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A systematic analysis of the factors that determine the strength of pre-mRNA splicing enhancers

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We find that the strength of splicing enhancers is determined by the relative activities of the bound serine–arginine (SR)-rich splicing factors, the number of SR proteins within the enhancer complex and the distance between the enhancer and the intron. Remarkably, the splicing activity of the bound SR proteins is directly proportional to the number of RS tetrapeptide sequences within the RS domain. Quantitative analysis of the effects of varying the distance between the enhancer and the intron revealed that the splicing efficiency is directly proportional to the calculated probability of a direct interaction between the enhancer complex and the 3′ splice site. These data are consistent with a model in which splicing enhancers function by increasing the local concentration of SR proteins in the vicinity of the nearby intron through RNA looping.
Keywords: pre-mRNA splicing/RNA looping/splicing enhancers/SR proteins

Introduction

Pre-mRNA splicing enhancers play a critical role in the regulation of alternative splicing and in correct splice site recognition of constitutively spliced pre-mRNAs (Reed, 1996; Hertel *et al*., 1997; Grabowski, 1998). Although splicing enhancers usually are located downstream of the affected introns (Fu, 1995; Manley and Tacke, 1996), they have also been observed within introns (Black, 1992; Lou *et al*., 1995) and upstream of regulated 5′ splice sites (Ryner *et al*., 1996; Heinrichs *et al*., 1998). Many of these RNA elements are recognized by members of the serine–arginine (SR) family of essential splicing factors (Zahler *et al*., 1992; Fu, 1995; Manley and Tacke, 1996). SR proteins contain one or two N-terminal RNA recognition motifs (RRM) and a variable length C-terminal arginine–serine (RS)-rich domain (Zahler *et al*., 1992). The RRMs are required for RNA binding (Cáceres and Krainer, 1992; Zuo and Manley, 1992) while the RS domains are required for protein–protein interactions with other components of the splicing machinery (Wu and Maniatis, 1993; Amrein *et al*., 1994; Kohtz *et al*., 1994) and serve as splicing activation domains (Graveley and Maniatis, 1998). SR proteins bound to splicing enhancers are thought to function by recruiting components of the general splicing machinery to the nearby intron (Reed, 1996; Hertel *et al*., 1997). Consistent with this proposal, splicing enhancers have been shown to stimulate the binding of the splicing factor U2AF to pre-mRNAs containing non-consensus 3′ splice sites (Wang *et al*., 1995; Zuo and Maniatis, 1996).

The relative activities of SR proteins are likely to be a function of their affinity for splicing enhancers and the strength of their activation domains. To uncouple binding and activation, we have made use of hybrid proteins in which RS domains from different SR proteins are fused to the bacteriophage MS2 RNA-binding protein (Graveley and Maniatis, 1998). Such fusion proteins can activate splicing of pre-mRNAs containing an MS2-binding site in place of a splicing enhancer. Thus, the splicing activities of different SR proteins can be studied independently of their affinities for RNA. Using this approach, we have found that the relative activities of different SR proteins are determined by the RS content of the RS domain. In fact, a careful quantitative analysis revealed that the potency of SR proteins is determined by the number of RS tetrapeptide sequences.

Most splicing enhancers are located within 100 nucleotides of the 3′ splice site but are not active when located further away (Lavigueur *et al*., 1993; Tian and Maniatis, 1994). However, the splicing enhancer in the *Drosophila melanogaster doublesex* pre-mRNA, the *dsx* repeat element (*dsx*RE), normally functions when located 300 nucleotides downstream of the regulated 3′ splice site (Tian and Maniatis, 1992). The *dsx*RE can even activate splicing when artificially inserted 500 nucleotides downstream from the 3′ splice site (Tian and Maniatis, 1994). The reason for this unusual activity is that the *dsx*RE is comprised of multiple enhancer elements, each of which is recognized by the splicing regulatory proteins Transformer (Tra) and Transformer 2 (Tra2), and an SR protein (Lynch and Maniatis, 1995). In the absence of Tra/Tra2, these elements are inactive when located 300 nucleotides downstream of the 3′ splice site (Lynch and Maniatis, 1995). However, in the presence of Tra/Tra2, a very stable enhancer complex is formed, allowing the enhancer to function at this distance (Lynch and Maniatis, 1995, 1996). Although it recently has been shown that multiple enhancer elements additively increase the efficiency of splicing (Hertel and Maniatis, 1998), the relationship between enhancer strength and the ability to function at a distance has not been approached systematically. Here we show that the activity of a given splicing enhancer decreases as the distance between the enhancer and the intron increases. Quantitative analysis of the data revealed that the splicing efficiency is directly proportional to the calculated probability of a direct interaction between the enhancer complex and the 3′ splice site. These studies provide the first quantitative analysis of the parameters that determine the strength of splicing enhancers, and they provide new insights into the mechanisms by which enhancers act.

Results

RS domains differ in their potency

The lengths of the RS domains of various SR proteins have been conserved from *D.melanogaster* to humans (Roth *et al*., 1991; Zahler *et al*., 1992). However, the basis for this conservation is unknown. One possibility is that the different RS domains vary in their ability to function as splicing activation domains. To test this idea, a series of hybrid proteins containing an RS domain and the MS2 bacteriophage coat protein was generated. These hybrid proteins were then tested for their ability to activate *in vitro* splicing of enhancer-dependent pre-mRNA substrates in which the normal splicing enhancer was removed and replaced by a binding site for the MS2 protein. Three previously described hybrids containing the RS domains of the human SR proteins SF2/ASF, SC35 and 9G8 (Graveley and Maniatis, 1998) were used, in addition to three new hybrids containing the RS domains of the human SR proteins SRp40, SRp55 (Screaton *et al*., 1995) and SRp75 (Zahler *et al*., 1993b) (Figure 1A). Each of these proteins was expressed in insect Sf9 cells using recombinant baculoviruses and purified under native conditions in a single step on Ni-NTA agarose. All of the hybrid proteins were phosphorylated in the insect cells as indicated by their interaction with the monoclonal antibody mAb104 (data not shown).

To ensure that differences observed in activator potency are due to interactions between the assembled enhancer complex and the splicing machinery rather than to effects on RNA binding, all of the experiments were carried out under conditions in which the MS2-binding sites are fully occupied by the hybrid proteins. Each hybrid protein was therefore titrated into splicing reactions using a substrate which contains a single MS2-binding site 70 nucleotides downstream of the *doublesex* 3′ splice site [*dsx*(70)M1]. Although the maximal level of splicing differed for each protein tested, the splicing efficiencies plateaued with increasing concentrations of hybrid protein, suggesting that the MS2-binding sites are saturated by the hybrid proteins (Figure 1B). The protein concentration required to reach half the maximal level of splicing ranged between 10 and 30 nM, a value that is close to the K_d for the binding of the MS2 protein to its site (10 nM) (Lowary and Uhlenbeck, 1987). Protein concentrations ~10 times the apparent K_d were used for all subsequent experiments (~200 nM).

To analyze the differences in RS domain potency, we examined the splicing of the *dsx*(70)M1 pre-mRNA and a substrate derived from the mouse immunoglobulin μ gene that contains a single MS2-binding site 20 nucleotides downstream of the 3′ splice site [IgM(20)M1]. Very low levels of splicing were observed in the absence of added protein (Figure 2A, lanes 1 and 9), or in the presence of MS2 lacking an RS domain (Figure 2A, lanes 2 and 10). Addition of the hybrid proteins to each reaction strongly activated RNA splicing (Figure 2A, lanes 3–8 and 11–16), but significant differences in the potency of each RS domain were observed (Figure 2B). For both substrates, the hybrids bearing the SRp55, SRp75 or 9G8 RS domains were the most potent, the hybrid bearing the SC35 RS domain had an intermediate potency, and the hybrid bearing the SF2/ASF RS domain was weakest (Figure 2B). Only the potency of the SRp40 RS domain differed with respect to the other RS domains between the two pre-mRNA substrates. These data clearly demonstrate that RS domains differ in their ability to activate weak 3′ splice sites.

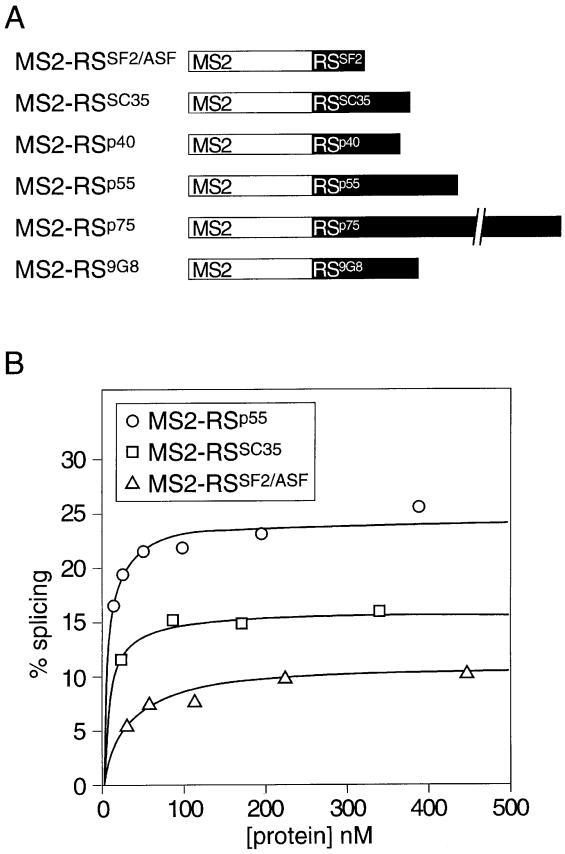

Fig. 1. Characterization of the MS2–RS hybrid proteins.
(**A**) Schematic diagram of the MS2–RS hybrid proteins. The open boxes labeled MS2 indicate the regions corresponding to the MS2 protein sequences containing the RNA-binding domain. The black regions correspond to the RS domains of the indicated human SR proteins. MS2–$RS^{SF2/ASF}$, MS2–RS^{SC35} and MS2–RS^{9G8} were described previously (Graveley and Maniatis, 1998). MS2–RS^{p40}, MS2–RS^{p55} and MS2–RS^{p75} contain residues 180–272 of SRp40 (Screaton *et al*., 1995), 184–344 of SRp55 (Screaton *et al*., 1995) and 179–494 of SRp75 (Zahler *et al*., 1993b), respectively.
(**B**) Comparative analysis of the splicing efficiencies of various hybrid proteins using the *dsx*(70)M1 pre-mRNA substrate. Different amounts of each hybrid protein were incubated in HeLa cell nuclear extracts with the *dsx*(70)M1 substrate for 2 h at 30°C. The data obtained with the MS2–$RS^{SF2/ASF}$ (△), MS2–RS^{SC35} (□) and MS2–RS^{p55} (○) proteins are shown. Similar results were obtained with MS2–RS^{p40}, MS2–RS^{p75} and MS2–RS^{9G8} (data not shown).

The activity of splicing enhancers decreases as a function of distance from the 3′ splice site

Splicing enhancers function when positioned close to a regulated intron, but are inactive when located at a distance (Lavigueur *et al*., 1993; Tian and Maniatis, 1994). In order to investigate the ability of enhancers to function at a distance, we generated a series of pre-mRNA substrates containing a single MS2-binding site located 70, 140, 200 or 300 nucleotides downstream of the *dsx* 3′ splice site (Figure 3A). Splicing reactions were carried out in HeLa cell nuclear extracts supplemented with saturating concentrations of each hybrid protein. As shown in Figure 3,

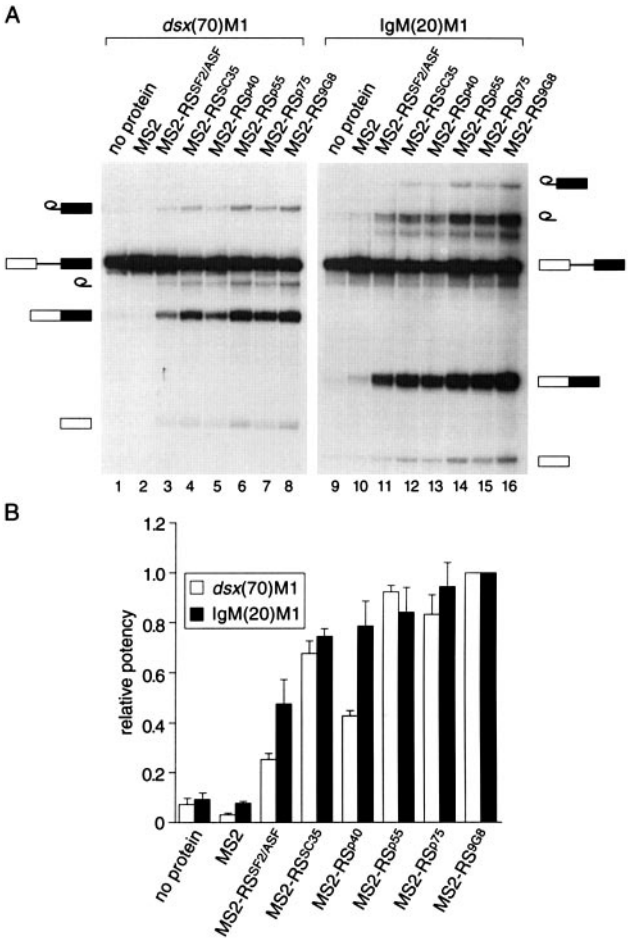

Fig. 2. Comparison of the splicing activation efficiencies with different hybrid proteins. (**A**) Splicing reactions were carried out for 2 h at 30°C. The reactions contained either no protein (lanes 1 and 9), MS2 (lanes 2 and 10) or saturating concentrations of each indicated hybrid protein (lanes 3–8 and 11–16). The reactions in lanes 1–8 contained the *dsx*(70)M1 pre-mRNA, while those in lanes 9–16 contain the IgM(20)M1 pre-mRNA. The reaction products were separated on a 12% denaturing polyacrylamide gel. The identities of the input, intermediates and products are indicated. (**B**) Quantitation of the data in (A). The potency of each RS domain is presented relative to MS2–RS^{9G8} which was the most potent activator for each RNA substrate and was arbitrarily set to 1.0. The white bars represent the data from the reactions using the *dsx*(70)M1 pre-mRNA while the black bars represent the data from the reactions using the IgM(20)M1 pre-mRNA. The data shown are the average of three separate experiments.

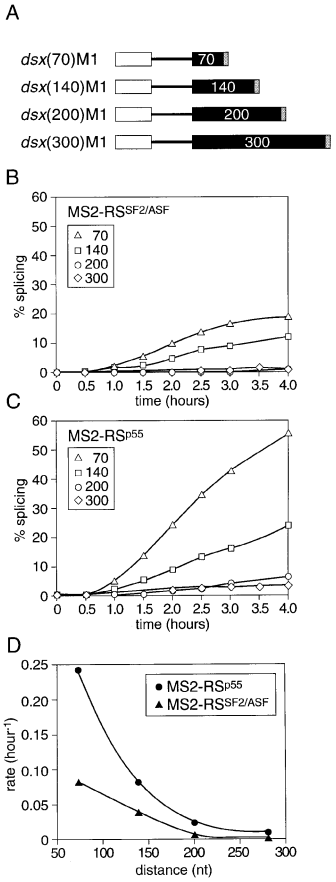

Fig. 3. The splicing activities of hybrid proteins are sensitive to the distance between the intron and the MS2 RNA-binding site. (**A**) RNA substrates. Four RNA substrates were generated that contained a single MS2-binding site (shaded box) 70 [*dsx*(70)M1], 140 [*dsx*(140)M1], 200 [*dsx*(200)M1] or 300 [*dsx*(300)M1] nucleotides downstream of the *dsx* 3′ splice site. (**B**) Quantitation of splicing data for reactions containing MS2–RS$^{SF2/ASF}$. Splicing reactions were conducted in HeLa cell nuclear extract supplemented with saturating concentrations of MS2–RS$^{SF2/ASF}$ and time points taken between 0 and 4 h. The percentage of spliced product is expressed as a function of time. (△) *dsx*(70)M1, (□) *dsx*(140)M1, (○) *dsx*(200)M1, (◇) *dsx*(300)M1. (**C**) Quantitation of splicing data for reactions containing MS2–RS^{p55}. Splicing reactions were performed as in (B) except that saturating concentrations of MS2–RS^{p55} were used. (△) *dsx*(70)M1, (□) *dsx*(140)M1, (○) *dsx*(200)M1, (◇) *dsx*(300)M1. (**D**) The rate of splicing (see Table I) promoted by MS2–RS$^{SF2/ASF}$ (▲) and MS2–RS^{p55} (●) was determined for each substrate and is plotted as a function of distance (nt) from the 3′ splice site.

splicing was most efficient when the MS2-binding site was located 70 nucleotides downstream of the 3′ splice site. The efficiency of splicing decreased when the MS2-binding site was positioned further away from the intron, and was nearly undetectable for the *dsx*(300)M1 pre-mRNA (Figure 3B and C). A comparison of the calculated rate constants for the hybrid proteins MS2–RS$^{SF2/ASF}$ and MS2–RS^{p55} clearly demonstrates that MS2–RS^{p55} is a much better activator than MS2–RS$^{SF2/ASF}$ at each distance tested (Figure 3D; Table I). For instance, when located 140 nucleotides from the 3′ splice site, MS2–RS^{p55} activates splicing as well as MS2–RS$^{SF2/ASF}$ located 70 nucleotides from the 3′ splice site. Thus a potent activator can function at a greater distance than a weak activator.

Strong natural enhancers function at a greater distance from the intron than weak natural enhancers

Using the hybrid protein approach, we have shown that strong activators can function at a greater distance from the 3′ splice site than weaker activators. In order to address the generality of this observation, we compared the ability of two natural splicing enhancers to activate splicing when located either 100 or 300 nucleotides downstream of the *dsx* 3′ splice site (Figure 4A). One enhancer was derived from avian sarcoma-leukosis virus (ASLV) (Katz and Skalka, 1990; Staknis and Reed, 1994) and the second enhancer we used was the purine-rich element (PRE) from the *dsx*RE (Lynch and Maniatis, 1995). Figure 4B shows that each enhancer functions well when located 100 nucleotides from the 3′ splice site,

Table I. Splicing rates promoted by MS2–RS hybrid proteins

RNA substrate	Hybrid protein				
	Single RS domain hybrids			Double RS domain hybrids	
	MS2–RS$^{SF2/ASF}$	MS2–RS^{SC35}	MS2–RS^{p55}	MS2–2RS$^{SF2/ASF}$	MS2–2RS^{SC35}
dsx(70)M1	0.08	0.20	0.24	0.25	0.32
dsx(70)M2	0.16	0.36	0.47	0.51	0.62
dsx(140)M1	0.04	0.05	0.08	0.07	0.09
dsx(140)M2	0.11	0.12	0.19	0.17	0.18
dsx(200)M1	0.005	0.015	0.023	0.015	0.024
dsx(200)M2	0.029	0.045	0.085	0.044	0.064
dsx(300)M1[a]	0.001	0.006	0.009	0.003	0.001
dsx(300)M2	0.012	0.015	0.023	0.019	0.014

All rate constants are expressed in units of h^{-1}. Each value was determined from a splicing reaction consisting of 7–8 time points over a 4 h time period. To derive the rate constants, the data at time points following the lag were fit to a first-order rate equation. Experimental error for the rate constants is within 15%.
[a]With the exception of MS2–RS^{p55}, splicing of the *dsx*(300)M1 substrate was not significantly above background.

although the ASLV enhancer is clearly more potent. When placed 300 nucleotides from the 3′ splice site, only the ASLV enhancer, and not the PRE enhancer, could activate splicing detectably (Figure 4B). Thus, the ability of a strong splicing enhancer to function at a greater distance from the 3′ splice site than weak splicing enhancers is a general property of splicing enhancers and not intrinsic to the hybrid proteins.

Multiple enhancer sites increase the probability of activating splicing

To assess the effect of enhancer site duplications on splice site activation, we generated substrates containing two MS2-binding sites at each distance used in Figure 3 (Figure 5A). As shown in Figure 5B and Table I, the percentage of spliced product was significantly greater for a substrate containing two MS2-binding sites than for substrates containing a single MS2-binding site. In fact, the rate of splicing at each distance increased ~2-fold for the RNA containing two binding sites compared with the corresponding RNA containing a single binding site (Figure 5C). Similar conclusions to those illustrated in Figure 5 can be drawn for the other hybrid proteins (Table I). These observations are in agreement with a recent study demonstrating that multisite enhancers increase the splicing efficiency proportionally to the number of enhancer elements present (Hertel and Maniatis, 1998).

Duplication of RS domains doubles the efficiency of splicing

The observation that splicing enhancers containing multiple binding sites for splicing activators increase the efficiency of splicing additively substantiates the proposal that only a single component of the enhancer complex at a time contacts the spliceosomal target (Hertel and Maniatis, 1998). A prediction of this model is that duplicating the number of splicing activation domains in a single hybrid protein should result in an additive increase in splicing efficiency. To test this prediction, we generated and tested two new hybrid proteins bearing either two SF2/ASF RS domains (MS2–2RS$^{SF2/ASF}$) or two SC35 RS domains (MS2–2RS^{SC35}) (Figure 6A). Saturating concentrations of these hybrid proteins were used to activate splicing reactions for each of the *dsx* pre-mRNAs shown in Figure

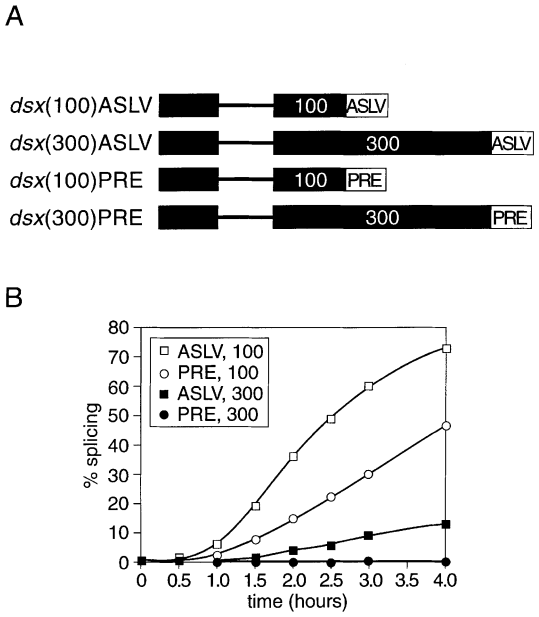

Fig. 4. A strong natural splicing enhancer functions at a greater distance than a weak natural splicing enhancer. (**A**) RNA substrates. The ASLV and PRE splicing enhancers were placed either 100 or 300 nucleotides downstream of the *dsx* 3′ splice site [*dsx*(100)ASLV, *dsx*(100)PRE, *dsx*(300)ASLV and *dsx*(300)PRE]. (**B**) Splicing time course of *dsx* substrates containing either the ASLV or PRE splicing enhancer 100 or 300 nucleotides downstream of the 3′ splice site. *dsx*(100)ASLV (□), *dsx*(100)PRE (○), *dsx*(300)ASLV (■), *dsx*(300)PRE (●).

5A. Figure 6B and Table I show that the rate of splicing for a single binding site substrate is ~2-fold greater when activated by a hybrid protein containing two RS domains. Similar results were obtained when comparing the *dsx* pre-mRNA substrates containing two MS2-binding sites (Table I). Therefore, duplicating the RS domain appears to double the potency of the splicing activator, an observation consistent with the proposal that SR proteins within splicing enhancer complexes do not interact with more than one target in the basic splicing machinery at a time.

RNA looping facilitates recruitment of the spliceosome

Splicing enhancer complexes are thought to function by interacting with a component of the basic splicing

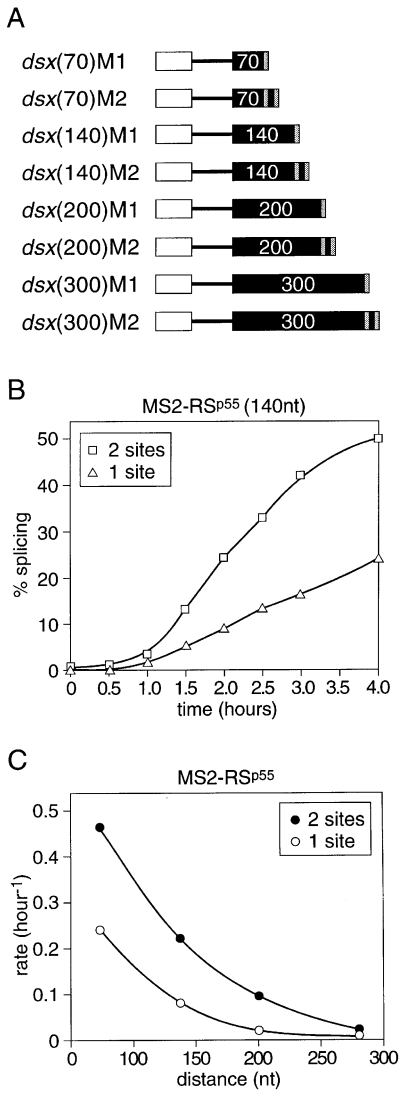

Fig. 5. Multiple MS2-binding sites increase the efficiency of splicing. (**A**) RNA substrates. Eight RNA substrates were tested that contained either one or two MS2-binding sites (shaded boxes) 70 [*dsx*(70)M1, *dsx*(70)M2], 140 [*dsx*(140)M1, *dsx*(140)M2], 200 [*dsx*(200)M1, *dsx*(200)M2] or 300 [*dsx*(300)M1, *dsx*(300)M2] nucleotides downstream of the *dsx* 3′ splice site. (**B**) Splicing reactions using *dsx*(140)M1 (△) and *dsx*(140)M2 (□) were carried out for the times indicated in HeLa cell nuclear extract supplemented with saturating concentrations of MS2–RS^{p55}. The percentage of spliced product is expressed as a function of time. (**C**) Comparison of the rates of splicing promoted by MS2–RS^{p55} on substrates containing either one (○) or two (●) MS2-binding sites as a function of distance (nt) from the 3′ splice site.

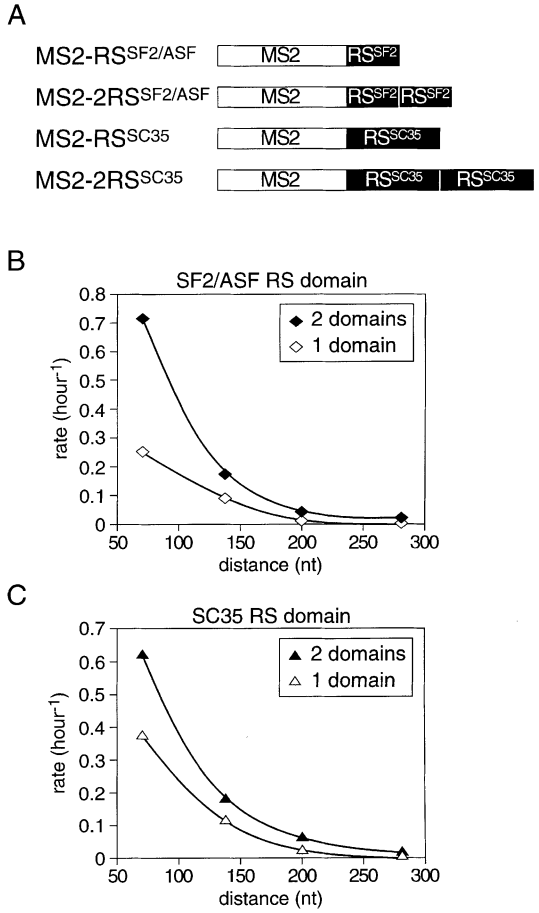

Fig. 6. Multiple RS domains increase the efficiency of splicing. (**A**) Schematic diagram of hybrid proteins containing either one or two RS domains from SF2/ASF (top) or SC35 (bottom). (**B**) Comparison of the rates of splicing promoted by saturating amounts of hybrid proteins bearing either one (◇) or two (◆) SF2/ASF RS domains on *dsx* substrates containing a single MS2-binding site at various distances from the 3′ splice site. (**C**) Comparison of the rates of splicing promoted by saturating amounts of hybrid proteins bearing either one (△) or two (▲) SC35 RS domains on *dsx* substrates containing a single MS2-binding site at various distances from the 3′ splice site.

machinery and recruiting the spliceosome to the intron. This model requires that the RNA between the enhancer and the intron loops out to facilitate recruitment. One prediction of the looping model is that the efficiency of splicing promoted by an enhancer complex at any given distance will be a function of the probability that the splicing enhancer complex is in close proximity to the site of action. One potential target of splicing enhancer complexes is U2AF, which binds to the 3′ splice site. It previously has been reported that splicing enhancers can stimulate the binding of U2AF to pre-mRNAs containing weak 3′ splice sites (Wang *et al*., 1995; Zuo and Maniatis, 1996). We used equations derived from DNA cyclization experiments (Wang and Davidson, 1966) to calculate the local concentration of the splicing enhancer complex in the vicinity of the intron, assuming that (i) the 3′ splice site is the target of the splicing enhancer complex, and (ii) the RNA between the enhancer and the 3′ splice site is largely unstructured and flexible (see Materials and methods). Interestingly, a plot of the rate of splicing promoted by each hybrid protein versus the calculated concentration of the enhancer at the 3′ splice site was fit best by a linear equation (Figure 7). This striking correlation suggests that the effect of distance upon splicing activation is strictly a function of the probability that the activators bound to the enhancer will be in the vicinity of the 3′ splice site. It should be noted that recent data (Rudner *et al*., 1998) have raised the question of whether U2AF is the true *in vivo* target of splicing enhancers. Nonetheless, the linear relationship between the rate of splicing and the calculated concentration of the enhancer complex holds true regardless of whether we use the 3′ splice site, the 5′ splice site or elsewhere within the intron as the target of the splicing enhancer in our calculations

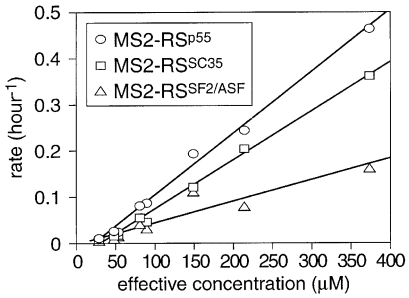

Fig. 7. Splicing enhancers recruit the spliceosome by RNA looping. The rate of splicing is shown as a function of the predicted concentration of the MS2-binding sites at the 3′ splice site (see Materials and methods). The data plotted include all of the rates from Table I calculated from the reactions containing MS2–RS$^{SF2/ASF}$ (△), MS2–RS^{SC35} (□) and MS2–RS^{p55} (○). The linear correlation suggests that splicing enhancers recruit the general splicing machinery by RNA looping.

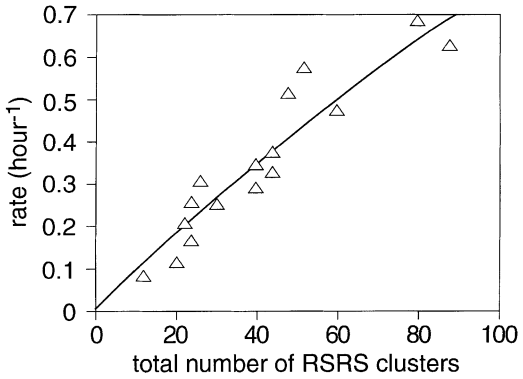

Fig. 8. The potency of splicing activators correlates with RS content. The rate of splicing promoted by each hybrid protein is plotted as a function of the number of clusters containing four consecutive alternating arginine and serine residues (either RSRS or SRSR) contained within the enhancer complex. No amino acid was counted more than once. Thus, five to seven consecutive alternating R and S residues count as only a single cluster, while a run of eight alternating residues counts as two adjacent clusters. The values are derived from splicing reactions with the *dsx*(70)M1 and *dsx*(70)M2 pre-mRNAs. Included in these data are all of the rates shown in Table I as well as those promoted by MS2–RS^{p75} [0.29 for *dsx*(70)M1 and 0.68 for *dsx*(70)M2], MS2–RS^{9G8} [0.30 for *dsx*(70)M1 and 0.57 for *dsx*(70)M2] and MS2–RS^{p40} [0.11 for *dsx*(70)M1 and 0.34 for *dsx*(70)M2]. Because MS2 binds RNA as a dimer, for the RNAs containing a single binding site, the RS content is twice that of each hybrid protein. Likewise, the RS content for the RNAs containing two binding sites is four times that of each hybrid protein.

(data not shown). These data are most consistent with a model in which recruitment of the spliceosome by splicing enhancer complexes is facilitated by RNA looping.

Discussion

RS domains from different SR proteins display distinct splicing activities

SR proteins differ in their abilities to complement splicing in SR protein-deficient S100 extracts (Lynch and Maniatis, 1996; Tacke *et al*., 1998), to commit pre-mRNAs to the splicing reaction (Fu, 1993; Tian and Maniatis, 1993; Lynch and Maniatis, 1996) and to promote the use of alternative splice sites (Zahler *et al*., 1993a). In these cases, differences in activity are attributable to the specificity of RNA binding rather than to strengths of the RS domains. The data presented here clearly demonstrate that RS domains have distinct potencies when activating a 3′ splice site from a downstream binding site. In contrast, a recent report showed no difference in RS domain potency using a splicing commitment assay (Chandler *et al*., 1997). It remains to be determined whether this apparent discrepancy is a consequence of the different assays used in the two studies.

The RS domains of different SR proteins contain different numbers of alternating arginine and serine residues, most of which are organized into distinct clusters. A quantitative analysis of the *in vitro* splicing results presented here revealed a near linear relationship between the number of arginine and serine residues present in an activator and its potency (Figure 8). This relationship exists when we consider the total number of arginine and serine residues (data not shown), RS dipeptides (data not shown) or RS clusters of four residues (Figure 8). However, the correlation no longer holds when we consider RS clusters of six residues or longer (data not shown). Interestingly, the best fit was obtained when the rate data were plotted against four-residue RS clusters, suggesting that four consecutive alternative arginine and serine residues may be a minimal functional independent unit. This observation is consistent with the recent finding that a single RS dipeptide is not sufficient for SR protein function *in vivo*, but that slightly larger stretches are active (Heinrichs and Baker, 1997). If we assume that each of these clusters can function as an independent unit, then RS domains are composed of multiple regions each capable of interacting with the target molecule. In agreement with this idea, duplicating the RS domains, which by definition doubles the number of independent interacting regions, leads to a proportional increase in the rate of splicing. Thus, potent activators contain a greater number of independent activating regions than weak activators.

It is interesting to note that the lengths of the RS domains of various SR proteins have been highly conserved from *Drosophila* to humans (Roth *et al*., 1991; Zahler *et al*., 1992). If these domains are functionally redundant, however, there should be no selective pressure to maintain the lengths of the RS domains. Perhaps RS domain length has been conserved throughout evolution because it is important that cells contain a variety of splicing regulators bearing splicing activation domains of different potencies in order to maintain proper balances in alternative splicing patterns.

Although RS content appears to be a major determinant of RS domain potency, protein structure and phosphorylation are also likely to affect potency. For example, while the 9G8 RS domain is the most potent activation domain we tested, it does not have the highest RS content. This indicates that the manner in which the RS clusters are displayed or the local environment of the RS clusters may have an impact on their ability to participate in protein–protein interactions. Phosphorylation is essential for the function of SR proteins (Cao *et al*., 1997; Xiao and Manley, 1997) and affects the strength of some protein–protein interactions. For instance, phosphorylation of SF2/ASF increases the strength of its interaction with U1-70K (Xiao and Manley, 1997; Wang *et al*., 1998). Further studies will be necessary to determine the roles of protein structure and phosphorylation in RS domain potency.

Multiple binding sites additively increase the efficiency of splicing

The inclusion of the female-specific exon in *dsx* depends on the presence of an exonic enhancer element (*dsx*RE) located ~300 nucleotides downstream of the regulated 3′ splice site. The *dsx*RE is composed of multiple individual enhancer elements which recently were shown to function as independent units in recruiting the general splicing machinery to the weak female-specific splice site (Hertel and Maniatis, 1998). Because multiple enhancer elements increased splice site usage additively, it was argued that only one enhancer complex is capable of interacting with the splicing machinery at a time. In agreement with the previous study, we observe that the duplication of enhancer sites, i.e. MS2-binding sites, increased the rate of splicing ~2-fold. Moreover, duplication of the RS domains also resulted in a 2-fold increase in splicing. Thus, our results support and extend the previous conclusion that only one spliceosomal target is available for multiple enhancer complexes, and demonstrate that the RS domain is involved in this critical protein–protein interaction.

The distance-dependent activity of splicing enhancers is a function of enhancer complex concentration

We have demonstrated that the activity of splicing enhancers decreases as they are positioned further from the regulated intron. Additionally, the ability of a splicing enhancer to promote splicing as a function of distance is related in a linear fashion to the calculated effective concentration of the splicing enhancer at the 3′ splice site. These data are consistent with a model in which RNA looping facilitates recruitment of the spliceosome by splicing enhancer complexes. They do not, however, exclude the possibility that splicing enhancer complexes recruit additional intermediate components (e.g. SR proteins) to the RNA, which in turn recruit the splicing machinery. One prediction of this latter model is that a physical blockade between the enhancer and the 3′ splice site should have a negative effect on the efficiency of splicing. To test this, we generated a pre-mRNA that contains an MS2-binding site between the 3′ splice site and a constitutive splicing enhancer. The addition of saturating amounts of MS2 protein, which lacks an RS domain, had no effect on the splicing efficiency of this pre-mRNA (B.R.Graveley and T.Maniatis, unpublished data). Thus, binding a neutral protein between the 3′ splice site and the enhancer complex does not affect the efficiency of pre-mRNA splicing. Taken together, these data are most consistent with the RNA looping model for splicing enhancer function.

It is important to understand the dramatic effect splicing enhancers have on the local concentration of the splicing activator at the 3′ splice site. While the concentration of hybrid proteins used in the reactions is ~200 nM, when they are bound to the RNA, the calculated effective local concentration increases more than three orders of magnitude. This remarkable increase in local concentration requires RNA flexibility. If, for example, the RNA was maintained as a stiff, inflexible rod, the concentration of the bound hybrid proteins in the vicinity of the 3′ splice site would be orders of magnitude lower than the concentration of the proteins added to the reaction. Thus, RNA flexibility must be an important component in modulating the activity of splicing enhancers.

The above calculations make the assumption that the RNA between the 3′ splice site and the enhancer of interest is quite flexible and mainly unstructured. While the former assumption is quite reasonable, the latter appears to contradict the general belief that RNA has a great potential to adopt higher order structures (Uhlenbeck, 1995). To our surprise, however, our data suggest that the RNA between the 3′ splice site and the splicing enhancer behaves as if it were linear. Regardless of the sequence content, the inclusion of RNA stretches to move the enhancer further downstream of the 3′ splice site always leads to a linear decrease in the activation potential of the enhancer. Thus, the *dsx* sequences spanning the 3′ splice site and the *dsx*RE have a linear character, or a similar average structural content, when incubated in nuclear extracts. This is quite surprising because computer algorithms predict the sequences we used to form secondary structures readily (data not shown). However, the structure of RNA in the absence of nuclear proteins may be very different from when it is incubated in nuclear extract. For instance, factors such as hnRNP proteins may bind to the RNA and remove some of the RNA structure, yet still allow the RNA to be quite flexible. Previous studies have shown hnRNP proteins rapidly associate with RNA to form H complex when pre-mRNA is added to nuclear extracts (Bennett *et al*., 1992). We note that a number of different RNA sequences have been tested between the *dsx*RE and the 3′ splice site, and no significant difference in enhancer activity was observed (Tian, 1993). Thus, with the exception of very large, stable structures, the linear behavior of RNA in nuclear extracts may be a general phenomenon.

Splicing and transcription

The similarities between transcription and splicing enhancers are striking (Hertel *et al*., 1997). In each case, proteins bind to enhancer elements and function to recruit components of the general machinery. Additionally, both transcription and splicing activators are modular, containing separable nucleic acid-binding domains and activation domains which are required for protein–protein interactions.

Here we show that splicing activation domains have distinct potencies and that potent splicing activators can function at a greater distance than weak splicing activators. These properties are also shared with transcription activators. For instance, certain transcription activation domains (i.e. VP16) are quite potent and can function at great distances from the promoter, while other transcription activation domains are weak (i.e. AH) and can only function efficiently when located near the promoter (Carey *et al*., 1990a). Moreover, we show that the potency of an RS domain correlates with its overall RS content, and to some degree the length of the RS domain. Likewise, a correlation between the potency of a transcription activator and its length has been demonstrated (Hope and Struhl, 1986; Ma and Ptashne, 1987; Wu *et al*., 1996). Additionally, the differences in potency were shown to be related directly to the strength of the interaction between the transcription activation domain and its target within the general transcription machinery (Wu *et al*., 1996). Here

we provide evidence that RNA looping may serve to facilitate interactions between bound splicing activators and the general splicing machinery. DNA looping is also involved in the interaction of transcription activators and the general transcription machinery (Rippe *et al*., 1995; Ptashne and Gann, 1997). Finally, we show that a splicing enhancer can be made stronger by increasing either the number of binding sites or the number of activation domains within each activator. Again, these properties are shared with transcription enhancers (Carey *et al*., 1990b; Ohashi *et al*., 1994).

Although transcription and splicing enhancers share many properties, there are striking differences between the mechanisms of activation directed by the two types of enhancers. While multiple transcription activators have been shown to cooperate synergistically to activate transcription, multiple splicing activators have only an additive effect on splice site activation. As previously discussed, the linear relationship between the number of splicing activators and splicing efficiency suggests that splicing enhancers target a single component of the splicing machinery (Hertel and Maniatis, 1998). Thus, although multiple enhancer-binding sites cooperatively can stabilize the formation of an enhancer complex (Lynch and Maniatis, 1996), splicing enhancers do not synergize functionally in spliceosome recruitment. It was shown recently by Carey and co-workers that increasing the number of activation domains in a transcription activator can result in a synergistic increase in transcriptional activity through domain synergy (Ohashi *et al*., 1994). In contrast to these observations, multiple RS domains do not show the signs of domain synergy. The proportional increase in activity upon multimerizing RS domains, therefore, is consistent with the conclusion above that splicing enhancer complexes can interact with only one target at a time. However, because SR proteins have been shown to interact with a number of general splicing factors including $U2AF^{35}$ (Wu and Maniatis, 1993), $U2AF^{65}$ (Zhang and Wu, 1996), U1-70K (Wu and Maniatis, 1993; Kohtz *et al*., 1994) and Urp (Tronchere *et al*., 1997), these conclusions do not exclude the possibility that splicing activators can synergize to activate splicing of some regulated introns by simultaneously contacting multiple components of the spliceosome.

Our results demonstrate that the potency of the activation domains contained within the enhancer complex, the number of individual enhancer elements, the distance between the enhancer and the site of action, and the flexibility of the intervening RNA affect the strength of a splicing enhancer complex. Each of these contributions is modular and can be altered independently of the other factors. The modularity thus provides an extensive repertoire for the evolution of splicing enhancers with extraordinary diversities.

Materials and methods

RNAs

The plasmid encoding *dsx*(70)M1 was described previously as p*dsx*-MS2 (Graveley and Maniatis, 1998). The plasmid p*dsx*(70)M2 was generated by ligating the annealed oligonucleotides 120-29c 5′-CGCGT-ACACCATCAGGGTACGAGCT-3′ and 120-29d 5′-CGTACCCTGAT-GGTGTACG-3′ into p*dsx*-MS2 digested with *Bst*BI and *Sac*I. The plasmid p*dsx*(300)M1 was generated by inserting an *Xho–Eco*RI fragment containing 84 nucleotides of the upstream exon, the full intron and 300 nucleotides of the downstream exon derived from D1 (Tian and Maniatis, 1992) into pSP72-MS2 (Graveley and Maniatis, 1998). The plasmid p*dsx*(200)M1 was synthesized by ligation of an *Xho–Eco*RI-digested PCR fragment (using a T7 primer and the oligonucleotide 5′-CGGA-ATTCACCGAAGCATTTAAGCTAATATGTG-3′) into p*dsx*(300)M1. p*dsx*(300)M2 and p*dsx*(200)M2 were generated as described above by inserting the annealed oligonucleotides 120-29c and 120-29d into p*dsx*(300)M1 or p*dsx*(200)M1, respectively. The plasmid p*dsx*(140)M2 was generated by ligation of an *Xho–Eco*RI-digested PCR fragment (using a T7 primer and the oligonucleotide 5′-CGGAATTCCGTTACT-CTAGTTAATGAAAAATT-3′) into p*dsx*(300)M2.

Digestion of p*dsx*(70)M2, p*dsx*(140)M2, p*dsx*(200)M2 or p*dsx*(300)M2 with *Pst*I or *Sac*I yielded the T7 RNA polymerase transcription templates for substrates containing one or two MS2-binding sites respectively, 70, 140, 200 or 300 nucleotides downstream of the regulated 3′ splice site.

Substrates containing the PRE located 100 or 300 nucleotides downstream of the regulated 3′ splice site were synthesized as described (Lynch and Maniatis, 1995). The plasmid *dsx*(100)ASLV is as described earlier (Staknis and Reed, 1994). The plasmid p*dsx*(300)ASLV was generated by blunt end ligation of a filled *Bgl*II–*Eco*RI fragment (Staknis and Reed, 1994) into the *Eco*RV site of D1 (Tian and Maniatis, 1992).

The IgM(20)M1 pre-mRNA was referred to previously as IgM-MS2 (Graveley and Maniatis, 1998).

Capped, ^{32}P-labeled RNAs were transcribed with either T7 or SP6 RNA polymerase and gel-purified prior to use.

Proteins

The expression and purification of MS2 protein was described previously (Graveley and Maniatis, 1998).

To facilitate simple shuffling of RS domain fragments, a new baculovirus transposition vector, pHIS-BIVT, was constructed. pHIS-BIVT was constructed by digesting pBluescript-KS(–) with *Spe*I and *Xba*I, trimming the termini and religating. The resulting plasmid was linearized with *Bam*HI and *Eco*RI and ligated to an oligonucleotide cassette (TGATATG-AGGGGTTCTCATCATCATCATCATATGGCCATG). This encodes a leader peptide MRGSHHHHHH and contains *Nde*I and *Nco*I restriction sites to facilitate the generation of in-frame fusions without many additional amino acids. This plasmid was digested with *Bss*HI and *Eco*RI, and the polylinker portion was ligated into a derivative of pFASTBAC-1 (Gibco-BRL) (in which the *Bam*HI site was destroyed by digestion, Klenow treatment and religation) at the *Bss*HI and *Eco*RI site, to create pHIS-BIVT. This plasmid can be used for *in vitro* translation using the T7 RNA polymerase promoter and for generating recombinant baculoviruses by transformation into the *Escherichia coli* strain DH10BAC. A PCR product encoding MS2 was generated that contained an *Nde*I site at the N-terminus and a flexible linker (GGGGGS), a *Bam*HI site, a TGA stop codon and a *Hin*dIII site at the C-terminus. This fragment was inserted into pHIS-BIVT at *Nde*I and *Hin*dIII to generate pHIS-BIVT-MS2.

The RS domain of each SR protein was amplified by PCR such that a *Bam*HI site was at the 5′ end and a *Hin*dIII site at the 3′ end following the stop codon. The PCR products were cloned into pBluescript-SK(–) and sequenced. The RS domains were then inserted into pHIS-BIVT-MS2 digested with *Bam*HI and *Hin*dIII to generate the corresponding baculovirus transfer vectors. To construct the duplicate RS domain proteins, the SF2/ASF and SC35 RS domains were amplified by PCR such that *Bam*HI restriction sites were present at both the 5′ and 3′ ends and the stop codon was eliminated. These PCR products were cloned into pBluescript-SK(–) and sequenced. These RS domain fragments were ligated into pHIS-BIVT-MS2-$RS^{SF2/ASF}$ or pHIS-BIVT-MS2-RS^{SC35} digested with *Bam*HI. All recombinant baculoviruses were generated as described by the manufacturer (Gibco-BRL). Each protein was expressed in infected Sf9 cells for 3 days and purified under native conditions as described (Graveley and Maniatis, 1998).

Splicing assays

Splicing assays were performed as described (Graveley and Maniatis, 1998), for the times indicated. All splicing gels were quantitated on a Fuji phosphorimager. The amounts of precursor (S) and spliced product (P) were determined and the percentage of splicing calculated using the equation: % splicing = P/(P+S). Rate constants were determined by fitting the data to a first-order rate equation.

Calculating the local concentration of the splicing enhancer complex at the 3′ splice site

The following equation yields a probable density that two points on a DNA molecule are in contact:

$$W = [3/(2\pi nl^2)]^{-1.5}$$

where l is the statistical segment length and n is the number of segments. The value n can be separated into the number of base pairs and the internucleotide distance per base pair (0.34 nm for DNA). To apply this to an RNA model, we used the values l = 4 nm [as measured for poly(U)] (Inners and Felsenfeld, 1970), an internucleotide distance of 0.65 nm per nucleotide and Avogadro's constant (6.022045×10^{23}), and simplified the equation to:

$$J_m(nt) = 0.13\times nt^{-1.5}[\text{mol/l}]$$

This equation is suitable to calculate the local concentration of two sites on an RNA molecule as a function of distance, for nucleotide distances >20 (Rippe *et al*., 1995; Van Gilst *et al*., 1997). For these calculations, we used the distance between the first nucleotide of exon 2 and the first nucleotide of the MS2 stem–loop. These values were as follows: *dsx*(70)M1 = 72 nucleotides, *dsx*(140)M1 = 138 nucleotides, *dsx*(200)M1 = 200 nucleotides and *dsx*(300)M1 = 283 nucleotides. For the RNAs containing two MS2-binding sites, the concentrations were calculated by the sum of the concentration of each MS2 site. The distance of the second MS2 binding site from the 3′ splice site was determined by counting the first MS2-binding site as two nucleotides (to account for the base pair at the base of the stem–loop). The distance of the second MS2-binding site for each RNA was as follows: *dsx*(70)M2 = 88 nucleotides, *dsx*(140)M2 = 154 nucleotides, *dsx*(200)M2 = 216 nucleotides and *dsx*(300)M2 = 299 nucleotides. Based on these values, the local concentration of the splicing enhancer at the 3′ splice site for each RNA is as follows: *dsx*(70)M1 = 213 μM, *dsx*(70)M2 = 370 μM, *dsx*(140)M1 = 80 μM, *dsx*(140)M2 = 148 μM, *dsx*(200)M1 = 46 μM, *dsx*(200)M2 = 87 μM, *dsx*(300)M1 = 27 μM and *dsx*(300)M2 = 52 μM.

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