Published in final edited form as:
J Biomed Sci. 2004 ; 11(3): 278–294.

Regulation of alternative RNA splicing by exon definition and exon sequences in viral and mammalian gene expression

Zhi-Ming Zheng
HIV and AIDS Malignancy Branch, Center for Cancer Research, National Cancer Institute, National Institutes of Health, Bethesda, MD 20892, USA

Abstract

Intron removal from a pre-mRNA by RNA splicing was once thought to be controlled mainly by intron splicing signals. However, viral and other eukaryotic RNA exon sequences have recently been found to regulate RNA splicing, polyadenylation, export, and nonsense-mediated RNA decay in addition to their coding function. Regulation of alternative RNA splicing by exon sequences is largely attributable to the presence of two major cis-acting elements in the regulated exons, the exonic splicing enhancer (ESE) and the suppressor or silencer (ESS). Two types of ESEs have been verified from more than 50 genes or exons: purine-rich ESEs, which are the more common, and non-purine-rich ESEs. In contrast, the sequences of ESSs identified in approximately 21 genes or exons are highly diverse and show little similarity to each other. Through interactions with cellular splicing factors, an ESE or ESS determines whether or not a regulated splice site, usually an upstream 3′ splice site, will be used for RNA splicing. However, how these elements function precisely in selecting a regulated splice site is only partially understood. The balance between positive and negative regulation of splice site selection likely depends on the cis-element's identity and changes in cellular splicing factors under physiological or pathological conditions.

Keywords

RNA; exons; introns; alternative RNA splicing; gene expression; RNA processing; splicing enhancers; splicing suppressors

RNA exon sequences were once thought to function simply as coding sequences. Recent advances in RNA research have indicated that the RNA exon sequence has a dual role: protein encoding and RNA processing through interactions with cellular proteins. Recent reviews have described the progress in several aspects of RNA processing research, including messenger RNA-binding proteins [46], network-coupling among gene expression [111], alternative pre-mRNA splicing and proteome expansion [112], exon point mutation and RNA splicing [19], intron elements in alternative splicing [90] and RNA splicing and human diseases [50]. This review will focus on the roles that exon definition and exon sequences play in the regulation of alternative RNA splicing.

Gene expression and RNA splicing

Most eukaryotic genes consist of exons and introns. Exons are segments of an interrupted gene that are represented in the mRNA, and introns are the sequences that intervene between exons. Each intron has at least three cis-elements: a 5′ splice site (5′ss), a branch site, and a 3′ splice site (3′ss) containing a run of 15 to 40 pyrimidines (usually Us) called a polypyrimidine tract

Zhi-Ming Zheng, M.D., Ph.D., HIV and AIDS Malignancy Branch, Center for Cancer Research, NCI/NIH, 10 Center Dr., Rm. 10 S255, MSC-1868, Bethesda, MD 20892-1868, USA, Tel. 301-594-1382, Fax 301-480-8250, E-Mail: zhengt@exchange.nih.gov.

(PPT). Most viral and mammalian introns are GU-AG introns, introns that start with a GU dinucleotide at intron 5′ ends and end with an AG dinucleotide at intron 3′ ends, with a few minor AU-AC introns [155;169]. Some exons are noncoding or partially coding exons, which are usually positioned at the 5′ or 3′ end of an RNA. With few exceptions, the majority of primary RNA transcripts (pre-mRNAs) of viral or mammalian split genes undergo multiple steps of nuclear RNA processing, including RNA 5′ capping, RNA splicing, and RNA 3′ end polyadenylation, before the mRNA is completely formed and exported to the cytoplasm for protein translation (Fig. 1). Recent studies have demonstrated that nuclear RNA processing is performed by a co-transcriptional network [52;111]. Eukaryotic and viral RNAs transcribed by RNA polymerase II (pol II) have m^7G caps added to their 5′ ends cotranscriptionally after about 20-30 nucleotides have been synthesized [27;124] recognizing and splicing the first intron on the nascent RNA transcript by cellular splicing machinery along the transcription elongation and emergence of the next exon and intron [53;89;119;172]. This co-transcriptional splicing of the first intron can be greatly enhanced by the RNA capping machinery [78;101;128], but alternative 5′ ss selection in co-transcriptional splicing can be influenced by the transcription rate of RNA pol II [38]. Finally, as a poly (A) signal emerges on newly synthesized RNA, addition of a poly(A) tail to the 3′ end of the RNA is initiated by polyadenylation factors carried along by the elongating Pol II [35;70], which in turn promotes splicing of the RNA 3′ terminal intron [102;120;161].

Pre-mRNA splicing involves five small U RNAs (U1, U2, U4, U5, and U6) and many splicing factors. Defining the exon-intron boundary is the first step in the accurate recognition of an intron 5′ ss and an intron 3′ ss. This involves interaction of the 5′ ss with U1 snRNP, of the branch site with U2 snRNP, and of the 3′ ss with $U2AF^{65}$ and $U2AF^{35}$ (a heterodimer of U2 auxiliary factors), modulated by many cellular splicing factors including serine/arginine-rich (SR) proteins [60]. SR proteins are a growing family of structurally related and highly conserved cellular splicing factors that are characterized by the presence of an RNA-recognition motif (RRM) and Ser/Arg (SR) dipeptides. Over the past two decades, a great deal has been learnt about how RNA splicing takes place by using a yeast system and RNAs containing strong introns. Unfortunately, the majority of viral and mammalian introns have suboptimal (weak) features with non-consensus sequences at each splice site and branch sites for these interactions. Sometimes an authentic splice site is indistinguishable from a cryptic splice site embedded in a viral or mammalian pre-mRNA and identification of its usage has to rely solely on experimental analysis. This raises many intriguing questions of how the cellular splicing machinery defines an authentic splice site in a “sea of similarity”.

Alternative RNA splicing and proteomic diversity

Constitutive RNA splicing describes the situation in which all introns on a pre-mRNA are spliced in the 5′ ss to 3′ss order and all exons are represented in the spliced message. In contrast, alternative RNA splicing implies that an RNA splice site is not used constitutively; instead, additional splice sites can be used as alternatives to it during RNA splicing. Alternative RNA splicing usually occurs when the RNA bears a weak splice site and presumably is dependent on the local availability of the correct forms of splicing factors. Thus, alternative splicings of a particular RNA can be found in different cell types, at different stages of cell differentiation, or at different stages of viral life cycles. The consequence of alternative splicing is the generation of different isoforms of the RNA for production of different proteins required for specific functions (Fig. 1). In general, there are four types of alternative RNA splicing (Fig. 2): alternative 5′ splice site selection, alternative 3′ splice site selection, intron retention, and exon exclusion and skipping. Although the example given for each category provides a simplified form for a clearer description, complex RNA splicing that includes all four modes could be found on a single pre-mRNA bearing multiple exons and introns. In a few unusual

cases, even exon repetition by trans-splicing and exon scrambling through RNA splicing and re-splicing have been described in mammalian gene expression [22;54;173].

Alternative RNA splicing has received little attention outside of RNA field since the discovery of exons and introns in the adenovirus in 1977 [9;28]. Recent reports of only 24,500 [92] to 26,588 [163] human genes, with an additional 5000-12,000 expected to be reported as gene-prediction programs improve, came as a surprise for most scientists, who had predicted that the human genome would have up to 120,000 genes [103]. Ironically, the number of human protein-coding genes is just twice that in the fly genome, and about 5 times larger than that in yeast (Table 1)! However, the finding that almost every human gene, on average, has 4.7 to 7.2 exons, with the largest number of exons, 234, identified in titin transcripts [163], was another surprise. This report from Celera also estimated that the decoded human genome has approximately 17,764 genes (66.8% of all human genes) that can produce alternatively spliced forms of the transcripts[163]. The number of human genes producing alternatively spliced transcripts is far more than that of any other species whose genome has been completely sequenced (Table 1). This is just one of many intriguing issues about the nature of genomic complexity. Conceivably, the complexity of a proteome is increased significantly by alternative RNA splicing rather than by transcriptional regulation. For example, alternative RNA splicing contributes over 1000 isoforms of neurexin transcripts that are expressed in distinct subsets of neurons [159]. As a result, it is an extreme challenge to sort out how neurons regulate such complex RNA splicing and the function each message specifies for a cell. A highly evolved mechanism must exist in the expression of a human gene to specify individual protein expression by precisely defining the selection of the correct isoform of the transcript. Recent studies have demonstrated that there are hundreds to thousands of cellular proteins that are associated with the regulation of RNA processing, and many of these are alternative RNA splicing factors [4;66;134;182]. Thus, it is understandable that, even before the human genome was completely decoded, aberrant RNA splicing was found to account for at least 15% of genetic diseases and some cancers [50].

Alternative RNA splicing has been extensively studied in viral gene expression ever since the discovery of RNA splicing. Viruses take advantage of alternative splicing to diversify their gene expression and thus to enlarge their proteomic potential with a limited genome. The adenovirus has served as a model since it was used in the discovery of RNA splicing [9;28]. Other well-understood systems are those of the papillomaviruses and HIV. Alternative viral RNA splicing in papillomavirus infection is associated with the virus life cycle and depends on the differentiation state of the keratinocyte host. HIV is a complex retrovirus that creates 46 different RNA transcripts by alternative splicing of a single genomic RNA for a total of 47 different mRNA species in the host [131]. Since viral RNA splicing utilizes the cellular splicing machinery (spliceosome) and is processed through the mammalian RNA splicing pathway, elucidation of viral RNA splicing control has provided much of our understanding of the regulation of mammalian RNA splicing over the past two decades, and will most likely continue to do so.

Exon definition and alternative RNA splicing

The definition of an exon presupposes that there is cross-talk between a 3′ ss and a 5′ ss over the exon, and that recognition of an upstream 3′ ss by the cellular splicing machinery can be strengthened by a downstream 5′ ss over the exon [8;71;76;88]. Accordingly, an oversized internal exon larger than 500 nucleotides (nts) would limit such cross-talk and splicing enhancement. This principle has been supported experimentally [148] and bioinformatically [174]. In fact, most mammalian pre-mRNAs contain very short exons, whereas the introns can be as large as tens of thousands of nts long. It is therefore often difficult to identify relatively small exons in the context of much larger non-coding intronic sequences.

The definition of an exon in the regulation of alternative RNA splicing can be exemplified by the splicing of Kaposi's sarcoma-associated herpesvirus (KSHV) K8 RNA. KSHV K8 is an early gene and encodes for a K-bZIP protein involved in viral DNA replication [104]. KSHV K8 pre-mRNA has four exons and three introns, with exon 3 bearing three alternative 5′ splice sites (Fig. 3) at nts 75838, 76155, and 76338 [153]. Utilization of the nt 75838 5′ ss stimulates splicing at the upstream nt 75645 3′ ss and predominates over the other two 5′ ss during RNA splicing. A primary reason that the nt 75838 5′ ss is preferentially selected over the other two 5′ ss is that the small size of exon 3 might make the cross-talk between the 3′ ss and 5′ ss accessible over the exon.

Removal of the first intron from a pre-mRNA has been found to be cap-dependent. We recently showed that the size of an RNA 5′-terminal exon also affects the cap-dependent RNA splicing. We have concluded from various approaches that efficient splicing of a pre-mRNA depends on the distance of the cap-proximal intron 5′ ss from the RNA 5′ cap, with an optimal distance of less than 304 nts (Zheng et al, unpublished observation). The large distance of a cap-proximal 5′ splice site from the RNA 5′ cap in AAV2 P5- and P19-generated transcripts may explain why this splice site is not recognized by the cellular splicing machinery [132]. Simply put, the cap-proximal 5′ ss will be easily selected if the cap-proximal exon is smaller than 304 nts. As a result, the RNA 5′ cap-binding complex (CBC) strengthens recruitment of U1 or U6 to a cap-proximal 5′ ss and mediates recognition of the cap-proximal 5′ ss. An extensive computational analysis of 2139 human genes consistently revealed that the mean length of a gene's first partially coding or non-coding exon is less than 348 nts [37].

Exonic splicing enhancers

As described above, the majority of viral and mammalian RNA introns are suboptimal, lacking consensus sequences in their splice sites. Instead of having a run of 15 to 40 pyrimidines (usually Us), the PPT between a branch site and a 3′ ss AG dinucleotide in viral or mammalian RNA introns is generally interspersed with purines and has low binding affinity for splicing factor U2AF, which is essential for 3′ ss recognition. How these suboptimal 3′ ss are selected correctly has been a major focus of splicing research in the past decade. This effort led to the discovery of exon recognition elements, which were later renamed exonic splicing enhancers (ESEs) [93;150;164;170]. The discovery of ESEs was exciting because they are distinct from the classical, intronic splicing signals.

Two classes of ESEs have been reported. The purine-rich ESEs are the most common and are usually located, with great vicinity, downstream of a suboptimal 3′ ss [49]. ESEs stimulate splicing of the upstream intron at both steps of the two-step trans-esterification splicing reaction [26;180]. The natural purine-rich ESEs found in viral and mammalian exons consist of a core motif of alternate As and Gs, generally 6 nts or longer (Table 2); an A-run or G-run sequence in an exon does not function as a splicing enhancer [152]. A purine-rich ESE usually has one or more SR binding sites. Through interactions with a subset of SR proteins, purine-rich ESEs recruit or strengthen the binding of U2AF to a suboptimal, upstream 3′ splice site and stimulate spliceosome assembly [61;93;150;184]. However, an exon sequence having one or more SR binding sites does not necessarily function as an ESE [178;179]and some of the exonic splicing suppressors (ESSs) also bind SR proteins [117;179]. A purine-rich element can also suppress splicing of a pre-mRNA when located in a regulated intron [56;84;181]. Thus, depending on the location and features of the regulated 3′ ss, a purine-rich ESE can function as an exonic splicing enhancer or an intronic splicing suppressor.

The other class of ESEs is the non-purine-rich ESE. This class includes the exonic AC-rich enhancer and exonic pyrimidine-rich enhancer (Table 2). The AC-rich enhancers were first identified by in vivo selection experiments and were found to stimulate splicing both in vivo

and in vitro [32]. AC-rich ESEs have been shown to be involved in the regulated splicing of both viral and cellular genes [32;57;181]. Recent studies demonstrated that the AC-rich ESEs interact with a cold-shock cellular protein, Y box protein 1 (YB-1), to function in a manner similar to that of the purine-rich ESEs [149]. Exonic pyrimidine-rich enhancers have been described in β-globin RNA [137] and other mammalian RNAs [45;145].

Exonic splicing suppressors

Exonic splicing suppressors or silencers (ESS) (Table 2) were discovered ten years ago in the course of exploring ESEs and have been identified in many pre-mRNAs [2;39;146;176]. These *cis*-elements negatively regulate the utilization of upstream 3′ splice sites. They are frequently located downstream of a juxtaposed ESE, but can also function upstream of an ESE [145; 166;178]. Unlike the ESEs, the sequences of the ESSs show little similarity to each other. However, each of the ESSs appears to contain a functional core motif that binds a number of cellular splicing factors. Seeking trans-acting factors for ESS function in the inhibition of RNA splicing was a major focus of research in the past six years, and the first breakthrough was the finding of multiple cellular splicing factors, the PTB, U2AF, and SR proteins, that bind to bovine papillomavirus type 1 (BPV-1) ESS1, a pyrimidine-rich ESS [179]. Subsequently, other cellular factors were also found to play a role in ESS function, including SC35 for the HIV-1 *tat* exon 3 ESS [117], hnRNP H for the rat β-tropomyosin exon 7 ESS [23], and hnRNP A1 for the FGFR 2 K-SAM ESS [41] and for the HIV-1 *tat* exon 2 ESS [17]. Moreover, the fibronectin EDA ESS has been also implicated in the maintenance of an RNA conformation that facilitates display of the adjacent ESE SR protein binding sequences [125].

Thus, ESSs may inhibit RNA splicing in vitro and in vivo through multiple mechanisms. Many ESSs appear to antagonize the activity of ESEs [83;176], and some of them are even both splice-site- and ESE-specific, such as BPV-1 ESS2 [181]; however, the exact mechanism by which ESSs act remains largely unknown. ESS splicing suppression in some viral or mammalian RNAs without notable ESEs, including an RSV src pre-mRNA [178] and FGFR-2 K-SAM [39;40], indicates that in some cases the function of the ESS is independent of an ESE. In addition, ESSs function, in most cases, only on suboptimal, alternative splice sites. Supporting this, the human β-globin pre-mRNA, which contains a constitutive, optimal (strong) 3′ ss, splices efficiently in the presence of a heterologous ESS [178], and optimization of a weak (suboptimal), alternatively regulated 3′ ss can also counteract the function of the ESS [142;146;180].

Regulation of alternative RNA splicing by ESEs and ESSs

Although ESEs and ESSs are thought to be present in most if not all alternatively spliced exons, the mechanisms that regulate alternative RNA splicing are only partially understood. The accepted principles on how ESEs and ESSs function in vivo have largely been deduced from in vitro studies. Currently, there are two simplified models of how an ESE functions in ESE-dependent RNA splicing. In the U2AF recruitment model, ESE-bound SR proteins interact with $U2AF^{35}$ and recruit $U2AF^{65}$ to a weak 3′ ss. In this model, $U2AF^{35}$ serves as a bridge between ESE-bound SR proteins and $U2AF^{65}$ through protein-protein interactions [62;184]. The existence of ESEs that exclusively bind SR proteins favors this model, but this model does not initially take ESSs into account, and disregards how an ESE overcomes the splicing suppression of an ESS. The second model, the ESE neutralization model, hypothesizes that the primary function of an ESE in an exon is to antagonize the function of an ESS within the same exon, rather than to recruit U2AF to an upstream 3′ ss [83]. This model is supported by evidence that removal of an ESS can enable splicing in the absence of the enhancer, that is, ESE-independent splicing can occur. However, alternative splice sites on viral and mammalian pre-mRNAs commonly have nonconsensus splicing signals and would not be recognized

efficiently by the cellular splicing machinery in the absence of an ESE, precluding the generality of the latter model. Along with the elucidation of the intrinsic properties of ESEs and ESSs, several excellent studies in model systems have provided convincing evidence that alternative RNA splicing regulated by ESEs and ESSs in physiological conditions depends on many variables, including the presence of multiple RNA cis-elements, the strength of the splicing signals, the cell type, and the cell activation and differentiation status, as well as the stage of viral infection. Thus, beside exon cis-elements on an RNA, the availability of trans-acting splicing factors and their physiological (modification) status in a defined environment play important roles in ESE-and ESS-regulated RNA splicing.

Regulation of alternative 3′ ss selection in splicing of BPV-1 late pre-mRNA

Regulation of alternative 3′ ss selection by ESEs and ESSs has been extensively studied in the BPV-1 late pre-mRNA. The primary transcripts expressed from the viral late promoter have a common late leader 5′ ss, but use a proximal or a distal 3′ ss for RNA splicing. Splicing at the proximal 3′ ss leads to the production of L2 mRNA, whereas splicing at the distal 3′ss results in the production of L1 mRNA. In situ hybridization studies demonstrated that the distal 3′ ss is utilized only in the granular cell layer of the epidermis, while selection of the proximal 3′ ss occurs in both the granular and spinous layers [6]. Recent studies show that this differentiation-specific alternative 3′ ss selection is related to the suboptimal features of both the proximal and distal 3′ss and is regulated by five viral cis-elements: three ESEs and two ESSs. Among these elements, three *cis*-acting elements, SE1, SE2, and ESS1, are positioned between the proximal and the distal 3′ ss, with ESS1 immediately downstream of SE1 and 122 nts upstream of SE2 (Fig. 4A). These elements regulate the selection of the proximal 3′ ss for RNA splicing [176; 177;179;180], whereas two other elements, SE4 and ESS2, are located downstream of the distal 3′ ss and control selection of the distal 3′ ss [181]. Thus, the five cis-elements interact in a coordinate fashion to specify which 3′ ss should be used. Any subtle changes that disrupt this coordinate balance can provoke a switch from one 3′ ss to another.

BPV-1 SE1 and SE2 are purine-rich ESEs and each has at least two ASF/SF2 binding sites, although others may exist [177]. BPV-1 ESS1 is composed of a 48-nt pyrimidine-rich sequence and has a U-rich 5′ region interacting with U2AF and PTB proteins, a C-rich central part binding 35- and 54-55 kDa SR proteins, and an AG-rich 3′ end favoring ASF/SF2 interaction [179]. Although it binds to PTB and $U2AF^{65}$, the 5′ U-rich region of ESS1 appears not to be essential for the inhibition of splicing in vitro [178;179] or in vivo [180]. Various approaches have shown that SE1 and SE2 synergistically promote the selection of the proximal 3′ ss over the suppression by ESS1 [180], presumably by interacting with SR proteins such as ASF/SF2, since mutation of either one of the two ASF/SF2 binding sites within either SE1 or SE2 switches the selection to the distal 3′ ss. Deletion or mutation of the ESS1central suppressor core GGCUCCCC, which also binds SR proteins but does not stimulate splicing [178;179], does not affect selection of the proximal 3′ ss. The same is true of ESS1 and SE2 double mutations; in this context, SE1 enhances the RNA splicing at the proximal 3′ ss in the absence of a functional ESS1 and SE2 [180]. The importance of ASF/SF2 in the selection of the proximal 3′ ss has been further verified under physiological conditions, where depletion of ASF/SF2 from cells decreases BPV-1 late RNA splicing at the proximal 3′ ss with a reciprocal increase in utilization of the distal 3′ ss [106]. This switch is also regulated by an AC-rich ESE, SE4, and an ESS2 downstream [181]. Accordingly, the mutation in SE4 can turn the distal 3′ ss usage back to the proximal 3′ss usage. In a BPV-1 late pre-mRNA containing a mutant SE2, a mutation in SE4 can cause the splicing machinery to revert to using the proximal 3′ ss instead of the distal 3′ ss (Liu and Zheng, et al, unpublished data), indicating that it functions in vivo. SE4 and ESS2 functionally resemble SE1 and ESS1 in the selection of an alternative, suboptimal 3′ ss, probably through interactions with the cellular protein YB-1 [149]. The over-expression of YB-1 by transient transfection increases utilization of the distal 3′ ss, supporting

this assumption (Zheng, et al., unpublished data). Although SE4 functions as an ESE for heterologous 3′ ss, BPV-1 ESS2 apparently operates in a 3′ splice site-specific and enhancer-specific manner [181]. It will be interesting to understand how ESS2 plays a suppressive role in the SE4-mediated RNA splicing. In summary, regulation of the usage of two alternative 3′ ss in the splicing of BPV-1 late pre-mRNAs is an intricate network of viral exonic cis-elements which is governed closely by cellular splicing factors in accordance with the stage of keratinocyte differentiation.

Regulation of alternative 5′ ss selection in splicing of adenovirus E1A pre-mRNA

Compared to the regulated selection of 3′ ss, there are only a few examples of ESE-regulated natural 5′ ss selection. Perhaps this is largely because recognition of the 5′ ss and the first step of RNA splicing are independent of the 5′ exon sequence. In fact, an adML RNA containing only 1 nt of 5′ exon sequence and having a downstream intron can undergo the first step of the splicing reaction in the presence of SR proteins [68]. This observation is consistent with several earlier reports that cellular splicing factors are involved in the regulation of alternative 5′ ss selection. This has been learnt largely from using ad2 E1A pre-mRNA as a model to analyze how SR proteins regulate alternative 5′ ss usage. Ad2 E1A pre-mRNA has three 5′ ss and one major and one minor 3′ ss. Alternative splicing of the three 5′ ss during early and late periods of the viral infection produces three main spliced RNA products: 9S, 12S, and 13S (Fig. 4B), among which the 13S RNA predominates during the early viral infection and the 9S RNA accumulates late in the infection. The 13S RNA encodes for a full-length oncoprotein, E1A. A number of studies have demonstrated that individual SR and non-SR proteins play roles in the selection of each alternative 5′ ss and in the shift from 13S to 9S RNA production. One interesting observation is that the 13S-to-9S transition can be triggered through a titration of the SR proteins 9G8, SC35, and ASF/SF2 by major-late transcripts that accumulate in nuclei late in the infection [69]. ASF/SF2 was the first of these that was found to relate to the selection of the 13S 5′ ss, but its role in this 5′ ss selection can be antagonized by overexpressed hnRNA A1 [14;116]. Later, SRp40 [138] and SRp54 [175], and more recently TLS [99] and RBM4 [91], were found to preferentially activate the 9S 5′ ss. However, lack of a specific binding site upstream of the 13S and 9S 5′ ss for these proteins suggests that individual splicing regulators of the 5 ‘ss selection site might function in an RNA sequence-independent manner (Fig. 4B). Interestingly, recent identification of a purine-rich ESE located immediately upstream of the 12S 5′ ss, and demonstration that such an ESE is involved in selection of the 12S 5′ ss through binding to SR protein 9G8, suggests that at least one of the 5′ ss on ad2 E1A pre-mRNA is preferentially selected in an ESE-dependent manner [11]. Likewise, a 32-nt ESE positioned between two regulated 5′ ss in exon 5 of the caldesmon pre-mRNA has been also found to stimulate the use of the upsteam 5′ ss and to regulate usage of a competing 5′ ss in a differential internal exon [48;75]. There have been no reports of ESS-regulated selection of alternative 5′ ss.

Remarks and perspectives

The hallmark discovery in the past decade of exon cis-elements involved in regulating RNA splicing has changed our view that the only function of exon sequences is to encode proteins. That the splicing of minor AU-AC introns in vivo [43] and in vitro [168] also responds to purine-rich enhancers indicates that ESE- and ESS-regulated RNA splicing has effects well beyond that of the regulated splicing of major GU-AG introns. Although this review mainly focuses on the roles of exon sequences in the regulation of alternative RNA splicing of major GU-AG introns, the many other functions displayed by exon sequences should not be ignored. For example, exon sequences have recently been found to be responsible for nonsense-mediated RNA decay (NMD) and for nuclear export of spliced mRNAs. An exon region 20-24 nts upstream of a splice junction provides a binding site for several cellular proteins of the exon

junction complex (EJC) that may function in NMD [86;109]. NMD degrades RNA that has a premature termination codon (PTC) >50-55 nts upstream of a 3′-most exon-exon junction [113;122], and is a means to prevent the synthesis of potentially harmful truncated proteins due to aberrant or alternative RNA splicing. Other components of the EJC, such as REF/Aly, could interact with the mRNA export receptor TAP to facilitate mRNA export [87;95;135; 183]. Taken together, there is no reason not to accept that alternative RNA splicing expands the diversity of the genome, while creating another layer in which to check the integrity of messages and to destroy unwanted ones before a protein harmful to the host is made. The finding that 35% of EST-suggested, alternatively spliced RNA isoforms carrying PTCs are apparent targets for NMD [100] makes this house-keeping hypothesis more plausible. Moreover, exon sequences on 3′ terminal exons have been also characterized as regulating RNA polyadenylation [15;151] and in defining the length of the RNA poly (A) tail [63;64].

Perhaps the most remarkable advance in the characterization of ESEs/ESSs is the finding that a single nt mutation within an ESE or ESS can lead to alternative RNA splicing that is associated with a genetic disorder or cancer. A single-base-change (C to U) at position +6 in SMN2 exon 7 converts an ASF/SF2-binding sequence [20] into an ESS that binds hnRNP A1 [85]. This mutation prevents efficient exon 7 splicing promoted by a downstream, Tra2-dependent, purine-rich ESE [72;107] and results in the skipping of exon 7 during the splicing of SMN2 RNA (Fig. 5). The BRCA1 gene in breast cancer has a G to U mutation at position +6 of exon 18 that disrupts the function of an ESE. This single-base mutation converts a high-score SF2/ASF motif (CUGAGUU) into a low-score SF2/ASF motif (CUUAGUU), and consequently leads to the skipping of exon 18 in the spliced BRCA1 mRNA and a C-terminal–truncated BRCA1 [21;105;118]. Together, these two studies exemplify the profound potential influence of single nucleotide polymorphisms on post-transcriptional events and the pathogenesis of genetic disorders and cancers. Thus, disease-related disruption and/or creation of ESE/ESS may well be a continuing, prospective focus in RNA splicing research.

The cell- or tissue-specific expression of splicing factors that regulate alternative RNA splicing has provided some fundamental insight into how alternative RNA splicing takes place in a cell- or tissue-specific manner. Regulation of the 4.1R exon 16 (E16) splicing switch by hnRNP A/B protein during erythropoiesis provides an excellent example. Expression of the human 4.1R gene is necessary for erythrocyte membrane formation. Erythrocyte membranes containing a 4.1R protein that lacks E16 coding sequences due to E16 skipping are very susceptible to shear forces, which could lead to hemolytic anemia. The 4.1R E16 contains an ESS that specifically interacts with hnRNP A/B proteins and inhibits E16 inclusion in the spliced 4.1R mRNA. Interestingly, this inhibition is relieved in differentiated erythroblasts. Declining expression of hnRNP A/B during erythroid differentiation has been correlated with increased E16 inclusion in the 4.1R mRNA [74] This indicates that natural developmental changes in hnRNP A/B proteins can affect physiologically important switches in pre-mRNA splicing. Gaining further understanding of how the expression of specific splicing factor(s) in specific tissues and cells contributes to the regulation of alternative RNA splicing will be a challenge, but may deliver the next wave of discoveries in RNA research.

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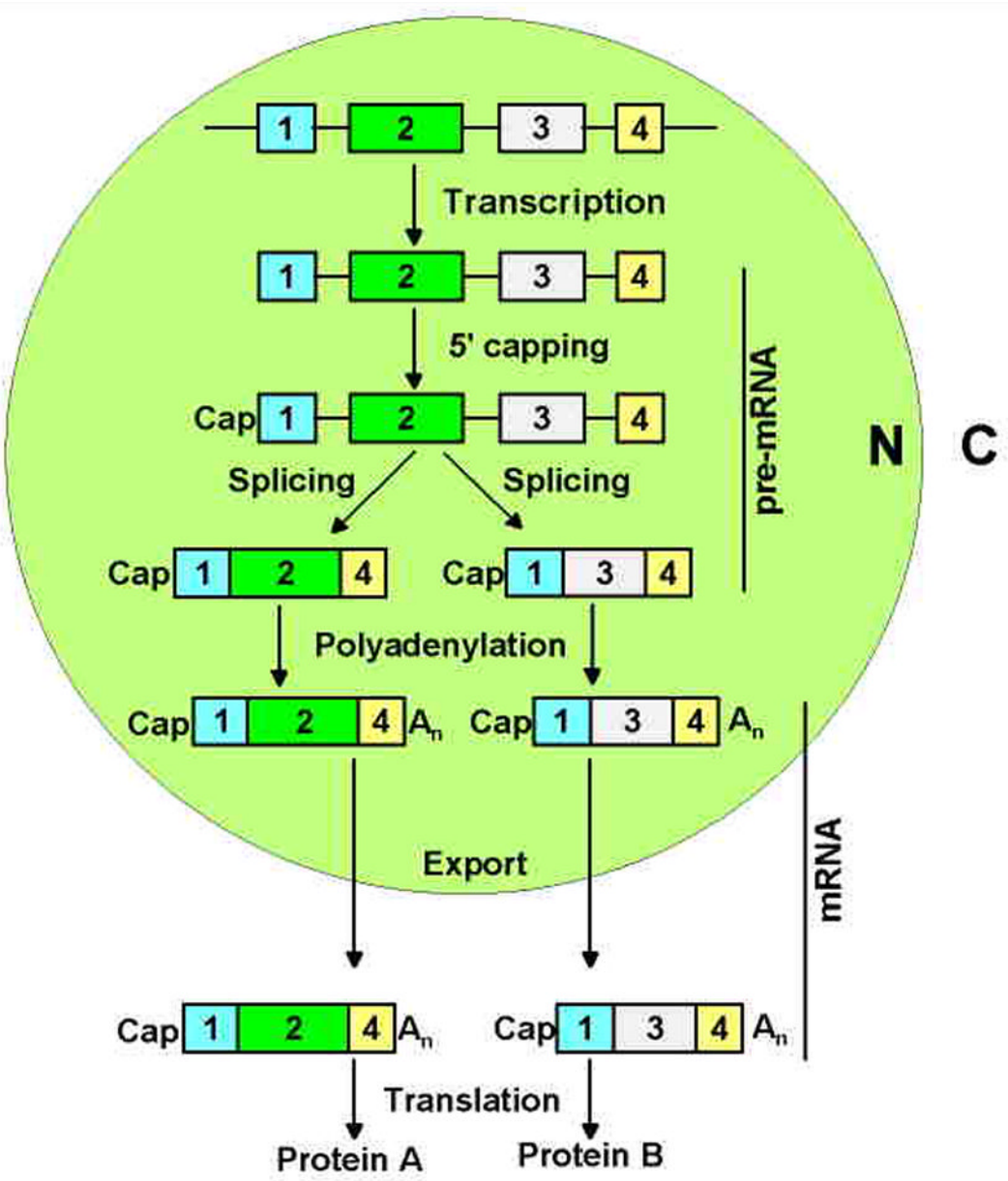

Fig. 1.

Viral and eukaryotic gene expression. Exons (boxes) and introns (lines) are indicated. The diagram illustrates the processing steps that occur before an mRNA is exported from the nucleus for translation. Alternative RNA splicing leads to the production of two isoforms of the message and consequently two different proteins. N, nucleus; C, cytoplasm.

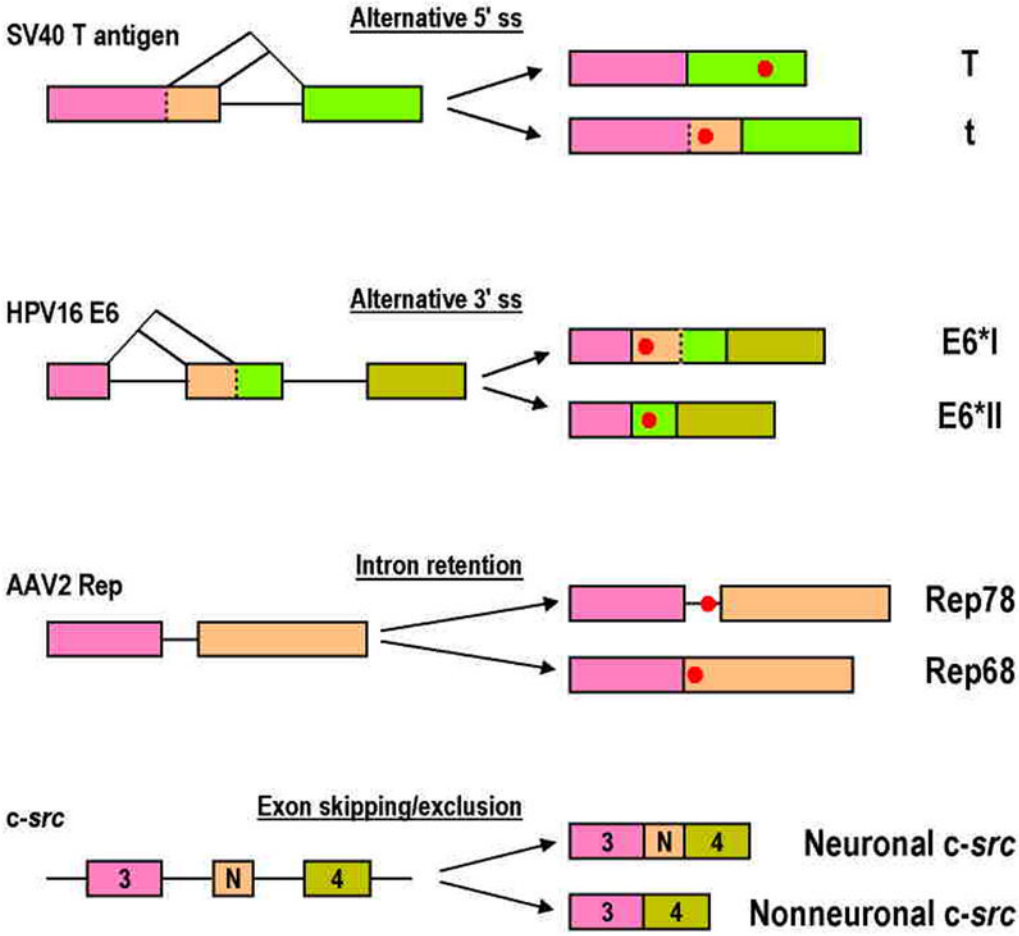

Fig. 2.

Types of viral and mammalian alternative RNA splicing. Examples of splice types occurring naturally in viral and mammalian RNA are shown. Exons (boxes) and introns (lines) are illustrated for each species of RNA. Alternative splice sites are indicated as vertical dashed lines. Red dots show stop codon locations on spliced RNAs. The names of the alternate spliced RNAs are indicated at the right. Drawings are not to scale. SV40 T antigen, simian virus 40 large T antigen RNA; HPV16 E6, human papillomavirus type 16 E6 bicistronic RNA; AAV2 Rep, adeno-associated virus type 2 Rep RNA [132]; mouse c-*src* RNA [136].

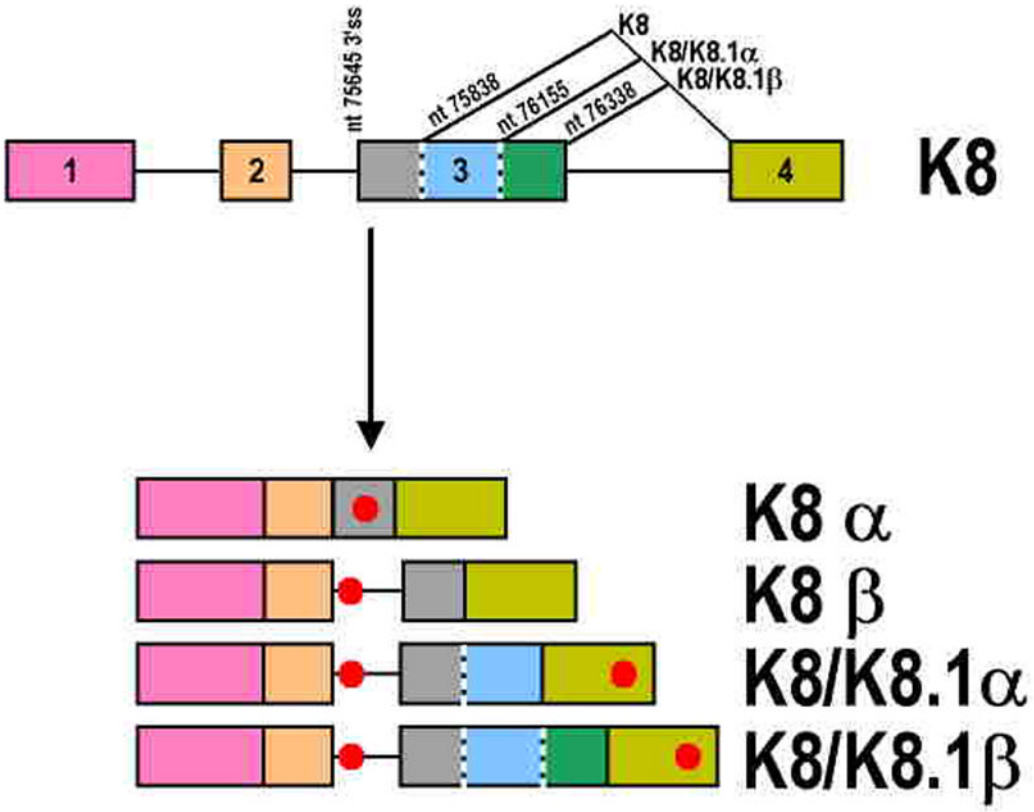

Fig. 3.

Alternative splicing of Kaposi's sarcoma-associated herpesvirus K8 RNA. KSHV K8 exon 3 has three alternative 5′ ss that share a single 3′ ss. See other descriptions in Fig. 2. It has been noted that removal of K8 intron 2 requires the selection of nt 75838 5′ ss and exon definition. An incomplete removal of intron 2 leads to production of K8β. Intron 2 retention is most common in the mRNAs with the use of the other two 5′ ss because selection of either one makes exon 3 larger than 500 nts, which restrains exon definition for recognition of upstream 3′ ss [153]. Drawings are not to scale.

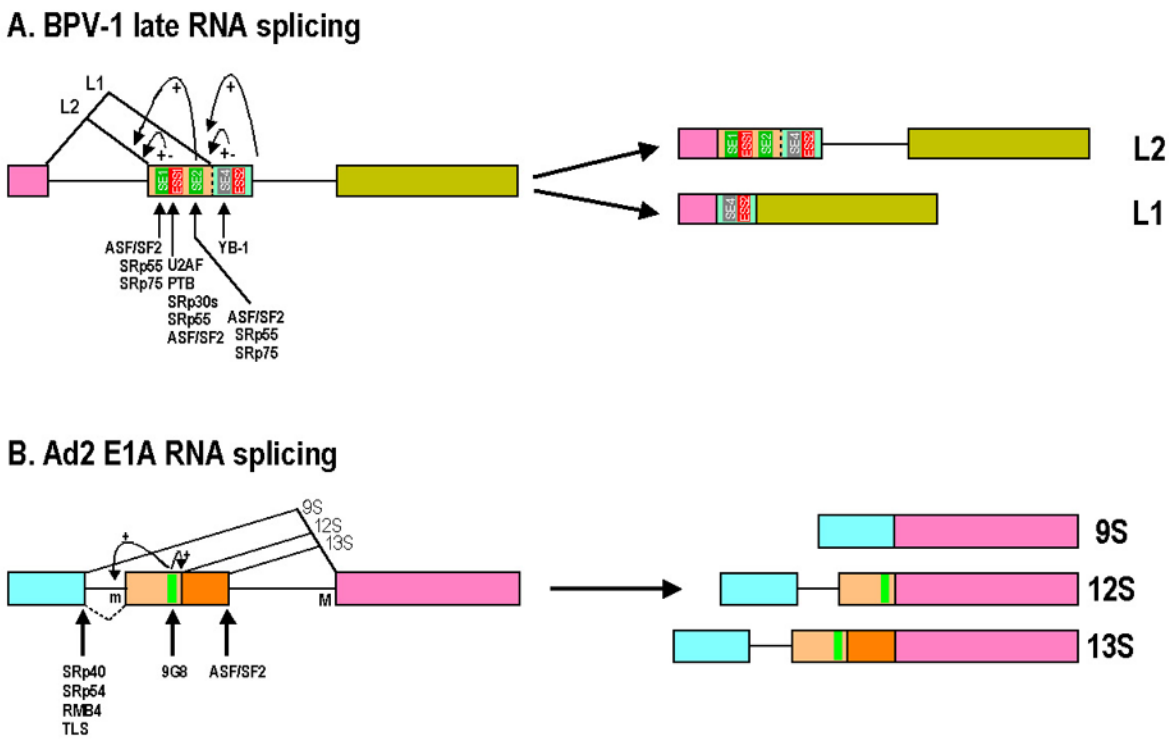

Fig. 4. Regulation of alternative splice site selection by ESE and ESS

A. SR and non-SR proteins (vertical arrows) regulate alternative 3′ ss selection on bovine papillomavirus (BPV-1) late pre-mRNA. BPV-1 late pre-mRNA has three exons and two introns, and exon 2 has two alternative 3′ ss. There are five cis-elements, three ESEs [two purine-rich (green boxes) and one AC-rich (grey boxes)] and two ESSs (red boxes), that control individual 3′ ss switching by interacting with cellular splicing factors, as indicated by the curved arrows. Two purine-rich ESEs (SE1 and SE2) between the two alternative 3′ ss synergistically promote the selection of the proximal 3′ ss over the suppression by the ESS1 positioned immediately downstream of the SE1. Similarly, an AC-rich ESE (SE4) coordinates with the downstream 5′ ss to overcome inhibition by another ESS (ESS2), also positioned immediately downstream of the AC-rich SE4, and to stimulate the distal 3′ ss selection. The alternative 3′ ss selection leads to production of L2 (proximal 3′ ss usage) or L1 (distal 3′ ss usage) mRNAs [106].

B. SR and non-SR proteins regulate alternative 5′ ss selection on ad2 E1A pre-mRNA. Ad2 E1A pre-mRNA has three alternative 5′ ss and one major (M) and one minor (m) 3′ ss whose modulation occurs during virus infection. Several SR and non-SR proteins have been associated with the regulation of the alternative 5′ ss switch as indicated by vertical arrows. The green box immediate upstream of 12S 5′ ss indicates a purine-rich ESE that functions bi-directionally as a splicing enhancer for selection of both the 12S 5′ss and the upstream, minor 3′ ss [11].

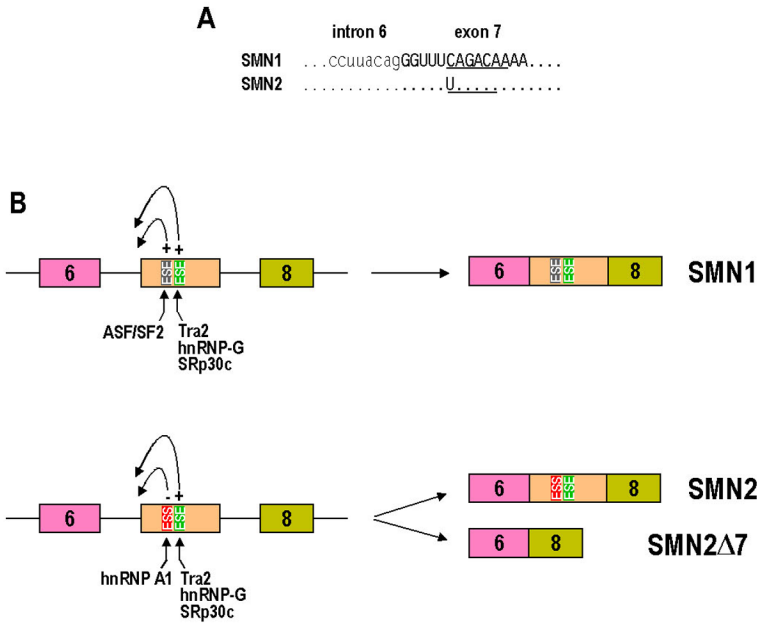

Fig. 5.

Regulation of alternative SMN2 RNA splicing by ESE and ESS. Two identical SMN (survival motor neuron) genes, telomeric SMN1 or SMNt and centromeric SMN2 or SMNc, are located in a 20-kb region on Chromosome 5q13 and each contains 9 exons and encodes a 38 kDa protein with 294 aa expressed at various levels in most tissues with high level in spinal cord [7;30;97]. SMN1 is a disease gene for proximal spinal muscular atrophy (SMA), a relatively common, neurodegenerative disorder in childhood, since 96% of SMA patients show homozygous absence of SMN1 caused by deletions or point mutations, in particular within exon 6, 7 and 8 of SMN1 [65;96;162]. SMN2 determines SMA severity and its expression level correlates with three types (I, severe; II, intermediate; and III, mild) of SMA [12;51] as SMN2 expression is capable of compensating for loss of SMN1. Although DNA and cDNA sequencing of the SMN1 and the SMN2 reveal only 5 nucleotide substitutions that do not alter the protein sequence, a single nt change, C to T, at codon 280 in exon 7 of the SMN2 (**A**) has been found to cause the exon to be skipped in majority of SMN2 mRNAs due to this mutation converting an ASF/SF2-binding site [20] into an ESS [85] that counteracts with an existing purine-rich ESE downstream [72;108], resulting in a spliced isoform (SMN2Δ7) (**B**) that encodes a truncated, nonfunctional protein missing the C-terminal 16 residues. Underlines in panel **A** show an ASF/SF2-binding site (ESE) in SMN1 and an ESS in SMN2 due to a C to U mutation at position +6 in exon 7 (capitalized letters).

Table 1

Genes and RNA splicing in eukaryotes

Organism	Estimated no. of genes	No. of genes with alternative splice form (%)	References
Human	26,588	17,764 (66.8)	[163]
Mouse	22,011	4750 (41)*	[129;165]
Worm	20,443	1,891 (10)	[127]
Fly	13,676	2,729 (20)	[121]
Yeast	6,034	3 (0.05)	[36]

* Data is based on analysis of 11,677 mouse transcripts [129].

Table 2

Exonic splicing enhancers and suppressors (silencers) in viral and mammalian RNAs

Name of RNA	Sequence motif	Interacting factors	References
Purine-rich ESE			
Ad2 E1A exon 2	GACGACGAG (Pu1) GAUGAAGAG (Pu2)	9G8 ASF/SF2	[11]
Ad2 E1A exon 3	?	?	[1]
ASV env	AAGAAGAAG	?	[152]
BPV-1 late exon 2	GAAGGA (SE1) GAAGAA (SE2)	SRp75, SRp55, SRp30a	[177]
BRCA1 exon 18	GCUGAGU	SF2/ASF	[105]
Caldesmon exon 5	GAGGAAGAGAAAAGGGCAGCAGAGGAGAGGCA	?	[48;75]
C/CGRP exon 3 (rat)	GAAGAAGAAG	SRp40, 37-kDa protein	[171]
C/CGRP exon 4	GCAGCACCUGGC (ESE B)	SRp55	[158]
Cardiac troponin T	GAGGAAGAA	SRp30a, SRp40, SRp55, SRp70	[126;133; 170]
CD44 exon v9	GGAAGAAGAUAAAGAC	9G8, ASF/SF2, SRp20	[55]
Exon 10(v6)	CCAGAAGGAAC	?	[167]
c-src N1 exon	GAGGAAGGUG	ASF/SF2, hnRNP A1,H,F	[136]
CFTR exon 13	AGAAAGAAGAAA	ASF/SF2, hTra2α	[5]
Dystrophin gene exon 27	AAGAAGAGG (DW1) AAGAAGCGAA (DW2)	?	[141]
EIAV tat/rev exon 3	AAGAAGAAAAAAGAAGAAA	ASF/SF2	[29;58]
FGFR-1 α exon	GGTGACCTGCTGCAG(ESE1) CTGCGGGACGATGTGCAGAG (ESE2)	SRp55 ?	[81]
FN EDA (EDI or EIIIA) exon	GAAGAAGA	ASF/SF2, 9G8	[16;33;93]
EIIIA exon (chicken)	GAAGAAGAC	?	[160]
FTDP-17 tau exon 10	AAGAAGAAG	Tra2β	[34;80]
G α_s exon 3	AAGAGGACCCGCAGGC	ASF/SF2, SC35?	[130]
β-globin exon 2	AGGACAA	ASF/SF2	[137]
exon 2	UGGACCCAGAGGU	?	[137]
GRH exon 4 (rat)	GAAGAGGAAG	Tra2α	[140]
GH-1 exon 3	GAAGAA	?	[123]
GH exon 5 (bovine)	GGAAGG	ASF/SF2	[44;150]
HIV-1 tat exon 2	GAAGAAGCGGAGACAGCGACGAAGA (ESE2)	ASF/SF2	[82]
tat-rev exon 3	GAAGAAGAA (ESE3)	ASF/SF2	[114;146; 154]
tev exon 6D	GGAGAAAGGAGAGA	ASF/SF2, SC35	[18;166]
HPRT exon 3	GAGAUGUGAUGAAGGAGAUGGGAGG	?	[147]
IGF-I exon 5	ATCCAGGAGGGGAACAGA	ASF/SF2	[144]
IgM exon M2 (mouse)	GAAGGACAGCA	?	[83]
MASγ-subunit exon 10	AAGAAGGAA	?	[77]
MVM NS-specific exon	AGAGAUCGAGGAGGAUUUGAGAG...(22nt)…GAAGAAAGA	?	[57]
NCAM exon 17	GGGGGGAAGCACACAGAGCCCAACGAGACCAC	SR protein	[31]
SMN1 exon 7	CAGACAA	SF2/ASF	[20]
exon 7	AAGAAGGAAGG	Htra2-β1, hnRNP-G, SRp30c	[72] [42;73]
SR protein 9G8 exon 3	GAAGAAGAA	9G8, SC35, ASF/SF2	[98]
SR protein 9G8 exon 4	AGAGGAAGGCGA (A)	9G8, SC35	[98]

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Name of RNA	Sequence motif	Interacting factors	References
	AGGAGCAGGGGACGAAG (B)		
α-TM exon 2 (rat)	AAGAGAAG	ASF/ SF2, SC35, SRrp86	[13;47]
β-TM exon 6 (rat)	GAGGAGGAG (6-1)	ASF/SF2	[139]
	GAGGAGGAG (6-2)		
exon 7	GAGGAGGAG	?	[23]
exon 8	GAAGAAGAG	ASF/SF2	[139]
XI collagen exon 6B	GAAGAAGA	?	[24]
AC-rich ESE			
BPV-1 late RNA	ACCACCACC (SE4)	YB-1	[181]
C/CGRP exon 4	ACUUCAACAAGUU (ESE A)	hTra2β	[157]
CD44 exon v4	CAACCACAA	YB-1	[149]
MVM NS2	CACCAUUCACGACACC	?	[57]
XI collagen exon 6B	CAAGCATCAGCAAAA	?	[24]
	GCCAAAC		
Pyrimidine-rich ESE			
FN EDA	UGUCGAUUCCA	?	[145]
β-globin exon 1	UGCCGUU	SC35	[137]
exon 2	UGCUGUU	SC35	[137]
MYPT1 (chicken)	UCCUACAUCCU	?	[45]
ESS			
4.1R Exon 16	UUUGAA	hnRNP A/B	[74]
BPV-1 late RNA	UCUUCUU (ESS1)	U2AF, PTB	[179]
	GGCUCCCCC (ESS1)	SRp55, SRp30s	[179]
	AGAGCAGG (ESS1)	ASF/SF2	[179]
	UGGU (ESS2)	?	[181]
BDV Polymerase exon	CUAGAUAUGGAUCC	?	[156]
CD44 exon v5	?	hnRNP A1	[115]
CD45 exon 4	GUGACCCCUUACCUACUCACACCACUGCAUUCUCACCCGCAAGCACCUUUG	?	[110]
DNA ligase III β exon	CCAAGUCAAAAUUUAC	37 Kd protein (?)	[25]
FGFR-2 K-SAM exon	UAGG	hnRNP A1	[39;41]
FGFR-2 BEK	UCUUUAGGUUCCCUUUCAAUUCU	PTB	[94]
FN EDA	CAAGGCC	?	[125]
FN EDA (chicken)	CAUGG	?	[160]
HIV-1 tat exon 2	CUAGACUAGA(ESS2)	hnRNP A1	[3;17;41; 142]
	UUGGGU(ESS2p)	hnRNP H	[79]
tat-rev exon 3	UUAG (ESS3)	hnRNPA1, SC35	[117;143; 146]
vif exon 3	PyUAG(ESSV)	hnRNP A/B	[10]
tev exon 6D	CCAAUAGUAGUAGCGGGAGAAUG	ASF/SF2, SC35	[18;166]
IgM exon M2 (mouse)	CUAGUAAACUUAUUCUUACGUCUUUCCUGUGUUGCCCUCCAGCUUUUAUCUCUGAGAUGGUCUUCUUUCUAGA	U2 snRNA	[83]
MASγ exon 9	AGUUCCA	?	[67]
MYPT1 (chicken)	UUAAACACAAGUU	?	[45]
SMN2 exon 7	UAGACA	hnRNP A1	[85]
α-TM SK exon	UAAGUGUUCUGAGCU	?	[59]
β-TM exon 7 (rat)	UGUGGGGGAC	hnRNP H	[23]

Ad2, adenovirus type 2; ASV, avian sarcoma virus; BDV, Borna disease virus; BRCA1, breast cancer 1 (breast cancer susceptibility gene); C/CGRP, calcitonin/calcinonin gene-related peptide gene; CFTR, cystic fibrosis transmembrane conductance regulator; EIAV, equine infectious anemia virus; FGFR, fibroblast growth factor receptor; FN, fibronectin; FTDP, frontotemporal dementia and Parkinsonism linked to chromosome 17; $G\alpha_S$, adenylyl cyclase stipulatory G-protein G alpha(s); GH, growth hormone; GRH, gonadotropin-releasing hormone; HPRT, hypoxanthine phosphor-ribosyltransferase; IGF-I, insulin-like growth factor I; MASγ, mitochondrial ATP synthase γ-subunit; MVM, minute virus of mice; MYPT-1, Myosin phosphatase targeting subunit 1; NCAM, neural cell adhesion molecule; SMN, survival of motor neuron; TM, tropomysin;