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Functional crosstalk between exon enhancers, polypyrimidine tracts and branchpoint sequences

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We recently identified enhancer elements that activate the weak 3′ splice site of α-tropomyosin exon 2 as well as a variety of heterologous weak 3′ splice sites. To understand their mechanism of action, we devised an iterative selection strategy to identify functional pyrimidine tracts and branchpoint sequences in the presence or absence of enhancer elements. Surprisingly, we found that strong pyrimidine tracts were selected regardless of the presence of enhancer elements. However, the presence of enhancer elements resulted in the selection of multiple, non-consensus branchpoint sequences. Thus, enhancer elements apparently activate weak 3′ splice sites primarily by increasing the efficiency of splicing of introns containing branchpoint sequences with less than optimal U2–branchpoint pairing arrangements. Comparison of consensus sequences from both our selection strategy and compilations of published intron sequences suggests that exon enhancer elements could be widespread and play an important role in the selection of 3′ splice sites.

Keywords: exon enhancers/iterative selection/pre-mRNA splicing

Introduction

Pre-mRNA splicing takes place in a large complex termed the spliceosome, which is composed of small nuclear RNAs (U1, U2, U4, U5 and U6) and multiple protein components (reviewed in Moore *et al.*, 1993; Madhani and Guthrie, 1994; Krämer, 1996). The precision with which intervening sequences are recognized and removed derives in large part from dynamic base-pairing interactions between U1 snRNA and the 5′ splice site, between U2 snRNA and the branchpoint region, between U5 snRNA and exon sequences, between U6 and the 5′ splice site, and between U2 and U6 snRNAs (reviewed in Nilsen, 1994). Association of U2 with the branchpoint occurs early during the splicing pathway and requires U2 auxiliary factor (U2AF) which binds to the adjacent polypyrimidine tract (Ruskin *et al.*, 1988; Zamore *et al.*, 1992). A series of elegant compensatory mutation studies in yeast and mammalian cell lines has shown that base pairing occurs between the branchpoint sequence and a conserved region of U2 snRNA (5′-GUAGUA-3′) (Parker *et al.*, 1987; Wu and Manley, 1989; Zhuang and Weiner, 1989). In yeast, the branch sequence is highly conserved (5′-UACUAAC-3′; the underlined adenosine indicates the branch nucleophile) and is apparently optimized for base pairing with U2. In mammals, the sequence is fairly degenerate (YNY-URAY) but sequences most closely resembling UAC-UAAC are preferentially used (Zhuang *et al.*, 1989). The branch nucleophile is typically the last adenosine in each sequence but splicing can proceed using adjacent adenosines or other nucleotides (Hornig *et al.*, 1986; McPheeters, 1996; Query *et al.*, 1996). Upon pairing with U2, it is thought that the branch nucleotide becomes bulged, presumably positioning the 2′-OH for nucleophilic attack on the 5′ splice site site (Schmelzer and Schweyen, 1986; Jacquier, 1990; Madhani and Guthrie, 1994; Query *et al.*, 1994).

The lack of conservation of branchpoint sequences in mammals enables regulation of alternative splicing through competition between branch sites of differing strength (reviewed in Smith *et al.*, 1989a; Valcárcel *et al.*, 1995; Adams *et al.*, 1996). However, weak branchpoint sequences can be offset by the strength of the adjacent polypyrimidine tract and vice versa, such that combinatorial action between the branchpoint and polypyrimidine tract can result in differential 3′ splice site selection (Fu *et al.*, 1988; Helfman *et al.*, 1990; Mullen *et al.*, 1991; Roscigno *et al.*, 1993; Norton, 1994; Singh *et al.*, 1995; Lin and Patton, 1995). Even in *Saccharomyces cerevisiae*, where there appears to be little or no alternative splicing and pyrimidine tracts are often unrecognizable, increasing the polypyrimidine content can increase the use of adjacent 3′ splice sites (Parker and Patterson, 1987; Patterson and Guthrie, 1991). Exon sequences have also been shown to act in combination with other splicing signals to increase the efficiency of splicing and enable greater regulation (Hoshijima *et al.*, 1991; Lavigueur *et al.*, 1993; Sun *et al.*, 1993; Watakabe *et al.*, 1993; Xu *et al.*, 1993; Tanaka *et al.*, 1994; Tian and Maniatis, 1994; Humphrey *et al.*, 1995). Generally, these so-called splicing enhancer elements are purine rich, but non-purine-rich sequences have also been shown to act as splicing enhancers (Staknis and Reed, 1994; Tian and Kole, 1995; Coulter *et al.*, 1997). It is thought that splicing enhancers bind to SR proteins to establish a network of pre-mRNA–protein and protein–protein interactions leading to commitment of a given set of splice sites (Fu, 1993; Staknis and Reed, 1994; Wu and Maniatis, 1993; Lynch and Maniatis, 1996). SR proteins form a family of splicing factors containing regions rich in serine and arginine residues

(reviewed in Fu, 1995; Valcárel and Green, 1996; Manley and Tacke, 1996). Despite experiments detecting interaction between various SR family members and between SR proteins and U1 snRNP, the exact mechanism by which SR proteins enhance splicing, particularly of weak splice sites, remains unclear.

We recently identified four purine-rich sequences in exon 2 of rat α-tropomyosin (α-TM) that act as enhancer elements. These elements bind SR proteins and function to activate the weak 3′ splice site of exon 2 as well as a variety of heterologous 3′ splice sites. To determine how these enhancers activate splicing, we characterized the 3′ splice site sequences selected after *in vitro* splicing of pre-mRNA substrates with randomized polypyrimidine tract sequences or randomized branchpoint sequences. Although enhancer elements can compensate for weak pyrimidine tracts in a variety of splicing substrates, strong pyrimidine tracts were selected following iterative selection techniques, regardless of the presence or absence of enhancer elements. In contrast, multiple, non-consensus branchpoint sequences were selected in the presence of enhancer elements, particularly when the accompanying pyrimidine tract was strong. Thus, enhancer elements can compensate for either a weak pyrimidine tract or a weak branchpoint sequence and the overall efficiency with which a given 3′ splice site is used depends on combinatorial action between the branchpoint, polypyrimidine tract and enhancer elements.

Results

Iterative selection strategy

Recent experiments using enhancer elements from exon 2 of α-TM showed that activation of weak 3′ splice sites, including heterologous 3′ splice sites, was due to combinatorial effects involving the strength of the branchpoint, polypyrimidine tract and enhancer elements. To assess more accurately the role of each of these elements in splicing, an iterative selection strategy was devised that uses pre-mRNA substrates containing either randomized branchpoint sequences or randomized pyrimidine tracts in the presence or absence of enhancer elements (Figure 1). In each case, a GAG at the 3′ splice site was incorporated to inhibit the second step of splicing and allow trapping of the lariat intermediate (Smith *et al.*, 1989b). Following splicing of these substrates, lariat intermediates were isolated, debranched and reverse transcribed. PCR was then used to amplify and reform the selected substrates followed by cloning and sequencing or by another round of splicing, selection and cloning. Using this strategy, the total pool of functional branchpoint sequences and pyrimidine tracts can be determined and the influence of enhancer elements in the selection of these sequences can be assessed and compared between otherwise identical pre-mRNA substrates.

Enhancer elements do not influence the sequence composition of the pyrimidine tract

To determine the sequence composition of the range of functional pyrimidine tracts, a pool of pre-mRNA substrates containing a consensus branchpoint sequence (UACUAAC) adjacent to 15 randomized nucleotides at the pyrimidine tract was spliced in the presence or absence

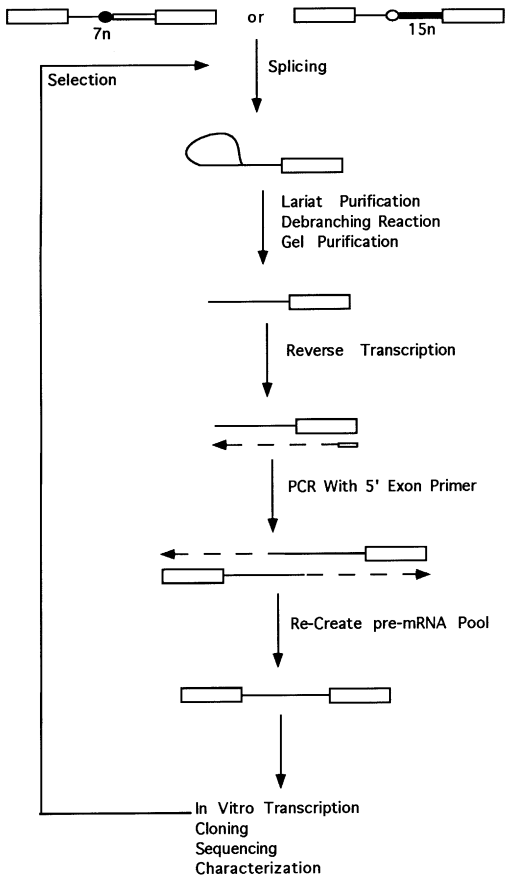

Fig. 1. Randomization/selection of functional pyrimidine tracts and branchpoints. Pre-mRNA substrates were prepared with either a 7 nt random branchpoint region or a 15 nt random polypyrimidine tract. Splicing was performed and lariat intermediates were isolated, debranched, gel purified, reverse transcribed and then amplified with primers that allow the substrate pool to be reassembled and re-spliced. After one or two rounds of splicing selection, the pool of sequences was cloned to identify functional pyrimidine tracts and branchpoints.

of enhancer elements. Compared with the accumulation of lariat intermediate with an identical substrate containing a strong pyrimidine tract (Dpy3, lane 1), only small amounts of lariat intermediates were detectable when the pools of pre-mRNA containing randomized pyrimidine tracts were spliced, even in the presence of enhancer element (Dpy15n and Dpy15n-E). However, after one and/or two rounds of selection, splicing efficiency dramatically increased in the presence of enhancer elements, as evidenced by the accumulation of lariat intermediates and free 5′ exon (Figure 2, lanes 6 and 7). In contrast, there was only a modest increase in splicing efficiency in the absence of enhancer elements (Figure 2, lanes 4 and 5). To illustrate that the dramatic increase in splicing efficiency was due to the enhancer elements, the pool of RNAs derived from two rounds of selection in the absence of enhancer elements was re-spliced in their presence (Figure 2, lane 8). In addition, the pool of RNAs selected after two rounds of selection in the presence of enhancer elements was re-spliced after their removal (Figure 2, lane 9). In both cases, the stimulatory effect of the enhancer elements is clear.

Rather than continuing with additional rounds of selection, resulting in the selection of those sequences optimal for splicing, sequencing of selected tracts was performed

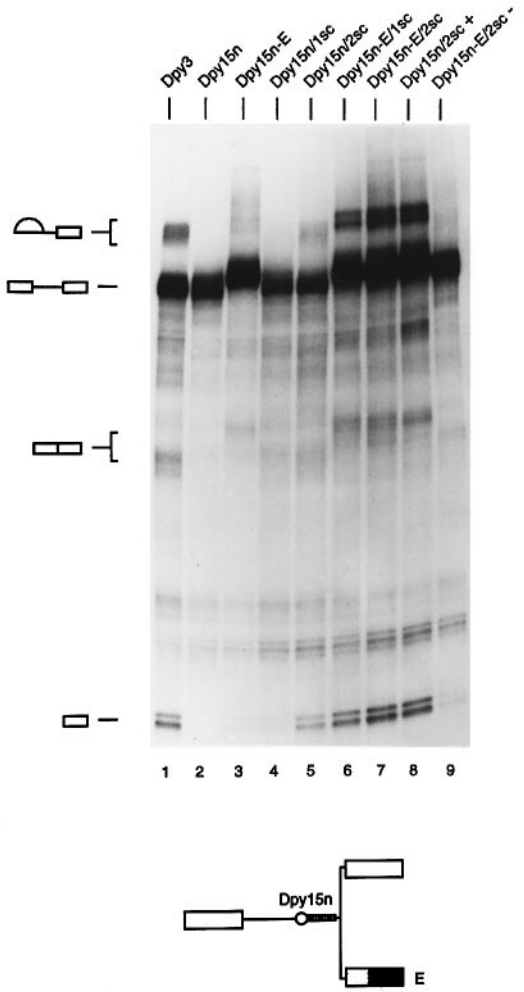

Fig. 2. Polypyrimidine tract selection. Pre-mRNA substrates carrying a 15 nt random pyrimidine tract (Dpy15n) were prepared in the presence or absence of the two central exon 2 enhancer elements (E). For control purposes, splicing was performed with a substrate containing a consensus branchpoint (UACUAAC) and a strong pyrimidine tract (Dpy3, 19 consecutive pyrimidines). Splicing was performed on the original pool of substrates (Dpy15n or Dpy15n-E) or after one (1sc) or two (2sc) rounds of selection cycles. To illustrate the requirement for the enhancer elements, the pool of RNAs selected in the presence of enhancer elements was re-spliced in their absence (Dpy15n-E/2sc–) and the pool selected in the absence of enhancer elements was re-spliced in their presence (Dpy15n/2sc+). The precursor, products and intermediates of splicing are indicated on the left.

after only one or two rounds of splicing in order to determine the entire range of pyrimidine tracts that can promote splicing. Comparison of the random regions from both pools showed that a strong preference for pyrimidine was selected following one round of splicing selection whereas uridines were predominantly selected after two rounds, regardless of the presence or absence of enhancer elements (Figure 3). The percentage base composition derived from statistical analysis of metazoan pyrimidine tracts (Senepathy *et al.*, 1990; Mount *et al.*, 1992) is shown in Figure 3, along with the percentage composition of pyrimidine tracts selected after one or two rounds of selection in the presence or absence of enhancer elements. The numbers are strikingly similar with perhaps less of a decrease in adenosine content within the pyrimidine tracts selected in the presence of enhancer elements. More importantly, simply increasing the overall pyrimidine composition from the initial 50% to 64% after only one round of selection resulted in the production of detectable splicing products, suggesting that this increase in pyrimidine content represented the minimal increase necessary to activate splicing, but only when coupled to the presence of enhancer elements. The same increase in pyrimidine content was detected in the absence of enhancer elements yet splicing was not detectable after one round of selection (Dpy15n/1sc). Thus, enhancer elements can activate the splicing of substrates containing weak pyrimidine tracts but do not affect the sequence composition of selected, functional pyrimidine tracts.

Selection of branchpoint sequences in the presence of enhancer elements

We next examined whether enhancer elements activate weak 3′ splice sites by affecting branchpoint selection. We again followed the strategy outlined in Figure 1 except that the seven nucleotides surrounding the branchpoint region were randomized. The randomized region was placed immediately upstream of one of two different pyrimidine tracts in the presence or absence of the α-TM exon enhancer elements (Figure 4). Dpy1 is a weak pyrimidine tract from upstream of α-TM exon 2 whereas Dpy3 is a stronger pyrimidine tract with 19 consecutive pyrimidines, identical to that used in Figure 2 as a control. From the four combinations of two pyrimidine tracts in the presence or absence of enhancer elements, only the combination of a weak pyrimidine tract, a randomized branchpoint region and no enhancer sequences (7n-Dpy1) was unable to undergo sufficient *in vitro* splicing to allow analysis (Figure 4).

For the remaining combinations, the levels of splicing before and after one round of selection, are shown in Figure 4. It is clear that in the presence of enhancer elements, the splicing of constructs containing randomized branchpoint regions was much more efficient, so much so, that splicing of the pool of RNA was clearly detectable even before selection with the combination of a strong pyrimidine tract and enhancer elements (7n-Dpy3-E). Indeed, the efficiency of splicing of such a pool of substrates was very similar to the splicing efficiency of a pre-mRNA with the same pyrimidine tract adjacent to a consensus branchpoint sequence (Dpy3). This raised the question of whether virtually any sequence can function as a branchpoint sequence in the presence of enhancer elements and suggests that factors that interact with enhancers stabilize U2 snRNA pairing to multiple branch sites. In contrast, the fact that a pool of substrates containing a randomized branchpoint region, a weak pyrimidine tract and enhancer elements (7n-Dpy1-E) did not splice efficiently suggests that a functional pyrimidine tract is also necessary to activate weak 3′ splice sites.

The pool of selected branchpoint sequences was cloned and sequenced after only one round of selection to identify all possible branchpoint sequences rather than the most optimal branchpoint sequence. The selected sequences for each combination are shown aligned with U2 snRNA in Figure 5. A variety of studies have suggested that bulging of the branchpoint facilitates nucleophilic attack on the 5′ splice site (Schmelzer and Schweyen, 1986; Weiner, 1993; Madhani and Guthrie, 1994; Query *et al.*, 1994).

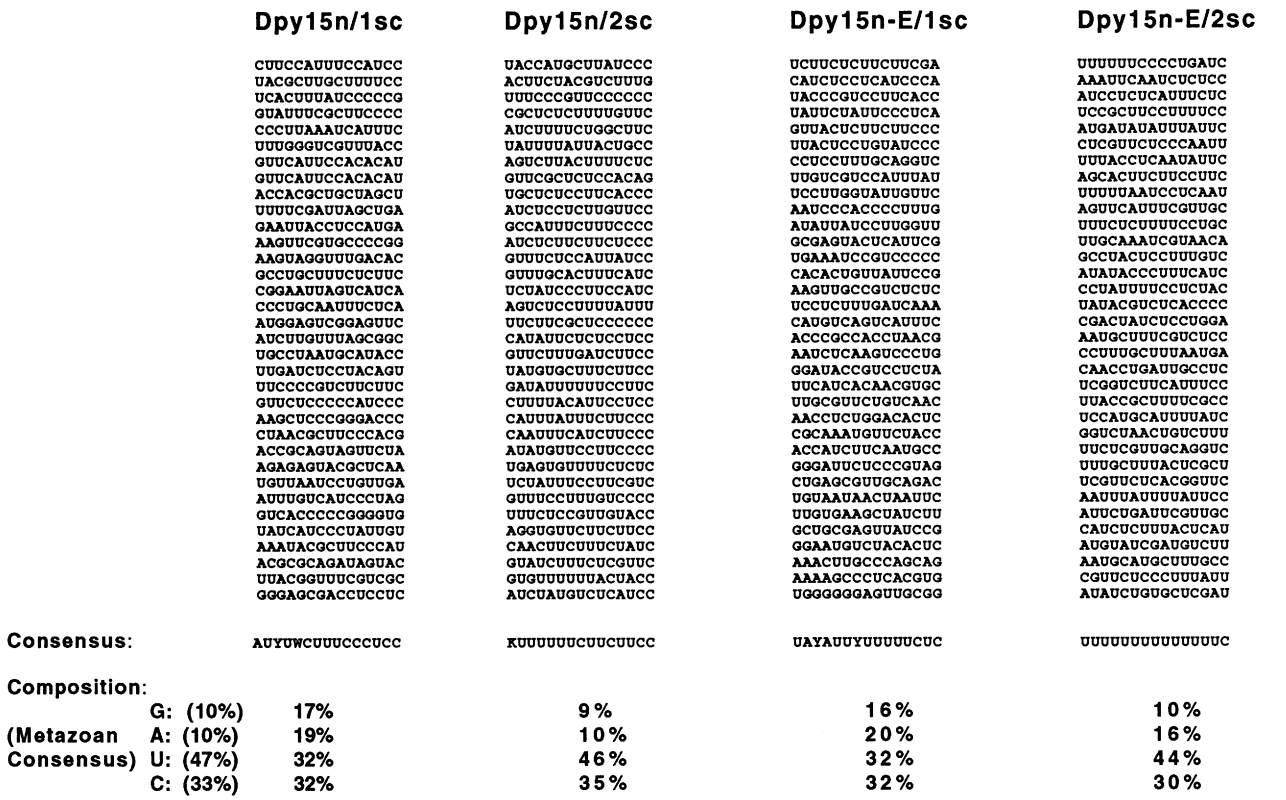

Fig. 3. Iterative selection of functional pyrimidine tracts. Pre-mRNA substrates containing a 15 nt randomized region corresponding to the polypyrimidine tract were spliced in HeLa nuclear extract. Lariat intermediates were isolated after one (Dpy15n/1sc and Dpy15n-E/1sc) or two (Dpy15n/2sc and Dpy15n-E/2sc) rounds of splicing selection and the sequence composition of the functional pyrimidine tracts was determined. The actual sequences and percentage composition obtained in the presence (E) or absence of enhancer elements is shown along with the metazoan consensus composition. Y = pyrimidine; K = G or U; W = A or U. Sequencing of the starting pool of random pyrimidine tracts confirmed that each of the four nucleotides was used with equal frequency.

Consistent with this, the majority of selected branchpoint sequences are predicted to pair most optimally with U2 by including a gap at the branchpoint, presumably indicative of a bulged branchpoint. This is particularly the case with a strong pyrimidine tract in the absence of enhancer elements (7n-Dpy3) and also with the combination of a weak pyrimidine tract and enhancer elements (7n-Dpy1-E). However, when the combination of splicing signals was stronger, more branchpoints were selected that have less potential to pair with U2 in the conventional manner (7n-Dpy3-E). For example, a subset of branchpoint sequences selected in the presence of enhancer elements contained the consensus sequence UAAU with a strong pyrimidine tract or CAAC with a weak pyrimidine tract. In both of these cases, multiple pairing arrangements with U2 are possible but the exact alignment is uncertain.

Enhancer elements promote splicing using multiple base pairing arrangements between U2 snRNA and the branchpoint sequence

Since the predicted pairing arrangements in Figure 5 were derived by computer alignment, it was necessary to determine precisely which nucleotide was acting as the branch nucleophile. Branchpoints were mapped by primer extension on selected substrates with the Dpy3 pyrimidine tract (Figure 6A). As expected, substrates containing branchpoints identical to yeast branchpoint sequences were spliced in the presence and absence of the enhancer elements with the third adenosine in the UACUAAC sequence chosen most of the time under both conditions. However, adjacent nucleotides could serve as the branchpoint, as shown by the presence of additional primer extension products. Prior to selection, branchpoint mapping with the pool of RNA containing enhancer elements and a randomized branchpoint region showed approximately equal use of any of the seven to nine nucleotides within the designated branch region (7n-E). Importantly, branch site selection for these constructs was restricted to the randomized region and cryptic branchpoints did not appear to be activated, consistent with the lack of primer extension products upstream or downstream of the randomized region. It should be stressed that in all of our constructs, the distance between the 5′ splice site and the branchpoint region was shortened to sterically hinder branch site selection except within the desired region to prevent cryptic branchpoint activation (Ruskin *et al.*, 1985; Smith and Nadal-Ginard, 1989).

We next chose selected constructs with dramatically different base pairing possibilities for analysis of splicing and for precise mapping of branchpoints used in the presence or absence of enhancer elements (Figure 6B and C). Specific substrates were chosen that contain branchpoint sequences that match consensus sequences shown in Figure 5 (UAAU, CAAC) or that contain a C preceding the hypothetical branchpoint adenosine (UACUCAC, UCAUCA). A ‘C’ preceding the branchpoint appeared to restrict branchpoint selection predominantly to the adjacent adenosine regardless of the presence of

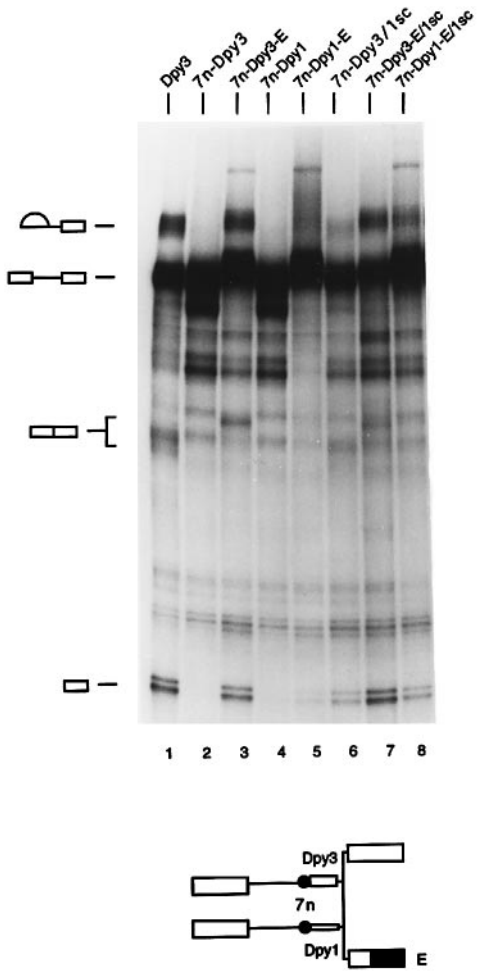

Fig. 4. Selection of branchpoint sequences. Pre-mRNA substrates containing a 7 nt random branchpoint region (7n) were spliced in the presence or absence of enhancer elements (E) with one of two different strength pyrimidine tracts (Dpy3 and Dpy1). Dpy3 is a relatively strong pyrimidine tract and Dpy1 a relatively weak one. Control splicing of a substrate containing a consensus branchpoint sequence (UACUAAC) with the Dpy3 pyrimidine tract is shown in lane 1. Splicing of the other substrates is shown before or after one round of selection (1sc). The combination of a randomized branchpoint and Dpy1 did not produce enough lariat intermediate for analysis. The splicing substrates and products are depicted on the left.

enhancer elements. In the case of the UCAUCA branchpoint sequence (UCA in Figure 6A and B), both adenosines were used with a slight preference for the upstream A. Similarly, base pairing with U2 in which double G–C base pairs flank the branch site (CAAC) appeared to restrict branch site selection to either of the two central adenosine residues. When double A–U residues flanked the branch site (UAAU), multiple branch sites were selected, either from the two central adenosine residues or from a G residue three nucleotides upstream.

Analysis of splicing of these constructs showed that combinatorial action between the branchpoint, polypyrimidine tract and enhancer elements determined overall splicing efficiency. With either the UAAU or CAAC branchpoint sequences, or with substrates containing a C preceding the branchpoint, splicing proceeded most efficiently in the presence of enhancer elements and a strong pyrimidine tract (Dpy3) with a continuing decrease in efficiency as these elements were removed or changed.

To test the splicing ability of substrates having perfect complementarity with U2 snRNA, a construct was prepared with UACUAC at the branch region which should perfectly pair with the GUAGUA sequence in U2. Splicing was only possible in the presence of enhancer elements and required a strong pyrimidine tract (Figure 6C). Branch site selection with this construct showed that the second adenosine was predominantly chosen as the branch nucleophile but additional surrounding residues could also serve as the branchpoint (Figure 6A). If all the nucleotides in this substrate are paired with U2, the branch nucleophile should not be bulged. Thus, enhancer elements may promote bulged branchpoints in a previously unexpected manner, other pairing arrangements may occur, or a bulged branch nucleophile may not be required for splicing in the presence of enhancer elements. Consistent with this, the selected branch regions shown in Figure 5 that predict perfect complementarity with U2 also required the presence of enhancer elements for splicing. Thus, it appears that enhancer elements can activate weak 3′ splice sites by promoting the splicing of substrates with non-consensus branchpoint sequences.

Discussion

Exon enhancers and polypyrimidine tracts

We have used substrates with randomized nucleotides at either the polypyrimidine tract or the branchpoint to identify those sequences that functionally activate splicing. Iterative selection techniques have been applied to exon sequences (Tian and Kole, 1995; Coulter *et al.*, 1997) and intron secondary structures (Libri *et al.*, 1995) but this paper describes the first attempt to apply these techniques to intron signals involved in 3′ splice site selection. We have found that, regardless of enhancer elements, the base composition of the selected polypyrimidine tracts was almost identical. However, even though the pyrimidine tract composition increased from 50% to 64% after one round of selection, splicing was only detectable in the presence of enhancer elements. Prior to selection, splicing was not detectable in either case. Thus, enhancer elements cannot entirely replace the pyrimidine tract but they can clearly increase the efficiency of splicing of substrates containing weak pyrimidine tracts. Since 3′ splice site selection is driven by competition between multiple polypyrimidine binding proteins, one possible explanation for the selection of pyrimidines, particularly uridines, after two rounds of selection is that strong binding sites for $U2AF^{65}$ are preferentially selected to enable more efficient entry of U2 into assembling spliceosomes and drive first step cleavage (Reed, 1989; Roscigno *et al.*, 1993; Lin and Patton, 1995; Singh *et al.*, 1995; Coolidge *et al.*, 1997).

Exon enhancers promote U2 snRNA pairing

Analysis of the pool of selected branchpoints revealed significant differences depending on the presence of enhancer elements. Computer prediction of base pairing between the selected branchpoint sequences and U2 snRNA showed that at least two different pools of branchpoints were selected, either consistent or inconsistent with a bulged branchpoint (see Figure 5). In addition, the presence of enhancer elements resulted in the selection of a subset of branchpoint sequences that showed conserv-

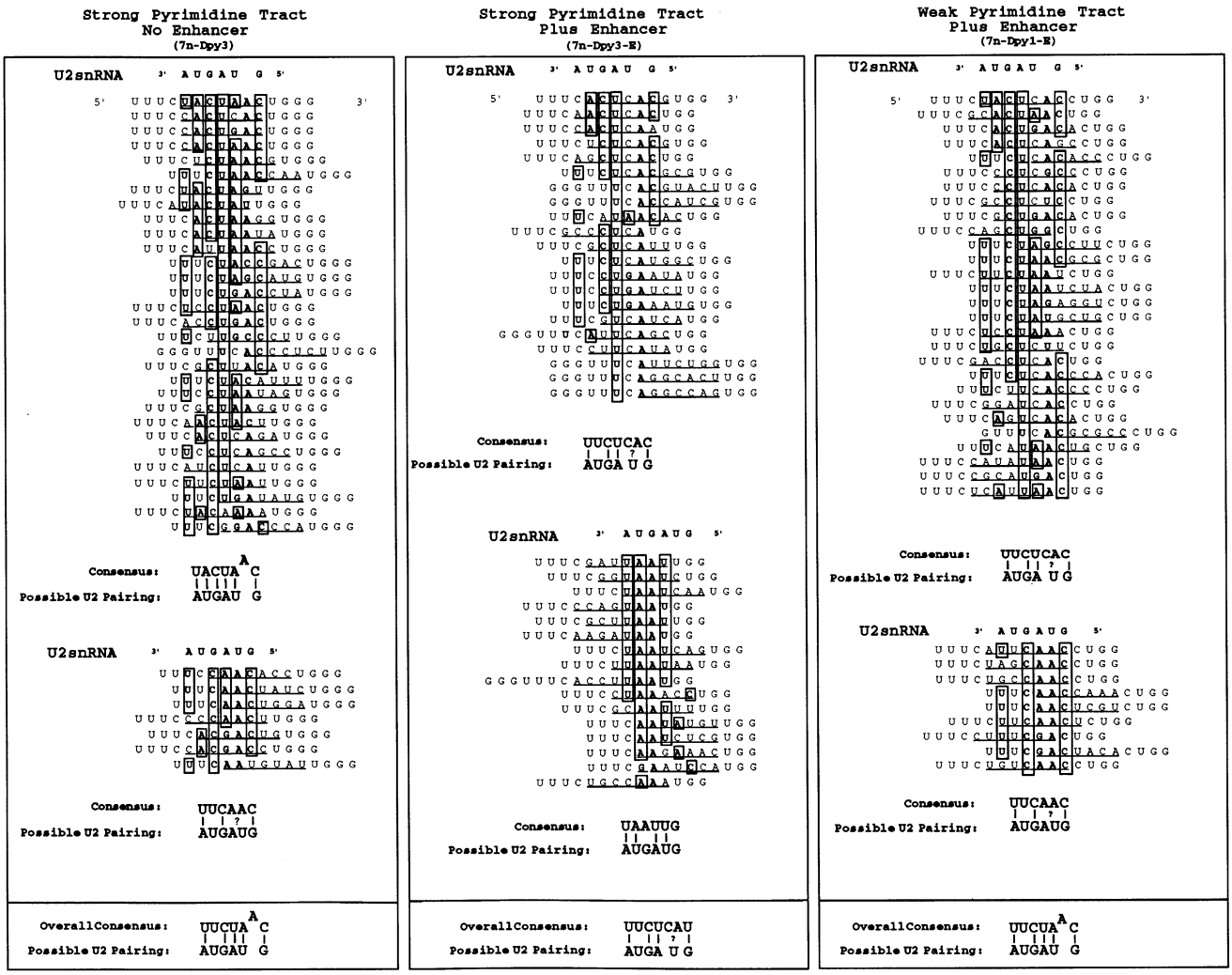

Fig. 5. Selection of functional branchpoint sequences. Pre-mRNA substrates containing a 7 nt randomized branchpoint region were spliced in HeLa extract in the presence or absence of enhancer elements (E) and either a strong (Dpy3) or weak (Dpy1) polypyrimidine tract. Following one round of iterative selection, functional branchpoints were sequenced and are shown in one of two predicted pairing arrangements with U2 snRNA. Nucleotides that can pair with U2 are boxed and a consensus sequence is displayed below each set as well as a consensus sequence derived from both groups. In the absence of enhancer elements, the combination of a weak pyrimidine tract and a randomized branchpoint region did not generate detectable levels of splicing to allow selection analysis.

ation of a C at position –1 relative to the branchpoint, at odds with the mammalian consensus sequence (YNYURAC; Senepathy *et al.*, 1990). However, algorithms that identify branchpoints based on alignment with U2 have detected conservation of a C at –1 (Lukashin *et al.*, 1992). While it is not clear why statistical analyses have identified differences at this position, one possible explanation is that enhancer elements may be widely dispersed allowing conservation of seemingly non-consensus branchpoints.

For the subset of branchpoint sequences selected in the presence of enhancer elements, the branchpoint may not be bulged. Alternatively, base pairing between U2 and these pre-mRNAs could occur using unusual arrangements, including bulged nucleotides within U2, that ultimately result in a bulged branchpoint. In addition, proteins associated with enhancer elements could promote branchpoint bulging in ways that are not readily predicted using computer alignment algorithms. Thus, whether these substrates use bulged or unbulged branch nucleophiles is not clear but an intriguing possibility is that enhancer elements and their associated proteins either obviate the need for a bulged branchpoint or use a mechanism other than typical base pairing with U2 to generate a bulged branch nucleophile. In addition, the strength of the accompanying pyrimidine tract can further modify pairing requirements between U2 and the pre-mRNA. With a strong pyrimidine tract, the selected consensus sequence directly flanking the branchpoint was UAAU whereas with a weaker pyrimidine tract CAAC was selected, resulting in a more stable pairing arrangement with U2 (Figure 5). The inference is that while enhancer elements can stabilize weaker pairing arrangements with U2, the strength of the accompanying pyrimidine tract can also affect pairing. Coupled with our analysis of the effect of these enhancer elements on α-TM splicing, both *in vitro* and *in vivo*, as well as other model pre-mRNAs (Adema *et al.*, 1988; Hartmuth and Barta, 1988; Hampson *et al.*, 1989; Yeakley *et al.*, 1993, 1996; Lou *et al.*, 1995; Zheng *et al.*, 1996), it seems clear that selection of 3′ splice sites is driven by the combinatorial and compensatory action of the three *cis*-acting signals that define 3′ splice sites: branchpoints, polypyrimidine tracts and enhancer elements.

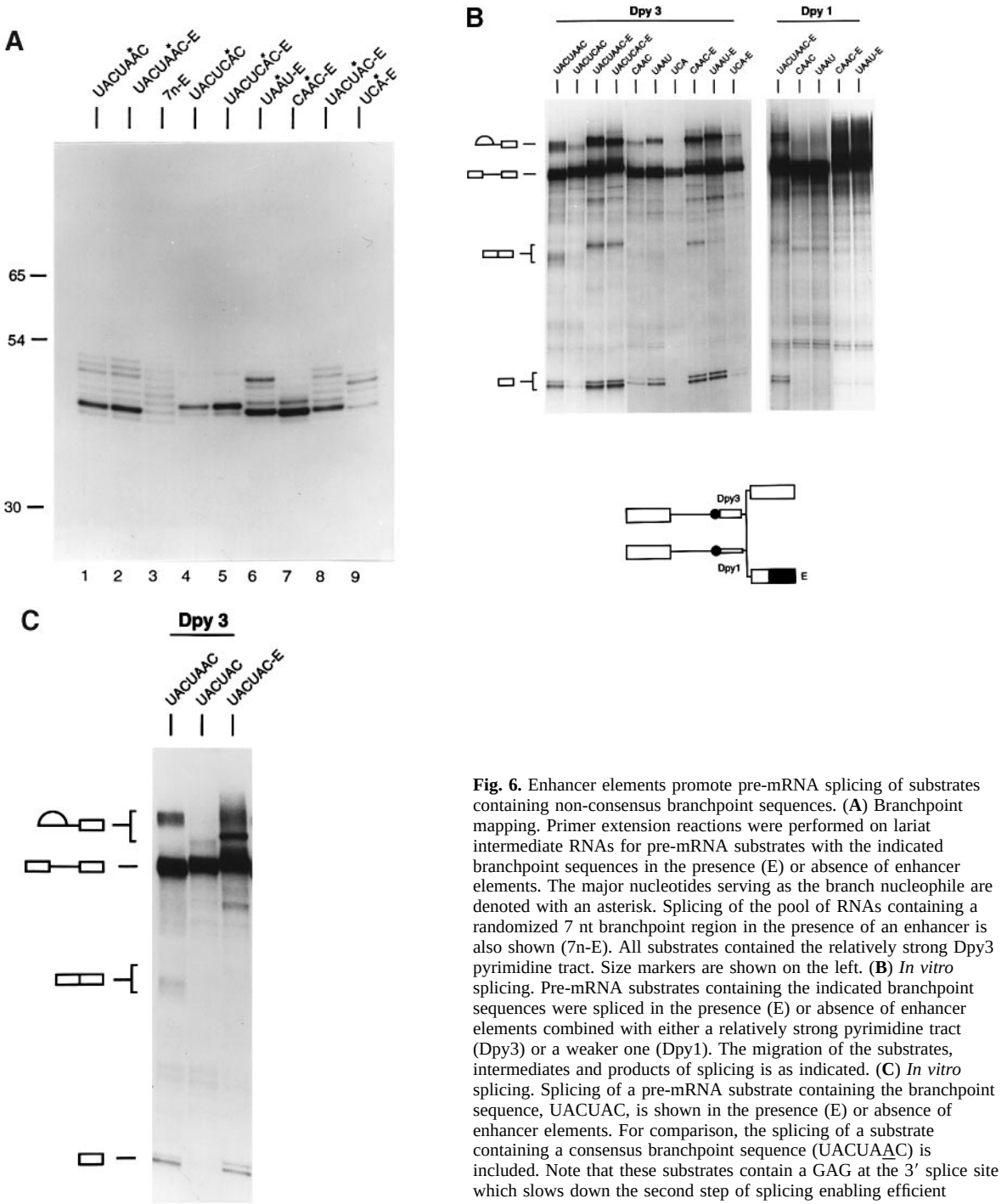

Fig. 6. Enhancer elements promote pre-mRNA splicing of substrates containing non-consensus branchpoint sequences. (**A**) Branchpoint mapping. Primer extension reactions were performed on lariat intermediate RNAs for pre-mRNA substrates with the indicated branchpoint sequences in the presence (E) or absence of enhancer elements. The major nucleotides serving as the branch nucleophile are denoted with an asterisk. Splicing of the pool of RNAs containing a randomized 7 nt branchpoint region in the presence of an enhancer is also shown (7n-E). All substrates contained the relatively strong Dpy3 pyrimidine tract. Size markers are shown on the left. (**B**) *In vitro* splicing. Pre-mRNA substrates containing the indicated branchpoint sequences were spliced in the presence (E) or absence of enhancer elements combined with either a relatively strong pyrimidine tract (Dpy3) or a weaker one (Dpy1). The migration of the substrates, intermediates and products of splicing is as indicated. (**C**) *In vitro* splicing. Splicing of a pre-mRNA substrate containing the branchpoint sequence, UACUAC, is shown in the presence (E) or absence of enhancer elements. For comparison, the splicing of a substrate containing a consensus branchpoint sequence (UACUAAC) is included. Note that these substrates contain a GAG at the 3′ splice site which slows down the second step of splicing enabling efficient trapping of the lariat intermediates.

Protein factors and the activation of weak 3′ splice sites

Our splicing selection assays identify those substrates that undergo the first step of splicing most efficiently, and stopping selection after only one or two rounds provided a unique opportunity to view the entire range of functional pyrimidine tracts and branchpoints. Nevertheless, all of the selected substrates must assemble early spliceosome complexes efficiently, recruit U2 snRNA to the branchpoint and then undergo the first catalytic step of splicing such that the sum total of each of these steps must proceed efficiently to enable selection. Prior to the entry of U2, the assembly of spliceosomes involves association of multiple factors with the pre-mRNA in discrete steps and complexes (Michaud and Reed, 1991, 1993; Rosbash and Seraphin, 1991; Jamison and Garcia-Blanco, 1992). It has been proposed that in mammalian systems, SR proteins promote protein–protein interactions across both introns and exons resulting in interaction between U2AF, U1 snRNP and SR proteins (Wu and Maniatis, 1993; Kohtz *et al*., 1994; Staknis and Reed, 1994; Tarn and Steitz, 1995; Lynch and Maniatis, 1996; Zuo and Maniatis, 1996). A similar bridging complex has been proposed in the yeast *S.cerevisiae* except that no SR proteins have been detected in these complexes and protein factors have been identified that interact directly with the branchpoint

(Abovich and Rosbash, 1997). While we have focused on possible base-pairing interactions between the branchpoint and U2 snRNA, it remains possible that a crucial rate limiting step in our splicing assays is the identification of the branchpoint, possibly through the binding of specific factors recognizing different branchpoint sequences. These factors would probably be recruited and stabilized by the interaction of SR proteins acting through enhancer sequences. However, it should be stressed that the first step of splicing ultimately requires base pairing with U2 and no matter what factors are involved in U2 recruitment, suitable pairing interactions must be accommodated. It seems clear from our results that suboptimal pairing arrangements require the action of enhancer elements and the factors that recognize these sequences.

Materials and methods

Construct preparation

All constructs used in this study were prepared using standard cloning techniques (Sambrook *et al.*, 1989). The α-TM single intron substrate (DS) and the β-globin substrate (RR108) were as described (Smith and Nadal-Ginard, 1989; Smith *et al.*, 1989a,b). Exon 2 mutants were prepared by inverse PCR (Imai *et al.*, 1991) on the DS template with appropriate oligonucleotide pairs.

For *in vitro* transcription, exon 2 templates were linearized and transcribed with SP6 RNA polymerase (Boehringer Mannheim). The alternative splicing constructs B2P2-E2mut, B3P3-E2mut and pGEN-E2mut were prepared by inverse PCR on the wild-type templates (Mullen *et al.*, 1991). Dpy1, Dpy2 and Dpy3 were derived from pPIP85 (Moore and Sharp, 1992) and contain a 30 nt deletion upstream of the branchpoint sequence (TACTAAC). Dpy1 contains a 21 nt polypyrimidine tract (CGCCCGTTTGTTGTGTGTCTC) derived from the intron upstream of α-TM exon 2. Dpy2 contains a 15 nt polypyrimidine tract (GCCCATTATTATCCA) and Dpy3 contains a 19 nt polypyrimidine tract (CCCCTTCTTCTTTTTCCCT) derived from PIP4 and PIP85, respectively (Roscigno *et al.*, 1993). Single-strand DNA templates for *in vitro* transcription were assembled using three synthetic oligonucleotides (A, B and C) ligated in the presence of two 28 nt bridging oligonucleotides (Tuerk and Gold, 1990). Oligonucleotide A (96 nt including the T7 promoter) and oligonucleotide B (38 nt) are common to all PIP85 derivatives. The C oligonucleotides contain differing pyrimidine tracts as well as the randomized regions used in the Tuerk and Gold SELEX experiments, 1990, and correspond to 101 nt for Dpy1, 95 nt for Dpy2 and 99 nt for Dpy 3. The sequence of Dpy 3 is as follows with lowercase letters indicating the T7 RNA polymerase promoter and uppercase letters corresponding to the transcribed RNA. The bold nucleotides denote the 5′ and 3′ splice sites: 5′-cggtaatacgactcactataGGGAGAGGGCGAATTCGAGCTCACTCTCTTCCGCATCGCTGTCTGCGAGGTACCCTA C-CAG**GT**GAGTATGGATCCCTCTAAAAGCGGGCATGACTTCTGGGGTTGTCCTGGGTTTCCGTGGGTTTCTACTAACTGGGCCCCTTCTTCTTTTTCCCTG**AG**GTCCTACACAACATACTGCAGGACAAACTCTTCGCGGTCTCTGCATGCAAGCTT-3′.

Transcripts carrying the two central purine-rich elements defined by the AX mutation (see Figure 1) were generated by PCR using oligonucleotides T7b (5′-CGGTAATACGACTCACTATAGGG-3′) and Sten II (5′-CCGCTCGTCCTCCGACGCCCGCAGCAGCTTCTCTTTTGCCTGCAGTATGTTGTGTAGGAC-3′).

In vitro transcription and splicing reactions

Capped pre-mRNA transcripts were prepared as described (Smith and Nadal-Ginard, 1989; Mullen *et al.*, 1991). Unlabeled competitor RNAs were prepared by decreasing the amount of [^{32}P]CTP 200-fold and adding unlabeled CTP to a concentration equal to that of the other three rNTPs. HeLa cell nuclear extracts were prepared as described by Abmayr *et al.* (1988) except that the final dialysis was stopped when equilibration with buffer E was reached as measured with a conductivity meter (Corning). Standard splicing reactions contained 20–40 fmol of ^{32}P-labeled RNA transcript, 2.2 mM $MgCl_2$, 0.5 mM ATP, 20 mM creatine phosphate, 1000 U/ml RNasin (Promega) and 30–40% nuclear extract, as indicated. Competitive inhibition experiments were performed by incubating the splicing reaction with the indicated amount of competitor RNA for 10 min before adding substrate RNA. Splicing reactions were fractionated by electrophoresis through either 5% or 16% polyacrylamide gels in the presence of 8 M urea followed by autoradiography. Detection of splicing complexes on native gels was in 4% Tris–glycine gels as described by Konarska and Sharp (1986). Samples supplemented with heparin (10 mg/ml) were incubated for 10 min on ice before electrophoresis.

In vitro selection

DNA templates used in the SELEX experiments were prepared as described for Dpy1, Dpy2 and Dpy3. After ligation and gel purification, ~300 ng of each randomized template pool were recovered and 20 ng were then subjected to PCR with T7b and 7cDNA (5′-AAGCTTGCATGCAGAGACCGC-3′) oligonucleotides to obtain double-stranded DNA templates for transcription by T7 RNA polymerase. For generation of the different RNA starting pools, ~140 ng of each gel-purified template (corresponding to 5.6×10^{11} different molecules) were transcribed with T7 RNA polymerase. After transcription, the reactions were treated with DNase (Promega) and phenol extracted and the RNAs were purified on an 8 M urea–8% polyacrylamide gel. The absence of DNA template was monitored by RT–PCR with T7 and 7cDNA oligonucleotides. The splicing selection (schematically depicted in Figure 1) was carried out for 50 min at 30°C using 40% HeLa nuclear extract and 150 fmol of each randomized transcript. After splicing, the reactions were loaded on to an 8 M urea–12% polyacrylamide gel and the lariat intermediates were purified. The lariat structures were then debranched in 60% HeLa S100 extract (Ruskin and Green, 1990) and repurified on an 8 M urea–8% polyacrylamide gel. Debranched RNA was reverse transcribed using the 7cDNA oligonucleotide and SuperScript Reverse Transcriptase (Gibco-BRL). The resulting cDNA was amplified with 7cDNA and 4cDNA (5′-GTGAGTATGGATCCCTCTAAAAG-3′) oligonucleotides and *Taq* DNA polymerase (Perkin–Elmer) for 28 cycles. Full transcripts were reconstructed by PCR using 109 cDNA (5′-cggtaatacgactcactataGGGAGAGGGCGAATTCGAGCTCACTCTCTTCCGCATCGCTGTCTGCGAGGTACCCTACCAGGTGAGTATGGATCCCTCTAAAAGCGGGC-3′) and 7cDNA oligonucleo- tides. After one or two rounds of selection, RT–PCR products were cloned and sequenced using an ABI 373A automated sequencer (Perkin–Elmer). Cloning of the initial pools were also carried out to verify that the nucleotide composition was representative and random. Sequences were analyzed using the Wisconsin Sequence Analysis Package (GCG v.8.1).

Branch site mapping

Branch sites were mapped by primer extension on purified lariat intermediates. Following splicing, the lariats were gel purified on 8 M urea–16% polyacrylamide gels and mixed with 100 fmol of 5′ end-labeled PE primer (5′-CTGCAGTATGTTGTGTAG-3′).

After denaturation at 80°C for 10 min, hybridization was performed at 45°C for 30 min. Primer extension was initiated by adding 10 U of reverse transcriptase (Gibco BRL). After 60 min at 42°C the RNA was degraded by treatment with NaOH at 55°C for 60 min. The products were analyzed on 8 M urea–12% polyacrylamide gels.

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