Synergistic interactions between two distinct elements of a regulated splicing enhancer

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Regulated alternative splicing of *doublesex* (*dsx*) pre-mRNA requires a splicing enhancer designated the *dsx* repeat element (*dsxRE*) that contains six copies of a 13-nucleotide repeat sequence. Previous studies have shown that the activity of the *dsxRE* requires the splicing regulators Transformer (Tra) and Transformer 2 (Tra2), and one or more members of the SR family of general splicing factors. In this paper we identify a purine-rich enhancer (PRE) sequence within the *dsxRE*, and show that this element functionally synergizes with the repeat sequences. In vitro binding studies show that the PRE is required for specific binding of Tra2 to the *dsxRE*, and that Tra and SR proteins bind cooperatively to the *dsxRE* in the presence or absence of the PRE. Thus positive control of *dsx* pre-mRNA splicing requires the Tra- and Tra2-dependent assembly of a multiprotein complex on at least two distinct enhancer elements.

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A well-characterized example of the regulation of alternative pre-mRNA splicing is the sex determination pathway in *Drosophila melanogaster* (for review, see Baker 1989; Steinmann-Zwicky et al. 1990; Maniatis 1991; Rio 1992). In the final step of this pathway the female-specific Transformer (Tra) protein, together with Transformer 2 (Tra2), a protein expressed in both males and females, positively controls the alternative splicing of *doublesex* (*dsx*) pre-mRNA (Nagoshi et al. 1988; McKown et al. 1988). The male (default) splicing of *dsx* pre-mRNA produces an mRNA consisting of exons 1–3, 5, and 6, encoding a transcription factor required for male sexual differentiation. Conversely, the female (regulated) pattern of *dsx* pre-mRNA splicing results in an mRNA containing exons 1–4, which encodes a transcription factor required for female sexual differentiation (Burtis and Baker 1989; Fig. 1A).

The Tra/Tra2 regulation of *dsx* pre-mRNA splicing provides a model for understanding positive control of alternative splicing. The female-specific 3' splice site immediately upstream of the fourth exon deviates significantly from the consensus sequence and thus in males is not recognized by the splicing machinery (Burtis and Baker 1989). Use of this splice site in females requires the proteins Tra and Tra2 as well as a regulatory element located within the fourth exon (Hedley and Maniatis 1991; Hoshijima et al. 1991; Ryner and Baker 1991; Tian and Maniatis 1992). This regulatory element, located 300 nucleotides downstream of the female-specific 3' splice site, is characterized by the presence of six 13-nucleotide repeat sequences (see Fig. 1B), and hence is

referred to as the *dsx* repeat element (*dsxRE*) (Burtis and Baker 1989; Nagoshi and Baker 1990; Tian and Maniatis 1993).

Tra2 binds specifically to the *dsxRE* (Hedley and Maniatis 1991), and both Tra and Tra2 are found in a multiprotein complex assembled on the *dsxRE* in vitro (Tian and Maniatis 1992; 1993). This complex also contains SR proteins (Tian and Maniatis 1993), a family of general splicing factors characterized by the presence of an RNA-binding domain (RNP domain) and a serine/arginine (SR)-rich region (for review, see Lamm and Lamond 1993; Horowitz and Krainer 1994). Similar to the SR proteins, Tra2 contains both an RNP and an SR domain, whereas Tra contains only an SR domain (Boggs et al. 1987; Amrein et al. 1988). The SR domain is required for the in vitro splicing activity of SR proteins (Caceres and Krainer 1993; Zuo and Manley 1993), and it has been implicated in both protein–protein interactions and subnuclear localization (Krainer et al. 1991; Fu and Maniatis 1992; Zahler et al. 1992; Wu and Maniatis 1993).

The involvement of SR proteins in the regulation of *dsx* female-specific splicing was directly demonstrated by in vitro splicing studies showing that SR proteins are required, in addition to Tra and Tra2, to form a functional complex that commits *dsx* pre-mRNA to the female-specific pattern of splicing (Tian and Maniatis 1993). Protein–protein interaction studies revealed that Tra and Tra2 interact with each other, with SR proteins, and with other general splicing factors (Wu and Maniatis 1993; Amrein et al. 1994). These observations suggest that the multiprotein complex assembled on the *dsxRE*

Figure 1. The sex-specific pattern of *dsx* pre-mRNA alternative splicing and the *dsx* splicing enhancer (*dsxRE*). (A) Open regions represent common exons, the light hatched region (exon 4) is the female-specific exon, and dark hatched regions (exons 5 and 6) are male-specific exons. Lines between the boxes represent introns, and the lines above and below the pre-mRNA illustrate the female- and male-specific patterns of alternative splicing. Sites of cleavage and polyadenylation are labeled poly A. (B) Enlargement of the female-specific exon 4 showing the organization of the *dsxRE*. Numbers above the fourth exon indicate the number of nucleotides from the beginning of the exon. Numbered regions indicate the positions of the repeat sequences, with the consensus sequence shown in the box below. The region labeled PRE indicates the position of the purine-rich region with the sequence indicated below.

facilitates the assembly of spliceosomal components on the weak female-specific 3' splice site through a network of protein-protein interactions (Tian and Maniatis 1993; Wu and Maniatis 1993; Amrein et al. 1994). The ability of the *dsxRE* to activate heterologous 3' splice sites led to the proposal that the *dsxRE* is a regulated splicing enhancer, similar in function to transcriptional enhancers (Tian and Maniatis 1993).

Constitutive splicing enhancers have been identified and characterized in a number of pre-mRNAs. These enhancers are located within exon sequences located downstream from introns containing a weak 3' splice site (Watakabe et al. 1993; Xu et al. 1993; Lavigne et al. 1993; Tanaka et al. 1994) and, in one case, a weak 5' splice site (Dirksen et al. 1994; Sun et al. 1993a,b). A characteristic feature of these enhancers is that they are rich in purines and, similar to the *dsxRE*, they are specifically recognized by SR proteins and U1 snRNP (Lavigne et al. 1993; Sun et al. 1993b; Staknis and Reed 1994). However, there are two important differences between the purine-rich and *dsx* splicing enhancers. First, the purine-rich enhancers are constitutively active, whereas the *dsxRE* requires the regulatory proteins Tra and Tra2. Second, purine-rich enhancers must be located

within 100 nucleotides of the regulated intron, whereas the *dsxRE* can function at least 500 nucleotides away (Tian and Maniatis 1994). Significantly, however, the *dsxRE* can function as a constitutive enhancer when located within 100 nucleotides of the regulated intron (Tian and Maniatis 1994).

The six 13-nucleotide repeat sequences in the *dsxRE* bear little resemblance to purine-rich enhancers. The consensus sequence of the *dsx* repeat is UCUUCAUACAACA, whereas a comparison of various purine-rich sequences (Tanaka et al. 1994) reveals a common motif of GAAGGA. A closer examination of the *dsxRE*, however, revealed a previously unnoticed near-consensus purine-rich sequence between repeats 5 and 6 (Fig. 1B). Thus the *dsxRE* may consist of two types of regulatory elements, the repeat sequences R1-6, and the purine-rich enhancer (PRE). In this paper we investigate the role of these two sequences in the function of the *dsxRE*. We show that either the R1-5 or the PRE alone can constitutively activate splicing when located near the intron, but both are required for efficient Tra/Tra2-dependent activation of female-specific splicing at a distance. In vitro binding studies with recombinant proteins show that both the repeats R1-5 and the PRE are required for

specific binding of Tra, Tra2 and SR proteins. We conclude that the *dsx*RE splicing enhancer complex is assembled through cooperative interactions between Tra, Tra2 and the SR proteins, and that these interactions require both the repeat sequences and the PRE.

Results

The dsx repeats R1-5 and the PRE are distinct constitutive splicing enhancer elements

The PRE includes two direct repeats of the sequence AAAGGAC, which differs at only one position from the GAAGGAC sequence found in the IgM M2 exon, the bGH exon 5 and many other purine-rich enhancers (Tanaka et al. 1994). We therefore investigated the role of the PRE in the constitutive and regulated activities of the *dsx* splicing enhancer. The constitutive splicing activity was assayed by placing the *dsx*RE containing or lacking the PRE within 100 nucleotides of the female-specific 3' splice site. The role of the PRE in the Tra- and Tra2-dependent activity of the *dsx*RE was examined by placing the same elements 300 nucleotides downstream (Tian and Maniatis 1994).

As shown in Figure 2 the *dsx*RE sequence containing both the repeats R2-5 and the PRE can function as a splicing enhancer in the absence of Tra and Tra2 when positioned close to the regulated intron (R2-5PRE; lanes 1–3). However, either the repeat sequences alone (R2-5; lanes 4–6), a synthetic repeat sequence (R2x4; lanes 7–9)

or a single copy of the PRE (*dsx*PRE; lanes 10–12) can constitutively activate the weak 3' splice site as efficiently as the intact *dsx*RE. We note that the presence of the PRE alone (*dsx*PRE) appears to stabilize this RNA during incubation in nuclear extract. As expected, little splicing is observed in the absence of any enhancer sequences (*dsx*ΔE; lanes 13–15), and the addition of Tra and Tra2 does not further stimulate the constitutive activation of splicing (Fig. 2, lanes 3, 6, 9, 12, 15; Tian and Maniatis 1994). We conclude that the *dsx*RE contains at least two distinct splicing enhancer elements that can function constitutively when positioned close to the *dsx* female-specific 3' splice site.

Both the dsx repeats R1-5 and the PRE are required for Tra- and Tra2-dependent splicing enhancer activity

In marked contrast to the constitutive enhancer activities described above, neither four repeat sequences (R2-5 and R2x4; Fig. 3A, lanes 10–12, 13–15) nor the PRE (*dsx*PRE; lanes 16–18) are sufficient to activate splicing when moved to their natural position 300 nucleotides downstream of the regulated 3' splice site, even in the presence of Tra and Tra2. Instead, both the *dsx* repeats and the PRE are required for efficient Tra/Tra2-dependent activation of female-specific splicing.

When five repeats are positioned at their wild-type spacing from the female-specific 3' splice site, deletion of the purine-rich element causes a significant decrease

Figure 2. Either the *dsx* repeat sequences or the PRE alone can function as constitutive splicing enhancers. As indicated by the schematic below the autoradiograph, all of the *dsx* RNA substrates contain part of exon 3, the entire regulated intron, and 100 nucleotides of exon 4. The substrates differ from each other by the presence or absence of different regions of the *dsx*RE. (R2-5PRE) The *dsx*RE lacking repeats 1 and 6; (R2-5) the *dsx*RE lacking repeats 1 and 6 and the PRE; (R2x4) a synthetic enhancer consisting of four copies of repeat 2; (*dsx*PRE) the PRE alone; (*dsx*ΔE) the RNA substrate without the *dsx*RE. Splicing intermediates were not detected because the splicing reactions were incubated for 2 hr. (NE) Nuclear extract; (– and +) pre-mRNA analyzed before or after the splicing reaction, respectively. Approximately one-tenth as much RNA was loaded in the NE – lanes as was used in the splicing reactions. Tra + Tra2 + and – indicate the presence or absence of these proteins, respectively, in the splicing reactions. The band directly below the precursor in lanes 11 and 12 is presumably a lariat intermediate. Arrowheads indicate spliced product.

Figure 3. Both the repeat sequences and the PRE are required for efficient Tra/Tra2-dependent activation of splicing. (A) As indicated by the schematics below the autoradiograph, the *dsx* RNA substrates are similar to those shown in Fig. 2 except that the *dsx*RE segments are located 300 nucleotides downstream from the 3' splice site. (R1-5PRE) The *dsx*RE lacking only repeat 6; (R1-5) the *dsx*RE lacking repeat 6 and the PRE. The indicated nanograms of Tra and Tra2 added to reaction are approximations based on Bradford assays. Spliced products resulting from the use of cryptic splice sites are labeled Cr. Arrowheads indicate correctly spliced product. (B) In vitro splicing reactions carried out with constructs containing either a wild type or mutant PRE. R1-5mPRE contains six point mutations in the PRE, thus changing the sequence from AAAGGACAAAGGACAAAA to AATCGACCATCGACAAA, with underlined residues indicating sites of mutations.

in the efficiency of splicing, especially at low concentrations of Tra/Tra2 (Fig. 3A; compare lanes 2 and 5). Consistent with previous results, splicing efficiency also decreases when the number of repeats is reduced from five to four in the presence of the PRE (R1-5PRE; Fig. 3A, lanes 1–3; compare to R2-5PRE, lanes 7–9). However, in the absence of the PRE, four repeats in their native context (R2-5; Fig. 3A, lanes 10–12) or as synthetic repeats (R2x4, lanes 13–15) are essentially inactive even in the presence of high levels of Tra/Tra2. We conclude that synergistic interactions between the repeat sequences and PRE are required for the Tra- and Tra2-dependent activity of the *dsx*RE.

A significant loss of activation efficiency was observed when the PRE was deleted. To determine whether the purine content of the PRE is required for its activity we introduced multiple base substitutions into the PRE (see legend to Fig. 3B). Pre-mRNA containing base substitutions in the PRE (Fig. 3B, R1-5mPRE, lanes 6–10) requires three- to fivefold more Tra and Tra2 than the wild-type RNA substrate (R1-5PRE, lanes 1–5; compare lanes 2 and 3 with 8 and 9, respectively) to achieve the same level of splicing activity. The failure of these base substitutions to completely inactivate the enhancer is likely because the primarily cytosine-substituted sequence has residual constitutive splicing enhancer activity (Tanaka et al. 1994). This possibility is consistent

with the observation that a nonpurine-rich sequence can function as a constitutive enhancer, albeit less well than the purine-rich sequence (Staknis and Reed 1994).

Deletion of the PRE decreases the affinity of Tra2 and SF2, but not SC35, for the dsxRE

To investigate the mechanism of the synergistic interactions between the *dsx* repeat sequences and the PRE we carried out in vitro nitrocellulose binding experiments. Previous studies demonstrated that Tra and Tra2 along with SR proteins (namely SC35 and SRp55) are sufficient to form a functional regulatory complex on the *dsx*RE (Tian and Maniatis 1993). We therefore studied the binding of purified recombinant Tra, Tra2, SC35 and SRp55, as well as SF2/ASF, to the *dsx*RE containing or lacking the PRE. It is important to note that these RNAs do not contain splice sites. Thus we do not address the question of how these proteins facilitate enhancer–3' splice site interactions.

Figure 4 shows the binding curves obtained with each protein and RNAs containing repeats 2–6 (R2-6), repeats 2–5 including the PRE (R2-5PRE), or repeats 2–5 lacking the PRE (R2-5). The affinity of Tra2 and SF2/ASF for R2-6 and R2-5PRE, which differ only in the number of repeats, is very similar. However, the affinity of both of these proteins drops by four- to fivefold upon deletion of

Figure 4. Tra2 and SF2/ASF, but not SC35, require the presence of the PRE for efficient binding to the *dsxRE*. Graphs showing the results of nitrocellulose filter-binding experiments in which the percentage of labeled RNA retained on a filter is plotted against the amount of protein added to the binding reaction. The protein concentration is varied, whereas RNA is held constant at a concentration of ~ 1 nM. The horizontal axis is protein concentration (molarity, M) as approximated by Bradford assays. The vertical axis is percent of RNA bound, which is measured as cpm retained on nitrocellulose filters relative to total cpm RNA in the binding reaction, minus cpm retained on filters in the absence of protein ($<3\%$ of input RNA). Binding reactions and filtration procedure are as described in Material and methods. $\bullet$ and solid lines correspond to the RNA R2-6; X and large dashed lines correspond to R2-5PRE RNA; ∇ and small dashed lines correspond to R2-5. Data were fitted to the standard binding equation $y = (100 \cdot x) / (\max_{1/2} + x)$, which is based on the Michaelis–Menton formula.

Figure 5. Binding specificities of Tra, Tra2, and SR proteins for the *dsxRE*. (A) Shown are average binding specificities derived from at least three independent experiments, except for SF2/ASF or SRp55 alone, which were based on two independent experiments. Because of inherent error, standard deviations of all reported averages are between 10 and 50%; therefore, all differences of at least twofold are considered significant. N/D notation indicates that the specificity of SF2 could not be measured because of the low binding affinity of this protein. (B) Representative data used in the calculation of binding specificities. The horizontal axis is RNA competitor concentration (M). The vertical axis is percent RNA bound to filter, relative to a reaction lacking any competitor. ($\diamond$ and dashed lines) Nonspecific competitor; ($\blacksquare$ and solid lines) specific competitor. Binding reactions are carried out in the presence of increasing amounts of specific or nonspecific competitor. In each experiment the specific competitor is identical to the probe, and the nonspecific competitor is a transcript of comparable length from a region of the fourth exon downstream of the *dsxRE*. This nonspecific competitor is the same as used in previous studies (D6 construct in Tian and Maniatis 1992, 1993). Probe and competitor are mixed before protein is added. When more than one protein is present in a reaction, proteins are also mixed prior to adding to probe and competitor. Inhibiting concentration (50%) (IC_{50}) for each competitor is calculated as the concentration of competitor needed to compete away 50% of binding, relative to a reaction in which no competitor is added. The calculated specificity is equal to IC_{50} (nonspecific) divided by IC_{50} (specific). We note that for SC35 alone a vast excess of specific competitor relative to probe is necessary for 50% inhibition of binding. We do not understand the reason for this but suggest that it may be a consequence of the tendency of SR proteins to aggregate, and of the low specificity of SC35 binding. For example, aggregates of SC35 may be formed on individual labeled molecules, and the competitor would have to dissociate the aggregate before competing with the binding of the labeled RNA. We emphasize that the calculated binding specificities are derived from internal comparisons of the IC_{50} s within a single experiment, and that because of variations between experiments meaningful comparisons cannot be made from isolated IC_{50} values between individual experiments. (C) Representative data used in the calculation of specificity of Tra2 binding to the R2-5PRE and R2-5 RNA probes. Competition experiments were performed and analyzed as described in B.

Figure 5. (See facing page for legend.)

the purine-rich region (difference between R2-5PRE and R2-5). In contrast, none of the deletions affect the binding of SC35 to the *dsxRE*. Thus, the repeat sequences alone are sufficient for SC35 binding whereas wild-type levels of binding of Tra2 and SF2/ASF require the PRE.

The relative affinities of Tra and SRp55 for the *dsxRE* decrease slightly (twofold) upon deletion of either a repeat sequence (compare R2-6 with R2-5PRE) or the purine-rich region (compare R2-5PRE with R2-5). This decrease in affinity could be a nonspecific effect resulting from changes in RNA secondary structure. Alternatively, the binding of Tra and SRp55 to the *dsxRE* may be mediated through both the repeat sequences and the PRE. We note that the affinity of SF2/ASF for any of the *dsxRE* constructs is somewhat less than that of any of the other proteins tested (20–60% bound with 1.5 μ M SC35 versus 5–20% bound with 1.5 μ M SF2). These small differences in affinity may explain why SC35 and SRp55, but not SF2/ASF, are able to form a committed regulatory complex with Tra and Tra2 in vitro (Tian and Maniatis 1993).

Both the dsx repeats and the PRE are required for cooperative binding of Tra, Tra2, and SR proteins to the dsxRE

We also determined the binding specificities of Tra, Tra2, and the SR proteins to *dsxRE* constructs containing (R2-5PRE) or lacking (R2-5) the PRE (Fig. 5A). Binding specificity was measured by performance of binding reactions in the presence of increasing amounts of either specific or nonspecific unlabeled RNA competitor. The binding specificity is calculated as the ratio of the amount of nonspecific versus specific competitor required for a 50% decrease in binding (see Fig. 5B for representative graphs). Consistent with previous studies, Tra2 bound to the R2-5PRE RNA with significant specificity (Hedley and Maniatis 1991; Tian and Maniatis 1992). In contrast, the SR proteins bound with little, although measurable, specificity. Tra also bound to R2-5PRE with some specificity, although less than that of Tra2. Previous results had indicated that Tra did not bind specifically to the *dsxRE* (Tian and Maniatis 1992). This apparent contradiction is explained by the fact that the nitrocellulose binding assay is more quantitative than the cross-linking assays used previously. However, it is important to note that the relative specificities of single and multiple proteins determined by filter binding are all qualitatively consistent with cross-linking studies (data not shown).

Interestingly, the specificity of binding of the combination of Tra and an SR protein is 10–15 times greater than that observed with either protein alone. Moreover, the addition of Tra2 to Tra plus either SC35 or SRp55 further increases the specificity of the Tra + SR complex by two- to threefold. This observation is consistent with previous results showing that levels of SR proteins bound to the *dsxRE* increase upon addition of Tra and Tra2 (Tian and Maniatis 1993, 1994) and strongly sug-

gests that these proteins bind cooperatively to the *dsxRE*.

A comparison of the binding specificities with the R2-5 RNA and the R2-5PRE reveals interesting differences. Consistent with the observed difference in binding affinities, the specificity of Tra2 decreased fivefold upon removal of the PRE. However, the specificity of Tra, SC35 and SRp55 remains essentially unchanged. The specificity of SF2/ASF for R2-5 could not be measured because the binding affinity of this protein is so low. Furthermore, although the combination of Tra + SC35 or SRp55 showed a marked increase in specificity for R2-5 over the specificity of either protein alone, this specificity was not further increased upon addition of Tra2. Thus the specificity of Tra2 alone, as well as its ability to bind along with Tra + SR, is significantly decreased in the absence of the PRE (see Fig. 5B,C).

We note that SF2/ASF behaves differently from SC35 and SRp55 in these binding specificity studies. SF2/ASF appears to interact with Tra on the R2-5PRE RNA, but Tra2 does not further stimulate the binding. This observation is also consistent with the fact that SF2/ASF does not form a committed regulatory complex with Tra and Tra2 (Tian and Maniatis 1993). The binding of Tra + SF2/ASF to the R2-5 RNA is the same as the binding of Tra alone. The addition of Tra2 does have a slight effect; however, the specificity is still less than any other proteins in combination. Thus we believe the effect of Tra2 addition to Tra + SF2 is unlikely to be a result of significant interactions among the three proteins.

Finally, we determined whether Tra, Tra2 and the SR proteins could bind directly to the PRE in isolation. Binding specificity measurements were made as described above, with the PRE as probe and a dimer of the repeat sequence as nonspecific competitor. This nonspecific competitor was used because it is of similar length to the isolated PRE, it contains a low purine content, and none of the proteins tested had any measurable specificity for even a repeat sequence tetramer (data not shown).

As shown in Table 1, Tra2 binds to the isolated PRE with 15-fold specificity. This is less than the specificity of Tra2 for R2-5PRE but at least threefold more than the

Table 1. Binding specificities of Tra, Tra2, and SR proteins for the PRE

Specificity for isolated PRE	
protein	specificity
Tra2	15
Tra	3
SC35	2
SF2/ASF	5

Average binding specificities are based on at least three independent experiments. As a result of inherent error, standard deviations of all reported averages are between 10% and 30%; therefore, all differences of at least twofold are considered significant.

specificity of Tra2 for R2-5. In contrast, the specificity of Tra for the isolated PRE is fourfold less than its specificity for either R2-5PRE or R2-5. Furthermore, SC35 binds to the isolated PRE with little or no specificity, whereas SF2 binds to the PRE alone with as much specificity as to R2-5PRE. These data are consistent with the idea that SF2 and Tra2 bind primarily to the PRE, whereas the PRE is not involved in the binding of Tra and SC35 to the *dsxRE*. The difference in the specificity of Tra2 for the R2-5PRE construct versus the isolated PRE may be due to a lack of defined secondary structure of the isolated PRE. Alternatively, the difference could reflect a minor component of Tra2 binding that is dependent on the repeat sequences.

Discussion

Previous studies demonstrated that Tra- and Tra2-dependent female-specific splicing of *dsx* pre-mRNA requires the repeat sequences located in the *dsxRE* (Hedley and Maniatis 1991; Hoshijima et al. 1991; Ryner and Baker 1991; Tian and Maniatis 1992). We show here that an additional enhancer motif located within the *dsxRE*, the PRE, is also essential for normal levels of Tra/Tra2-dependent splicing enhancer activity. Moreover, we show that the PRE is required for normal levels of Tra2 binding to the *dsxRE*.

The dsxRE contains two distinct enhancer elements

The PRE is essentially identical to previously defined purine-rich exonic enhancers, whereas the *dsx* repeat sequences are distinct. Interestingly, both of these sequences are independently able to constitutively activate splicing when placed in close proximity (100 nucleotides) to the weak female-specific 3' splice site. A third type of sequence capable of activating the *dsx* female-specific 3' splice site was identified in an attempt to inactivate a purine-rich enhancer by extensive base substitutions (Staknis and Reed 1994). This pyrimidine-rich sequence is active, and UV cross-linking studies revealed a switch in SR protein binding to the wild-type and mutant sequences. SRp40 was shown to bind to the purine-rich sequence, whereas the pyrimidine-rich sequence was recognized primarily by SRp30 (Staknis and Reed 1994).

On the basis of the observation that SR proteins bind specifically to the *dsxRE* in the absence of Tra and Tra2, it was proposed that the constitutive activity of this enhancer is mediated by SR proteins (Tian and Maniatis 1994). Here we show that deleting the PRE does not decrease the affinity of SC35 for the *dsxRE* nor does it affect its constitutive activity of the enhancer. Thus the repeat sequences alone may be sufficient for recognition by SC35, and the constitutive activity of the R2-5 and R2x4 elements may therefore require SC35. In contrast, the binding of SF2/ASF to the *dsxRE* was dramatically decreased by deletion of the PRE. Thus SF2/ASF may be required for the constitutive splicing enhancer activity of the PRE. This possibility is consistent with the fact

that SF2/ASF has been shown to bind to other purine-rich splicing enhancers (Sun et al. 1993b), and that SF2/ASF binds to the isolated PRE with fivefold specificity.

Tra/Tra2-regulated activation of splicing requires both enhancer elements

When the *dsxRE* is distant (300 nucleotides) from the female-specific 3' splice site, it is no longer able to constitutively activate splicing, but instead requires the activity of Tra and Tra2 (Tian and Maniatis 1994). Although either the repeat sequences or the PRE can constitutively activate splicing, neither alone is sufficient to mediate the normal Tra/Tra2-dependent activation of splicing. Low levels of splicing activity can be detected when the PRE is removed from the *dsxRE*, but at least five times as much Tra and Tra2 is required to observe splicing activity. When fewer repeat sequences are present, the requirement for the PRE is even greater.

Contrary to the in vitro results reported here, a dimer of the consensus repeat sequence located 300 nucleotides from the female-specific 3' splice site was shown to be sufficient for Tra/Tra2-dependent female-specific splicing of *dsx* in vivo (Inoue et al. 1992). This difference could be due to the fact that the latter experiments were carried out in cultured *Drosophila* cells where the efficiency of splicing would be expected to be greater than that observed in the in vitro system used in our studies. Alternatively, additional factors that compensate for the loss of the PRE may be present in the cultured *Drosophila* cells.

The PRE is required for Tra/Tra2-dependent splicing and binding of Tra2

Tra and Tra2 play a critical role in the assembly of a multiprotein regulatory complex on the *dsxRE*. This complex includes SR proteins and possibly other splicing factors (Tian and Maniatis 1993, 1994). In this paper, we provide direct evidence that the assembly of this complex involves cooperative interactions between Tra and SR proteins and between a complex of these two proteins and Tra2. We also demonstrate that deletion of the PRE decreases the specificity of Tra2 binding to the *dsxRE* and eliminates the cooperative binding between Tra2 and the Tra/SR protein complex. Moreover, we show that Tra2 binds specifically to the isolated PRE. These observations suggest that the PRE functions as a high affinity Tra2-binding site that is required for cooperative interactions with Tra and SR proteins, and that this cooperativity is required for the assembly of the multicomponent splicing enhancer complex. This possibility is consistent with the observations that both Tra and Tra2 can interact with each other and with SR proteins, and that SR proteins interact with each other (Wu and Maniatis 1993; Amrein et al. 1994). The cooperative binding of proteins containing SR domains may also have more general implications regarding mechanisms of splice site selection. Although individual SR proteins bind to RNA with low specificity (Caceres and Krainer 1993; Zuo and

Manley 1993), cooperative interactions between different SR proteins could lead to the formation of highly specific RNA–protein complexes on pre-mRNA. Thus, the diversity of RNA sequences recognized by heteromultimers of SR proteins could be enormous.

Two previous reports have shown that Tra2 binds to isolated repeat sequences (Hedley and Maniatis 1991; Inoue et al. 1992). In one study Tra2 was shown to bind to a dimer of the repeat consensus sequence, but not to a repeat sequence containing point mutations that inactivate the element *in vivo*. However, the affinity of Tra2 for the synthetic repeats was not compared with that for the intact *dsxRE* (Inoue et al. 1992). In the other study Tra2 was shown to bind weakly to a multimerized repeat sequence, but binding to such a construct was at least 10-fold less than the binding of Tra2 to a construct containing repeats 2–6 (inclusive of the PRE) (Hedley and Maniatis 1991). Furthermore, we have found that neither Tra nor Tra2 bind specifically to the R2x4 repeat in filter-binding experiments (data not shown).

On the basis of the results presented here we propose that the complexity of the *dsxRE* may be a general characteristic of regulated splicing enhancers. In the case of constitutive enhancers, which act in close proximity to the 3' splice site, a single type of element may be sufficient for their activity. By contrast, the ability of the regulated enhancer to function at a distance in response to specific regulatory proteins may require a more complex organization. Like regulated transcriptional enhancers (Tjian and Maniatis 1994), these splicing enhancers may require synergistic interactions between multiple protein components which combine to achieve a high level of specificity and stability of the regulatory complex.

Materials and methods

RNA

The splicing substrates in which the *dsxRE* is located 100 nucleotides downstream from the female-specific 3' splice site were derived from the D16 plasmid (Tian and Maniatis 1994), which contains 84 nucleotides of *dsx* exon 3, the complete 114-nucleotide intron between exons 3 and 4, and the first 70 nucleotides of the fourth exon followed by polylinker sequence. D16 linearized with *Bam*HI was used to synthesize the *dsxΔE* transcript. Construct R2-5PRE was generated by subcloning an *Mlu*I–*Fsp*I fragment of the *dsxRE* into the polylinker of D16 and linearizing with *Dra*I for *in vitro* transcription. R2-5 contains a *dsxRE* fragment beginning at *Mlu*I and ending at a PCR-generated *Bam*HI site between the fifth repeat and the PRE. R2x4 contains four copies of the repeat containing sequence GGA-TCCGTCTTCAATCAACATACGCGAGATCT multimerized into the *Bam*HI to *Bgl*II site of D16. The *dsxPRE* construct was also generated by insertion of the PRE-containing oligo GGATCCAAAGGACAAAGGACAAAATGTAGATCT into the *Bam*HI/*Bgl*II sites of D16.

The splicing substrates in which the *dsxRE* sequences are placed 300 nucleotides away from the 3' splice site were generated from plasmid D1 (Tian and Maniatis 1992), which contains the entire wild-type *dsx* fourth exon. When linearized with *Dra*I, this construct is the template for the R1-5PRE transcript.

To facilitate subcloning, the polymerase chain reaction (PCR) was used to introduce an *Eco*RI site just upstream of the first repeat sequence in the D1 construct. R1-5 was derived by substitution of an *Eco*RI–*Bam*HI fragment of the *dsxRE* (by use of the previously described PCR-generated sites) for the fourth exon fragment downstream of the *Eco*RI site in D1. Inserts R2-5PRE, R2-5, R2x4, and *dsxPRE* were also subcloned into this *Eco*RI site in D1. The construct R1-5mPRE was derived from R1-5PRE by PCR mutagenesis to change the sequence of the PRE from AAAGGACAAAGGACAAAA to AATCGACCATC-GACCAAAA.

The probes R2-5PRE and R2-5 used for binding studies correspond to inserts R2-5PRE and R2-5 subcloned directly into SP73 for *in vitro* transcription. Binding probe R2-6 was generated by linearization of the *Mlu*I–*Fsp*I R2-5PRE construct downstream of the *Fsp*I site. The nonspecific competitor was transcribed from construct D6 (Tian and Maniatis 1994) linearized with *Apa*LI. The isolated PRE probe for binding studies was generated by removal of the *dsx* exon and intron sequences from the *dsxPRE* construct. The nonspecific competitor, R2x2, was transcribed from an SP72 construct containing a dimer of the repeat sequence oligo described above. None of the probes or competitors used for binding studies contained any splicing sites.

Splicing substrates and binding probes were uniformly labeled with [³²P]UTP, with a 1:18 molar ratio of [³²P]UTP to cold UTP. Competitors for binding studies were transcribed without the addition of any labeled nucleotides.

In vitro splicing reactions

In vitro splicing reactions were carried out as described in Tian and Maniatis (1992).

Recombinant proteins

Recombinant baculoviruses expressing Tra and Tra2, and purification of Tra and Tra2 were as described in Tian and Maniatis (1992). Recombinant viruses expressing SC35, SF2/ASF, and SRp55, and purification of these proteins were as described in Tian and Maniatis (1993).

Nitrocellulose filter binding assays

Binding reactions to be analyzed by filtration were done in 145 mM KCl, 3.2 mM MgCl₂, 1 mM ATP with 50 μg/ml BSA and 0.5 mg/ml yeast tRNA in a total volume of 10 μl. Probe concentration was ~1 nM in all reactions. For binding specificity experiments the amount of protein used was in the linear range of the binding curve whenever possible. Binding reactions were incubated at 30°C for 20 min, and then returned to ice. Reactions were diluted before loading with 90 μl of buffer containing 145 mM KCl and 3.2 mM MgCl₂ at 4°C, and then filtered immediately through a 0.45 μm pore nitrocellulose filter sandwiched in a filtration minifold (Schleicher & Schuell). Dilution was done to ensure that reactions were loaded evenly on filters. Filters were washed once with 200 μl of dilution buffer, then the vacuum was broken and the filters allowed to air dry briefly. Retention of labeled RNA on filters was analyzed by liquid scintillation. Percentage of RNA bound to filters relative to input RNA was corrected for retention of probe in the absence of protein. For all of the reported experiments this background retention was <3% of input RNA.

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