The Relative Strengths of SR Protein-mediated Associations of Alternative and Constitutive Exons Can Influence Alternative Splicing*

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Jeremy M. Stark‡§, Thomas A. Cooper¶, and Mark B. Roth‡¶

From the ‡Division of Basic Sciences and Molecular and Cellular Biology Program, Fred Hutchinson Cancer Research Center, Seattle, Washington 98104 and the ¶Departments of Pathology and Cell Biology, Baylor College of Medicine, Houston, Texas 77030

We have characterized the functional role of SR protein-mediated exon/exon associations in the alternative splicing of exon 5 of chicken cardiac troponin T (cTnT). We have previously shown that SR proteins can promote the association of the alternative exon 5 with the flanking constitutive exon 6 of this pre-mRNA. In this study, we have shown that when exons 2, 3, and 4 of the cTnT pre-mRNA are spliced together, the composite exon 2/3/4 contains an additional SR protein binding site. Furthermore, we have found that SR proteins can also promote interactions between the pairs of exons 2/3/4-5 and 2/3/4-6. We then asked whether the SR protein binding sites in these exons play a role in cTnT alternative splicing in vivo. We found that the SR protein binding sites in exons 2/3/4 and 6 promote exon 5 skipping, and it has previously been shown that the SR protein binding site in exon 5 promotes exon 5 inclusion. Consistent with these results, we find that the SR protein-mediated association of exon 2/3/4 with 6 is preferred over associations involving exon 5, in that exons 2/3/4 and 6 are more efficient than exon 5 in competing an SR protein-mediated exon/exon association. We suggest that the relative strengths of SR protein-mediated associations of alternative and constitutive exons play a role in determining alternative splicing patterns.

During splicing of some precursor messenger RNA (pre-mRNA), splice sites that are short and poorly conserved must be properly chosen and brought together across introns, some as large as 100 kilobases (1). The selection of any pair of splice sites is the result of interactions of each individual splice site with the splicing machinery combined with the pairing of the selected splice sites with one another across introns. The relative strength of these interactions appears to vary greatly between splice site pairs during alternative splicing, in which as many as hundreds of different mRNAs are formed from single genes (2, 3). To understand how splice site pairs are chosen, research has focused on identifying the factors that are responsible for this variation during alternative splicing.

SR1 proteins are a family of splicing factors that appear to play an important role in alternative splicing in that individual SR proteins can influence the selection of distinct alternative splice sites (4–6). Insight into the mechanism of SR protein function has come through the identification of RNA sequences to which they bind and appear to influence splicing. These sequences are characteristically composed of purine-rich sequences that reside within an alternatively spliced exon, and when these sequences are found to be important for the selection of the alternative exon where they reside, they are termed exon splicing enhancers (1, 5). SR proteins appear to function at exon splicing enhancers by promoting a variety of interactions that result in the interaction of exons across introns. SR proteins, bound to exon enhancers, appear to promote interactions of both the U1 and U2 snRNPs1 with adjacent splice sites (7–10). These splicing snRNPs may interact with those bound to a flanking exon and in this way promote interactions of exons across introns (11). As well, SR proteins, independent of other factors, can promote a specific association between an exon enhancer-containing alternative exon and a flanking constitutive exon, which also has an SR protein binding site (8). SR proteins may perform this function on a number of pre-mRNAs, because sequence analysis reveals that when purine-rich SR protein binding sites are found in alternative exons, they tend to also be found in one or more of the constitutive exons that flank the alternative exon, based on sequence comparisons (8).

The finding that SR proteins can promote exon/exon associations between alternative and constitutive exons suggests that SR protein-mediated exon/exon associations could be important for the selection of exon pairs during alternative splicing. To address this hypothesis, we have analyzed a splicing event that involves SR protein binding sites in contiguous exons: the alternative inclusion of exon 5 into the chicken cardiac troponin T (cTnT) mRNA. Exon 5 is included in mRNAs in embryonic skeletal and cardiac muscle and is excluded from mRNAs in the adult (12). It has previously been shown that exon 5 contains an SR protein binding site that is essential for exon 5 inclusion (13) and that SR proteins can promote the association of the alternative exon 5 with the flanking constitutive exon 6 of this pre-mRNA (8). In this study, we show that SR proteins can also promote interactions between the pairs of exons 2/3/4-5 and 2/3/4-6, where exon 2/3/4 is the composite of exons 2, 3, and 4 spliced together. We also show that SR protein binding sites in the flanking constitutive exons, exons 2/3/4 and 6, appear to promote exon 5 skipping in vivo. Consistent with

1 The abbreviations used are: snRNP, small nuclear ribonucleoprotein; cTnT, cardiac troponin T; PAGE, polyacrylamide gel electrophoresis; WT, wild type; PCR, polymerase chain reaction.
these results, we find that the SR protein-mediated association of exon 2/3/4 with 6 is preferred over associations involving exon 5, in that exons 2/3/4 and 6 are more efficient than exon 5 in competing an SR protein-mediated exon/exon association. We suggest that the relative strengths of SR protein-mediated associations of alternative and constitutive exons play a role in determining alternative splicing patterns.

**EXPERIMENTAL PROCEDURES**

**UV Cross-linking**—UV cross-linking of RNA to purified SR proteins was performed similar to that previously described (15). Exon 2/3/4 RNA was transcribed from a synthetic oligonucleotide template with T7 polymerase and labeled using [α-32P]GTP. 3 pmol of radiolabeled exon 2/3/4 RNA was incubated with 500 ng of total calf thymus SR proteins and 50 pmol of non-specific competitor RNA (13) under splicing conditions for 10 min at 30 °C. To some samples other nonradiolabeled RNAs were added as competitors at two concentrations each (10 and 30 pmol/reaction). These exon RNAs (2/3/4, 2/3/4 m3/m4, 2, 3, and 4) were transcribed from a synthetic oligonucleotide template with T7 polymerase (14). RNAs were purified from 5% urea-PAGE gels and quantified using a spectrophotometer. Their sequences are shown in Fig. 1A except for the complete sequence of exon 2, which is UAGCUCCUGACAUUG- CGGACUCUGAAGGUGCGUUGAAGGAUACGACGA.

The exon 2/3/4 RNAs were placed on ice and UV irradiated at 120,000 μJ for 7 min at 254 nm (Stratalinker 1800, Stratagene, La Jolla, CA). Following UV irradiation, each sample was incubated with 1 μl of RNase A and T1 (Ambion) at 37 °C for 30 min, mixed with an equal volume of protein sample buffer, incubated at 90 °C for 3 min, and resolved by 10% SDS-PAGE. The gels were exposed to a PhosphorImager, and the relative signals were quantified with Image/Quant (Molecular Dynamics, Sunnyvale, CA).

**RNA Affinity Chromatography**—RNA affinity chromatography assays for SR protein- mediated exon/exon associations were performed as described previously (8). RNA affinity columns containing exon 5/UP or exon 6 were assembled using R17-exon fusion RNAs. For each column, 8 μg of R17-exon RNA was bound to 10 μg of R17-glutathione S- transferase protein affinity affinity chromatography. The RNA affinity columns were incubated for 1 h at 30 °C in a volume of 220 μl in 20 mM Heps, pH 7.6, 220 mM KCl, 3.6 mM MgCl2, 3.6 mM ATP, and 4.5 mM creatine phosphate with 100 μl of a solution of 20 μg/ml bovine serum albumin, 10 μg/ml Escherichia coli tRNA, with or without 2.6 μg of calf thymus or HeLa SR proteins. The final concentration of SR proteins in the defined system was 12 μg/ml, whereas the SR protein concentration in standard splicing reactions with HeLa nuclear extract is approximately 30 μg/ml (15).

Radiolabeled RNAs were added to the columns at the same time that RNA affinity columns containing exon 5/UP or exon 6 were assembled using R17-exon fusion RNAs. For each column, 8 μg of R17-exon RNA was bound to 10 μg of R17-glutathione S- transferase protein affinity affinity chromatography. The RNA affinity columns were incubated for 1 h at 30 °C in a volume of 220 μl in 20 mM Heps, pH 7.6, 220 mM KCl, 3.6 mM MgCl2, 3.6 mM ATP, and 4.5 mM creatine phosphate with 100 μl of a solution of 20 μg/ml bovine serum albumin, 10 μg/ml Escherichia coli tRNA, with or without 2.6 μg of calf thymus or HeLa SR proteins. The final concentration of SR proteins in the defined system was 12 μg/ml, whereas the SR protein concentration in standard splicing reactions with HeLa nuclear extract is approximately 30 μg/ml (15).

Radiolabeled RNAs were added to the columns at the same time that SR proteins were added. Control RNA was transcribed from Bluescript KS+ linearized with XbaI (small) or XhoI (large). The exon 4, exon 2/3/4, and exon 2 m3/m4 RNAs (sequence shown in Fig. 1A) were transcribed from synthetic oligonucleotide templates. All of these RNAs were transcribed with T7 polymerase and labeled using [α-32P]GTP (14). The RNAs for a given experiment were transcribed in parallel with the ratios of labeled GTP/cold GTP set such that all the RNAs would have the same molar-specific activity. RNAs were purified from 5% urea-PAGE gels and quantified using a scintillation counter.

Equal amounts of the exon/intron RNAs were mixed with a set amount of control RNA to make normalized RNA samples. A part of these samples was saved as preload, and the rest was added in equal amounts to RNA affinity columns. To some columns competitor RNAs were added (exon 5 WT, exon 2/3/4, or exon 6), which were synthesized with T7 polymerase and were not radiolabeled (14). In these experiments where competitors were added (Fig. 4), the radiolabeled 2/3/4 RNA concentration was 10 pmol/reaction, and the RNA concentration was 20 μg/ml. Following incubation, columns were washed four times at 4 °C in Buffer S (8). One-third of each sample was loaded onto a 6 M urea, 5% acrylamide gels. The gels were exposed to a PhosphorImager, and the relative signals were quantified with Image/Quant (Molecular Dynamics). The percent exon 5 inclusion from a given PCR reaction was computed by dividing the signal from the exon 5 inclusion product by the sum of the signals of exon 5 included and exon skipped product (multiplied by 100). The percent exon 5 inclusion for a given minigenie is the mean of the percent exon 5 inclusion from at least three independent transfections.

**RESULTS**

**Exon 2/3/4, the Composite of Exons 2, 3, and 4 Spliced Together, Can Bind to SR Proteins**—To analyze SR protein associations with the cTnT pre-mRNA, we asked whether SR proteins could interact with exons that are upstream of exon 5. In addition to studying the biochemical properties of exon 4 alone, we considered that exon 4 may be part of a larger exon, of exons 1, 2, 3, and 4 spliced together, when it is spliced to exon 5. Exons 2 and 3 are moderately purine-rich and, when spliced to exon 4, could generate an SR protein binding site and thereby influence the splicing pattern of exon 4. Thus, we have analyzed the SR protein binding characteristics of the composite of exon 2, 3, and 4 spliced together, which we refer to as exon 2/3/4 (Fig. 1A).

To test whether exon 2/3/4 can bind SR proteins, we asked whether exon 2/3/4 could form a UV cross-linked species with SR proteins. Radiolabeled exon 2/3/4 (3 pmol) was incubated with purified SR proteins and nonspecific competitor RNA (50 pmol) under splicing conditions and subsequently exposed to
UV irradiation at 254 nm, which can create covalent bonds between protein and RNA (13). The reactions were then treated with RNase A and T1 to displace uncleaved RNA, and the radiolabeled proteins were resolved by 10% SDS-PAGE. In these experiments, exon 2/3/4 formed cross-linked species with several SR proteins (Fig. 1B, lane 1). To determine the specificity of the cross-link, identical reactions were performed in the presence of five different competitor RNAs: exon 2/3/4; exon 2 m3m4/6, which is similar to exon 2/3/4 except it has 13 purine to pyrimidine mutations in the exon 2 and 3 region (Fig. 1A); full-length exon 2; exon 3; and exon 4. Each competitor was tested at two concentrations: 10 and 30 pmol/reaction. Addition of the exon 2/3/4 competitor RNA led to a decrease in the cross-linking efficiency of radiolabeled exon 2/3/4 to all the SR proteins (lanes 2 and 3). The exon 2 m3m4/6, full-length exon 2, exon 3, and exon 4 competitor RNAs, in contrast, were significantly less efficient at inhibiting the cross-linking efficiency (lanes 4–11). For example, the cross-link to SRp55 was inhibited 4- and 12-fold by 10 and 30 pmol of exon 2/3/4 competitor, respectively, 1.3- and 1.6-fold for the same amounts of exon 2 m3m4, 1.6- and 1.8-fold for exon 2, 1- and 2-fold for exon 3, and 1.5- and 3-fold for exon 4. These results suggest that an efficient SR protein binding site is created by the splicing together of exons 2, 3, and 4.

**SR Proteins Are Sufficient to Promote the Associations of Exon 2/3/4 with Exon 6 and Exon 2/3/4 with Exon 5/UP**—In a previous study, we found that SR proteins are sufficient to promote a specific association of exon 6 with exon 5/UP, an exon 5 mutant that has an enhanced SR protein binding site (18). Thus, we wondered whether SR proteins could also promote associations of exon 2/3/4 with exons 5 and/or 6. To test this prediction, we assayed the affinities of 1) radiolabeled exon 2/3/4 and exon 4 RNAs for exon 5/UP affinity columns and 2) radiolabeled exon 2/3/4 and exon 2 m3m4/6 for exon 6 affinity columns. In these experiments, the radiolabeled RNAs were incubated with the affinity columns along with fixed amounts of radiolabeled control RNAs both in the presence or absence of SR proteins. To assay for relevant interactions, the concentration of SR proteins in these experiments is 12 μg/ml, which is on the same order as that present in standard splicing reactions with HeLa nuclear extract (15). Following several washes, RNA was extracted from the columns and resolved on a 5% urea-PAGE gel. Note that in Fig. 2 the long exon 2/3/4 and 2 m3m4 RNAs labeled E are incubated with a short control RNA (labeled C), and the short exon 4 RNA (labeled E) is incubated with a long control RNA (labeled C).

In these experiments (Fig. 2), SR proteins reproducibly resulted in a >5-fold increase in the amount of exon 2/3/4, which bound to both exon 5/UP columns (lanes 2 and 3) and exon 6 columns (lanes 10 and 11). In contrast, the addition of SR proteins did not result in an increase in the amount of exon 4, which bound to exon 5/UP columns (lanes 7 and 8), or in the amount of exon 2 m3m4/6, which bound to exon 6 columns (lanes 13 and 14). Analysis of supernatant fractions from these incubation reactions shows that the amounts of RNAs present at the end of the incubations were the same with or without SR proteins (lanes 4 and 5, and data not shown). This result indicates that SR proteins do not influence the stability of the these RNAs in the binding reaction. The supernatant signal does not diminish with SR proteins because radiolabeled exon RNAs are in molar excess of column exon RNA. These results suggest that SR proteins are sufficient to promote the associations of exon 2/3/4 with exon 5/UP and exon 2/3/4 with exon 6.

**SR Protein Binding Sites in Exons 2/3/4 and 6 Promote Exon 5 Skipping**—The findings that SR proteins can promote exon/exon associations between the pairs of exons 5/UP-6 (8), 2/3/4-5/UP, and 2/3/4-6 suggested that the SR protein binding sites in each of these exons may play a role in cTnT splicing. The SR protein binding sites in alternatively included exons, such as exon 5, are generally found to be essential for inclusion of the alternative exon (1, 13). However, the functional role of the SR protein binding sites in constitutive exons, such as exons 2/3/4 and 6, remained unexplored.

To characterize the role of purine-rich SR protein binding sites in exon 2/3/4 and exon 6 in the splicing of cTnT, we have mutated these sites and assayed their effects on cTnT splicing in vivo. Four minigenes were constructed (Fig. 3A): WT, which contains wild-type cTnT genomic sequence; 2 m3m, which has the mutations of purine residues in exons 2 and 3 described above (see Fig. 1A); 6 m, which has mutations of purine residues in exon 6 (8); and 2 m3m6 m, which has a combination of the mutations made in exons 2, 3, and 6. The 2 m3m and 6 m mutations can be expected to affect the SR protein binding capacity of exons 2/3/4 and 6 in vivo (Refs. 8 and 13, Figs. 1 and 2), although it is formally possible that these mutations could also affect the associations of additional factors. These minigenes were transfected into QT35 fibroblasts and primary chicken embryo skeletal muscle cultures. RNA was subsequently isolated from the transfected cells, and the splicing patterns of each minigene were assayed by reverse transcriptase-PCR. In these experiments, the mutations affected the relative ratio of mRNAs including and excluding exon 5. In QT35 cells, where the wild-type cTnT minigene exhibits exon 5 inclusion levels of 42%, both the 2 m3m and 6 m minigene exhibit increased exon 5 inclusion levels in these cells to 59 and 61%, respectively (Fig. 3A). The combination mutant 2 m3m6 m exhibited an exon 5 inclusion level of 73% in QT35 cells, which is higher than either of the individual mutants. Single mutations in exon 2 and exon 3 each show a partial increase of exon 5 inclusion at 47 ± 4% and 47 ± 3%, respectively, although it is not clear whether these increases
The SR Protein-mediated Exon/Exon Associations

Exon 6 Is Preferred over Associations Involving Exon 5—

The SR protein-mediated interaction of exon 2/3/4 with exon 6 in vitro is preferred over interactions involving exon 5. A and B, the competition of the SR protein-mediated association of exon 2/3/4 with exon 6. The relative amount of exon 2/3/4 and control RNA added to exon 6 affinity columns is shown (A and B, lane 1). The signals from exons 2/3/4 are shown from complexes assembled in either the absence (A and B, lane 2) or presence (A and B, lane 3) of SR proteins. The signals from complexes assembled in the presence of SR proteins that also contain various competitor RNAs are shown: 30 and 300 pmol/reaction of exon 5/WT (A, lanes 4 and 5, respectively), 30 and 300 pmol/reaction of exon 2/3/4 (A, lanes 6 and 7, respectively). 15 and 150 pmol/reaction of exon 5/WT (B, lanes 4 and 5, respectively), and 15 and 150 pmol/reaction of exon 6 (B, lanes 6 and 7, respectively). The migration of the control RNA is indicated to the left of each panel by a C. C, a model of SR protein-mediated exon/exon associations in the cTnT pre-mRNA. The SR protein-mediated association of exon 2/3/4 with exon 6 (left) is shown in competition with the SR protein-mediated associations of both exon 2/3/4 with exon 5 (lower right) and exon 6 with exon 5 (upper right). Although SR proteins are depicted as directly promoting these interactions, it is likely that they also promote these associations in cooperation with other factors such as snRNPs.

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FIG. 3. The purine residues in exons 2, 3, and 6 promote efficient skipping of exon 5. Illustrated are minigenes that express a genomic fragment of chicken cTnT from exon 1 to exon 6 with exon 6 fused to a genomic fragment of skeletal actin. The shaded boxes indicate mutation of purine residues within that exon. The mutations in exons 2 and 3 are identical to those shown in Fig. 1A. The mutation in exon 6 replaces a 58-nucleotide purine stretch with exon 2 of skeletal troponin I (13). The minigenes were transfected into QT35 fibroblasts and primary chicken embryo skeletal muscle cultures. Subsequently, splicing was assayed by reverse transcriptase-PCR with the oligonucleotides. For each minigene, the percentage of spliced mRNA that includes exon 5 is shown. Each percentage was averaged from at least three independent transfections. The standard deviations from the mean are indicated to the right of each percentage. Also shown are reverse transcriptase-PCR products from one transfection for the WT, 2 m3m, 6 m, and 2 m3m6 m minigenes. The film exposures were chosen such that the lanes would exhibit similar overall intensities to allow easy comparison. Although these calculations are unaffected by exposure times to the PhosphorImager, the percentages were calculated from approximately the same exposure times for each transfection. The standard deviations are less than 5%, because the relative ratio between exon 5 included RNA, and exon 5 skipped RNA, which is internal to each sample, is isolated from pipetting errors. These ratios also have been found to be unaltered by changes of the number of cycles of the PCR reaction and are the same as that observed by primer extension (data not shown). The reverse transcriptase-PCR products, which include exon 5 and skip exon 5, are indicated to the left by inc and skp, respectively.

are significant. This result indicates that at least two purine-rich regions in exon 2/3/4 need to be mutated to significantly affect its splicing. In muscle cells, the mutant minigenes 2 m3m, 6 m, and 2 m3m6 m showed slight increases in exon 5 inclusion at 94, 98, and 95%, respectively, where the WT exon 5 inclusion is already high at 89%. These results indicate that the purine-rich sites in exons 2, 3, and 6 promote exon 5 skipping. Alternatively, the mutated forms of exons 2, 3, or 6 could possibly be acting as inhibitors of exon 5 inclusion. The simplest interpretation of all of these results is that SR proteins bound to exons 2/3/4 and 6 promote exon 5 skipping.

The SR Protein-mediated Association of Exon 2/3/4 with Exon 6 Is Preferred over Associations Involving Exon 5—

The finding that the SR protein binding sites in exons 2/3/4 and 6 promote exon 5 skipping suggests that SR proteins may prefer to promote the association of exon 2/3/4 with exon 6 instead of associations of exons 2/3/4 or 6 with exon 5. To test this hypothesis, we performed an in vitro competition assay for exon/exon associations where we assayed the relative ability of exons 2/3/4, 5, and 6 to disrupt the SR protein-mediated association of exon 2/3/4 with exon 6. In these experiments, radiolabeled exon 2/3/4 was incubated with exon 6 columns and SR proteins in the presence of exon 5/WT, exon 2/3/4, or exon 6 as competitor RNAs. Following several washes, RNA was extracted from the columns and resolved on a 5% urea-PAGE gel.

In the first experiment (Fig. 4A), we compared inhibition capacity of exon 5/WT with that of exon 2/3/4. In this experiment, exon 5/WT inhibited the 2/3/4-6 interaction 3- and 7-fold with 30 and 300 pmol/reaction, respectively (lanes 4 and 5), whereas exon 2/3/4 inhibited the 2/3/4-6 interaction 6- and 7-fold with a 30 and 300 pmol/reaction, respectively (lanes 6 and 7). In the second experiment (Fig. 4B), we compared the inhibition capacity of exon 5/WT with that of exon 6. In this experiment, exon 5/WT inhibited the 2/3/4-6 interaction 1.3- and 3-fold with a 15 and 150 pmol/reaction, respectively (lanes...
performed these experiments. These results suggest the SR consistently 1.8-fold more efficient than exon 5/WT at disrupting and 5.5-fold with a 15 and 150 pmol/reaction, respectively and exon 2/3/4 and 6 promote alternative exon 5 skipping in vivo. We suggest that the relative strengths of SR protein-mediated associations between exons 2/3/4, 5, and 6 play a role in cTnT alternative splicing (Fig. 4C).

DISCUSSION

The relative strengths of exon/exon associations appear to vary between distinct exon pairs during alternative splicing (2, 3). To understand how alternative splicing patterns are established, it will be critical to identify the molecular mechanisms that give rise to this variation. In this study, we have characterized the role of SR protein-mediated exon/exon associations in this process. Previous results have shown that SR proteins, bound to exons, may function to promote the interactions of exons across introns, both by promoting exon/exon associations directly and as well by promoting snRNP associations with splice sites (7–10). In this study, we have analyzed the chicken cardiac troponin T pre-mRNA, which has SR protein binding sites in multiple contiguous exons: the constitutive exons 2/3/4 and 6 and the alternative exon 5. With this pre-mRNA, we show that the SR protein binding sites in the constitutive exons appear to promote alternative exon skipping in vivo. These results are consistent with our additional findings that the SR protein-mediated in vitro association of the two constitutive exons (exon 2/3/4 with exon 6) is preferred over SR protein-mediated associations involving the intervening alternative exon (exon 5). We suggest that the relative strengths of SR protein-mediated associations of alternative and constitutive exons play a role in determining alternative splicing patterns.

SR protein binding sites are not likely to be the only elements that influence the relative strengths of exon/exon associations during cTnT alternative splicing, as is suggested by the finding that the SR protein binding sites in exons 2/3/4 and 6 are not absolutely essential for exon 5 skipping. For example, the efficiency of association of splicing snRNPs with a particular exon, which can interact with snRNPs bound to another exon, is also likely to influence the efficiency of particular exon/exon associations (1, 11, 19). These mechanisms play a role in cTnT splicing, because exon 5 inclusion can be enhanced by improving the base complementarity of its 5′-splice site for the U1 snRNA (16, 20). Another element that likely influences exon/exon associations is the constitutive preference for splicing proximal exons. This appears to be a common feature of RNA splicing, because, in general, the most abundant mature RNAs contain exons spliced in order (2). The mechanisms that underlie this preference are unknown, although the co-transcriptional nature of splicing suggests that the pairing of two proximal exons could be finished before the synthesis of another distal exon (21, 22). The finding that exon 5 skipping in cTnT is not simply a default splicing pathway indicates that these mechanisms are at work even in cases of the splicing of weak, alternative exons. Thus, the order that the exons are organized in the pre-mRNA, the relative strengths of the splice sites of each exon, and the relative strengths of SR protein-mediated exon/exon associations likely act in combination to affect the relative efficiencies of particular exon/exon associations during alternative splicing.

We have also found that the assembly of SR protein binding sites can depend upon previous splicing events; exon 4 appears to bind efficiently to SR proteins only when it is spliced to exons 2 and 3 in the composite exon 2/3/4. The apparent involvement of at least two purine-rich regions in the splicing of exon 2/3/4 suggests that the long purine-rich sequence in exon 2/3/4 may bind a complex of multiple SR proteins. Plus, the finding that this SR protein binding site influences exon 5 inclusion indicates that the alternative splicing of exon 5 is linked to other splicing events that are distant on the pre-mRNA from the alternative splicing event. It follows that part of the regulation of exon 5 inclusion could occur through controlling the relative timing of these distant splicing events. For example, delayed splicing of exons 2, 3, and 4, which synthesizes the SR protein binding site in exon 2/3/4, could increase the chance that exon 5 could compete with exon 4 for splicing to exon 6. Thus, it appears that regulation of exon 5 inclusion is not limited to intronic elements previously shown to promote muscle-specific exon inclusion (17, 23). Clearly, an assumption in this model is that exons 2, 3, and 4 are spliced together when the purine residues in exons 2 and 3 affect exon 5 inclusion, which remains to be determined. There are other examples where the synthesis of a splicing element is dependent upon a previous splicing event. In the pre-protachykinin pre-mRNA, the splicing of exons 4 and 5 places the strong 5′-splice site proximal to exon 4, which promotes the splicing of exon 4 to exon 3 (19). In addition, the splicing of exons 6 and 8 of the β-tropomyosin pre-mRNA creates an exonic splicing enhancer that is essential for the splicing of exons 6 and 5 (24, 25). In another case, the splicing of intron 3 of the tumor necrosis factor-β gene is dependent upon the presence of an additional upstream intron (26). Together, these results underscore the importance of studying an alternative splicing event in the context of the whole pre-mRNA.

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