplasmic RNA trafficking, and cap-dependent translation. It has high affinity for, amongst others, a RNA sequence with homology to a region found in 3’ splice site selection elements (Landsberg et al., 2006). hnRNP A2 is believed to be involved, via up-regulation or mislocalization, in may human cancers and is found in many tumor-derived cell lines (Moran-Jones et al., 2009).
In a certain exon splicing silencer element, ESS3 of the HIV-1 tat pre-mRNA, the binding of hnRNP A1 is followed by cooperative binding of the secondary domain along the exon, which enables the hnRNP A1 to displace the SR protein SC35 from its ESE, which prevents splicing of that exon (Figure 6). It has been shown that hnRNP A1 can unwind the RNA secondary structure through cooperative binding. This involves the displacement of a GST-MS2 protein that is bound to a hairpin structure, which results in unwinding of the hairpin. The hnRNP A1 then spreads over the exon through cooperative binding in the 3’ to 5’ direction, thus displacing the SC35. This has not been shown with the SR protein ASF/SF2. This suggests that the effect depends on the strength of the SR protein interaction with its ESE and the abundance of the SR and hnRNP proteins in the nucleus (Okunola and Krainer, 2009).

In the exon definition model of splice site recognition the splice sites at each end of an exon surrounded by introns cooperate to establish the splicing of the nearby introns. When stimulating or inhibiting splicing at a proximal 5’ or 3’ splice site, ASF/SF2 promotes exon inclusion and hnRNP A1 promotes exon skipping respectively. In the APRT gene exon 4 it has been shown that the 3’ splice site upstream of this exon is weakened when there is a mutation in the 5’ splice site downstream of the exon, which creates interference with the exon definition. Mutations in either the 3’ or 5’ splice sites usually leads to exon skipping, however, in the case of the APRT exon 4 the mutation in the 5’ splice site only reduced the splicing at the 3’ splice site, it did not eliminate it. If hnRNP A1 is removed some of the exon inclusion is restored since it allows more use of the 3’ splice site, even though it is no longer well defined by a downstream 5’ splice site. This suggests that hnRNP A1 have a role in exon definition by inhibiting splicing at 3’ splice site. hnRNP may also be able to prevent the juxtaposition of the ends of a short exon which is necessary for the exon definition (Bai et al., 1999).

Figure 6. Description of the mechanism which hnRNP A1 utilizes to displace SC35 and inhibit exon splicing. 1: hnRNP A1 binds to the ESS while MS2 coat protein binds to the hairpin structure and SC35 binds to the ESE. 2-3: hnRNP A1 displaces the MS2 protein through cooperative spreading. 4-5: The hairpin is unwind. 6-7: Cooperative spreading of hnRNP A1 displaces bound SC35 from the ESE and inhibits splicing. From Okunola and Krainer (2009).
hnRNP A3

hnRNP A3 is a poorly understood protein of the hnRNP family. It has a similar structure to the hnRNP A1 and A2 and certain isoforms are known to co-localize with hnRNP A1 in neuronal transport granules and interact with nuclear actin, together with hnRNP A2 (Makeyev et al., 2005). It has recently been discovered that hnRNP A3 also is a telomere-binding protein. It has been shown that an hnRNP A3 mutant, hnRNP dA3, has a DNA-binding activity that is specific to the G-rich strand (Huang et al., 2008). Even though it is not involved in the regulation of alternative splicing, it undergoes alternative splicing itself. The **hnRNPA3** gene has homologous sequences, pseudo exons, on many chromosomes, but only the sequence on chromosome 2 has introns and shows the features of an active gene locus. A sequence that was thought to be the real gene has been proved to be a processed pseudo exon called **HNRPA3P1**. This pseudo exon contains a frame shift mutation that results in premature termination of translation and a non-functional protein. The real **hnRNP A3** gene has four isoforms that are created through alternative splicing where three of them lack nucleotides either in the beginning or in the middle. This suggests that there are two optional ways of the alternative splicing. However, the mechanisms behind the alternative splicing remain largely unknown (Makeyev et al., 2005).

hnRNP H

hnRNP H can, in contrast to many other hnRNP proteins, act both as an activator and as a repressor of splicing. An activation effect has been seen in, amongst others, the regulation of exon 6D of HIV-1 and the apoptotic mediator Bcl-x. In HIV-1, hnRNP H binds to a glycine rich domain and is required for the association of U1snRNP to exon 6D, which enhances exon inclusion. hnRNP is involved in the Bcl-x production by directing the 5’ splice site choice. Repression on the other hand has been detected at the 5’ splice sites in the NF-1 and THSβ genes. It can bind to G-rich 5’ splice sites and block U1snRNP binding, which leads to exon skipping. This especially takes place when there are mutations that impair U1snRNP pairing. The fact that hnRNP H can both activate and repress splicing by binding to GRDs suggests that in addition to the binding sites, the sequence and the position of the sites are important for the splicing activity (Crawford and Patton, 2006).

hnRNP L

hnRNP L is a nuclear RNA binding protein with many functions and is involved in mRNA export, mRNA stability and splicing and it has four RNA-recognition motifs. When it is interacting with introns, hnRNP L can act either as an activator or a repressor in alternative splicing, depending on the splice site proximity. hnRNP L is able to regulate its own expression through alternative splicing. Excess hnRNP L can bind to CA rich motifs located in exon 6 with high affinity. This leads to inclusion of exon 6A and in this case, the CA rich domain acts as an intronic enhancer of exon 6A inclusion. The inclusion of exon 6A brings with it a premature stop codon, which leads to a reduction of functional hnRNP L mRNA. It is believed that the CA rich domain contains different clusters and that several hnRNP L molecules binds to each cluster parts which may induce conformational changes which generates an hnRNP L concentration-dependent splice-regulatory signal. This role of activation contrasts to the usual repression activity of hnRNP proteins (Rossbach et al., 2009).

The CD45 gene encodes a hematopoiesis-specific transmembrane protein tyrosine phosphatase. Three variable exons of the **CD45** gene are skipped during T-cell activation which leads to a decreased phosphatase activity and maintenance of T-cell homeostasis. An exonic splicing silencer, ESS1, has been identified in the **CD45** exon 4. This element mediates both partial exon repression in resting cells and increased exon skipping during T-cell stimulation. The partial exon repression is the result of the binding of at least five hnRNP
proteins to the ESS1 element with hnRNP L acting as the main functional regulator. This stalls the assembly of the U1 and U2snRNP components which inhibits the formation of the spliceosome. The activation of the T cells leads to two changes in the composition of the ESS1 and the hnRNP L complex. A posttranslational modification of hnRNP L increases the silencing activity slightly while addition of a PTB-associated splicing factor, PSF, to the complex have a more prominent effect on exon skipping. The posttranslational modifications of hnRNP L is not associated with increased nuclear concentrations or changed affinity for ESS1 but may be related to phosphorylation or decreased acetylation. The levels of PSF are the same in both resting and in activated T cells, which suggests that the binding of PSF to the ESS1 element is not regulated by protein expression or localization. One suggestion for the mechanism of PSF regulation involves signal-induced changes in PSF association with other nuclear proteins and PSF is known to be a binding partner of PTB (discussed below), other nuclear proteins, DNA and RNA. This indicates that PSF may exist in a protein complex in the nucleus of resting T cells and upon activation of the cell, PSF is released and then recruited to the ESS1 complex. The functions of hnRNP L and PSF suggests that hnRNP L plays a major role in the basal level of exon repression under resting conditions and the combined effects of hnRNP L and PSF regulates the ESS1-dependent activation-induced silencing (Melton et al., 2007).

TDP-43
Nuclear factor TDP-43 is a multifunctional RNA binding protein that is a member of the hnRNP family. It is involved in transcription, pre-mRNA splicing, alternative splicing, mRNA stability and mRNA transport. It is a 414 amino acid protein and it has four distinct regions; an N-terminal sequence which contains a nuclear localization signal and two RNA recognition motifs (RRM). The RRM1 of TDP-43 mediates its RNA binding ability and RRM2 is needed for correct assembly. The fourth region is a glycine rich C-terminal domain. The GRD has been shown to be essential for the protein to function as a splicing silencer of exon 9 of the CFTR gene. This region is also responsible for the capability of forming complexes with other hnRNPs (Bose et al., 2009; D’Ambrogio et al., 2009). It has also been reported that this region is required for the ability of TDP-43 to act as a transcriptional insulator for a mouse gene. It has been shown that TDP-43 is able to bind to hnRNP A2. Since the very same region is necessary for the TDP-43 splicing inhibitory activity it is likely that TDP-43 is required to form an hnRNP complex through its C-terminus to inhibit exon splicing (D’Ambrogio et al., 2009). TDP-43 does not only interact with hnRNP proteins, but also with RNA helicases, splicing factors, translation regulatory proteins and proteins involved in mRNA transport and stability. TDP-43 has recently been identified as an important protein in diseases, such as ALS (Amyotrophic Lateral Sclerosis), FTLD-TDP (Frontal Temporal Dementia-TDP), and IBMFD (Inclusion Body Myopathy, Paget's disease and Frontotemporal Dementia). The pathology is typically characterized by clearance of TDP-43 from the nucleus and accumulation in the cytoplasm of affected cells, which indicates that the diseases involve either loss of TDP-43 nuclear function or toxic TDP-43 accumulation in the cytoplasm (Freibaum et al., 2009).

Spinal muscular atrophy, SMA, is a common autosomal recessive disorder that is caused by degeneration of motor neurons. The survival of motor neuron gene (SMN) is spliced into two, almost identical transcripts, SMN1 and SMN2. The difference is a shift from C to T at position 6 in exon 7 in SMN2. Homozygous loss of SMN1 cannot be compensated by SMN2 since the change in nucleotides at position 6 causes exon 7 skipping through alternative splicing. There has been two different suggestions to this splice pattern; the first is loss of an ESE and the second is gain of an ESS. Several trans-acting factors are involved in the exon 7 splicing, such as SR proteins and hnRNP proteins. The SR proteins act as positive regulators
through the AG-rich ESE element in SMN exon 7 and hnRNP A1 acts as a negative regulator by binding to the ESS element that is created by the change from C to T in the SNM2 exon 7. However, it is now believed that over expressed TDP-43 can promote exon inclusion of exon 7, which gives it a dual role in alternative splicing (Figure 7). Both RRM are needed for this regulation as well as interactions with an hnRNP protein and Htra2-β1 (a serine-protease). It seems that TDP-43 interacts with the RNA in a sequence-independent manner, however evidence suggest that the exon inclusion is mediated through complex formation at an ESE element in exon 7, thus inhibiting the negative effect by hnRNP A1 (Bose et al. 2008).

![Figure 7. The difference between SMN1 and SMN2. In this model the exon gains an ESS for hnRNP A1 in SMN2. The hnRNP A1 blocks inclusion of exon 7. When TDP-43 is over expressed it inhibits the effects of hnRNP A1, possibly by forming a new complex with the other proteins involved in this reaction that is resistant to the negative effect of hnRNP A1. From Bose et al. (2008).]

**PTB**

The polypyrimidine tract binding protein, PTB or hnRNP I, is an RNA-binding protein that binds to polypyrimidine tracts, such as those located at or upstream of 3’ splice sites. This can regulate alternative splicing by creating a region of silencing (Clower et al., 2010). PTB has the ability to interfere with exon definition but it seems that the PTB binding sites are not sufficient for exon silencing. Some exons require other silencer sequences or weak splice sites to achieve inhibition of exon inclusion. In the case of IIIb exon of FGF-R2 a weak polypyrimidine tract and an ESS that interact with hnRNP A1 is also needed to promote exon skipping. This suggests that PTB acts together with co-repressors to mediate exon silencing. PTB binding sites sometimes overlap binding sites for U2AF and this competition could be one reason for the inhibitory action of PTB. The majority of exons that are silenced by PTB are flanked by PTB binding sites on both nearby introns and it is suggested that PTB bind across the exon. In neuronal cells PTB is responsible for exon silencing, but there are still exons that are only expressed in neuronal cells. Lower levels of PTB in these cells and also the presence of a neuronal type of PTB, nPTB is thought to be responsible for this. nPTB can compete with PTB for binding but has a weaker repressive effect (Wagner and Garcia-Blanco, 2001).
Conclusions

Alternative splicing is a very complex mechanism that is responsible for the human diversity, large proteome and phenotypic complexity. This has become clearer during the last decade and it is now believed that up to 90% of the human genes undergo alternative splicing. Both constitutional and alternative splicing is catalyzed by the spliceosome. The spliceosome consists of five UsnRNPs and many, up to hundreds, of proteins. The recruitment and affinity of the spliceosome components are often dependent on regulatory proteins and sequences. In alternative splicing there are mainly four ways to create the alternative transcripts. There is the possibility of choosing an alternative 5’ or 3’ splice site, skipping of whole exons and intron retention. However, there is some debate about the intron retention mechanism, if it should fall under alternative splicing or not.

The regulatory system consists of, amongst others, regulatory SR proteins and hnRNP proteins. The SR proteins usually promote exon inclusion, such as ASF/SF2, SC35 and SRrp37, however there are exceptions. In the case of the β-tropomyosin gene SC35 antagonizes ASF/SF2 which leads to exclusion of exon 6A, which would give SC35 an inhibitory role. hnRNP proteins tends to inhibit exon inclusion such as hnRNP A1, hnRNP A2, hnRNP H, hnRNP L, TDP-43 and PTB. But there are exceptions among the hnRNPs as well and one such exception is hnRNP H which can act both as an activator and as a repressor of splicing. This regulatory activation can be seen in exon 6D of HIV-1 and in Bcl-x. The regulation of alternative splicing is also dependent on exonic splicing regulatory sequences and intronic regulatory sequences. These can be either enhancer or silencer, depending on where and how they act. The enhancer sequences are targets for SR proteins and the silencer sequences are targets for hnRNP proteins.

Alternative splicing relies on a complex mechanism that involves many components and interactions. It is also quite sensitive to mutations. If a mutation changes the affinity for regulatory proteins, creates or removes regulatory sequences or creates or changes the affinity for splice sites it changes the dynamic of the alternative splicing. Exons can be included, pseudo exons can be included and the length of the exons can be altered. This can lead both to nonfunctional proteins and proteins that cause disease. In the case of ataxia telangiectasia there is a mutation on an ISPE element on the ATM gene. This leads in several steps to the inclusion of a pseudo exon, which causes the disease. Pyruvate dehydrogenase complex deficiency is a genetic disorder of mitochondrial metabolism. A mutation in exon 7 of the E1α subunit leads to the use of a cryptic 5’ splice site instead of the constitutive splice site. The mutation creates or enhances an enhancer element for SC35. SC35 activates this cryptic splice site via the enhancer element, which leads to retention of intronic sequences. SMA, or spinal muscular atrophy, is dependent on the SMN gene. This gene has two very similar transcripts and the difference is a nucleotide shift in the second variant that creates an ESS element. Binding of TDP-43 to this element causes exon exclusion and disease due to nonfunctional SMN.

Due to all this further understanding of alternative splicing and its regulation is a very important step in managing and preventing disease. If the regulation of alternative splicing could be altered, diseases that are now incurable could get successful treatment. Alternative splicing is involved in different cancers and if the splicing could be inhibited or altered in the tumors, more efficient tumor control could be managed. Alternative splicing is an area where more research is needed, both for the understanding of human complexity and for disease management.
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