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Enhancer-dependent 5′-Splice Site Control of fruitless Pre-mRNA Splicing*

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The Drosophila fruitless (fru) gene encodes a transcription factor that essentially regulates all aspects of male courtship behavior. The use of alternative 5′-splice sites generates fru isoforms that determine gender-appropriate sexual behaviors. Alternative splicing of fru is regulated by TRA and TRA2 and depends on an exonic splicing enhancer (fruRE) consisting of three 13-nucleotide repeat elements, nearly identical to those that regulate alternative sex-specific 3′-splice site choice in the doublesex (dsx) gene. dsx has provided a useful model system to investigate the mechanisms of enhancer-dependent 3′-splice site choice. However, little is known about enhancer-dependent regulation of alternative 5′-splice sites. The mechanisms of this process were investigated using an in vitro system in which recombinant TRA/TRA2 could activate the female-specific 5′-splice site of fru. Mutational analysis demonstrated that one 13-nucleotide repeat element within the fruRE is required and sufficient to activate the regulated female-specific splice site. As was established for dsx, the fruRE can be replaced by a short element encompassing tandem 13-nucleotide repeat elements, by heterologous splicing enhancers, and by artificially tethering a splicing activator to the pre-mRNA. Complementation experiments showed that Ser/Arg-rich proteins facilitate enhancer-dependent 5′-splice site activation. We conclude that splicing enhancers function similarly in activating regulated 5′- and 3′-splice sites. These results suggest that exonic splicing enhancers recruit multiple spliceosomal components required for the initial recognition of 5′- and 3′-splice sites.

Alternative pre-mRNA splicing is commonly used to regulate the expression of genes and to enrich the proteomic diversity of higher eukaryotic organisms (1–4). Current estimates suggest that ~60% of human genes are alternatively spliced (5). Enhancer-dependent alternative 5′-splice site choice, genetic approaches have already provided this information for alternative 5′-splice site choice in the

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The abbreviations used are: dsx, doublesex; TRA, Transformer; TRA2, Transformer2; ESE, exonic splicing enhancer; RS, Arg/Ser-rich; SR, Ser/Arg-rich; dsxRE, dsx splicing enhancer repeat element; snRNPs, small nuclear ribonucleoprotein; U2AF, U2 auxiliary factor; ASF, alternative splicing factor; SFS, splicing factor-2; fru, fruitless; fruRE, fru splicing enhancer repeat element; PRE, purine-rich element.
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Drosophila melanogaster fruitless (fru) gene. The detailed study of fru alternative splicing therefore provides an excellent opportunity to expand our limited knowledge on the mechanisms that govern ESE-dependent 5′-splice site regulation. fru is expressed in ~0.5% of the neurons in the central nervous system (44, 45). Cloning of fru demonstrated that sex-specific alternative splicing of the fru pre-mRNA determines the majority of male courtship behavior (38, 46, 47). Like formation of similar, if not identical, enhancer complexes. In contrast to requires TRA and TRA2 to switch from the male-specific pattern (38, 46). However, in contrast to dnx, fru alternative splicing involves the activation of a female-specific 5′-splice site (38, 40, 46). This activation requires an ESE located immediately upstream of the female-specific 5′-splice site (40). Surprisingly, the fru enhancer, designated fruRE, contains three copies of similar 13-nucleotide repeat elements that regulate the female-specific alternative 3′-splice site in dnx (see Table I). Thus, TRA and TRA2 regulate both dnx and fru alternative splicing, presumably through the formation of similar, if not identical, enhancer complexes. In support of this suggestion, it was previously shown that the fruRE is capable of functionally replacing the dnxRE using heterologous pre-mRNAs in transfection assays (40). Interestingly, whereas the dnx enhancer complex activates an alternative 3′-splice site, the fru enhancer complex activates an alternative 5′-splice site. Initial recognition of exon/intron junctions is achieved by several splicing factors (48). U1 snRNP binds to the 5′-splice site by a combination of RNA/RNA and protein/RNA interactions (49, 50). The 3′-splice site is initially recognized by U2AF. The activation of 5′- or 3′-splice sites thus requires the recruitment of a different set of splicing factors to their respective exon/intron junction. Previous studies have demonstrated that each dnxRE interacts with only one spliceosomal target (51). However, in the manner in which the alternative splicing of the fru pre-mRNA is regulated suggests that the TRA/TRA2-dependent dnx and fru ESEs may recruit distinct spliceosomal components to the adjacent intron.

This hypothesis was tested using an in vitro splicing system that allowed an in-depth investigation of the splicing enhancer that controls the recognition of the fru female-specific 5′-splice site. Mutational analysis demonstrated that one of the 13-nucleotide repeat elements present in the fruRE is sufficient to activate the regulated female-specific splice site. As was demonstrated for dnx, the fruRE can be replaced efficiently by a short element encompassing tandem 13-nucleotide repeat elements, by previously characterized heterologous ESEs, and by artificially tethering a splicing activation domain to the pre-mRNA. Finally, complementation experiments demonstrated that SR proteins facilitate ESE-dependent 5′-splice site activation. These experiments show that the fruRE and dnxRE function similarly in activating regulated 5′- and 3′-splice sites, respectively, and suggest that the fru and dnx ESE complexes are capable of recruiting targets that contain multiple spliceosomal components required for the initial recognition of 5′- and 3′-splice sites.

EXPERIMENTAL PROCEDURES

RNAs—To generate the in vitro splicing substrate fruF-long, two fragments generated from fruM′-FwT (a gift from Bruce Baker) (40) were inserted into the same restriction sites of SP73 (Promega): a PraI fragment that contains the common 3′-exon and an EcoRI fragment containing the female-specific 5′-splice site and the upstream splicing enhancer. Substrate fruF was derived from fruF-long by deleting ~560 nucleotides of intronic sequences (XbaI and NcoI digest, followed by blunt ligation). Mutant substrates fruFmut1, fruFmut2, fruFmut3, fruFmut23, and fruFmut123 are derivatives of construct fruF. In all these constructs, the 13-nucleotide repeat sequences have been changed from NNNTCAATGCAAC to NNNGGACGCTTC. To generate these mutations, a three-step PCR method was used. Primers spanning each repeat in forward (fwd) and reverse (rev) directions were used in combination with a reverse primer at the downstream EcoRI site (fruFrev) and an SP6 primer, respectively. The PCR fragments so generated were then annealed together and amplified. After digestion with EcoRI, mutated fragments were inserted into EcoRI-digested fruF backbone, replacing the wild-type fruF EcoRI fragment. The fru primers used were 5′-ACTGGCACGCTACTACCCAAA-3′ (fruFmut1fwd), 5′-GTACGGTCCAGATTTGGTTCCTT-3′ (fruFmut1rev), 5′-TCTGGACTGTACCAATTGCACCTTT-3′ (fruFmut2fwd), 5′-GTAACGTGCACCAAGCACGGCTCTCCGGCA-3′ (fruFmut2rev), 5′-TCAAGCCAGTACTTCAGTCCATG-3′ (fruFmut3fwd), 5′-GTAACGTGCACCAAGCACGGCTCTCCGGCA-3′ (fruFmut3rev), 5′-GTCGGTGTTAACATTGCCTCTTT-3′ (fruFrev). The cryptic splice site, aaag/tgtag (the slash indicates the exon/intron junction), was removed by PCR-based site-directed mutagenesis, resulting in the deletion of nucleotides a/gt. The primers used for site-directed mutagenesis were as follows: 5′-ATTAGGGACAGTATAA-3′ (primer 1fwd), 5′-GCCGCGACACATTTAGTCTCTTTTACATTAGTGTCGCGGCCG-3′ (primer 2fwd), 5′-TAAAGAAGGACTGATTAGGTGGCCGCGCCG-3′ (primer 3fwd), and 5′-GCTGCGAGATGGTTCTCGATCAGG-GTCC-3′ (primer 4rev). The insert was XhoI/SalI-digested and subcloned into fruF+ F1mut, an insert containing the fru male-specific exonic and 5′-splice site sequences was generated by PCR amplification using fruF and fruBglI and cloned into the SstI-XhoI site of pSP72-MS2 (23) digested with XhoI and BamHI. The identities of all constructs were verified by DNA sequencing.

With the exception of fruF/0M1, all constructs were digested with XhoI to yield the SP6 mRNA polymerase transcription templates. Capped, 2P-labeled RNAs were transcribed with SP6 RNA polymerase (Promega). The fruF/0M1 construct was digested with T7 RNA polymerase, Transcripts were gel-purified on a 7 M urea and 4% polyacrylamide gel before use.

In Vitro Splicing Assays—Conditions for in vitro splicing reactions were 30% HeLa nuclear extract (52), 1 mM ATP, 20 mM creatine phosphate, 3.2 mM MgCl2, 5 units of RNasin (Promega), 1 mM dithiothreitol, 72.5 mM KCl, 3% polyvinyl alcohol (30–70 KDa), and 12 mM Hepes (pH 7.9). Reactions for TRA/TRA2-dependent substrates were performed in the absence or presence of 500 nM TRA/TRA2, unless otherwise stated. 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. Bands were visualized and quantitated using PhosphorImager analysis (Bio-Rad). Percent spliced is defined as follows: spliced products/unsliced product + spliced products). The background was determined individually for each lane because the total number of substrate and product counts varied throughout a time course. The fold activation of the female-specific spliced product was calculated by dividing the percent female-specific spliced product in the presence of 500 nM TRA/TRA2 by the percent female-specific spliced product in the absence of TRA/TRA2. Comparison of female- and male-specific (or cryptic) splice site strength was performed by taking the ratio of percent female-specific spliced product to percent cryptic spliced product in fruF. S100 complementation experiments were performed essentially as described above, except that the HeLa cell cytoplasmic extract was digested with S100 (500 units/mL) and 200 U/mL RNase in 500 mM KCl, 20 mM Hepes (pH 7.9). Reactions were complemented with varying amounts (0–800 nM) of the baculovirus-produced SR protein 9G8. In vitro splicing reactions using the MS2-RS fusion proteins were carried out using 15% HeLa cell nuclear extract as described (53), except that the reac-
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In Vitro Splicing of fru Minigenes—As observed for dax, the products of the sex determination genes tra and tra2 switch fru splicing from the male-specific pattern to the female-specific pattern through the activities of an ESE that contains multiple, near-identical 13-nucleotide repeat elements (38). However, whereas the dax enhancer complex activates an alternative 3′-splice site, the fru enhancer complex activates an alternative 5′-splice site. To investigate the mechanisms of this process, an in vitro splicing system was established in which recombinant TRA/TRA2 could activate the female-specific 5′-splice site of fru. Similar to the successful approach taken to unravel the mechanisms of enhancer-dependent 3′-splice site activation in dax, this assay relies on spliceosomal activities supplied from HeLa cell nuclear extract supplemented with the Drosophila-specific recombinant splicing factors TRA and TRA2 (28).

RESULTS

To evaluate the efficiency of TRA/TRA2-dependent splice site activation, a series of titration experiments were performed. As demonstrated in Fig. 1C (upper panel), high concentrations of TRA/TRA2 resulted in significant activation of the female-specific 5′-splice site. At lower concentrations of TRA/TRA2, the female-specific 5′-splice site was only marginally activated. These observations suggested that TRA and TRA2 cooperatively influence 5′-splice site choice. Indeed, quantitation of the data in Fig. 1C (lower panel) clearly illustrates the sigmoidal nature of the binding curve, characteristic of synergistic assembly of multicomponent complexes. These observations mirror the TRA/TRA2-dependent activation profile determined for dax (51), thus providing support for the notion that the daxRE and fruRE assemble into highly related splicing complexes. Supplementation of splicing reactions with TRA alone did not lead to any measurable activation of the female-specific splice site (data not shown). The dax pre-mRNA was used as a control to evaluate the activity of the recombinant proteins as well as to compare the efficiencies of TRA/TRA2-dependent splice site activation in the dax and fru systems (data not shown). PhosphorImager analysis indicated similar splicing efficiencies between the fruF and dax pre-mRNAs (∼50% spliced/unspliced at 2 h). However, the efficiency of fruF-long pre-mRNA splicing was observed to be ∼25%. Together, these results demonstrate that fru female-specific 5′-splice site activation can be faithfully recapitulated in vitro using HeLa cell nuclear extracts that are complemented with the Drosophila recombinant splicing activators TRA and TRA2.

Relative Contribution of Each 13-Nucleotide Repeat Element to Female-specific 5′-Splice Site Activation—To study the relative contribution of each repeat element, a series of mutant fruF substrates were tested in the presence or absence of saturating concentrations of TRA/TRA2 (300 nM). The fruFmut1, fruFmut2, fruFmut3, fruFmut23, and fruFmut123 pre-mRNAs were derived from fruF by mutating the sequence of each 13-nucleotide repeat element (Fig. 2A) (see “Note Added in Proof”). In the absence of TRA/TRA2, the female-specific 5′-splice site was not utilized in any of these constructs (Fig. 2B, lanes 1–6). However, in the presence of TRA/TRA2, the female-specific 5′-splice site was activated for each pre-mRNA containing at least one 13-nucleotide repeat element (lanes 7–11). Moreover, there was no significant difference in the levels of TRA/TRA2-dependent female-specific splicing, regardless of which repeat was mutated (Fig. 2, B and C). Importantly, TRA and TRA2 did not activate female-specific splicing of the fruFmut123 pre-mRNA, in which all three 13-nucleotide repeats have been mutated (Fig. 2B, lane 12), indicating that at least one repeat is necessary for TRA/TRA2-dependent splicing. By comparing the difference in the observed -fold activation of the female-specific spliced product for the tested substrates when TRA/TRA2 was added (Fig. 2C), it can be inferred that the presence of a single repeat is both necessary and sufficient for maximal activation of the female-specific 5′-splice site. We conclude that the 13-nucleotide repeat elements are the only splicing regulatory elements that significantly contribute to the activation of the fru female-specific 5′-splice site.

Regulation of the fru Female-specific 5′-Splice Site Requires Competing Splice Sites—Because TRA is expressed only in female flies (54), a possible mechanism of regulation is that the female-specific 5′-splice site is weak and requires ESEs for activation (40). Thus, in male flies, in which TRA is not expressed, the default splicing pathway favors the stronger male-specific splice site. In support of this hypothesis, the sequences for the male-specific, cryptic, and female-specific 5′-splice site are TAG/gtaag, Aaa/gtaag, and GCC/gtaag, respectively. Compared with the 5′-splice site consensus sequence (YAG/gttag), we predicted that the male-specific splice site would be the strongest, followed by the female-specific splice site and finally the cryptic splice site. However, in vitro splicing reactions of fruF indicated that the cryptic 5′-splice site 150 nucleotides upstream of the female-specific splice site, positioned in between the first and second repeat elements (Fig. 3A), was efficiently utilized (Figs. 1B, 2B, and 3B). Therefore, in the case of fru, splice site usage does not correlate well with splice site strength based on a consensus sequence match (55). Quantitation of the female-specific spliced product over the cryptic spliced product revealed that the cryptic splicing pathway was dominant and independent of TRA/TRA2 (Fig. 3B, lanes 1–3). Upon addition of TRA/TRA2, the ratio of female-specific splice site choice to cryptic splice site choice increased by ∼65-fold (Fig. 3C). To investigate TRA/TRA2-dependent regulation of the female-specific 5′-splice site without interference from constitutive splice sites, the cryptic 5′-splice site was deleted by site-directed mutagenesis (Fig. 3A). Surprisingly, removal of the competing splice site resulted in loss of regulation by TRA/TRA2 (Fig. 3B, lanes 4–6). Quantitation showed equal efficiency of female-specific splice site activation in the presence and absence of TRA/TRA2 (Fig. 3, B and D), even at decreased levels of nuclear extract (data not shown). These results indicate that activation of the fru female-specific 5′-splice site
requires the presence of competing 5'-splice sites. Although the fru female-specific 5'-splice site presumably provides a suboptimal binding site for spliceosomal components, recognition of the female-specific 5'-splice site by the spliceosome occurs efficiently in the absence of competing 5'-splice sites. Therefore, alternative 5'-splice site choice in the fru pre-mRNA is potentiated in part by the relative activities of competing splice sites.

Other Well Characterized ESEs Can Replace the fruRE—Two tandem 13-nucleotide repeat elements have been shown to be sufficient to activate the TRA/TRA2-dependent 3'-splice site in dsx (51, 56) or to trigger constitutive activation when moved closer to the regulated 3'-splice site (30, 57). In addition, many other ESEs have been shown to activate the weak female-specific 3'-splice site of dsx (58) and other pre-mRNAs. To test if other ESEs can substitute for the fru RE, we prepared two different pre-mRNAs that contained the male- and female-specific 5'-splice sites. As outlined in Fig. 4A, the fruRE was replaced in the context of competing male- and female-specific 5'-splice sites either with two tandem 13-nucleotide repeat elements, fruM+F2Rs, or with an ESE containing both the well

![Diagram](image-url)
Figure 3. **fru female-specific 5′-splice site strength.** A, schematic of the fruF pre-mRNA construct as described in the legend to Fig. 1, B, autoradiograph of in vitro splicing reactions performed with fruF constructs. Lanes 1–3, fruF; lanes 4–6, fruF with the cryptic splice site deleted. The reactions in lanes 1 and 4 contained unspliced pre-mRNAs. The reactions in lanes 2 and 5 contained no TRA/TRA2. The reactions in lanes 3 and 6 were complemented with 500 nM TRA/TRA2. The identities of unspliced and spliced RNAs are indicated on the right. C, bar graph comparing female-specific and cryptic splice site activation in the fruF construct. D, bar graph depicting the fold activation of the female-specific spliced product upon addition of TRA/TRA2 in fruF and in fruF with the cryptic splice site deleted. *nt*, nucleotides.

Figure 4. **Heterologous splicing enhancers activate the fru female-specific 5′-splice site.** A, RNA substrates used. Symbols are as described in the legends to Figs. 1 and 2. The black boxes represent the upstream male-specific exon. M and F indicate the positions of the male- and female-specific 5′-splice sites, respectively. 2Rs indicates two 13-nucleotide (nt) repeat elements placed 10 nucleotides upstream of the fru female-specific 5′-splice site. The box 624/PRE indicates replacement of the fruRE with a pyrimidine (624)/purine (PRE)-rich enhancer. B, left panel, autoradiograph of in vitro splicing reactions. The identities of the spliced products are indicated on the right. The female-specific (*) and male-specific (×) products are indicated. Right panel, bar graph showing the fold activation of female- to male-specific 5′-splice site usage. Each value was normalized to the ratio observed for fruM+F1mut. C, autoradiograph of S100 complementation reactions performed with fruM+F2Rs. The recombinant SR protein 9G8 was added to S100 splicing reactions to final concentrations of 200, 400, and 800 nM. The identities of the spliced products are indicated on the right. *NE*, HeLa cell nuclear extract.

Characterized dax purine-rich element (PRE) and a pyrimidine-rich enhancer element isolated from in vitro selection experiments (designated 624), fruM+F624/PRE (59). As a control, we tested fruM+F1mut, which contains a single mutated repeat element. These ESES were placed 10 nucleotides upstream of the regulated 5′-splice site to promote constitutive activation by SR proteins present in HeLa cell nuclear extracts. As expected, male-specific 5′-splice site usage was by far the predominant splicing pathway of fruM+F1mut in the absence of TRA and TRA2 (Fig. 4B, lane 2). Replacement of the fruRE either with tandem 13-nucleotide repeats (fruM+F2Rs) or with a purine- and pyrimidine-rich ESE (fruM+F624/PRE) significantly activated the female-specific 5′-splice site (lanes 4 and 6). Quantitative analysis revealed that the ratio of female- to male-specific splicing changed up to 150- and 30-fold for fruM+F2Rs and fruM+F624/PRE, respectively, compared with the fruM+F1mut pre-mRNA. Thus, the 2Rs ESE in fruM+F2Rs is clearly more potent in directing the splicing machinery to the female-specific splice site compared with the 624/PRE ESE in fruM+F624/PRE. To test if activation of the female-specific splice site is SR protein-dependent, we tested fruM+F2Rs splicing in SR protein-deficient cytoplasmic extracts (S100). As illustrated in Fig. 4C, the addition of the recombinant SR protein 9G8, which interacts with the 13-nucleotide repeat element, complemented S100 experiments in a concentration-dependent manner (lanes 5–7). We conclude that heterologous ESES can qualitatively substitute for the fruRE. As demonstrated for ESE-dependent 3′-splice sites, the activation of the female-specific 5′-splice site in fru can be mediated by at least one member of the SR protein family.

Different RS Domains Vary in Their Ability to Activate the fru 5′-Splice Site—Similar to dax, TRA and TRA2 complement

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*a* K. J. Hertel, unpublished data.
the **fru** RE, and heterologous ESEs recognized by SR proteins can efficiently activate the **fru** female-specific 5′-splice site *in vitro*. It has previously been shown that hybrid proteins containing the MS2 bacteriophage coat protein fused to a human RS domain can activate the *dsx* female-specific 3′-splice site when artificially tethered to the pre-mRNA (53). Thus, the MS2-RS fusion proteins can functionally substitute for the *dsx* ESE complex. To test whether the same is true for the **fru** ESE, we generated **fru**(70)M1, in which the **fru** enhancer has been replaced by a single MS2-binding site (Fig. 5A). This pre-mRNA was incubated in HeLa cell nuclear extract in the presence or absence of saturating concentrations of a variety of MS2 fusion proteins (Fig. 5B, left panel). The **fru**(70)M1 pre-mRNA was poorly spliced in the absence of fusion protein (lane 1) and in the presence of the MS2 protein lacking an RS domain (lane 2). In contrast, the addition of any of three different fusion proteins containing a natural RS domain (MS2-RE, MS2-SP23RS, or MS2-RSp55) activated splicing of the **fru**(70)M1 pre-mRNA (lanes 3–5). Similar results were obtained with the same proteins on the *dsx*(70)M1 pre-mRNA (Fig. 5B, right panel, lanes 1–5). The relative potency of each fusion protein was calculated in comparison with the most potent activator of each pre-mRNA, MS2-RS<sup>GR</sup>, which was arbitrarily set at a value of 1.0. This analysis revealed that the relative potency of each activator was similar on each of the two pre-mRNAs (Fig. 5C). This suggests that the RS domains within enhancer complexes may be involved in similar types of protein/protein interactions to activate 5′- or 3′-splice sites.

**DISCUSSION**

The TRA/TRA2-dependent activation of the *dsx* 3′-splice site has served as an excellent model system to study the mechanisms of enhancer-dependent 3′-splice site activation. Similarly, our analysis of the TRA/TRA2-dependent regulation of **fru** alternative splicing should provide an analogous mechanistic framework for the regulation of enhancer-dependent alternative 5′-splice site choice. Overall, the structural and functional resemblance of the **fru**RE and *dsx*RE is remarkable. Both ESEs consist of multiple, highly conserved 13-nucleotide repeat elements that are dispersed throughout a stretch of ~300 untranslated nucleotides. However, the distance between the first repeat element and the regulated splice site is noticeably different: only 38 nucleotides separate the enhancer and the splice site in **fru** compared with 300 nucleotides in *dsx*. As observed for *dsx*, heterologous ESEs that function in the absence of TRA and TRA2 can substitute for the **fru**RE. In addition, previous work demonstrated that the **fru**RE could activate heterologous 5′- and 3′-splice sites (40).

Thus far, the only discernible difference between the activities of the *dsx*RE and **fru**RE appears to be the manner in which multiple repeat elements affect splice site activation. Our data indicate that only one repeat is both necessary and sufficient to maximally activate female-specific splicing of the **fru** pre-mRNA (Fig. 2). This differs from the situation in *dsx*, where splicing efficiency is directly proportional to the number of repeat elements present (51). These observations do not necessarily indicate that multiple repeat elements are not able to further augment spliceosomal recognition of the female-specific 5′-splice site. However, they demonstrate that multiple repeats do not further increase the measured rate-limiting step that is reached by a single repeat element *in vitro*. Thus, the recruitment of the splicing machinery to the female-specific 5′-splice site in **fru** may require less assistance from splicing enhancers. One repeat element could then be sufficient to lower the activation barrier. However, the use of a slightly different pre-mRNA substrate design in the splicing assays might provide an alternative explanation for this difference. Unlike the *dsx* substrates used, all **fru** pre-mRNAs tested in Fig. 2 contain a competing cryptic splice site. As has been argued recently, it is very likely that the nature of the splice site competition amplifies relatively small changes in ESE-induced splice site strength (60).

Mutation of all three repeat elements led to the loss of TRA/TRA2-dependent 5′-splice site activation. These results are in agreement with earlier cell transfection studies (40) and demonstrate that the 13-nucleotide repeat elements are the only cis-acting sequences that significantly contribute to female-specific 5′-splice site activation in **fru**. In addition, the conservation of the repeat elements (Table I), the thermody-
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Table I
Comparison of 13-nucleotide repeat elements in dsx and fru

| Repeat no. | dsx            | fru            |
|------------|----------------|----------------|
| 1          | UCUUCAAUCAACA  | ACUUCAUCAUCAACA |
| 2          | UCUUCAAUCAACA  | UCUUCAAUCAACA  |
| 3          | UCUUCAAUCAACA  | UCUUCAAUCAACA  |
| 4          | UCUUCAAUCAACA  | NA             |
| 5          | UCUUCAAUCAACA  | NA             |
| 6          | UCUUCAAUCAACA  | NA             |

FIG. 1. NA, not applicable.

Mechanism of ESE-dependent Splice Site Activation—Given the extensive analogies to enhancer-dependent activation of 3′-splice sites, it is reasonable to suggest that the fru splicing enhancer complex increases the local concentration of the splicing machinery at the regulated fru female-specific 5′-splice site. It is well established that 3′- and 5′-splice sites are initially recognized by different components of the splicing machinery (61). How then is it possible that the seemingly identical TRA and TRA2 enhancer complexes assembled on the daxRE and fruRE recruit different targets and with different directionality? One potential explanation stems from the domain structure of TRA2, one of the protein components of the enhancer complex (31). TRA2 contains two separate RS domains, one on the N terminus and the other on the C terminus (62). It is conceivable that each of the bipartite RS domains is responsible for the recruitment of specific factors to either the 5′- or 3′-splice site (Fig. 6A). In three-dimensional space, these RS domains could be located on opposing ends of TRA2, therefore providing directionality. However, the demonstration that the fruRE complexes can be replaced by other RS proteins or by MS2-RS fusion proteins that do not have bipartite RS domain structures does not lend strong support for this explanation. An alternative model proposes the presence of cell-specific splicing factors that mediate interactions between the enhancer complex that activates the 5′-splice site and particular components of the splicing machinery (Fig. 6B). Although we cannot rule out this model, it is not very likely to occur because all heterologous ESEs tested activated the female-specific 5′-splice site, suggesting that the presumed additional factor interacts with any RS domain indiscriminately. In addition, all MS2-RS fusion proteins tested activated both 5′- and 3′-splice sites to similar extents.

Comparison of the fundamental principles that govern dsx and fru alternative splicing demonstrated striking parallels between the action of ESEs on regulated 5′- and 3′-splice sites. These observations could imply that splicing enhancers employ the same mechanisms to increase 3′- or 5′-splice site recognition. Therefore, an attractive interpretation of the data presented here suggests a model in which the daxRE and fruRE recruit identical components not specifically to splice sites, but to the regulated exon (Fig. 6C). Consistent with the proposal that ESEs simultaneously assist 3′- and 5′-splice site recogni-
tion (63), these components might be the metazoan version of a pre-assembled pentas spliceosome similar to the one recently described in yeast (64, 65). The prespliceosome must contain, at the minimum, the components required for initial recognition of the 5′- and 3′-splice sites. Alternatively, the enhancer complex might interact with a master assembly factor, such as the splicing coactivator SRm160 (22), that may provide a more stable platform for the assembly of the spliceosome. No doubt, further work is necessary to evaluate ESE-dependent occupancy at the regulated 5′-splice site and to determine the direct interactions by which SR protein-dependent splicing enhancer complexes activate regulated splice sites.

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Note Added in Proof—Since acceptance and on-line publication of this report we have discovered in a follow-up experiment of the mutational analysis described in Fig. 2 that the substrates fraFmut23 and fraFmut1 did not contain the anticipated mutations within the repeats. To rectify this discrepancy, fraFmut23 and fraFmut1 were remade. After sequence verification, the in vitro splicing experiments described in Fig. 2 were carried out three times. The results obtained with the corrected mutant substrates were significantly different, suggesting an alternate interpretation of the mutational analysis of the fraRE. Unlike what was observed in Fig. 2, activation of the female-specific 5′-splice site was detected at maximal levels for fraFmut23 in the absence of TRA and TRA2 (lane 5), while all other substrates tested behaved as illustrated in Fig. 2 (lanes 1–4 and 6). The presence of TRA and TRA2 increased female-specific 5′-splice site usage only slightly for fraFmut23 (1.3-fold) but significantly for wild type (6.5-fold), fraFmut3 (6-fold), and fraFmut2 (4-fold) (lanes 7–12). Similar to the triple mutant fraFmut123, fraFmut1 was not able to support female-specific splice site activation even in the presence of TRA and TRA2 (lanes 5, 6, 11, and 12). Efficient cryptic splice site usage depended on the presence of at least one upstream 1193-base repeat element. Thus, cryptic splice site choice was severely decreased for fraFmut23 and fraFmut123 (lanes 5, 6, 11, and 12).

The repeated experiments agree with the conclusion of our study that one repeat element is sufficient to promote female-specific 5′-splice site usage. However, the new data do not support the notion that each repeat element is equally efficient in this activity. Rather the data suggest that repeats 2 and 3 do not directly participate in female-specific splice site activation as their enhancement appears to be solely concentrated on activating the cryptic 5′-splice site. These observations are consistent with the following model. The 13-nucleotide splicing enhancer elements 3 and 2 almost exclusively activate the cryptic splice site, while repeat 1 is responsible for the activation of the female-specific 5′-splice site. This observation is significant as it further demonstrates that the location of splicing enhancer elements relative to potential splice sites dictates the nature of exon definition. The new data, consistent with the conclusion that the nucleotide repeat elements are the only regulatory elements that promote activation of the female-specific splice site, however, with the added complexity that repeats 2 and 3 establish the preference of a competing splicing pathway. The fact that fraFmut23 activates female-specific splicing in the absence of TRA and TRA2 is also consistent with the observations from dax studies that 13-nucleotide repeat elements have constitutive enhancing potential as long as they are within close proximity to the regulated splice site and as long as no alternate splicing pathways of preference exist.

While the repeated experiments influence the interpretation of the mutational analysis described in Fig. 2, they do not alter the major conclusions of the study as outlined in the abstract.