Novel Exonic Elements That Modulate Splicing of the Human Fibronectin EDA Exon*

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Three exons in the fibronectin primary transcript are alternatively spliced in a tissue- and developmental stage-specific manner. One of these exons, EDA, has been shown previously by others to contain two splicing regulatory elements between 155 and 180 nucleotides downstream of the 3'-splice site: an exon splicing enhancer and a negative element. By transient expression of a chimeric β-globin/fibronectin EDA intron in COS-7 cells, we have identified two additional exonic splicing regulatory elements. RNA generated by a construct containing the first 120 nucleotides of the fibronectin EDA exon was spliced with an efficiency of approximately 50%. Deletion of most of the fibronectin EDA exon sequences resulted in a 20-fold increase in the amount of spliced RNA, indicative of an exon splicing silencer. Deletion and mutagenesis studies suggest that the fibronectin exon splicing silencer is associated with a conserved RNA secondary structure. In addition, sequences between nucleotides 93 and 118 of the EDA exon contain a non-purine-rich splicing enhancer as demonstrated by its ability to function in a heterologous context.

Higher eukaryotes have evolved alternative splicing as a common means of producing several different mRNAs and polypeptides from a single primary transcript (for review, see Ref. 1). Transcripts that undergo alternative splicing can produce mRNAs and proteins with different structures, properties, and functions. The utilization of alternative splice sites can be regulated to prevent the inappropriate accumulation of one or more alternative products. To date, several examples of cis-acting regulatory elements, distinct from bona fide splice sites, have been identified which modulate splicing in a diverse assortment of genes from viruses (2–10), Drosophila (11–14), and higher eukaryotes (15–33).

The fibronectin gene is a classical example of a gene that undergoes alternative splicing (34, 35). The fibronectin gene is evolutionarily conserved and is predicted to yield up to 20 different mRNAs in human cells. The generation of this remarkable number of different mRNAs, and consequently polypeptides, is made possible by alternative splicing in three different coding regions of the fibronectin primary transcript (for review, see Refs. 36 and 37). One of the alternatively spliced regions encompasses the EDA (also referred to as EDI or EDI) exon. This exon is excluded selectively in fibronectin mRNAs produced by hepatocytes, but it is included to various extents by other cell types (Fig. 1A). EDA exon splicing is also subject to developmental regulation as EDA exon inclusion generally declines with age and differentiation (36, 37). In addition to tissue-specific and developmental regulation, inclusion of the EDA exon is modulated during the process of tissue repair and in certain diseases.

For these reasons, regulation of fibronectin EDA exon splicing has been the subject of several studies. In fact, the EDA exon was the first exon with which it was demonstrated that proper alternative splicing could occur in the context of transfected minigenes (38). Using the same experimental approach, it was determined subsequently that the central 81 nucleotides of the 270-nucleotide EDA exon were required for its inclusion into mRNA (31). Using a chimeric adenovirus/fibronectin EDA intron substrate in an in vitro splicing system, Laviguer et al. (32) further mapped the element required for EDA exon inclusion to a 9-nucleotide purine-rich motif (5’-GAAGAAGAC-3’). Caputi et al. (33) identified the same purine-rich sequence as a positive cis-acting element by transfection experiments. Similar exonic purine-rich motifs have been demonstrated to stimulate splicing (39–41) and have been termed exon recognition sequences or exon splicing enhancers (ESEs).1 A negative element (5’-CAAGG-3’) involved in down-regulation of EDA exon inclusion was also identified within the EDA exon just downstream of the ESE (33). Although the ESE within the EDA exon has been demonstrated (32) to interact with splicing factors belonging to a family of serine/arginine-rich RNA-binding proteins collectively termed SR proteins (42), it is not yet known if trans-acting factors interact with the negative element.

Using a chimeric intron composed of sequences flanking the 5'-splice site (5'ss) of the first intron of human β-globin and sequences flanking the 3'-splice site (3'ss) of the human fibronectin EDA exon, we observed that deletion of most of the fibronectin EDA exon sequences resulted in a dramatic increase in splicing efficiency. We have carried out mapping experiments to characterize this novel exon splicing silencer (ESS) element. Closer examination of the ESS revealed an adjacent positive element that is able to stimulate splicing when placed downstream of a heterologous intron. This novel ESS and adjacent positive element represent the third and fourth splicing regulatory elements to be identified within the 270-nucleotide EDA exon. The presence of so many different regulatory elements is of particular interest because they may

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1 The abbreviations used are: ESE(s), exon splicing enhancer(s); 5'ss, 5'-splice site; 3'ss, 3'-splice site; ESS, exon splicing silencer; PCR, polymerase chain reaction; CAT, chloramphenicol acetyltransferase; bp, base pair(s); HIV-1, human immunodeficiency virus type 1.
EXPERIMENTAL PROCEDURES

Plasmid Constructions—All plasmids are derivatives of pSVβFN-(2). Truncation of fibronectin exon sequences (accession no. X07718) was carried out by PCR using the sense primer FN3’s, 5′-CGT CGA CAAG AGA AAA TGG TAT CTG C-3′ (nucleotides 1146–1164) and the following antisense primers: Δ, 5′-CCG GAT CCA ATG AGA (nucleotides 1255–1272); Δ1, 5′-CCG GAT CCC ACC CTG TCG GA-3′ (nucleotides 1313–1345); Δ2, 5′-CCG GAT CCA ACT TGC CCC TGT GG-3′ (nucleotides 1316–1330); Δ3, 5′-CCG GAT CCG CCT TCC CAA CCA AT-3′ (nucleotides 1301–1315); and Δ4, 5′-CCG GAT CCT TTG ATG GAA TGA AC-3′ (nucleotides 1286–1300) to generate the respective constructs. The mod series of fibronectin exon mutations was carried out by PCR-mediated site-directed mutagenesis as described previously (45) using the FN3’s sense primer and the antisense primer CATR1 (described below) along with the following mutagenic primers: mod1, 5′-CAC AGG GGC AGA atc TAC AGG TGT ACC CCA CCA ACC GAAG-3′; mod2, 5′-CAT CAA AAT TGC ggag atc CAC GAC GGC AAG-3′ (nucleotides 1294–1328); and mod3, 5′-CAC TGA TGG GAT ggctg TCC AAA ATT GCT T-3′ (nucleotides 1273–1303) to generate respective constructs. Lowercase letters indicate nucleotides introduced to create a unique BglII restriction enzyme site (underlined). The single stem-loop 1 mutation (SL1B) was generated in a similar fashion using the mutagenic primer SL1B, 5′-ATC CAT ATG TGG TAC TCG ATT CCA ATG GAC-3′, which overlaps the EcoRI restriction endonuclease site of the chloramphenicol acetyltransferase (CAT) open reading frame. 50 fmol of Plasmid AAT-1-linearized template DNA and 1 pmol of 5′-end-labeled CATR1 primer were subjected to 15 thermal cycles (1 min each at 94 °C, 30 s at 61 °C, and 1.5 min at 72 °C) with 2 units of Taq DNA polymerase in a 20-μl reaction. Free CATR1 primer was removed by agarose gel purification of the extended primer. S1 nuclease protection assays were carried out as described previously (43) using 10 μg of total RNA and 5–10 × 10^6 cpm (approximately 50–100 fmol) of the appropriate probe. S1-resistant probe DNA fragments were subjected to electrophoresis on 4% polyacrylamide, 8 M urea gels and subsequently visualized and quantitated using a PhosphorImager (Molecular Dynamics). All cited ratios of spliced to unspliced RNAs are averages of results obtained in at least three independent experiments.

RESULTS

Fibronectin EDA Exon Sequences Are Implicated in Repression of Splicing—In previous studies, our laboratory generated a chimeric gene composed of sequences flanking the 5′-ss of the first intron of the human β-globin gene and sequences flanking the 3′-ss upstream of the EDA exon of the human fibronectin gene (2). The fibronectin sequences within the chimeric construct include 99 nucleotides of the intron and the first 118 nucleotides of the EDA exon (Fig. 1B). The previously characterized purine-rich ESE and the adjacent negative element, which are located between 155 and 180 nucleotides downstream of the EDA 3′-ss, are not present in pSVβFN– and thus do not contribute to the level of splicing observed. S1 analysis of RNA isolated from transfected COS-7 cells showed that splicing of pSVβFN– RNA is relatively inefficient and results in the accumulation of roughly equal amounts of spliced and unspliced RNA (Fig. 1C, lane 1). In an attempt to study the positional effect of a heterologous ESS element, we generated a truncated form of the EDA exon (designated pSVβFNΔ; see Fig. 2) in which sequences from +28 to +118 were deleted. Deletion of these sequences resulted in a 20-fold increase in the ratio of spliced to unspliced (S:U) RNA (Fig. 1C, lane 2). This finding indicates that a splicing silencer element may exist within this region of the fibronectin EDA exon and that the EDA 3′-ss may be intrinsically efficient.

Mapping the Fibronectin EDA ESS—To map the fibronectin ESS element, we generated sequential 3′-truncations of the EDA exon sequences (Fig. 2). S1 analysis of RNA from COS-7 cells transfected with pSVβFN1, Δ2, Δ3, or Δ4 (Fig. 3) revealed that, with the exception of Δ1 (lane 2), splicing efficiency steadily increased as fibronectin EDA exon sequences were progressively shortened (lanes 3–5); the ratio of spliced to unspliced RNA approached that obtained with pSVβFNΔ. The Δ1 truncation defines the 3′-boundary of the fibronectin ESS as nucleotide +101 relative to the EDA 3′-ss. The correlation between the size of the truncation and the splicing efficiency observed with Δ2, Δ3, and Δ4 suggests that the fibronectin ESS may be comprised of multiple smaller elements dispersed within the first 100 nucleotides of the EDA exon or that its function is dependent upon the secondary structure.

However, based on the effect of these 3′-deletions alone, we could not exclude the possibility that the progressive increase in splicing is caused by the positioning of CAT sequences closer to the EDA 3′-ss and not by disruption of an ESS within the EDA exon. To test this possibility, we used site-directed mutagenesis to introduce internal deletions in the EDA exon within the context of pSVβFNΔ (Fig. 2). The net effect of these internal modifications is a 5′-nucleotide deletion in pSVβFNΔmod1 and pSVβFNΔmod2 and a 7′-nucleotide deletion in pSVβFNΔmod3. All three mutations resulted in a moderate (4–8-fold) increase in splicing efficiency (Fig. 4). These results suggest that the effects seen in Fig. 3 are caused by deletion of a splicing silencer element between nucleotides +28 and +101 within the context of the EDA exon.
EDА Exonic Splicing Regulatory Elements

Identification of a Non-purine-rich Splicing Enhancer within the EDА Exon—The complete abrogation of splicing observed with the pSVβFNΔ1 construct suggests that the sequences deleted in pSVβFNΔ1 (nucleotides 102–118) harbor a splicing enhancer element. However, if that is the case, it differs from "classical" ESE elements (39–41) in that it is not purine-rich. To test if these EDА exon sequences could stimulate splicing when placed in a heterologous context, nucleotides 93–118 of the EDА exon were inserted into pSVCBSB (Fig. 6A) downstream of a chimeric β-globin/HIV-1 intron. The pSVCBSB chimeric intron is not spliced (43) since critical HIV-1 exon splicing regulatory elements have been deleted (2). We have demonstrated previously that splicing of this intron can be stimulated significantly by several classical purine-rich ESEs, including the ESE within the fibronectin EDА exon. Consistent with our previously published results (43), only trace amounts of spliced RNA were detected with pSVCBSB (Fig. 6B, lane 1). Insertion of nucleotides 93–118 of the EDА exon in the sense orientation (pSVCBSBFs) stimulated splicing at least 25-fold (Fig. 6B, lane 2). Conversely, insertion of the same EDА sequences in the antisense orientation did not have a stimulatory effect on splicing (Fig. 6B, lane 3).

The region associated with enhancer activity contains the sequence 5′-AGGTTAGCC-3′, which bears homology (a 6 out of 9 match; underlined) to the 5′-ss consensus sequence, 5′-C(A/G)GTTAG(C/A)GT-3′. One model to explain how this novel enhancer functions is that this pseudo-5′-ss element recruits U1 snRNP to stimulate recognition of the EDА 3′ss much like authentic 5′-splice sites have been demonstrated to stimulate exon recognition (46, 47). To examine this possibility, we introduced a point mutation that converted the critical GT dinucleotide of the pseudo-5′-ss to CT (designated +1C in Fig. 6C), deleted the majority of the pseudo-5′-ss (Fig. 6C, Δ5′ss), or deleted the AC-rich region immediately downstream of the pseudo-5′-ss (Fig. 6C, ΔAC). Analysis of the effect of these mutations revealed that none of the mutations tested resulted in loss of enhancer function. Rather, in all cases the mutation resulted in a further enhancement in the ratio of spliced to unspliced RNA (Fig. 6D, compare lanes 3 vs lane 2). Therefore, the splicing enhancer activity associated with this non-purine-rich sequence cannot be attributed solely to the 5′-ss-like element.

The Fibronectin ESS Can Potentially Form a Stable, Conserved Secondary Structure—The relatively large size of the fibronectin ESS prompted us to examine its ability to form a secondary structure. The 118 nucleotides of the EDА exon present in pSVβFN– are predicted to form three stem-loops (Fig. 7A). Each of the two stem-loops closest to the EDА 3′ss contains a hairpin loop of 4 nucleotides and an internal loop that interrupts the 13-bp stem. The third and most distal stem-loop is predicted to consist of a stem of only 6 bp and a hairpin loop of 4 nucleotides (Fig. 7A, ΔAC). Therefore, we examined whether the first 118 nucleotides of the EDА exon of other species retained the potential to form a secondary structure similar to that in Fig. 7A. Fibronectin sequences of different species were obtained from sequence data bases and aligned (Fig. 7B). Compared with the human fibronectin sequence, the
EDA exons from fibronectin of other mammals such as dog, rat, and mouse differ by only a few nucleotides, whereas those of chicken, newt, and frog are slightly more divergent. As expected, most of these nucleotide substitutions are silent at the amino acid level. The interesting observation is the occurrence of covariance and conservative substitutions.

The region comprising step-loop 1 is completely conserved among human, dog, and rat fibronectin sequences. Chicken, newt, and frog fibronectin sequences contain several substitutions that do not compromise the ability of this region to form step-loop 1. An example of covariance is seen in chicken fibronectin involving positions 123 and 144. As depicted in Fig. 7A, the nucleotides at these two positions are predicted to form a G-C bp in stem-loop 1. In chicken fibronectin, the nucleotide at 123 is replaced by an A, and a compensatory C-T substitution occurs at nucleotide 144, thus maintaining the ability to form a canonical Watson-Crick bp. Several examples of conservative substitutions involving putative base pairing between G and U residues are also observed. In both chicken and frog, a G-U bp in stem-loop 1 is converted to a canonical A-U bp by a G → A substitution at position +24. Examples in which canonical Watson-Crick bp are replaced by a wobble bp are also observed. In frog, a U-A bp is converted to a U-G bp by an A-G substitution at +40, and a G-C bp is converted to a G-U bp by a C → T substitution at +44. Taken together, these examples of covariance and conservative nucleotide substitutions support the notion that the stem-loop 1 secondary structure depicted in Fig. 7A is absolutely conserved and is indicative of selective
Transient transfection of COS-7 cells with a plasmid containing a chimeric gene containing the first 118 nucleotides of the fibronectin EDA exon and upstream intron sequences has led to the identification of an ESS and an adjacent positive element within that part of the EDA exon. The construct used in this study places the 3′ss of the alternatively spliced EDA exon in the context of a terminal exon. It has been demonstrated that even in this simplified context, recognition of the EDA 3′ss remains responsive to regulatory exon sequences both in vitro (32) and in vivo (2). Therefore, we believe that conclusions drawn from this chimeric gene should be applicable to authentic EDA exon splicing regulation. Deletion studies mapped the 3′-boundary of the fibronectin ESS to nucleotide +101 of the EDA exon. Actinomycin D time course experiments failed to detect a significant effect of exonic deletions on RNA stability; therefore, it can be concluded that the fibronectin ESS and the adjacent enhancer act at the level of splicing. Secondary structure prediction of the human EDA sequence and the alignment of the first 118 nucleotides of the EDA exon from various species combined with the mutagenesis data presented here implicate conserved secondary structures as critical determinants of ESS activity.

Because the Δ1 truncation (which defines the 3′-boundary of the ESS) eliminates the third stem-loop, stem-loop 3 cannot be essential for ESS activity. In fact, we demonstrate that the region comprising stem-loop 3 harbors an ESE activity. This is discussed in more detail below. The mod3 mutation, which affects base pairs in stem-loop 1, resulted in a moderately increased (8-fold) level of splicing. Therefore, it can be concluded that the region comprising stem-loop 1 is required for maximal ESS activity. However, the relatively high splicing levels observed with the Δ4 truncation indicate that sequences comprising stem-loop 1 have little ESS activity on their own in COS-7 cells. The SL1 mutations provide strong evidence that formation of the stem-loop 1 secondary structure rather than the primary sequence per se is essential for complete abrogation of splicing in the context of the Δ1 truncation. Therefore, stem-loop 1 is required but not sufficient for maximal ESS activity in COS-7 cells.

Results with truncation mutants Δ2, Δ3, and Δ4 revealed that splicing efficiency increased as increasing amounts of stem-loop 2 were removed. Both the mod1 and mod2 mutations, which affect base pairs in stem 2, resulted in a moderately increased (4–6-fold) splicing efficiency. These results support the conclusion that the region comprising stem-loop 2 is an important determinant of ESS activity; however, they do not provide direct evidence that the predicted stem-loop 2 secondary structure is functionally important. The SL2 mutations, which target the lower stem of stem-loop 2, were designed to address this question. Analysis of these mutations revealed that formation of the lower stem of stem-loop 2 is not essential for ESS activity. Consistent with this observation, the ability to form the lower stem of stem-loop 2 is not well conserved in fibronectin sequences of chicken, newt, and frog. In these species, several disruptive substitutions are present on one strand of the predicted helix (Fig. 7B). In contrast to the results obtained with the SL2B mutation, two other mutations that map to the base of stem-loop 2, mod1 and A2, decreased ESS activity significantly. Because the mod1 mutation is in the context of FN–, some of the observed increase in splicing efficiency may be partly attributable to the downstream ESE, making the direct comparison of mod1 to A2 and SL2B difficult. The fact that the mod1 mutation increases splicing and that...
the Δ2 and SL2B constructs, both of which do not contain the downstream ESE, have markedly different splicing efficiencies suggests that the affected nucleotides sequence at the base of putative stem-loop 2 plays an important role in ESS function. Computer prediction analysis of possible secondary structures carried out on the Δ2 and SL2B mutations (data not shown) reveals that both destroy the ability to form putative stem-loop 2. However, in the case of the SL2B mutation, an alternate stable stem-loop containing extensive base pairing can form in its place. In contrast, no equally stable structure is predicted to form with the Δ2 deletion. Therefore, it is possible that in addition to the primary sequence, certain base pairing interactions within the region of stem-loop 2 may also be important for ESS function. However, we do not yet understand the structural requirements of this region for ESS function, and in the absence of significant evolutionary covariation or conclusive experimental evidence we should emphasize the putative nature of stem-loop 2. Nonetheless, the data presented here clearly demonstrate that the region comprising putative stem-loop 2 displays very strong ESS activity in COS-7 cells.

FIG. 6. Evidence for a novel, non-purine-rich ESE within the EDA exon. Panel A, strategy to test if sequences adjacent to the ESS can stimulate splicing in a heterologous context. The plasmid pSVCBSB is identical to pSVCBS except that it contains the HIV sequences flanking the tat/rev 3'ss (47 nucleotides of intron and 5 nucleotides of exon sequences; depicted in black) instead of the fibronectin sequences flanking the EDA 3'ss. Sequences between +93 and +118 of the EDA exon (depicted in gray) were inserted in either the sense or anti-sense orientation into pSVCBSB to generate pSVCBSBFs and pSVCBSBFas, respectively. Panel B, S1 nuclease protection analysis was carried out using 10 μg of total RNA from transfected COS-7 cells. Each sample was hybridized with the homologous probe as depicted in Fig. 1B. The probe used for analysis of pSVCBSB RNA extended into the β-globin exon sequences; hence, unspliced RNA appears as a band migrating slightly slower than that of its derivatives. Positions of the protected probe fragments are indicated on the left for pSVCBSB and the right for pSVCBSBFs and pSVCBSBFas: U, unspliced RNA; S, spliced RNA. Ratios of spliced to unspliced RNA are shown at the bottom of each lane. Panel C, summary of constructs used to study the non-purine-rich enhancer. Sequences are shown in the 5’→3’ orientation. The last three nucleotides of intron sequences are in lowercase Italic. The position of the 3'ss is depicted by the arrow. Exon sequences are in uppercase letters. The BamHI restriction endonuclease site and the BgIII/BamHI junction are in lowercase letters to emphasize the boundaries of HIV-1, fibronectin, and polylinker sequences. Shown from top to bottom are the parent pSVCBSB, which contains no insert (indicated by the gap), pSVCBSBFs, pSVCBSBFs+1C, pSVCBSBFsΔ5'ss, and pSVCBSBFsΔAC. The pseudo-5'ss discussed in the text is underlined. The G → C mutation introduced at position -1 of the pseudo-5'ss is in thin type. Panel D, S1 nuclease protection analysis was carried out using 10 μg of total RNA from transfected COS-7 cells. Each sample was hybridized with the homologous probe as depicted in Fig. 1B. The probe used for analysis of pSVCBSB RNA extended into the β-globin exon sequences; hence, unspliced RNA appears as a band migrating slightly slower than that of its derivatives. Positions of the protected probe fragments are indicated on the left for pSVCBSB and the right for its derivatives: U, unspliced RNA; S, spliced RNA. Ratios of spliced to unspliced RNA are shown at the bottom of each lane.
of the adjacent ESS. This organization of splicing control elements is reminiscent of the adjacent enhancer and silencer elements identified in the terminal HIV-1 tat/rev exon (2). Unlike most exonic splicing enhancers identified to date (39–41), the sequence associated with this particular splicing enhancer is not purine-rich and thus not a classical ESE. Nevertheless, this novel enhancer is functionally equivalent to classical ESEs because it too can stimulate splicing of an ESE-dependent intron in an orientation-dependent fashion. Sequence analysis of this region raised the possibility that the enhancing activity could be attributed to the presence of a pseudo-5' ss. In other systems, 5' ss-like elements have been shown to contribute to modulation of splicing both negatively as in the case of the human calcitonin/calcitonin gene-related peptide gene (17). However, analogous regions of homology to the 5' ss consensus can also be identified within the antisense orientation, which was found to have no ESE activity. Disruption of the pseudo-5' ss by either a point mutation or deletion did not abolish ESE activity; rather, the mutations resulted in a marked enhancement of splicing. These results indicate that the 5' ss-like element is not required for enhancer activity of this sequence. In contrast, the result obtained with the DAC derivative suggests that the region comprising the 5' ss-like element can stimulate splicing in the absence of downstream fibronectin sequences. Therefore, it is possible that two distinct non-purine-rich stimulatory elements are present between nucleotides +93 and +118 of the fibronectin EDA exon. A similar non-purine-rich exon element that stimulates splicing of the upstream intron by stabilizing the interaction between U2AF
and the polypyrimidine tract has been described in troponin T exon 16 (48). Interestingly, the two mutations designed to alter the 5’s-like element increase the overall A/C content of the test sequence. Therefore, this newly identified positive element may be an example of a class of A/C-rich enhancer elements which has been described to be functionally equivalent to purine-rich ESEs (49). It is noteworthy to point out that most of these substitutions that are observed between +86 and +118 in fibronectin sequences of chicken, newt, and frog also increase the overall A/C content. We speculate that in these organisms perhaps the strength of the enhancer is increased at the expense of the strength of the silencer.

At least two general mechanisms could explain the modulatory effect of the elements identified in the EDA exon: (i) they contribute to a secondary structure that simply impedes proper recognition of the 3’ss; or (ii) specific cellular trans-acting factors interact with these elements to modulate the efficiency with which the 3’ss is recognized. The sequences at the intron/exon junction surrounding the EDA 3’ss have previously been proposed to form an alternate secondary structure involving base pairing between intronic and exonic sequences to form a single stem-loop interrupted by several bulges (50). Within this structure, the AG dinucleotide of the EDA 3’ss is buried within the predicted secondary structure. Data presented in this study are inconsistent with the structure reported previously as being of functional importance. The SL1 mutations provide compelling evidence that stem-loop 1 as predicted in Fig. 7 is inconsistent with the structure reported previously as being of functional importance. The SL1 mutations provide compelling evidence that stem-loop 1 as predicted in Fig. 7 is not recognized. The sequences at the intron/exon junction surrounding the EDA 3’ss have so many cis-acting elements that it must involve more complex tertiary interactions that cannot be predicted by the folding programs used.

We describe here the initial identification of an exon element within the EDA exon of human fibronectin which has a dramatic down-regulatory effect on splicing. This novel ESS lies adjacent to a non-purine-rich region that is demonstrated to stimulate splicing in several contexts. Why should an alternatively spliced exon have so many cis-acting elements that modulate its inclusion into mRNA? In the case of the fibronectin EDA exon, it is possible that several distinct elements regulate its inclusion in both a tissue-specific and developmentally stage-specific fashion as well as in response to certain external stimuli. Therefore, we can envision several parallel splicing regulatory circuits that interact with various regulatory trans-acting factors to bring about the spectrum of EDA exon inclusion observed in different tissues at different stages under different conditions. Now that several cis-acting modulatory elements have been identified, it will be of interest to identify the trans-acting factors and determine their individual as well as their collective mechanisms of action.

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