REPORT

A general role for splicing enhancers in exon definition

BIANCA J. LAM and KLEMENS J. HERTEL

Department of Microbiology and Molecular Genetics, College of Medicine,
University of California, Irvine, California 92697-4025, USA

ABSTRACT

Exonic splicing enhancers (ESEs) facilitate exon definition by assisting in the recruitment of splicing factors to the adjacent intron. Here we demonstrate that suboptimal 5' and 3' splice sites are activated independently by ESEs when they are located on different exons. However, when they are situated within a single exon, the same weak 5' and 3' splice sites are activated simultaneously by a single ESE. These findings demonstrate that a single ESE promotes the recognition of both exon/intron junctions within the same step during exon definition. Our results suggest that ESEs recruit a multicomponent complex that minimally contains components of the splicing machinery required for 5' and 3' splice site selection.

Keywords: spliceosome; splice site recognition; splicing regulation; U1 snRNP; U2AF

INTRODUCTION

The removal of introns in higher eukaryotes requires the assembly of the spliceosome to accurately recognize short, poorly conserved 3' and 5' splice sites (Reed & Palandjian, 1997; Burge et al., 1999). The presence of weakly conserved splice sites allows extensive alternative pre-mRNA splicing to occur, one of several processes that exponentially enrich the proteomic diversity of higher eukaryotic organisms (Black, 2000; Graveley, 2001). Current estimates indicate that more than 60% of human genes are alternatively spliced, sometimes leading to hundreds of different mRNA isoforms from a single gene. However, poorly conserved splice sites can lead to aberrant splicing, often compromising the expression of the correct gene products (Krawczak et al., 1992; Nakai & Sakamoto, 1994; Carstens et al., 1997; Lee & Feinberg, 1997; Stoppa-Lyonnet et al., 1997).

Based on the observation that the majority of exons are significantly shorter than introns, it has been proposed that exons are basic units of recognition in higher eukaryotes (Berget, 1995). Studies on regulated alternative splicing have identified exonic *cis*-acting elements, referred to as exonic splicing enhancers (ESEs),

that facilitate the process of exon definition. These elements are capable of activating weak splice sites in adjacent introns. It is now appreciated that ESEs are not only components of regulated exons but also of constitutively spliced exons (Mayeda et al., 1999; Schaal & Maniatis, 1999). Generally, ESEs are binding sites for members of the serine/arginine-rich (SR) protein family of essential splicing factors. The assembled SR proteins, in turn, are thought to directly or indirectly recruit individual components of the spliceosome to the exon prior to the removal of adjacent introns (Fu, 1995; Manley & Tacke, 1996; Tacke & Manley, 1999; Blencowe, 2000; Graveley, 2000).

The *doublesex* (*dsx*) repeat element (*dsxRE*) is a well-characterized ESE consisting of six nearly identical 13-nt repeat elements that are binding sites for Transformer (Tra), Transformer 2 (Tra2), and an additional SR protein. When fully assembled, the *dsxRE* complex activates the recognition of a weak, sex-specific 3' splice site (Ryner & Baker, 1991; Tian & Maniatis, 1992; Lynch & Maniatis, 1996; Tacke & Manley, 1999). It was demonstrated that each 13-nt element additively increases *dsx* splicing efficiency, implying that each enhancer complex interacts with a single target during spliceosomal assembly, presumably U2AF (Hertel & Maniatis, 1998; Graveley, 2001). Recently, Tra and Tra2 have also been shown to activate female-specific 5' splice site choice in *fruitless* (*fru*; Ryner et al., 1996;

Reprint requests to: Klemens J. Hertel, Department of Microbiology and Molecular Genetics, College of Medicine, University of California, Irvine, California 92697-4025, USA; e-mail: khertel@uci.edu.

Heinrichs et al., 1998). Interestingly, three of the same 13-nt enhancer elements directing alternative splicing in *dsx* were identified to regulate female-specific splicing in *fru*, most likely through recruitment of U1 (Jamison et al., 1995; Zahler & Roth, 1995; Graveley, 2000). In contrast to the conclusions drawn from the *dsx* studies, a comparison of the *fru* repeat element (*fruRE*) and *dsxRE* activities suggested that certain ESEs may interact with more than one spliceosomal target for the recognition of 3' and 5' splice sites. Further support for this suggestion comes from recent investigations describing the ESE-dependent activation of alternative exons that are flanked by suboptimal splice sites (Bourgeois et al., 1999; Selvakumar & Helfman, 1999). However, the interpretations of these latter results were limited by the fact that multiple ESEs appear to be involved in the activation of the regulated exons.

In this study, we set out to determine if the stimulatory activity of an individual ESE is limited to the activation of one of the splice sites, or if a single ESE simultaneously promotes the recognition of both exon/intron junctions. Using heterologous substrates for *in vitro* splicing, we demonstrate that the activation of weak splice sites by ESEs located on different exons occurs synergistically. In agreement with the exon definition model, our results demonstrate that the recognition of each suboptimal exon constitutes an independent rate-limiting step during spliceosome assembly. However, when the ESEs are situated within one exon that is flanked by suboptimal splice sites, the activities of both ESEs were observed to be additive. These results demonstrate that an individual enhancer complex is sufficient to activate both weak splice sites. We conclude that an ESE recruits a complex that minimally contains factors necessary for both 3' and 5' splice site recognition. Our data is consistent with a model in which components of the splicing machinery that define the boundaries of exon/intron junctions are assembled prior to recruitment to the exon.

RESULTS

Splicing enhancers act as synergistic splice site activators when located on different exons

Sex-specific splicing of *dsx* and *fru* depends on the activities of multiple conserved 13-nt ESE elements that are recognized by Tra and Tra2. Because the *dsxRE* and *fruRE* activate the recognition of a 3' and a 5' splice site, respectively, it is possible that certain ESEs recruit more than one spliceosomal component to the exon. To test this hypothesis, we generated a heterologous pre-mRNA (*fru5'ss/dsx3'ssMS2*) gene construct that contains the regulated female-specific *fru* 5' splice site and the weak female-specific *dsx* 3' splice site on different exons (Fig. 1A). Because each of these

FIGURE 1. Synergistic activation of intron removal. **A:** Schematic of *fru5'ss/dsx3'ssMS2* pre-mRNA containing enhancer dependent splice sites on separate exons. Boxes represent exons. Light gray boxes within exons represent the Tra/Tra2-dependent *fruRE*. Black boxes within exons represent the MS2-RS dependent enhancer. Lines between the boxes represent introns. Solid lines above the pre-mRNA illustrate the splicing pathway. Slashes represent the junction between *fru* and *dsx* sequences. **B:** Representative autoradiograph showing splicing efficiencies after 2 h of incubation, rate constants, and fold activation for *fru5'ss/dsx3'ssMS2* splicing with 25% HeLa nuclear extract. Lane 1 contains no complementation. Lane 2 is supplemented with 400 nM Tra/Tra2. Lane 3 is supplemented with 200 nM MS2-RS^{9G8}. Lane 4 is supplemented with 400 nM Tra/Tra2 and 200 nM MS2-RS^{9G8}. Identities of unspliced and spliced RNAs are indicated on the right. Rate constants and fold activation are indicated on the bottom. **C:** Bar graph representing the fold activation of rate constants. X-axis corresponds to the lanes in **B**. Y values represent the fold activation as shown in **B**.

splice sites is poorly recognized in its natural context and limits the efficiency of spliceosome assembly (Ryder & Baker, 1991; Tian & Maniatis, 1992; Heinrichs et al.,

TABLE 1. Rate constants and fold activation for *fru5'ss/dsx3'ssMS2* and *dsx3'ssMS2/fru5'ss*^a

Substrate	None (h ⁻¹)	Tra/Tra2 (h ⁻¹)	MS-RS ^{9G8} (h ⁻¹)	Tra/Tra2/MS-RS ^{9G8} (h ⁻¹)
<i>fru5'ss/dsx3'ssMS2</i>	$0.5 \pm 0.1 \times 10^{-2}$	$4 \pm 2 \times 10^{-2}$	$9 \pm 2 \times 10^{-2}$	$89 \pm 9 \times 10^{-2}$
Fold activation	1	8 ± 4	18 ± 5	170 ± 17
<i>dsx3'ssMS2/fru5'ss</i>	$1 \pm 0.1 \times 10^{-2}$	$4 \pm 0.5 \times 10^{-2}$	$7 \pm 1.5 \times 10^{-2}$	$13 \pm 0.3 \times 10^{-2}$
Fold activation	1	4 ± 1	7 ± 2	13 ± 2

^aEach value was determined from at least two independent splicing reactions consisting of six time points over a 2-h time period. Experimental error for each rate determination is within 20%. Rates determined from different experiments varied less than 50%.

1998), intron removal from the *fru5'ss/dsx3'ssMS2* construct requires the juxtaposition of two suboptimal splice sites. In this configuration, the weak female-specific *fru* 5' splice site in *fru5'ss/dsx3'ssMS2* is activated by its native ESE (*fruRE*) that contains three 13-nt repeat elements recognized by Tra, Tra2, and an SR protein (Heinrichs & Baker, 1995; Lynch & Maniatis, 1996). Similar to the experimental design utilized in previous studies (Graveley & Maniatis, 1998), the weak 3' splice site of *dsx* in the *fru5'ss/dsx3'ssMS2* construct is activated by specific interactions between MS2-RS fusion proteins and the MS2 binding site located approximately 70 nt downstream of the 3' splice site. Thus, the design of the *fru5'ss/dsx3'ssMS2* construct permits the independent activation of the *fru* 5' or the *dsx* 3' splice sites by the addition of recombinant Tra/Tra2 and/or the MS2-RS fusion protein MS2-RS^{9G8} to HeLa cell nuclear cell extracts.

In vitro splicing assays were performed in the presence or absence of saturating amounts of Tra/Tra2 and/or MS2-RS^{9G8} to evaluate the splicing efficiency of *fru5'ss/dsx3'ssMS2*. For each set of conditions, the splicing reaction was followed over a period of up to 2 h. Splicing efficiencies were derived from rate measurements as previously described (Hertel & Maniatis, 1998). Although no measurable spliced products were detected in the absence of these recombinant proteins, the addition of either the Transformer or MS2-RS fusion proteins resulted in a small but significant activation over background levels (Fig. 1B, lanes 1–3). Kinetic analysis indicated that each enhancer complex increased intron removal by a factor of at least 10-fold over background levels (Fig. 1C; Table 1). This observation is surprising because it suggests that an ESE located on one exon can positively influence the recognition of a weak splice site on a neighboring exon. By contrast, constitutive splice sites that conform to consensus sequences without an ESE have no significant influence on weak splice sites of neighboring exons. For example, the common 3' splice site in *fru* cannot positively influence recognition of the upstream weak female 5' splice site (Ryner et al., 1996; Heinrichs et al., 1998). Similarly, the constitutive 5' splice site of *dsx* exon 3 cannot compensate for the subopti-

mal 3' splice site of exon 4 (Ryner & Baker, 1991; Tian & Maniatis, 1992). We conclude that ESEs are capable of activating not only weak splice sites within the same exon, but also weak splice sites across the intron. Importantly however, the activation of both ESEs by the addition of both the Tra/Tra2 and MS2-RS^{9G8} proteins further increased the accumulation of the spliced product considerably to levels greater than two orders of magnitude over background activity (Fig. 1B, lane 4, 1C).

Analysis of the processing kinetics shown in Figure 1 clearly indicates synergistic activation when both enhancer complexes are allowed to form (Fig. 1B,C). These results suggest that the activity of each ESE accelerates a different rate-limiting step during exon definition, presumably the recruitment of U1 snRNP to the 5' splice site and the U2AF heterodimer to the 3' splice site. Alternatively, the observed synergy could arise from cooperative binding interactions between enhancer elements across the intron. To test for possible cooperative binding interactions between the enhancer complexes, we examined the assembly efficiency of the MS2-RS^{9G8} enhancer complex in the presence or absence of the Tra/Tra2-dependent enhancer complex. Although the overall efficiency of intron removal was, as expected, more efficient in the presence of both enhancers, first-order kinetics were observed and the concentration of MS2-RS^{9G8} required for half-maximal activation remained nearly unchanged (Fig. 2). This finding demonstrates that the synergistic activation of intron removal is not caused by advantageous binding interactions between the ESEs tested. Thus, in agreement with previous studies, we conclude that each enhancer complex assembles independently (Hertel & Maniatis, 1998).

Individual enhancer elements activate the recognition of both exon splice sites within the same step of spliceosome assembly

Although the synergistic effects of the *fru5'ss/dsx3'ssMS2* splicing reaction are consistent with the recruitment of different components of the splicing machinery, this result might also be explained by the in-

FIGURE 2. Titration of MS2-RS^{9G8} on *fru5'ss/dsx3'ss*MS2 pre-mRNA. Schematic of pre-mRNA is shown in Figure 1A. **A:** MS2-RS^{9G8} titration in 25% HeLa nuclear extract with or without 400 nM Tra/Tra2. Identities of unspliced and spliced RNAs are indicated on the right. Splicing efficiency is indicated on the bottom. **B:** Splicing efficiency was graphed as a function of MS2-RS^{9G8} concentration and then fitted to an isotherm. Dissociation constants are indicated in the graph.

dependent recruitment of a common component to the ESEs of each exon. In other words, because each exon contains a weak splice site, its recognition might constitute a different rate-limiting step during exon definition. We therefore questioned whether ESE-dependent synergy would be observed if the enhancer-dependent splice sites and enhancers were located on the same exon. To address this question, we generated a three-exon substrate (*dsx3'ss*MS2/*fru5'ss*) that contains both enhancer-dependent splice sites and their respective enhancers all within the internal exon (Fig. 3A). Synergistic activation of internal exon inclusion would be expected if each ESE complex independently activates its flanking weak splice site. The processing efficiency of this pre-mRNA was determined as described for the *fru5'ss/dsx3'ss*MS2 construct. In the absence of recombinant proteins, low background activities were detected (Fig. 3B, lane 1), and as anticipated, addition of the Tra/Tra2 or MS2-RS^{9G8} proteins significantly increased intron removal by 4 and 7-fold, respectively (Fig. 3B, lanes 2 and 3). Unexpectedly, the activation of both ESEs by the addition of the Tra/Tra2 and MS2-

RS^{9G8} proteins did not lead to a dramatic increase in splicing efficiency as observed for *fru5'ss/dsx3'ss*MS2 (Fig. 3B, lane 4). Only a 13-fold activation over background levels was detected. A comparison of splicing efficiencies, initial rates, and fold activation revealed that the presence of both enhancer complexes results not in a synergistic, but in an additive increase in these parameters of intron removal (Fig. 3B,C; Table 1). We therefore conclude that each ESE in *dsx3'ss*MS2/*fru5'ss* activates the same rate-limiting step during exon definition.

DISCUSSION

Splicing enhancers activate both exon splice sites within the same step of exon definition

The formation of the early spliceosomal E complex containing U1 snRNP, U2AF, and SF1 commits the pre-mRNA to the splicing pathway and is therefore considered a crucial step during exon definition (Reed,

FIGURE 3. Additive effects in splice site activation. **A:** Schematic of *dsx3'ssMS2/fru5'ss* pre-mRNA containing enhancer-dependent splice sites on the same exon. Boxes represent exons. Dark gray boxes are constitutive exons. Boxes within exons represent enhancers as described in Figure 1A. The middle exon represents a fusion of *dsx* and *fru* exons to generate weak 3' and 5' splice sites within the same exon. A slash indicates the junction between *dsx* and *fru* sequences. Lines between the boxes represent introns. Numbers on top of the lines differentiate the two introns. The solid lines above the pre-mRNA illustrate the splicing pathway. **B:** Representative autoradiograph showing splicing efficiencies after 2 h of incubation, splicing efficiency, initial rates, and fold activation for *dsx3'ssMS2/fru5'ss* splicing in 20% HeLa nuclear extract. Lane 1 contains no complementation. Lane 2 is supplemented with 400 nM Tra/Tra2. Lane 3 is supplemented with 200 nM MS2-RS^{9G8}. Lane 4 is supplemented with 400 nM Tra/Tra2 and 200 nM MS2-RS^{9G8}. Identities of unspliced and spliced RNAs are indicated on the right. Splicing efficiencies and fold activation are indicated on the bottom. **C:** Bar graph representing the fold activation of rate constants. X-axis corresponds to the lanes in **B**. Y values represent the fold activation as shown in **B**.

1996; Reed & Palandjian, 1997; Burge et al., 1999). ESEs assist early spliceosomal complex formation by interacting with components of the splicing machinery that make up E complex. By introducing suboptimal 5' and 3' splice sites into test pre-mRNAs, exon definition was made dependent on the activities of two ESEs. The results presented in Figures 1 and 3 demonstrate that the contribution of two ESEs towards exon definition is contingent on whether the suboptimal splice sites they activate are located within the same or on different exons. Based on current views of exon definition, each exon should be recognized by the splicing machinery as an independent unit (Berget, 1995). In agreement with this interpretation, we observe synergistic splicing kinetics with the *fru5'ss/dsx3'ssMS2* construct (Fig. 1). Each exon in the *fru5'ss/dsx3'ssMS2* construct contains a suboptimal splice site that requires the activity of an ESE for sufficient recognition by the splicing machinery. Thus, the initial definition of each exon constitutes an independent and rate-limiting step in intron removal (Fig. 4A).

We were surprised to observe that the activation of a single ESE in the *fru5'ss/dsx3'ssMS2* pre-mRNA led

to any measurable splicing activity (Fig. 1B). In their natural context, the *fru* and *dsx* alternative exons are flanked by exons that contain constitutively active splice sites. Therefore, functional flanking splice sites cannot compensate for the splicing deficiency at the regulated exon. Splicing of the *fru5'ss/dsx3'ssMS2* pre-mRNA, however, requires the activation of two weak juxtaposing splice sites. The observed increase in intron removal by the activation of only one of the two ESEs demonstrates that splicing enhancers can positively influence the recognition of a weak splice site on a neighboring exon. In other words, this result shows that ESEs are capable of activating not only weak splice sites within the same exon, but also weak splice sites flanking adjacent exons. These data extend earlier findings that demonstrated that the *dsxRE* is able to suppress 5' splice site mutations across the intron (Tian & Maniatis, 1993). Further studies will be required to determine if the magnitude of this cross-intron rescue is dependent on intron length.

Our analysis using the *dsx3'ssMS2/fru5'ss* pre-mRNA allows us to conclude that each ESE activates the same rate-limiting step during exon recognition when

A

B

C

FIGURE 4. See legend on facing page.

one exon with two suboptimal splice sites is defined (Fig. 3). These observations strongly suggest that ESE-assisted exon definition, and therefore the recognition of both exon/intron junctions, occurs within the same step during the assembly of the spliceosome. In addition, our results suggest that each ESE is capable of suppressing deficient 5' and 3' splice site signals, most likely by recruitment of U1 snRNP and U2AF. For this to occur within the same rate-limiting step implies that a single ESE complex can simultaneously recruit components of the splicing machinery to both exon/intron junctions (Fig. 4B). Thus, we conclude that ESE complexes can function bidirectionally during exon definition. That is, ESE complexes enhance exon definition by the concurrent recruitment of splicing factors required for the recognition of both 5' and 3' splice sites.

Based on the use of suboptimal splice sites, it is reasonable to assume that the recruitment of U1 snRNP to the 5' splice site and U2AF/U2 snRNP to the 3' splice site is impaired in the test substrates (Blencowe, 2000; Graveley, 2000; Graveley et al., 2001). Because of their well-documented RS domain interactions, SR proteins, such as SC35 and ASF/SF2, have been proposed to act as a bridge between U1 snRNP and U2AF (Wu & Maniatis, 1993; Zuo & Maniatis, 1996). The ESE complexes used here contain at least one SR protein or RS-fusion protein. As it is currently unclear if the proposed interactions between U1 snRNP/U2AF and the enhancer complex (Fig. 4) are direct or indirect, additional SR proteins present in HeLa cell nuclear extracts may be required. Thus, ESEs may nucleate the assembly of protein/protein and protein/RNA networks that ultimately lead to the simultaneous recruitment of U1 snRNP and U2AF (Reed, 1996).

Splicing enhancers recruit a pre-spliceosome to the exon

Previous studies supported a model in which the spliceosome assembled in a stepwise manner onto splice site signals of the pre-mRNA. These observations led us to our first hypothesis that ESEs recruit different splicing factors at distinct steps of assembly. However, our *in vitro* splicing results on *dsx3'ssMS2/fru5'ss* question this model because the kinetic data suggests simultaneous recruitment of splicing factors to both exon/intron junctions. An attractive alternative explanation

consistent with the data presented here would be the ESE-dependent recruitment of a pre-spliceosomal complex containing, at least, U1 snRNP and U2AF (Fig. 4C). Support for the proposed model comes from the recent identification and characterization of a functionally significant particle in yeast containing more than 60 splicing factors, including all five spliceosomal snRNPs (Nilsen, 2002; Stevens et al., 2002). This complex was isolated by glycerol gradient sedimentation and appeared to form the precursor of the active spliceosome independently of the pre-mRNA. In fact, addition of soluble factors from micrococcal nuclease-treated yeast extract allowed the penta-snRNP to become active. Furthermore, it was hypothesized that the nuclease-treated extract is only providing certain pre-mRNA recognition factors and ATPases needed for conformational changes that would allow this pre-spliceosome to become active (Stevens et al., 2002). Due to their ability to interact with several spliceosomal factors, it is likely that SR proteins are components of the metazoan version of this higher order snRNP complex. According to this scenario, SR proteins could tether U1 snRNP, U2AF, U2 snRNP, and other splicing factors in a multiprotein complex to the pre-mRNA in a single step through their interactions with ESEs or exonic splicing enhancer complexes. Future experiments will concentrate on testing the validity of this model.

MATERIALS AND METHODS

RNAs used in splicing reactions

The minigene *fru5'ss/dsx3'ssMS2* contains the female-specific *fru* 5' splice site and the weak *dsx* 3' splice site on different exons, each activated by independent enhancers. MS2-RS^{9G8} activates the *dsx* 3' splice site by recognition of a downstream MS2 hairpin. Tra/Tra2 binds the *fruRE* element to activate the female-specific 5' splice site of *fru*. To construct *fru5'ss/dsx3'ssMS2*, a *Bst*XI fragment from *dsx(70)M2* (Graveley & Maniatis, 1998) was inserted into the *Sma*I/*Pvu*II digested *fruF* minigene (A. Bakshi, B. Lam, F.Y. Ekinci, J.K. Webb, B. Graveley & K. Hertel, in prep.). The minigene *dsx3'ssMS2/fru5'ss* contains the same splice sites and respective enhancers within one internal exon. *dsx3'ssMS2/fru5'ss* was generated by fusing the *dsx* and *fru* minigenes. To construct *dsx3'ssMS2/fru5'ss*, an *Eco*RV/*Pvu*II fragment of the *FruF* minigene was inserted into the

FIGURE 4. Model for enhancer-dependent exon definition. Exons, exonic splicing enhancers, SR proteins, and selected splicing factors are indicated. Suboptimal splice sites are denoted by GU and Py-AG. Arrows denote interactions between or recruitment of splicing factors, SR proteins, and/or splice sites. **A:** Synergy arises when suboptimal splice sites are present on different exons. Enhancer complexes are recruited independently to each exon and activation of each splice site constitutes different rate-limiting steps in exon definition. **B:** When suboptimal splice sites are located within the same exon, each ESE complex activates the same rate-limiting step of exon definition. Each ESE complex simultaneously recruits components of the general splicing machinery required for the recognition of the 5' and 3' splice sites. **C:** SR proteins tethered to exonic splicing enhancers may recruit a higher order spliceosomal complex during exon definition.

SacI site of *dsx*(70)M2 (Graveley & Maniatis, 1998). Insertion at the SacI site of *dsx*(70)M2 leaves two MS2 hairpins in the resulting construct. *fru*5'ss/*dsx*3'ssMS2 was linearized with *Pst*I, in vitro transcribed with SP6 RNA Polymerase (Promega), uniformly labeled with ³²P, and gel purified on 7 M urea polyacrylamide gel. *dsx*3'ssMS2/*fru*5'ss was linearized with *Hind*III, in vitro transcribed with T7 RNA Polymerase (Promega), uniformly labeled with ³²P, and gel purified on 7 M urea polyacrylamide gel.

Recombinant proteins

Recombinant baculoviruses expressing Tra, Tra2, MS2-RS^{9G8}, and their purification were as described previously (Tian & Maniatis, 1992; Lynch & Maniatis, 1996).

In vitro splicing reactions

In vitro splicing reactions for *fru*5'ss/*dsx*3'ssMS2 were performed in 25% HeLa nuclear extract, 1 mM ATP, 20 mM creatine phosphate, 3.2 mM MgCl₂, 5 U RNasin (Promega), 1 mM DTT, 72.5 mM KCl, and 20 mM HEPES, pH 7.9. No proteins, 400 nM Tra/Tra2 and/or 200 nM MS2-RS^{9G8} was supplemented as indicated in Figure 1. For titration experiments, MS2-RS^{9G8} was added from 0–200 nM without Tra/Tra2 or in the presence of 400 nM Tra/Tra2. Total reaction volume was 25 μL. Unless otherwise indicated, splicing reactions were performed for 2 h at 30 °C. Following incubation, reactions were proteinase K digested, phenol chloroform extracted, and ethanol precipitated prior to PAGE separation. In vitro splicing reactions for *dsx*3'ssMS2/*fru*5'ss were performed as for *fru*5'ss/*dsx*3'ssMS2, except 20% HeLa nuclear extract was used.

Quantitation and analysis of splicing reactions

Bands were visualized and quantitated using PhosphorImager analysis (BioRad). Percent spliced is defined as spliced products/(unspliced product + spliced products). To derive kinetic rate constants for *fru*5'ss/*dsx*3'ssMS2, time points (0.75, 1, 1.25, 1.75, and 2 h) were fit to a first-order rate description for product appearance. Rate constants for *dsx*3'ssMS2/*fru*5'ss were derived from initial rates assuming complete conversion of pre-mRNAs to spliced products. Titration data were fit to a single binding isotherm. The background was determined individually for each lane, as the total number of substrate and product counts varied throughout a time course.

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