Cell

Dynamic Regulation of Alternative Splicing by Silencers that Modulate 5′ Splice Site Competition

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DOI 10.1016/j.cell.2008.10.046

SUMMARY

Alternative splicing makes a major contribution to proteomic diversity in higher eukaryotes with ~70% of genes encoding two or more isoforms. In most cases, the molecular mechanisms responsible for splice site choice remain poorly understood. Here, we used a randomization-selection approach in vitro to identify sequence elements that could silence a proximal strong 5′ splice site located downstream of a weakened 5′ splice site. We recovered two exonic and four intronic motifs that effectively silenced the proximal 5′ splice site both in vitro and in vivo. Surprisingly, silencing was only observed in the presence of the competing upstream 5′ splice site. Biochemical evidence strongly suggests that the silencing motifs function by altering the U1 snRNP/5′ splice site complex in a manner that impairs commitment to specific splice site pairing. The data indicate that perturbations of non-rate-limiting step(s) in splicing can lead to dramatic shifts in splice site choice.

INTRODUCTION

In higher eukaryotes, the majority of pre-mRNAs are subject to alternative splicing, a process that can be regulated according to developmental stage or cell type, or in response to signal transduction pathways (reviewed in Black, 2003; Blencowe, 2006; House and Lynch, 2008). Splicing patterns can be remarkably complex, with some pre-mRNAs processed to yield dozens or even thousands of distinct isoforms (reviewed in Black and Graveley, 2006).

Intensive bioinformatic and experimental analyses have begun to identify specific sequence elements that either positively (splicing enhancers) or negatively (splicing silencers) influence splice site choice and, in many cases, specific *trans*-acting factors that recognize these elements have been characterized (reviewed in Black and Graveley, 2006; Blencowe, 2006; Wang and Burge, 2008).

With regard to silencers, there is evidence in support of several distinct mechanisms by which these elements exert their inhibitory effects. One straightforward mechanism is "bind and block" wherein a protein factor binds to a silencing element and sterically prevents the binding of a splicing factor (e.g., Kanopka et al., 1996; Mayeda and Krainer, 1992; Merendino et al., 1999; Shin et al., 2004; Ule et al., 2006; Valcarcel et al., 1993; Wagner and Garcia-Blanco, 2001; Zheng et al., 1998, Zhu et al., 2001). A second mechanism is silencer-promoted formation of nonfunctional or "dead end" complexes (e.g., Agris et al., 1989; Kan and Green, 1999; Giles and Beemon, 2005; House and Lynch, 2006; Labourier et al., 2001). Such complexes apparently contain all of the factors necessary for splicing but are unable to execute the reaction, presumably because a crucial conformation or conformational change is blocked. A third mechanism is blockage of communication between splice sites either by looping out of the affected site (e.g., Blanchette and Chabot, 1999) or by binding of a repressive complex downstream of the regulated site (e.g., Nagengast et al., 2003; Sharma et al., 2005; Sharma et al., 2008). In these cases, splice site recognition does not appear to be affected, but productive association of 5′ and 3′ splice sites is prevented by mechanisms that have not yet been elucidated. There are also numerous examples of silencers that have been shown to bind specific *trans*-acting factors; but how these proteins exert their negative effects is largely unknown (reviewed in Black and Graveley, 2006; Fu, 2004; Hastings and Krainer, 2001; Matlin et al., 2005).

Although the mechanisms of splicing silencers are beginning to be elucidated in the context of regulated exons, it is not clear whether similar elements or mechanisms are operative in the repression of "splice sites" that are never used in splicing. In this regard, it is well established that potential 5′ splice sites (including those that perfectly match the consensus recognition site for U1 snRNP) far outnumber authentic 5′ splice sites (Senapathy et al., 1990; Sun and Chasin, 2000). Furthermore, it is not clear why "pseudoexons," exon-sized sequences that are

bounded by sequences indistinguishable from functional 3′ and 5′ splice sites, are ignored by the splicing machinery (Cote et al., 2001; Sun and Chasin, 2000).

To gain further insight into potential molecular mechanisms by which splice sites are silenced, we employed a randomization-selection (SELEX) (Tuerk and Gold, 1990) strategy in vitro designed to identify all possible sequences at specific intronic and exonic positions that could silence a consensus 5′ splice site. Because the experimental design demanded that a "perfect" proximal 5′ splice site be silenced in the presence of a weakened distal 5′ splice site, we anticipated that we would recover elements that would inactivate or occlude the proximal site through formation of stable protein-RNA complexes. Focusing only on elements that conferred the strongest silencing phenotypes, we identified two exonic and four intronic motifs whose presence caused nearly complete inhibition of proximal splicing and concomitant activation of the weak upstream site. Remarkably, none of these motifs functioned by sequestering or inactivating the strong 5′ splice site. Rather, in the absence of the upstream 5′ splice site, the consensus site remained fully functional. These results demonstrate that kinetic effects on non-rate-limiting steps can elicit dramatic differences in splicing patterns and might help to explain alternative splicing phenotypes observed when the levels of many splicing factors, including basal components of the spliceosome (e.g., Karni et al., 2007; Olson et al., 2007; Park et al., 2004), are even modestly altered.

RESULTS

Identification of Intronic and Exonic Splicing Silencers Using a Randomization-Selection Approach

To identify potential splicing regulatory signals that could silence a strong 5′ splice site, we generated a synthetic pre-mRNA containing two alternative 5′ splice sites: a weak upstream 5′ splice site and a strong proximal 5′ splice site (see Figure 1 and Experimental Procedures). In in vitro splicing assays, the strong proximal 5′ splice site was used almost exclusively (Figure 1A). Importantly, the weak distal 5′ splice site remained functional because inactivation of the proximal 5′ splice site by mutation resulted in distal splicing (Figure 1A). We used this base construct to generate pre-mRNAs containing completely randomized regions either upstream or downstream of the strong 5′ splice site. The pool randomized at positions +11 to +22 relative to the proximal site was used to identify intronic silencers, whereas the pool randomized at the positions −18 to −7 was used to identify exonic silencers (see Experimental Procedures). Twelve nucleotides were chosen for randomization because this length represents at least 2-fold coverage of the binding site size (6 nt) of most RNA binding proteins (see Fairbrother et al., 2002) and the total pool ($\sim 10^7$ variants) can be easily accommodated in standard in vitro splicing reactions (3 ng RNA contain $\sim 10^{10}$ molecules of substrate). The positions for insertion of the randomized sequences were chosen so as not to overlap with the minimal binding site of U1 snRNP (−6 to +10 relative to the splice junction; P.A.M., J.A.D., and T.W.N., unpublished data; Mount et al., 1983). To evaluate the quality of each pool, in vitro transcribed RNAs were subjected to primer extension sequencing; equal distribution of all bases in the randomized regions indicated no sequence bias (data not shown; see Figure S4A [available online]). To facilitate the selection (see below), the 3′ splice site was mutated from AG/G to UC/C such that splicing was arrested after the first *trans*-esterification reaction (e.g., Reed, 1989), resulting in accumulation of lariat-3′ exon intermediates. This 3′ splice site mutation did not affect 5′ splice site choice (compare Figure 1A with 1B). When the pools were assayed for splicing in vitro, they spliced identically to the unsubstituted RNAs; that is, the proximal site was used almost exclusively, indicating that most sequences within the pool had no effect on splice site choice (Figure 1B, compare lanes 1 and 3; see Figure S4B).

To identify those sequences that could modulate use of the proximal site, we used the selection strategy outlined in Figure 1C. In brief, the pools of templates were transcribed in vitro and because we considered it likely that the most common "silencing elements" in the pool would be sequences that could base pair with the 5′ splice site and thus occlude it through formation of stable secondary structure, we introduced a step to exclude such elements prior to splicing assays. Because purified U1 snRNP binds stably to consensus 5′ splice sites (the strong site) but not to splice sites mutated at the +5+6 positions (the weak site) (P.A.M., J.A.D., and T.W.N., unpublished data), we could use U1 snRNP binding to enrich for RNA molecules in which the strong proximal 5′ splice site remained accessible. Accordingly, we mixed purified U1 snRNP (Hochleitner et al., 2005) with the starting pools and recovered bound RNAs using anti-U1A antisera (see Experimental Procedures). Recovered RNAs were then deproteinized and used as substrates for in vitro splicing in HeLa cell nuclear extract.

After splicing, the RNAs were separated on denaturing polyacrylamide gels and the region containing distal lariat intermediates was excised from the gel. Following debranching (see Experimental Procedures), the linear RNA molecules containing the proximal 5′ splice site and surrounding sequence were amplified by RT-PCR. These molecules were then used to regenerate the starting constructs by overlapping PCR (Figure 1C).

As shown in Figure 1D (lane 0), there was negligible distal splicing in the starting pools but the proportion of distal splicing intermediates rose rapidly after iterative rounds of selection; maximum accumulation of distal intermediates was reached after only four rounds of selection for the intronic position (Figure 1D, lanes 1–4) and seven rounds for the exonic position (data not shown). At this point, the populations were recovered and intact templates generated as described above. These pools of DNA were then cloned and propagated as libraries and pre-mRNAs transcribed from individual clones were analyzed for their splicing behavior. Although there was clone-to-clone variance, the majority demonstrated a pronounced preference for the distal splice site (data not shown; see Figure 2B). By this type of analysis, we recovered 106 clones containing intronic silencers and 52 clones containing exonic silencers that displayed predominant use of the distal 5′ splice site; clones that demonstrated substantive but less dramatic shifts in splice site choice were not analyzed further.

All clones that showed dramatic splicing phenotypes were sequenced. The 89 unique intronic silencers (12-mers) and the

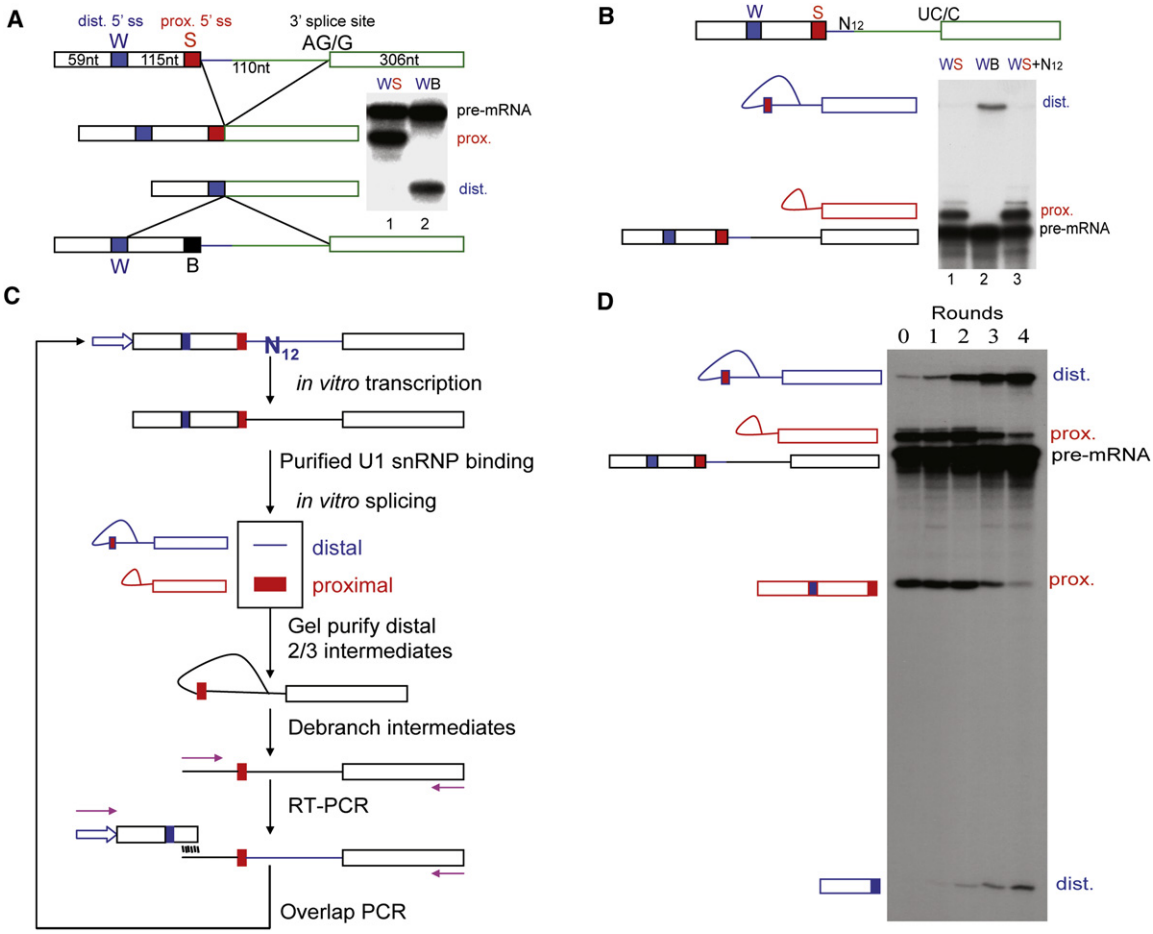


Figure 1. Functional Selection of Splicing Silencers

(A) Schematic representation of the base construct used for functional SELEX; the distal 5′ splice site (AUG/GUAAAC) is weak (W) relative to the proximal strong (S) 5′ splice site (AUG/GUAAGU). Only the proximal 5′ splice is used when this pre-mRNA is spliced in vitro (lane 1), whereas the distal site is activated when the proximal 5′ splice site is inactivated by a block mutation (AUC/CAUUCAUA, lane 2).

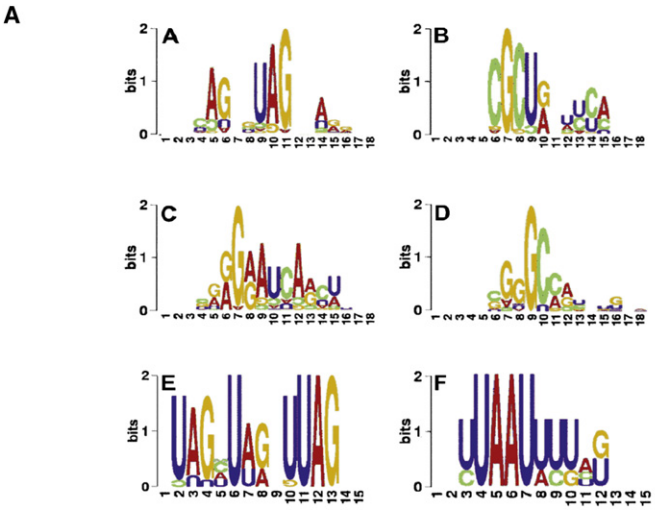
(B) In vitro splicing of the same pre-mRNA as in (A) except that the 3′ splice site was changed from AG/G to UC/C to arrest splicing after the first catalytic step. Lane 1, splicing of the pre-mRNA with a wild-type proximal 5′ splice site; lane 2, splicing when the proximal 5′ splice site was inactivated by mutation; lane 3, splicing when the proximal 5′ splice site was wild-type but a randomized 12 nt sequence was inserted downstream from positions +11 to +22 (see text).

(C) Schematic of the functional SELEX strategy; for a detailed description of individual steps, see the text and Experimental Procedures.

(D) Using the strategy illustrated in (C), the pool of body-labeled pre-mRNAs randomized at positions +11 to +22 relative to the proximal 5′ splice site were spliced in vitro and analyzed after zero (lane 0), one (lane 1), two (lane 2), three (lane 3), or four (lane 4) rounds of selection for lariat-3′ exon intermediates resulting from use of the distal 5′ splice site. The positions of splicing intermediates, free 5′ exon, and lariat-3′ exon are indicated.

47 unique exonic silencers (12-mers), together with 2 nt flanking regions, were then hierarchically clustered to extract groups of similar sequences. Four intronic motifs and two exonic motifs were identified (see Table S1 for a complete list of the sequences). The six distinct motifs are presented as logos in Figure 2A and the splicing behavior of a specific representative of each class is shown in Figure 2B. Importantly, none of the silencing motifs markedly reduced overall splicing efficiency because a reciprocal relationship was observed between the reduction in proximal splicing and enhancement of distal splicing.

To assess the general significance of the selected silencer elements to splicing of human pre-mRNAs, they were compared with three sets of previously characterized splicing silencer motifs (Wang et al., 2004, 2006; Zhang et al., 2003; Zhang and Chasin, 2004). In particular, motifs A, C, E, and F, but not B or D, showed highly significant similarities (for details, see Figure S1A and Table S2). The potential relevance of the silencer motifs was then examined further by determining their occurrence on a genome-wide scale. We found that intronic motifs A and C are highly enriched downstream of pseudo 5′ splice sites relative to constitutive 5′ splice sites ($p < 10^{-121}$ and $p < 10^{-74}$); the exonic motifs E and F were 3 to 4 times more abundant in pseudoexons compared with real exons ($p < 10^{-7}$, for details, see Figure S1B). These results are consistent with a role for several of the identified motifs in repressing pseudo 5′ splice sites.



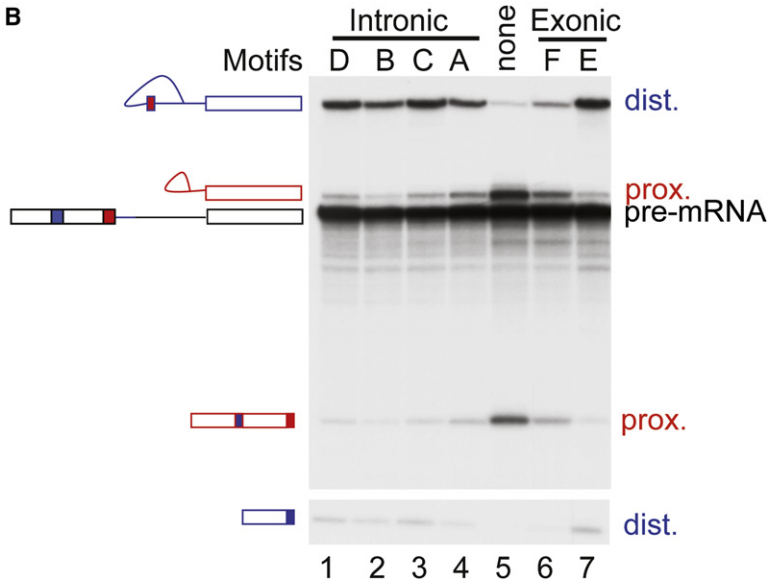


Figure 2. Splicing Silencers Identified via Functional SELEX

(A) The 106 intronic and 52 exonic sequences that demonstrated strong silencing activity (see text) were grouped into six classes (A, B, C, D intronic; E, F exonic) based on hierarchical clustering for sequence similarity and are presented as logos created using RNA structure logo (Gorodkin et al., 1997; Schneider and Stephens, 1990). All individual unique sequences, including those that did not fall into the six classes, are listed in Table S1 with repeated sequences deleted.

(B) Pre-mRNAs, each containing a specific representative of one of the six classes (motif D, GGGCCACTTGGA, lane 1; motif B, CGCTGGTCATTC, lane 2; motif C, GAGGATCA GCTT, lane 3; motif A, CGTTAGAGTAGC, lane 4; motif F, CTT AATTTTAGT, lane 6; motif E, TAGTTTAGTTAG, lane 7) of silencer was spliced in vitro. Lane 5 is a splicing reaction with a pre-mRNA that does not contain a splicing silencer. The positions of splicing intermediates (free 5′ exon and lariat-3′ exon) are indicated.

Splicing Silencers Identified In Vitro Function in Intact Cells

Because the informatic analyses indicated that the silencing elements identified in vitro were highly likely to be relevant in vivo, specific representatives of each class, in the same context as that assayed in vitro, were introduced into a mammalian expression vector and transfected into HeLa cells; the functional 3′ splice site (AG/G) was used in vivo. As shown in Figure 3A, the splicing behavior of the base construct was identical to that observed in vitro; that is, nearly exclusive use of the proximal strong 5′ splice site in the wild-type construct (Figure 3A, lane 5) and exclusive use of the distal site when the proximal site was inactivated by mutation (Figure 3A, lane 8). Importantly, all of the silencers increased distal splicing (Figure 3A, lanes 1–4, 6, 7). The most dramatic effect was observed with exonic motif E (Figure 3A, lane 7); the in vivo splicing phenotypes of exonic motif F and the four intronic elements were more modest than those observed in vitro. In addition, random sequences other than the selected silencers were tested and they did not alter the 5′ splice site choice (Figure S4B).

Although reduction in the magnitude of regulation in vivo could result from several potential variables, we hypothesized that the preference for use of the proximal splice site might be stronger in vivo than in vitro. In this case, weakening of the proximal site would be predicted to maintain the preferential use of the proximal site and might allow more effective silencing. Indeed, when we weakened the proximal site to make it identical to the distal site, proximal splicing predominated (Figure 3C, lane 5). Strikingly, the potency of all of the silencing elements was greatly enhanced under these conditions (Figure 3C, lanes 1–4, 6, 7); all now caused an almost complete shift to distal splicing.

If the silencing elements functioned by occluding or otherwise inactivating the affected 5′ splice site, it would be predicted that overall splicing would be drastically impaired when the elements were present at both 5′ splice sites in the dual splice site construct. However, this expected behavior was not observed. Remarkably, when the silencing elements were inserted at both 5′ splice sites, the wild-type pattern of splicing was restored (Figures 3B and 3D). Importantly, there was no substantive reduction in the overall extent of splicing; quantitation showed that the overall splicing efficiency of the dual silencer constructs ranged from 83% (motif E) to 100% (motif C) of that measured for the construct lacking silencer elements.

We then asked if similar patterns could be observed in vitro. As shown in Figure 3E, this was indeed the case; in the absence of silencing elements, there was exclusive use of the proximal site, and when silencing elements were placed at both sites the wild-type pattern was restored. As was the case in vivo, the presence

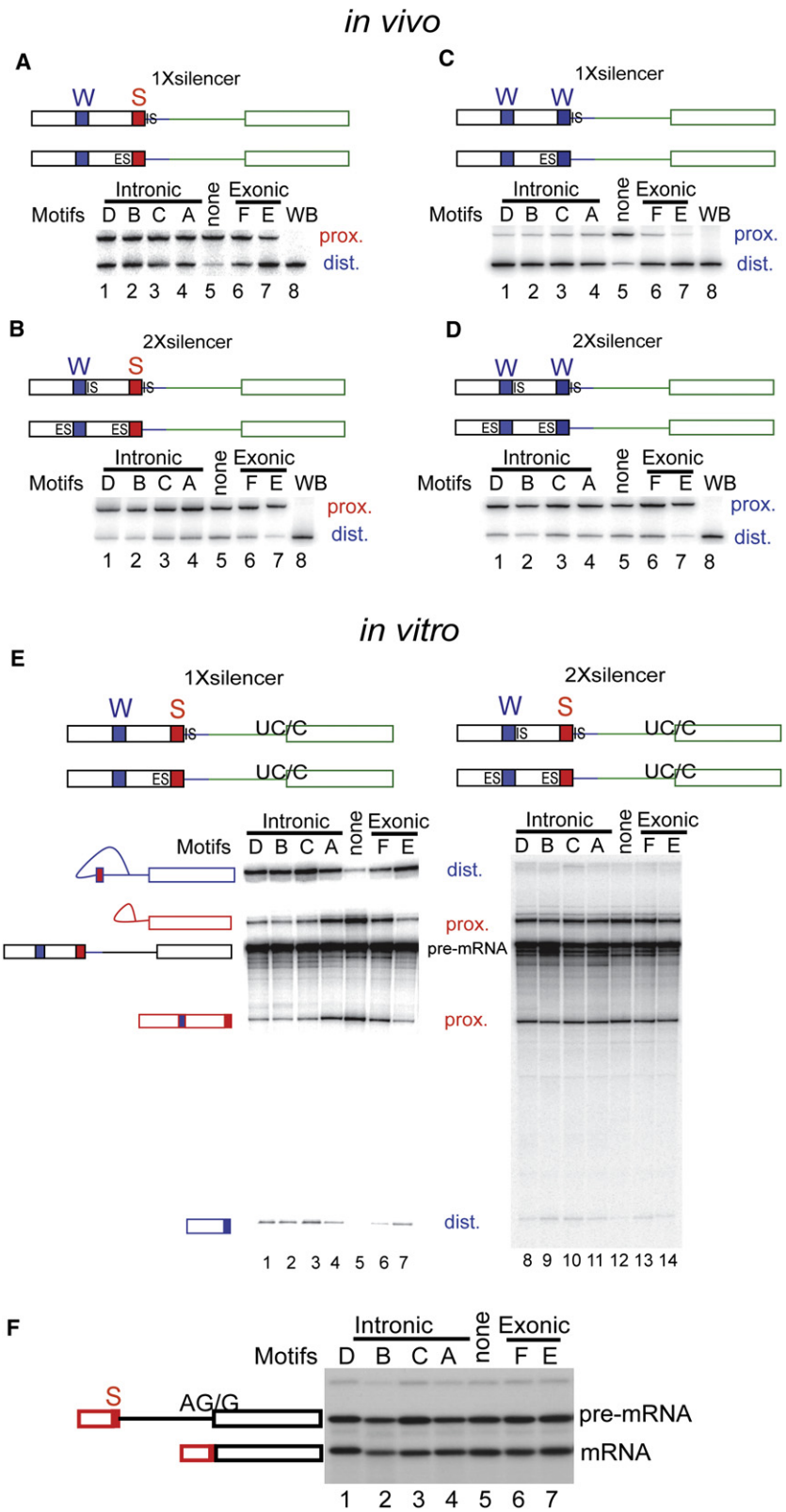


Figure 3. Effects of Silencing Elements Both In Vivo and In Vitro

(A) HeLa cells were transfected with plasmids expressing the same pre-mRNAs as those assayed in Figure 2 except that the 3′ splice site was wild-type. Splicing was assayed by semiquantitative RT-PCR; products generated from use of the proximal or distal 5′ splice sites are indicated. Lane 1, motif D; lane 2, motif B; lane 3, motif C; lane 4, motif A; lane 5, no silencer; lane 6, motif F; lane 7, motif E; lane 8, pre-mRNA lacking a silencer in which the proximal 5′ splice site was inactivated by mutation as in Figure 1A.

(B) In vivo splicing of pre-mRNAs identical to those in (A) except that the silencing motif was inserted at positions +11 to +22 (intronic) or −18 to −7 (exonic) relative to the distal 5′ splice site as appropriate; thus each silencing motif was present twice in each pre-mRNA at the same position relative to both the proximal and distal 5′ splice sites. Lane designations are as in (A).

(C) In vivo splicing of pre-mRNAs with duplicated weakened 5′ splice sites (see text). As in (A) each pre-mRNA contained one specific silencing motif upstream or downstream of the proximal 5′ splice site. Lane designations are as in (A).

(D) In vivo splicing of pre-mRNAs with duplicated weakened 5′ splice sites with duplicated silencing motifs. As in (B), the specific silencer motifs were inserted upstream or downstream as appropriate of the distal 5′ splice site when the same motif was upstream or downstream of the proximal 5′ splice site. Lane designations are as in (A).

(E) Body-labeled dual 5′ splice site pre-mRNAs identical to those assayed for splicing in vivo in (A) and (B) except that the 3′ splice site was inactivated by mutation, were spliced in vitro. Each pre-mRNA contained the specific silencing motifs described in the legend to Figure 2 either upstream or downstream of the proximal 5′ splice site. Lane 1, motif D; lane 2, motif B; lane 3, motif C; lane 4, motif A; lane 5, no silencer motif; lane 6, motif F; lane 7, motif E. For lanes 8–14, specific silencer motifs were inserted either upstream or downstream of the distal 5′ splice site as appropriate such that each pre-mRNA contained identical silencer motifs at the same position relative to both 5′ splice sites. Lane 8, motifs D; lane 9, motifs B; lane 10, motifs C; lane 11, motifs A; lane 12, no silencers; lane 13, motifs F; lane 14, motifs E.

(F) In vitro splicing of pre-mRNAs lacking the distal 5′ splice site in the presence or absence of silencing elements. The same pre-mRNAs as in (E), lanes 1–7, lacking the distal 5′ splice site with a wild-type 3′ splice site and with the specific silencer elements present upstream or downstream of the remaining 5′ splice site were spliced in vitro. Lane 1, motif D; lane 2, motif B; lane 3, motif A; lane 5, no silencer; lane 6, motif F; lane 7, motif E.

of silencing elements at both sites did not markedly affect the overall level of splicing; quantitation showed that the overall splicing efficiency of the dual silencer constructs ranged from 70% (motif E) to 92% (motif C) of that measured for the pre-mRNA lacking silencer elements. An important and unexpected conclusion that emerged from both the in vivo and in vitro experiments was that the silencers did not function by preventing use of the affected site, but rather changed in some way the ability of the affected site to compete with the unsilenced site (see Discussion).

The results described above predicted that the silencing elements would have little if any effect on splicing in single 5′ splice site constructs (i.e., in the absence of a competing 5′ splice site). As shown in Figure 3F, this prediction was borne out by experiment; when the upstream 5′ splice was removed, none of the silencing elements had a pronounced effect on the accumulation of spliced product.

Because the in vivo results indicated that the silencer elements were more potent when the affected 5′ splice site was weakened (see Figure 3C), it was of interest to determine if this increased activity would translate into observable kinetic effects on splicing rate. Accordingly, we carried out time courses of splicing using single 5′ splice site constructs where the site was strong or weak with or without each of the silencing elements; we also measured the rate of splicing of each of these constructs in the presence of a strong 3′ splice site or when the 3′ splice site was weakened by purine substitutions into the polypyrimidine tract (Tian and Maniatis, 1994) (Figure 4A). Figure 4B shows the results of the 60 min time point for the panel of constructs with two of the silencing elements (motifs D and E); Figures 4C–4F show the time course of splicing for all of the constructs, and Figure 4G summarizes the data obtained at the 40 min time points.

The data reveal several interesting points. First, there were perceptible but subtle decreases in splicing rate in the presence of any of silencing elements when both splice sites were strong (Figure 4C); essentially the same kinetics were observed when only the 3′ splice site was weakened (Figure 4D). Second, when the 5′ splice site was weakened in the presence of the strong 3′ splice site, there was a marked decrease in splicing rate observed with four of the silencing elements (D, B, A, and E); this effect was not observed with motifs C and F (Figure 4E). Third, weakening of the 3′ splice site amplified the kinetic effects of the four intronic silencing elements, but had little if any effect on the two exonic silencers (Figure 4F). Because recognition of the weakened 3′ splice site relies on participation of the 5′ splice site (e.g., Barabino et al., 1990), the simplest explanation for these results is that the intronic silencers make a weak 5′ splice site less able to facilitate loading of factors at the 3′ splice site. Taken as a whole, the kinetic data strongly suggest that the silencing elements exert their effects through modulation of the affected 5′ splice site and when that site is strong, the effects on splicing are essentially invisible.

Mechanism of Silencer Function

Because recognition at the 5′ splice site by U1 snRNP is almost surely the first step required for splicing in general (e.g., Grabowski et al., 1985; Jamison et al., 1992; Seraphin and Rosbash, 1989), it seemed unlikely that the silencing elements could function by affecting this step of the reaction (Reed and Maniatis, 1986). Indeed, when we measured U1 snRNP occupancy of the strong 5′ splice site by psoralen crosslinking or immunoprecipitation with anti-U1A antisera, we did not observe any differences between RNAs lacking or containing any of the silencer elements (data not shown). Although these approaches are useful for measuring the extent of U1 snRNP occupancy, they are not informative regarding potential differences in the manner in which U1 snRNP engages the affected 5′ splice site. To address this issue, we examined 5′ splice site recognition in the presence or absence of silencing elements using a nuclease protection assay of RNAs containing a single labeled phosphate 5′ of the uridine in the Gp*U at the 5′ splice site (Figure 5A and Maroney et al., 2000b). We have shown previously (Maroney et al., 2000a) that the binding of U1 snRNP to the strong 5′ splice site in the absence of any known splicing control element produces a characteristic pattern of nuclease resistant fragments (Figure 5B, none). Essentially identical patterns of protection were observed in the presence of silencing elements C and F (Figure 5B, motifs C and F) indicating that these motifs did not markedly perturb U1 snRNP binding, at least as judged by this assay. However, strikingly distinct patterns of protection were observed in the presence of silencing elements A, B, D, and E. All of the protections both in the control RNA and present in RNAs containing silencers B, C, D, and F were strictly dependent upon U1 snRNP binding, because no protected fragments were observed when the 5′ end of U1 snRNA was blocked with a complementary 2′ Ome oligonucleotide. Extensive analyses, including RNA affinity approaches, have failed to reveal any candidate proteins that bind to these silencing elements (data not shown); observations that suggested the possibility that the U1 snRNP/5′ splice site interaction itself might be altered. Figure 5C provides direct evidence that this is the case for silencing element D; the distinctive pattern of protected fragments observed in nuclear extract was closely recapitulated using highly purified U1 snRNP (Hochleitner et al., 2005) (Figure 5C). The fact that the control RNA and two of the silencing elements (motifs C and F) yield "wild type" patterns of protection clearly indicates that not all sequences elicit distinct nuclease resistant species and highlights the functional significance of those that do (see Discussion).

Intronic silencer A and exonic silencer E also showed altered patterns in the nuclease protection assay (Figure 5B, motifs A and E, lanes +−), but with these RNAs we observed accumulations of U1 snRNP-independent fragments (Figure 5B, motifs A and E, lane ++). Both of these silencers contain UAG motifs, characteristic of potential hnRNP A1 binding sites (e.g., Burd and Dreyfuss, 1994) and RNA affinity purification indicated that RNAs containing either silencer bound hnRNP A1 (Figure S5A and data not shown). Further analyses were conducted with exonic silencer motif E. Figures 6A and 6B show that a GST-hnRNP A1 fusion protein (Blanchette and Chabot, 1999) specifically pulled down U1 snRNP only when motif E was present and only when U1 snRNP was allowed to bind to the 5′ splice site. Because the fusion protein specifically crosslinks to an RNA containing element E (Figure 6C), it was possible to perform the reciprocal pull down. Figure 6D shows that the crosslinked

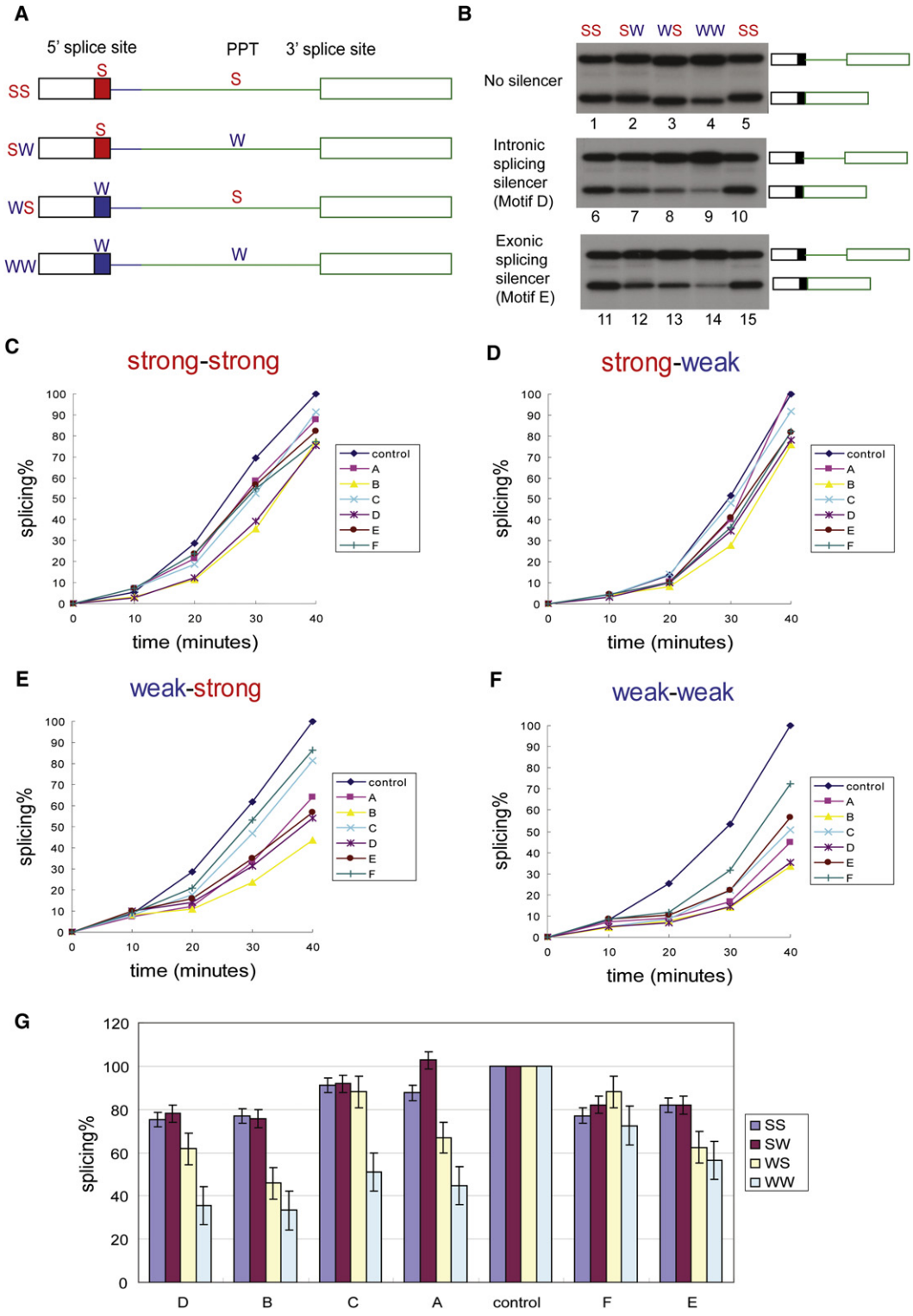


Figure 4. Individual Silencer Motifs Have Distinct Kinetic Effects on Splicing of Single 5′ Splice Site Pre-mRNAs

(A) Schematic representation of pre-mRNAs used to measure kinetic effects on splicing of individual silencer motifs. The strong and weak 5′ splice sites are the same as in Figure 1. The strong 3′ splice site construct has an unaltered polypyrimidine tract whereas the weak 3′ splice site contains purine substitutions in the polypyrimidine tract (Tian and Maniatis, 1994). For each panel of four constructs, individual silencing motifs as described in the legend to Figure 2 were inserted upstream or downstream of the 5′ splice site as appropriate.

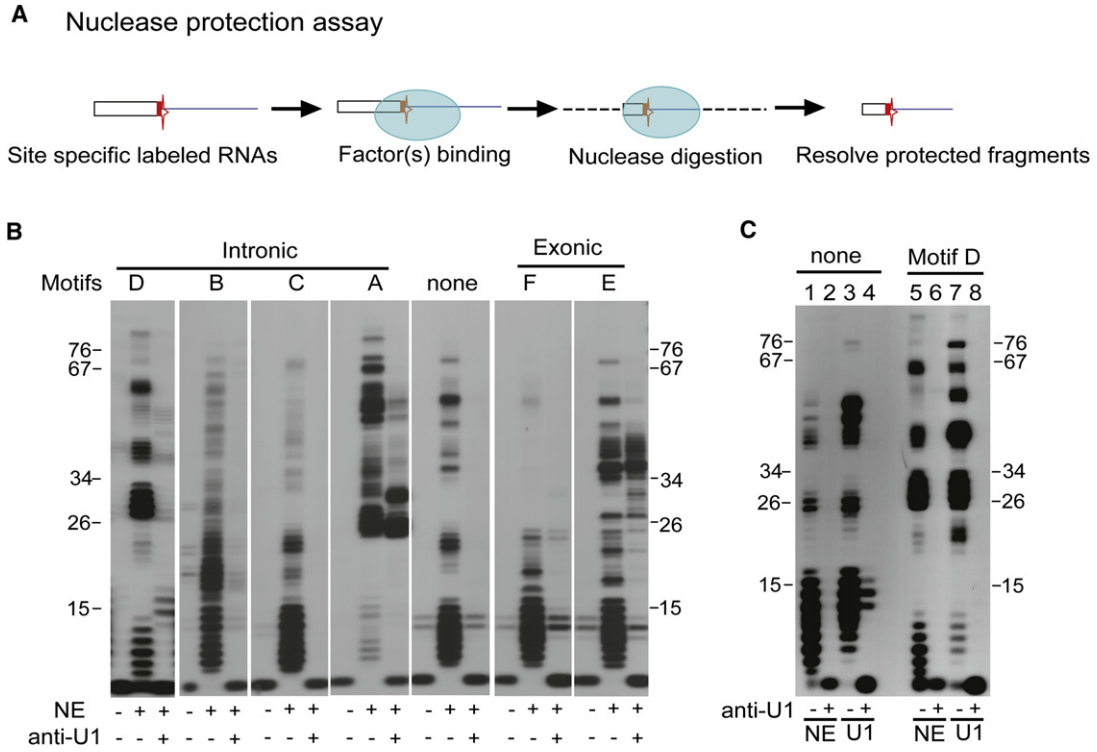


Figure 5. Nuclease Protection Analyses Reveal that Some Silencing Motifs Alter the U1 snRNP/5′ Splice Site Complex

(A) The nuclease protection experimental strategy is shown schematically. RNA molecules containing a uniquely labeled phosphate are incubated with protein(s). After binding, the RNAs are digested with micrococcal nuclease and nuclease-resistant fragments are visualized after fractionation on denaturing gels (Maroney et al., 2000b).

(B) RNA transcripts spanning −45 to +45 relative to the 5′ splice site were site specifically labeled at the phosphate between the G*U of the 5′ splice site. Each transcript contained a specific silencing motif as indicated or lacked any motif (lanes none). After incubation in HeLa cell nuclear extract, reactions were diluted and digested with micrococcal nuclease (see Experimental Procedures). Following deproteinization, resistant fragments were visualized after gel fractionation. NE is nuclear extract and anti-U1 is a 2′Ome oligonucleotide which hybridizes to the 5′ end of U1 snRNA. The positions of markers of known size are indicated.

(C) Nuclease protection of control or motif D containing RNAs using purified U1 snRNP. The site specifically labeled RNAs in (B) corresponding to none and motif D were either incubated in nuclear extract, NE (lanes 1, 2 and 5, 6) or with purified U1 snRNP (lanes 3, 4 and 7, 8) in the absence (lanes 1, 3 and 5, 7) or presence (lanes 2, 4 and 6, 8) of the anti-U1 2′Ome oligonucleotide. Following incubation, reactions were processed as in (B) with some modifications (see Supplemental Data).

protein was immunoprecipitated by anti-U1A antisera but not by control antisera. These results provide direct evidence that U1 snRNP and hnRNP A1 bind to the same RNA molecules.

DISCUSSION

Here, we have employed a functional SELEX strategy in vitro to identify intronic and exonic sequence elements that silence nearby 5′ splice sites. Importantly, the silencers we characterized function both in vitro and in vivo. The data support several conclusions. First, effective silencing can be achieved without occluding or inactivating the affected site. Second, two of the six motifs (B and D) recovered from the selection might function idiosyncratically because they are not enriched in or near pseudoexons compared with real exons, yet still act as silencers in the construct used for selection. This result suggests the possibility that many splicing control elements are relevant only in their specific pre-mRNAs and thus invisible to informatic techniques that rely on statistical analyses of motif frequencies. Third, informatic analyses indicate that four of the identified motifs (A, C, E, and F) play a role in suppressing the use of both pseudo 5′ splice sites and pseudoexons (see Supplemental Data). Fourth, the results strongly suggest that intrinsic features of RNA sequences (e.g., motif D and perhaps motifs B, C, and F) themselves

(B) In vitro splicing of the panel of four constructs either lacking any silencer element (no silencer, lanes 1–5) or containing either intronic silencing motif D (lanes 6–10) or exonic silencing motif E (lanes 11–15). Autoradiograms of the 60 min time points of each reaction are shown.

(C–F) Time courses of splicing of the indicated pre-mRNAs containing or lacking the indicated silencing motif. Each data point is the average of three independent experiments. The percentage of splicing of indicated pre-mRNAs lacking any silencer motif was set to 100%. Values were determined by quantitating the extent of splicing [product/(product + precursor)] for each reaction and are expressed as percent of the extent of splicing of a pre-mRNA lacking any silencing elements.

(G) Quantitation of the extent of splicing at 40 min time points from three independent time courses for each panel of pre-mRNAs containing the indicated silencing motif. Values expressed as percent were calculated as in (C)–(F). Error bars represent standard deviations of quantified values.

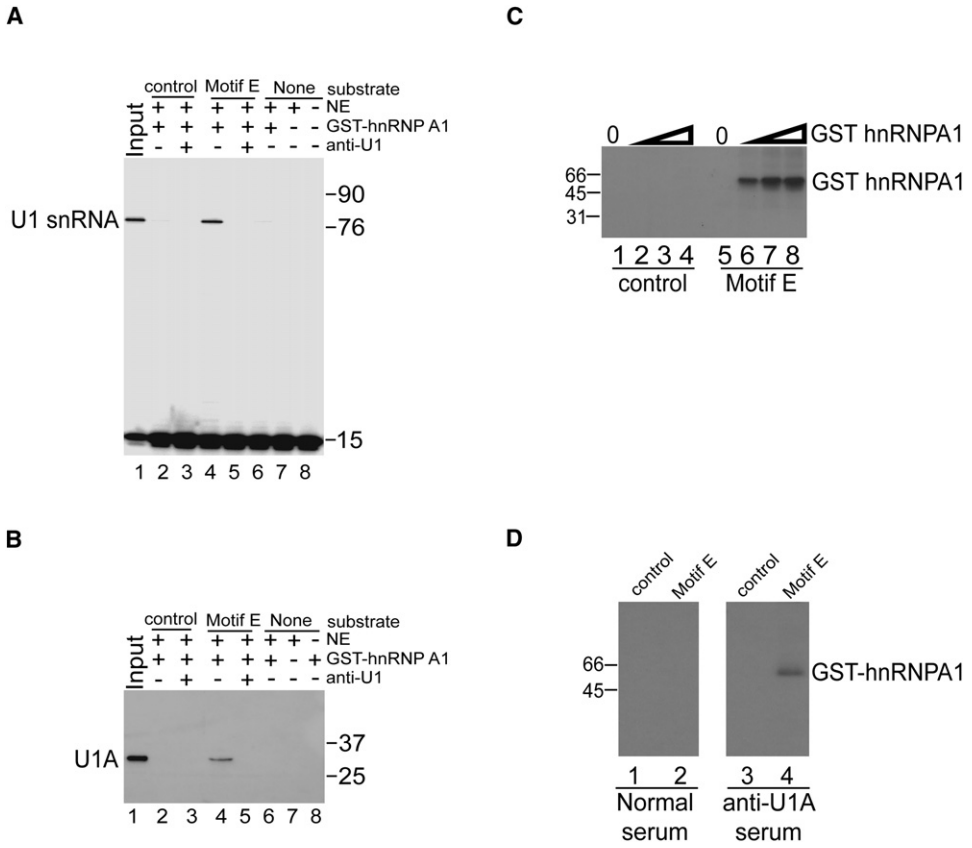


Figure 6. U1 snRNP and hnRNP A1 Bind Simultaneously to the 5′ Splice Site Region in the Presence of Silencer Motif E

(A) GST-hnRNP A1 pull down of U1 snRNA only in the presence of silencer motif E. Unlabeled RNA transcripts composed of regions −45 to +45 relative to the 5′ splice site either lacking a silencing motif (lanes 2, 3) or containing silencing motif E (lanes 4, 5) were incubated in HeLa cell nuclear extract (lanes 2–7) supplemented with 500 ng GST-hnRNP A1 fusion protein (Blanchette and Chabot, 1999) as indicated (lanes 2–6) either in the absence (lanes 2, 4, 6, 7, 8) or presence (lanes 3, 5) of a 2′Ome oligonucleotide complementary to the 5′ end of U1 snRNA. In lanes 6, 7, and 8, no RNA was added. Following incubation, reactions were diluted then bound and eluted from glutathione beads. The eluates were deproteinized and primer extension was performed (Takacs, et al., 1988) with an oligonucleotide complementary to bases 62 to 78 of U1 snRNA. Input (lane 1) denotes a primer extension reaction on RNA extracted from 25% of an aliquot of nuclear extract equivalent to that used in lanes 2–8.

(B) Exactly as in (A) except that after elution from the glutathione beads, proteins were fractionated on a denaturing polyacrylamide gel, blotted, and probed with anti-U1A antibody.

(C) The same transcripts described in (A), either lacking a silencer (control, lanes 1, 2, 3, 4) or containing exonic silencer motif E, were site-specifically labeled at position −14 relative to the splice site. Transcripts were then incubated with nuclear extract (GST-hnRNP A1, lanes 1 and 5) or presupplemented with increasing amounts of recombinant GST-hnRNP A1 proteins (lane 2, 3, 4 and 6, 7, 8) and UV crosslinked to detect interacting proteins. Proteins were selected with glutathione beads, digested with RNases, and resolved by SDS-PAGE and visualized by autoradiography (see Figure S5B for the total input proteins before selection). The amount of GST-hnRNP A1 added: 125 ng (lanes 2,6), 250 ng (lanes 3, 7), 500 ng (lanes 4, 8).

(D) Aliquots of the UV cross-linking reactions (lanes 4 and 8 from Figure 7C) were immunoprecipitated with either anti-U1A antiserum or normal rabbit serum. Bound complexes were then digested with RNases, passed through glutathione sepharose, and eluted with reduced glutathione before fractionation by SDS-PAGE. Labeled proteins were visualized by autoradiography.

(without the participation of ancillary proteins) can influence the ability of 5′ splice sites to compete with each other. Fifth, we demonstrate that silencers can alter the U1 snRNP/5′ splice site complex in a manner that renders the affected site to be at a competitive disadvantage with respect to 5′ splice sites occupied by an "unaltered" U1 snRNP/5′ splice site complex (Figure 7). Sixth, and perhaps most importantly, we show that effects on non-rate-limiting kinetic steps in splicing can lead to dramatic shifts in splice site choice.

In our experimental design, we focused on one construct under one set of conditions in extracts prepared from one cell type and analyzed only those motifs that gave the strongest phenotypes. It is important to note that we ignored sequences that produced substantive but less dramatic splice site switching. Although we do not know how many sequence motifs would yield intermediate phenotypes, it seems likely that it is much larger than six. Furthermore, only one intronic and one exonic position were analyzed. It will be of interest to determine which sequences would emerge if the selections were carried out with the randomized sequence inserted at different positions or in different contexts. It will also be of interest to determine if sequences that could overcome the silencing effects could be

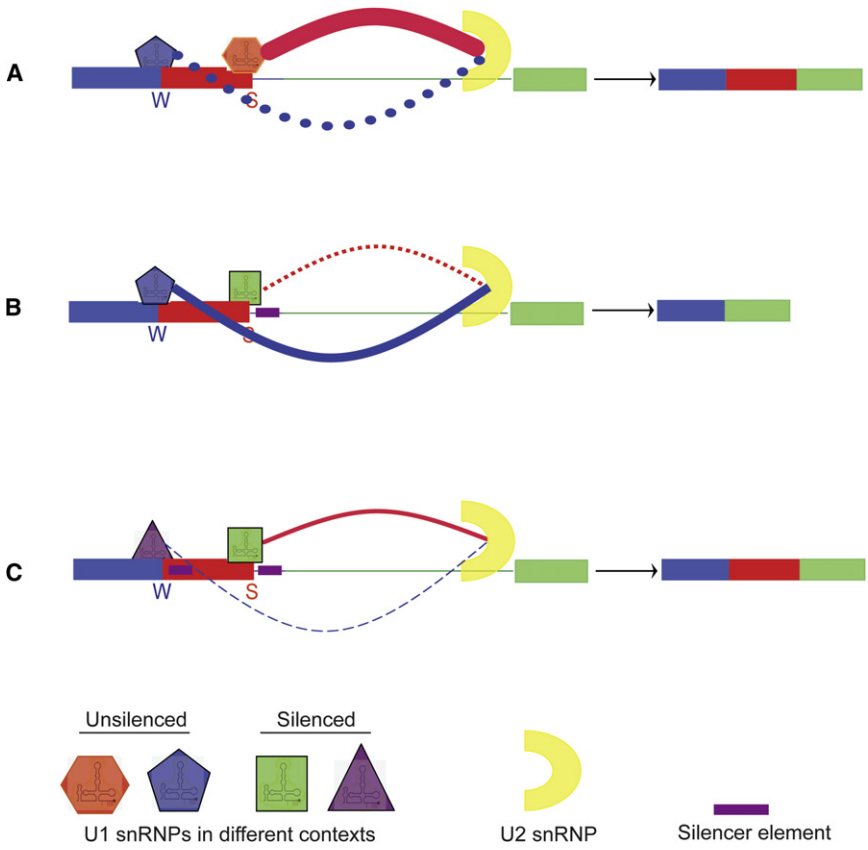


Figure 7. Model for Dynamic Regulation of Splice Site Choice by Splicing Silencers

(A) U1 snRNP bound to the proximal strong 5′ splice site (hexagon) adopts a conformation that can efficiently engage U2 snRNP. In this situation, U1 snRNP bound to the strong site outcompetes U1 snRNP bound at the weak site (pentagon) resulting in the use of the proximal site. Relative efficiencies of engagement of U1 and U2 snRNPs are depicted schematically by the thickness of the lines connecting them; the line from the unused site is dashed.

(B) In the presence of a splicing silencer adjacent to the proximal site, the interaction of U1 snRNP (square) is altered such that it less efficiently interacts with U2 snRNP. Accordingly, use of the distal site is observed. When both splice sites are "silenced" simultaneously (C), the proximal site regains its competitive advantage; in both illustrations, the line from the unused site is dashed.

selected in silencer containing constructs. Despite the inherent limitations of our current study, the results suggest that the regulation of splicing might be remarkably subtle and the "splicing code" (e.g., Black, 2003; Fu, 2004; Hertel, 2008; Matlin et al., 2005) correspondingly complex.

Intuitively, it would be expected that the most effective splicing silencers would sequester or otherwise inactivate the affected splice site. The silencers we have selected clearly do not function in this manner because the affected site remains fully functional but is not used when a competing site is present. When interpreted from a kinetic perspective, our observations rule out any scenario in which the silencers affect a slow or rate-limiting step. If this were the case, presence of the silencers would cause reductions in the ability of the affected site to function proportional to the extent of activation of the competing site.

To account for the observed data, the silencers must affect a fast kinetic step to allow action of the silencer without substantive effects on the overall reaction rate. The fast reaction step has to be more than one order of magnitude faster than the slow reaction step when slowed by the silencer, and, consequently, several orders of magnitude faster than the rate-limiting step when unaffected by the silencer. Introduction of the silencers at both splice sites would slow the fast reaction step at both sites and re-establish the original pattern of splice site selection, as we have observed (see Figures 3 and 7).

We suggest that such a fast step could be the joining of 5′ and 3′ splice sites in a complex committed to splice site choice. This interpretation is consistent with the findings of Lim and Hertel (2004) who showed that commitment to the general splicing pathway and commitment to specific splice site pairing are kinetically separable. Specifically, they demonstrated that commitment to splice site pairing became irreversible only upon formation of A complex, when U2 snRNP is locked onto the pre-mRNA. We suggest that the silencers function by altering the way in which U1 snRNP engages the 5′ splice site in such a way that it is less able to efficiently engage U2 snRNP, and thus the unaffected site obtains a competitive advantage. This interpretation is consistent with our nuclease protection data (see Figure 5) and with recent studies using site-directed hydroxyl-radical footprinting (Dönmez et al., 2007) that demonstrated U1 and U2 snRNPs are in close proximity in early spliceosomal complexes. These studies suggested that the two snRNPs engage in a direct spatially fixed interaction wherein the 5′ end of U2 snRNP is located on a specific "side" of U1 snRNP. Any distortion or misorientation of U1 snRNP caused by a silencer could negatively affect its ability to establish proper contact with U2 snRNP. The notion that appropriate alignment of U1 and U2 snRNPs early in spliceosome assembly is crucial for commitment to splice site choice provides a possible explanation for the observation that some of the silencers affect "weak" 5′ splice sites more than "strong" ones. We suggest that U1 snRNP bound to "weak" sites (i.e., those with fewer base pairs) are already somewhat "misaligned" and thus more susceptible to further distortion by the silencers.

In addition, it seems likely that hnRNP A1 might exert its silencing function in a similar manner; that is, by affecting the alignment of U1 and U2 snRNPs. There have been several distinct models proposed for the mechanism by which hnRNP A1 can silence (reviewed in Black, 2003; Black and Graveley, 2006; Matlin et al., 2005). Although all of these mechanisms might be valid in certain contexts, our finding that the protein binds simultaneously with U1 snRNP suggests that it might

directly interfere with the ability of U1 snRNP to interact correctly with U2 snRNP, either by altering the conformation of the U1 snRNP particle or perhaps by shielding important surfaces on the snRNP necessary for establishing correct alignment with U2 snRNP.

In summary, we suspect that a large fraction of examples of alternative and regulated splicing events will be dictated by kinetic parameters similar to the ones we have described. In this regard, there are several examples where the function of splicing control elements is only evident in the presence of competing splice sites (e.g., Cheah et al., 2007; Lam et al., 2003; Reed and Maniatis, 1986) and additional poorly understood examples where sequence context determines the use of competing sites (e.g., Chen and Helfman, 1999; Haj Khelil et al., 2008; Krawczak et al., 2007; Manabe et al., 2007; Mayeda and Ohshima, 1988; Nelson and Green, 1988; O'Neill et al., 1998, Ule et al., 2006).

In any multi-intronic pre-mRNA, each internal 5′ splice site is in competition with multiple 5′ splice sites as is each 3′ splice site. Myriad cross-exon and cross-intron interactions ensure that correct sites are paired. However, it is not hard to imagine that minor perturbations of any of these interactions (e.g., by tissue-specific or developmental-stage-specific changes in the levels of splicing factors [Karni et al., 2007; Olson et al., 2007; Park et al., 2004]), rates of transcription, and/or chromatin structure (reviewed in House and Lynch, 2008; Kornblihtt, 2006; Maniatis and Reed, 2002) could alter a delicate kinetic balance and result in the very complex patterns of splice site choice that are observed. Such a view makes sense from an evolutionary perspective in that small advantageous changes in intronic or exonic sequence could rapidly lead to the expansion of proteomic diversity seen in higher eukaryotes.

EXPERIMENTAL PROCEDURES

Pre-mRNA Substrates and In Vitro Splicing

The construction of pre-mRNA templates containing duplicated 5′ splice sites comprised of portions of the rat preprotachykinin and *Drosophila* doubesex gene is described in detail in the Supplemental Data, as is the introduction of fully randomized regions either +11 to +22 or −18 to −7 relative to the proximal 5′ splice site. All in vitro splicing reactions were conducted with body labeled substrate for 60 min in HeLa cell nuclear extract (Dignam et al., 1983) as described elsewhere (Maroney et al., 2000a) except where indicated differently in the text or figure legends. For the functional SELEX studies, the entire randomized pools of templates were transcribed; full-length transcripts were gel purified and bound to purified U1 snRNP in NET-2 buffer. After immunoprecipitation with polyclonal anti-U1A antibody (Kambach and Mattaj, 1992), pre-mRNAs were deproteinized and added to in vitro splicing reactions. For debranching, lariat-3′ exon intermediates were gel purified and incubated with HeLa cell cytoplasmic S100 extract under splicing conditions for 30 min at 30°C as described previously (Ruskin and Green, 1985).

In Vivo Splicing Analysis

Full-length DNA fragments were cloned into pcDNA3.1 (Invitrogen) under the control of the CMV promoter and transfected into HeLa cells using Lipofectamine2000 (Invitrogen) according to the manufacturer's protocol. Twenty-four hours after transfection, the extent and position of splicing was determined by semiquantitative RT-PCR as described in the Supplemental Data.

Nuclease Protection Assays

RNA fragments spanning the region from −45 to + 45 relative to the proximal 5′ splice site containing each of the individual silencer motifs or lacking any silencer were uniquely labeled at G*U of the 5′ splice site. Site-specific labeling, oligonucleotide inhibition, and nuclease protection assays followed the procedures described by Maroney et al. (2000a, 2000b). For details, see the Supplemental Data.

GST-hnRNP A1 Pull Downs and U1 snRNP Immunoprecipitation

Incubations containing nuclear extract either with or without added GST-hnRNP A1 as indicated in the figure legends were mixed with prewashed glutathione sepharose 4B (Amersham Biosciences) in NET-2 for 1 hr at 4°C. The bound complexes were washed three times, eluted with reduced glutathione (50 mM glutathione in 50 mM Tris-HCl [pH 8.0]), and subjected to further analysis.

For the analysis of cross-linking to site-specifically labeled RNAs, reactions were irradiated with 254 nm UV light (see Supplemental Data) and then selected with glutathione sepharose as described above. The eluant was digested with 2 μg RNase A at 37°C for 30 min before proteins were fractionated on 10% polyacrylamide SDS gels.

For immunoprecipitation of cross-linked proteins, incubations, treated as above with UV light, were mixed with polyclonal anti-U1A antibody (Kambach and Mattaj, 1992) or control antibody, prebound to protein A agarose beads in NET-2 buffer supplemented with RNasin (Promega) for 1 hr at 4°C. The bound complexes were washed three times, digested with RNase A, and then selected on glutathione sepharose. Proteins were eluted from the beads at 95°C with SDS running buffer. Isolated proteins were then resolved via SDS-PAGE and visualized by autoradiography.

SUPPLEMENTAL DATA

Supplemental Data include five figures and two tables and can be found with this article online at http://www.cell.com/supplemental/S0092-8674(08)01384-6.

ACKNOWLEDGMENTS

We thank J. Bruzik, B. Chabot, and P. Grabowski for providing constructs, and S. Gunderson and I. Mattai for anti-U1A antiserum. We also thank D. Black, C. Burge, M. Caprara, X.D. Fu, B. Graveley, K. Hertel, B. Konforti, A. Krainer, K. Lynch, J. Manley, D. Rio, and J. Steitz as well as the anonymous reviewers for constructive comments, and A. M. Micenmacher for preparing the manuscript. The research was supported by a grant from the Sixth Framework Program of the European Union via The Network of Excellence "EURASNET" to R.L. and NIH grants to E.J., L.A.C., and T.W.N.

Received: April 11, 2008
Revised: August 20, 2008
Accepted: October 29, 2008
Published: December 24, 2008

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