The EMBO Journal Vol.17 No.20 pp.6049–6060, 1998

Analysis of the functional specificity of RS domains *in vivo*

Brigitte Dauwalder and William Mattox[1]

Department of Molecular Genetics, University of Texas, MD Anderson Cancer Center, 1515 Holcombe Blvd, Box 45, Houston, TX 77030, USA

[1]Corresponding author
e-mail: wmattox@mdacc.tmc.edu

A number of splicing factors contain extensive regions that are rich in arginine and serine (RS domains). These domains are thought to facilitate protein–protein interactions that are critical in the regulation of alternative splicing. Using a domain swap strategy, we have tested the ability of RS domains from several proteins to substitute *in vivo* for an essential RS domain in the *Drosophila* splicing regulator TRA-2. By several criteria, RS domains were found to vary significantly in their ability to support the splicing regulation functions of TRA-2. The RS domain of dU2AF^{50} functioned efficiently, while that of the dSRp55 protein did not. Moreover, we find similar differences in the ability of RS domains to direct fusion proteins to discrete subnuclear sites at which TRA-2 associates with spermatocyte chromosomes. These results indicate that RS domains are not all functionally equivalent *in vivo*.

Keywords: alternative splicing/*Drosophila* sex determination/nuclear localization/spermatogenesis/SR proteins

Introduction

Alternative pre-mRNA splicing is an important regulatory mechanism that is widely used among eukaryotic genes, but relatively little is known about the factors responsible for tissue-specific regulation of splicing. Studies on several alternatively spliced pre-mRNAs have demonstrated that proteins from the SR superfamily of splicing factors play important roles in controlling the recognition of specific splice sites (Fu, 1995; Manley and Tacke, 1996). During splicing of constitutive introns, several SR proteins are known to facilitate both the initial recognition of splice sites and the assembly of pre-spliceosomal complexes (Ruskin *et al*., 1988; Kohtz *et al*., 1994; Staknis and Reed, 1994). In *Drosophila*, SR proteins are known to affect alternative splicing when they form developmentally specific splicing enhancer complexes with TRANSFORMER (TRA) and TRANSFORMER-2 (TRA-2), two regulatory proteins that are themselves closely related to the SR proteins. These complexes form on splicing enhancers in pre-mRNAs from the sex determination gene *doublesex* (*dsx*) and direct its sex-specific processing (Tian and Maniatis, 1993; Lynch and Maniatis, 1995). *In vitro* studies indicate that TRA and TRA-2 directly facilitate binding of the SR protein RBP1 as well as several other SR proteins to specific regions of the *dsx* pre-mRNA (Lynch and Maniatis, 1996). These complexes function to recruit the U2AF protein and other components of the general splicing machinery to the female-specific branch-point/polypyrimidine-tract region of the *dsx* RNA (Zuo and Maniatis, 1996). The local recruitment of these factors favors use of the female-specific *dsx* 3′ splice site to form an mRNA encoding the female-specific *dsx* protein. In the absence of TRA or TRA-2, an alternative 3′ splice site is used by default, giving rise to mRNA encoding the male specific *dsx* protein (Nagoshi *et al.*, 1988; Ryner and Baker, 1991).

Both SR proteins and a number of related splicing regulators contain regions rich in arginine and serine (RS domains) that are necessary for interactions between these factors (Wu and Maniatis, 1993; Kohtz *et al*., 1994). However, it has remained unclear whether the RS domains from individual proteins differ significantly in function or whether they play a generic, non-specific role. *In vitro* studies have suggested that RS domains can be interchanged between splicing factors or replaced by artificially synthesized RS rich sequences without substantial effects on protein function (Valcarcel *et al*., 1996; Chandler *et al*., 1997b). While this suggests that RS domains play a generic role, only a few studies have yet tested how RS domain replacements affect protein function *in vivo* (Li and Bingham, 1991; Caceres *et al*., 1997).

The *Drosophila* sex determination system provides an opportunity to study the contribution of RS domains to the function of splicing regulators *in vivo*. Because the interactions of TRA, TRA-2 and SR proteins are required for formation of a functional splicing enhancer complex on *dsx* pre-mRNA, mutations affecting these interactions are predicted to result in both a switch in the *dsx* splicing pattern and in dramatic changes in the sexual phenotypes of adult flies. Here we have used this system to examine whether functional specificity is conferred by the essential RS domain of the TRA-2 protein. TRA-2 is of particular interest because it functions as a specific regulator in *dsx* splicing, has no effect on viability and is organized in a manner that closely resembles core SR proteins (Amrein *et al.*, 1988; Goralski *et al.*, 1989). Moreover, in addition to *dsx* pre-mRNA, TRA-2 also affects alternative splicing of several other specific pre-mRNAs. For instance, in the nervous system TRA-2 is required for sex-specific splicing of *fruitless* (*fru*) pre-mRNA (Ito *et al*., 1996; Ryner *et al*., 1996; Heinrichs *et al*., 1998), while in the male germline it affects sex-specific processing of pre-mRNAs from *exuperantia* (Hazelrigg and Tu, 1994), *att* (Madigan *et al*., 1996) and the *tra-2* gene itself (Mattox and Baker, 1991; Mattox *et al*., 1996). This system thus offers the opportunity to study the effects of RS domains on regulation of multiple pre-mRNA targets *in vivo*.

To assess the ability of RS domains from other proteins to replace the TRA-2 RS2 domain we have generated transgenic fly strains expressing fusion proteins. We find that while some RS domains are functionally interchangeable, others confer only partial or target-specific function. These data argue that RS domains found in different SR proteins are not all functionally equivalent to the TRA-2 RS2 domain *in vivo*.

Results

In order to determine whether the RS domains from other RS proteins can substitute for the TRA-2 RS2 domain, we first deleted RS2 [amino acids (aa) 193–264] to create TRA-2ΔRS2. The RS domains of the *Drosophila* TRA, SRp55 (dSRp55), U2AF^{50} (dU2AF^{50}) and human SC35 proteins were then inserted in place of RS2 to generate the fusion genes TRA-2/TRA RS, TRA-2/dSRp55 RS, TRA-2/dU2AF^{50} RS and TRA-2/SC35 RS (Figure 1). In addition, we also inserted the C-terminal part of the TRA-2 RS2 domain which has been shown to be more conserved between *Drosophila melanogaster* and *Drosophila virilis* than other parts of the RS2 domain (Chandler *et al.*, 1997a), generating TRA-2/RS$_{carb}$. All changes were made in the context of the genomic sequences containing the native *tra-2* promoter, introns and flanking sequences. The constructs were inserted into the CaSpeR *Drosophila* transformation vector and used to generate transgenic flies by P-element mediated germline transformation of the *tra-2* mutant strain *w^{1118}/B^{S}Y; tra-2^{B}/CyO*. The transgenic lines were examined to determine the effects of the transgenes on sexual differentiation and RNA splicing patterns in *tra-2* mutant backgrounds.

Epitope-tag fusions at the C-terminus, but not the N-terminus of the TRA-2 proteins specifically disrupt male germline functions

To follow the expression of RS domain fusion proteins, we added sequences coding for foreign epitopes to them. To identify an epitope tagging strategy that would allow detection of the expressed proteins while not affecting their function *in vivo*, we initially tested epitopes fused at both the N- and C-terminus of the full-length TRA-2 protein. In two of these, the FLAG epitope tag (9 aa) was fused either at the C-terminus (C-FLAG) or just inside the N-terminus (N-FLAG) of the TRA-2^{226} isoform. In the third construct the *Escherichia coli* β-galactosidase protein was fused to the C-terminus of TRA-2 (C-*lacZ*). It should be noted that the C-terminal epitope fusions mark all three TRA-2 isoforms, while the N-terminal epitope tag labels only the two major isoforms TRA-2^{264} and TRA-2^{226}, omitting a minor isoform TRA-2^{179} (Figure 1).

All three epitope fusion constructs were able to rescue somatic sex determination functions when introduced into *tra-2* mutant flies as judged by several cuticular features that are sexually dimorphic in males and females (described in detail below). When germline functions were examined, however, we found that only N-FLAG restored male fertility to normal levels (Table I). The fact that both C-FLAG and C-*lacZ* failed to function only in the germline demonstrates that it is possible to inactivate the germline function of the gene without inactivating somatic function. This difference is unlikely to be due to the presence of the untagged TRA-2^{179} present in N-FLAG bearing flies since this isoform has previously been shown to be neither necessary nor sufficient for germline function (Mattox

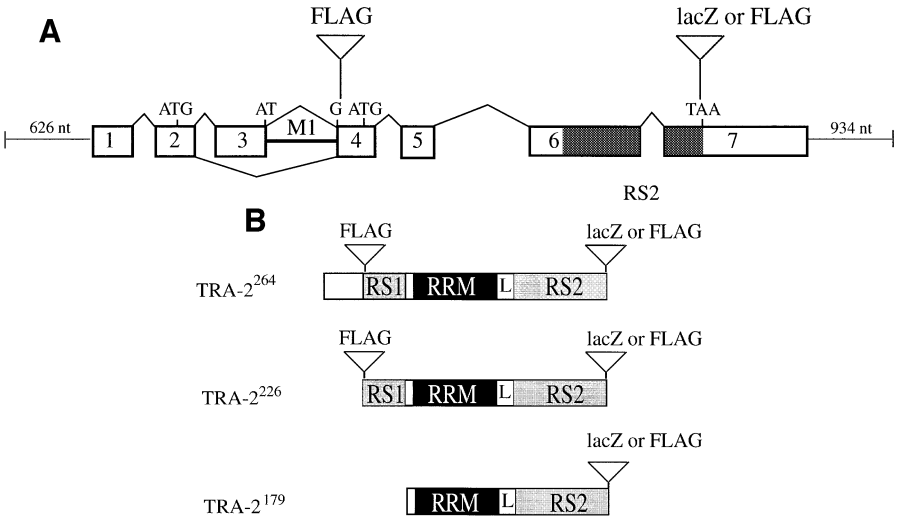

Fig. 1. Structure of the fusion constructs. The organization of the *tra-2* genomic fragment used to generate transgenic flies is shown in (**A**). Exons are indicated by open boxes. The autoregulated M1 intron is shown by a horizontal line, all other introns are indicated by blank regions between the exons. The gray areas in exons 6 and 7 indicate the coding region for RS2. ATG start codons for the three different protein isoforms and the stop codon used by all isoforms are shown. The M1 intron splits the ATG start codon for the TRA-2^{226} isoform. For N-FLAG tagged proteins, sequences coding for the FLAG epitope were inserted five amino acids downstream of the ATG for the TRA-2^{226} isoform. C-terminally tagged proteins were fused to *lacZ* or to FLAG sequences immediately upstream of the stop codon. TRA-2 protein isoforms and location of epitope tags are shown in (**B**). The gray areas labeled RS1 and RS2 indicate RS domains. The black area labeled RRM indicates the RNA binding domain and the open box labeled L indicates the linker region. Note that the N-terminal FLAG epitope labels the TRA-2^{264} and TRA-2^{226} isoforms only, whereas the C-terminal tags label all three protein isoforms. RS2 was deleted in TRA-2ΔRS. In fusion proteins RS2 (aa 193–264) was precisely replaced by RS domains of other proteins.

et al., 1996). Although *tra-2* transgenes with all three types of epitope tags conferred rescue of *dsx* splicing, the C-terminal FLAG epitopes could not be detected in immunostaining experiments. We therefore used both the N-FLAG and C-*lacZ* fusions for the analysis of somatic functions. In the germline we focused on N-FLAG fusion proteins for the analysis of male germline function.

RS domains from other SR proteins can substitute for the TRA-2 RS2 domain in somatic female sexual differentiation

Chromosomally female (XX) individuals lacking TRA-2 function are sexually transformed in somatic tissues and develop into adults that are identical in appearance to males (Watanabe, 1975). Our initial assay for the function of the different transgenes was to determine whether they could rescue this somatic phenotype. Several visible cuticular features that are sexually dimorphic in males and females were examined for each line. These include abdominal pigmentation, the number and position of sex comb bristles (a group of bristles on the basitarsus of the first leg), the number of sixth sternite bristles and the genitalia. For each fusion construct we generated a number of independent transgenic lines to control for variations due to the insertion site of the transposon. The phenotypic analysis for all lines of *XX; tra-2* flies with one dose of the transgene is summarized in Table II. As expected, most TRA-2ΔRS transgenic lines do not show rescue of the *tra-2* phenotype and are still male in appearance. In a few lines we observed a very slight rotation of the otherwise male genitalia which is an indicator of intersexuality. This suggests a small amount of residual function in these lines. The full-length TRA-2 constructs that were used as a positive control provided complete rescue in a majority of the lines examined, resulting in *XX; tra-2* animals with wild-type female appearance. Among the RS fusion constructs tested, the most consistent strong rescue to fully differentiated females was observed with TRA-2/dU2AF^{50} RS. Intermediate rescue producing mostly female-like intersexes was observed for both TRA-2/TRA RS and TRA-2/SC35 RS, suggesting that these RS domains function less well than RS2. Although the above results are qualitatively the same for constructs tagged with the FLAG epitopes and with the *lacZ* transgene, variable results were observed for TRA-2/dSRp55 RS fusions which did not confer rescue in the FLAG-tagged constructs, but did give rise to somatically rescued flies as a *lacZ* fusion gene. The cause for this discrepancy is unknown, but it has previously been observed that *lacZ* fusions stabilize some proteins during *Drosophila* embryogenesis and it is possible that this is responsible for the observed difference. Rescue similar to that of the full-length construct was observed with the TRA-2/RS$_{carb}$ transgene which contains only the C-terminal part of the TRA-2 RS2 domain. A few lines (from otherwise rescuing constructs) that did not rescue most likely reflect insertions of the P-element into chromosomal positions where the gene is not sufficiently expressed at the appropriate time in sexual development.

Two representative lines carrying each construct were chosen for detailed phenotypic analysis, as summarized in Table III and Figure 2. This confirmed the above conclusions and revealed that individual external characteristics follow the same general trend. For example, both lines carrying TRA-2/dU2AF^{50} RS rescued all aspects of cuticular differentiation examined, including abdominal pigmentation, number of sternite bristles, the number of

Table I. Comparison of phenotypic rescue using different TRA-2 epitope fusions

Epitope fusion	Rescue of sexual differentiation observed	Rescue of male fertility observed	Immunostaining
N-FLAG	yes	yes	+++
C-FLAG	yes	no	−
C-*lacZ*	yes	no	+++

Table II. Somatic sexual differentiation in transgenic lines

			Number of lines with various sex phenotypes				
Epitope	RS domain	Total lines	Female (complete rescue)	Female-like intersexes	Intersexes	Male-like intersexes	Male (no rescue)
C-FLAG	none (ΔRS)	16	0	0	0	2	14
C-FLAG	TRA-2 RS2	16	6	3	0	0	7
C-FLAG	TRA	14	0	6	5	1	2
C-FLAG	SC35	31	8	9	5	4	5
C-FLAG	dU2AF^{50}	20	8	3	3	4	2
C-FLAG	dSRp55	15	0	0	0	2	13
N-FLAG	none (ΔRS)	31	0	0	0	12	19
N-FLAG	TRA-2 RS2	19	13	3	1	2	0
N-FLAG	SC35	23	1	12	3	1	6
N-FLAG	dU2AF^{50}	20	10	2	4	2	2
N-FLAG	dSRp55 (full length)	14	0	0	0	6	8
N-FLAG	dSRp55 (fragment A)	17	0	0	0	11	6
N-FLAG	dSRp55 (fragment B)	15	0	0	0	10	5
N-FLAG	RS2$_{carb}$	15	9	1	2	0	3
lacZ	none (ΔRS)	14	0	0	0	0	14
lacZ	TRA-2 RS2	10	4	3	0	0	3
lacZ	dU2AF^{50}	9	4	2	0	3	0
lacZ	dSRp55	20	6	6	1	6	1

Table III. Detailed phenotypic analysis of chromosomally female *tra-2* individuals from representative transgenic lines

Epitope	RS Domain	Line	Rescue of female abdominal pigmentation	Average number of sixth sternite bristles	Rescue of female genitalia	Sex combs	Rescue of female fertility
No transgene			++++	18.3 ± 1.3	++++	4.3 ± 0.5	yes
N-FLAG	none (ΔRS)	85.1	−	0	−	8.8 ± 0.9	no
		44.2	−	0	−	8.2 ± 1.0	no
N-FLAG	TRA-2 RS2	28.2	++++	18.9 ± 1.1	++++	4.0 ± 0	yes
		43.2	++++	18.9 ± 0.7	++++	4.0 ± 0	yes
N-FLAG	SC35	31.3	+++	19.0 ± 1.6	++++	4.6 ± 0.5	yes
		28.3	++	8.9 ± 8.6	++	6.4 ± 1.7	no
N-FLAG	dSRp55 (full length)	26.1	−	0	−	8.5 ± 0.5	no
		24.1	−	0.2 ± 0.6	−	8.3 ± 1.1	no
N-FLAG	dSRp55 (fragment A)	70.1	−	0	−	7.5 ± 0.8	no
		17.1	−	0	−	7.5 ± 0.5	no
N-FLAG	dSRp55 (fragment B)	73.1	−	0.1 ± 0.3	−	6.9 ± 0.6	no
		65.1	−	1.4 ± 1.9	−	8.0 ± 1.1	no
N-FLAG	dU2AF^{50}	25.1	++++	18.6 ± 1.1	++++	4.0 ± 0	yes
		17.4	++++	18.5 ± 1.1	++++	4.0 ± 0	yes
N-FLAG	RS2$_{carb}$	17.7	++++	19.0 ± 0.9	++++	4.0 ± 0	yes
		32.2	++++	18.6 ± 1.5	++++	4.0 ± 0	yes
C-FLAG	TRA-2 RS2	15.1	++++	20.2 ± 1.6	++++	4.1 ± 0.3	yes
		45.1	++++	18.4 ± 1.4	++++	4.9 ± 0.7	yes
C-FLAG	TRA	41.1	++	19.1 ± 1.1	++	6.7 ± 0.7	no
		64.2	++	18.5 ± 1.4	++	6.5 ± 0.9	no
*lac*Z	none (ΔRS)	17.3	–	0	−	9.1 ± 0.3	no
		60.1	–	0	−	8.8 ± 0.6	no
lacZ	TRA-2 RS2	3.3	++++	19.5 ± 1.5	++++	4.0 ± 0.0	yes
lacZ	dU2AF^{50}	12.2	++++	19.9 ± 1.4	++++	4.0 ± 0.0	yes
		16.1	++++	19.5 ± 1.5	++++	4.0 ± 0.0	yes
lacZ	dSRp55	7.1	++++	19.5 ± 1.3	++++	3.9 ± 0.3	yes
		74.2	++++	15.0 ± 1.8	+++	4.8 ± 0.4	no

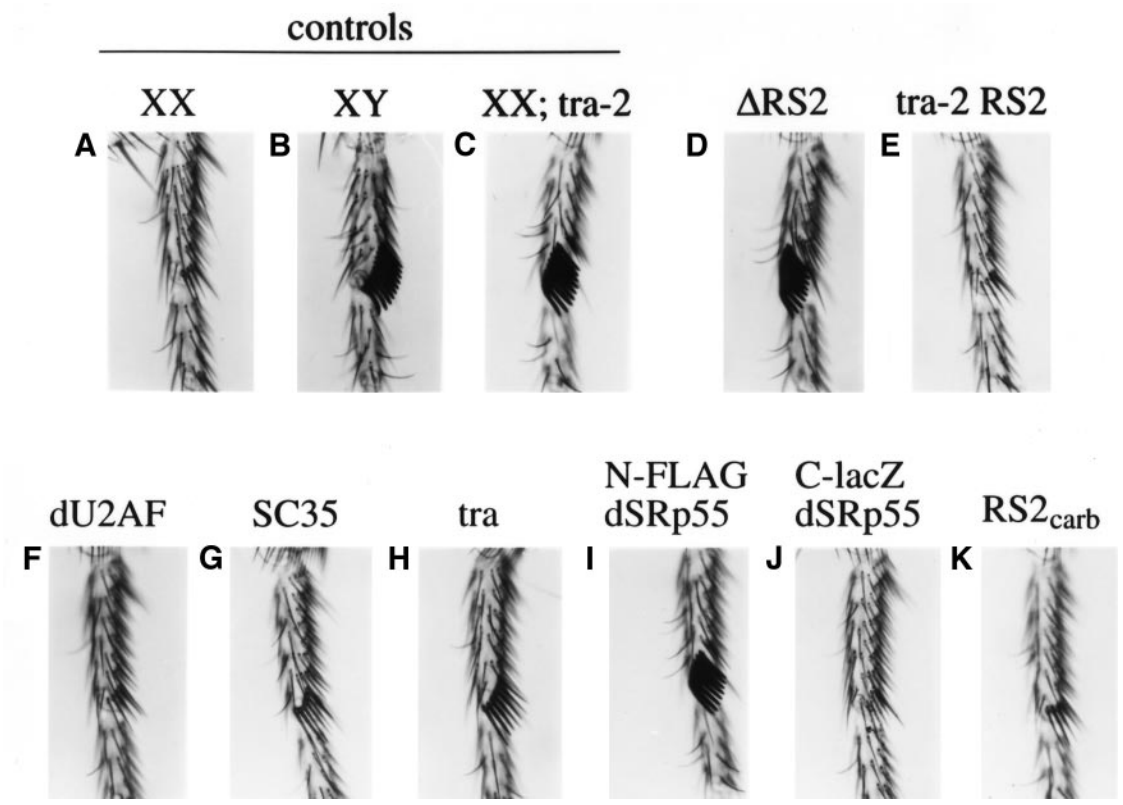

Fig. 2. Differentiation of sex combs in transgenic lines. Male sex combs and analogous female bristles from the basitarsus of the first leg are shown in various control and transgenic fly strains. The genotype for all transgenic lines is *(XX) w^{1118}/w^{1118}; $tra\text{-}2^{B}/tra\text{-}2^{B}$*. The identity of the RS domains replacing the TRA-2 RS2 domain is indicated. Unless otherwise indicated, fusion proteins in the lines shown were epitope tagged at the N-terminus with FLAG.

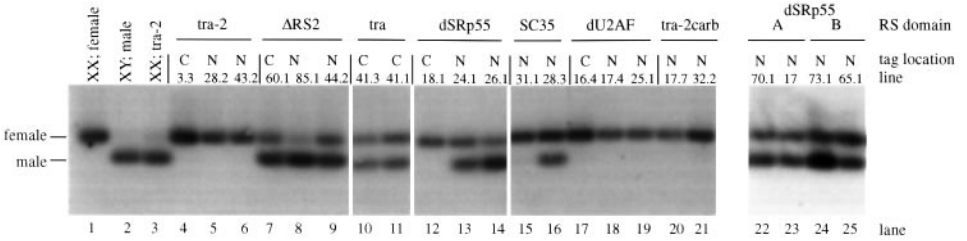

Fig. 3. Alternative splicing of *dsx* pre-mRNAs in transgenic lines. RT–PCR was performed using primers specifically amplifying male- and female-specific *dsx* mRNAs. The PCR products were visualized by Southern blot hybridization with primers that span the male- and female-specific splice junctions. The genotype of control flies in lanes 1–3 is as indicated, the genotype for all transgenic lines is XX; *tra-2* (lanes 4–25). The identity of the RS domains replacing the TRA-2 RS2 domain is as indicated. Proteins were epitope tagged at the C-terminus (C) with β-gal or at the N-terminus (N) with FLAG. The identity of individual transgenic lines assayed is indicated by their line number.

sex combs and the morphology of genitalia. Intermediate rescue (as in the case of TRA-2/SC35 RS and TRA-2/TRA RS) is evidenced by intermediate morphology of sex combs, number of sternite bristles, pigmentation and genital morphology. Together, the above results indicate that RS domains from different SR proteins substitute for TRA-2 RS2 to varying degrees.

TRA-2 RS domain substitutions can regulate dsx and fru splicing

Female sex determination requires TRA-2-dependent female specific splicing of the *dsx* pre-mRNA. The observed somatic phenotypes should therefore be reflected in the ability of the transgenes to direct female specific splicing of the *dsx* mRNA. We used RT–PCR on RNA from *XX; tra-2* animals carrying one dose of the transgenes to examine splicing of the *dsx* RNA (Figure 3). Each lane corresponds to RNA from a representative independent line. Figure 3, lanes 1 and 2, shows the RT–PCR products from control wild-type females and males, respectively. As shown in Figure 3, lane 3, *XX; tra-2* mutant animals produce predominantly the male specific splice form. Figure 3, lanes 4 to 21, shows the *dsx* splicing patterns in *tra-2* mutant animals carrying the different transgenes. TRA-2ΔRS lines produce predominantly male RNA (Figure 3, lanes 7–9) and a smaller amount of the *dsx* female specific transcript, consistent with the relatively small amount of female differentiation that occurs in these flies. TRA-2/dU2AF^{50} RS fusions (Figure 3, lanes 17–19) and TRA-2/RS$_{carb}$ (Figure 3, lanes 21–24) produce exclusively the female specific splice form, indistinguishable from the control transgene with the native TRA-2 RS2 domain (Figure 3, lanes 4–6). Fusions that give intermediate phenotypic rescue show both male- and female-specific *dsx* transcripts (TRA-2/SC35 RS, TRA-2/TRA RS). Splicing in TRA-2/dSRp55 RS varied again depending on the epitope used. In all the RS fusions, the amount of female specific *dsx* splicing corresponds well with the phenotypic sexual development we observed; we find the expected direct correlation between the amount of female specific *dsx* splicing and the rescue of female morphology in the different transgenic lines.

Female-specific, alternative splicing of the *fru* pre-mRNA in parts of the nervous system has been shown to be TRA-2 dependent. We therefore used a subset of the same lines to examine how deletion of the RS2 domain and its replacement with other RS domains affected *fru* splicing. RNA was isolated from *XX; tra-2* animals carrying the transgenes and analyzed by RT–PCR

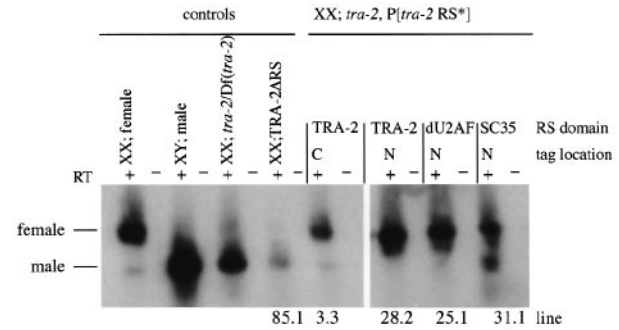

Fig. 4. Alternative splicing of *fru* pre-mRNA in transgenic lines. RT–PCR was performed using primers that specifically amplify male- and female-specific *fru* mRNAs. PCR products were visualized by Southern blot hybridization with sex specific *fru* probes generated by PCR using a second set of primers that amplify regions of the male and female *fru* exon. The genotypes of flies and the identity of the RS domains replacing the TRA-2 RS2 domain are indicated. Proteins were epitope tagged at the C-terminus with β-gal or at the N-terminus with FLAG. The identity of individual transgenic lines assayed is indicated by their line number below the lanes. RT–PCR reactions were performed in either the presence (+) or absence (–) of reverse transcriptase (RT).

(Figure 4). *fru* mRNA from *XX; tra-2/Df(tra-2)* mutant animals undergoes male specific splicing that can be reversed completely to female by full-length TRA-2 transgenes with an epitope tag either at the C-terminus (C) or N-terminus (N). Transgenes lacking the RS2 domain failed to rescue *fru* splicing. TRA-2/dU2AF^{50} RS on the other hand directed female-specific splicing, indistinguishable from the full-length TRA-2 protein, whereas TRA-2/SC35 RS gave rise to both male- and female-specific splice products. These results parallel our findings on *dsx* splicing, showing that the RS2 domain of TRA-2 is required for *fru* splicing in the nervous system and can be functionally replaced by RS domains from other proteins.

In summary, while our results demonstrate that there are differences in the extent of somatic rescue with various RS substitutions, we find that in general the RS domains tested were able to at least partially substitute for RS2. At least one RS domain (that from dU2AF^{50}) substituted fully for the TRA-2 RS2 domain.

The fusion proteins function differently in the male germline

In addition to its role in somatic sex determination, TRA-2 affects splicing of multiple targets in the male germline and is required for male fertility. We therefore examined the ability of various transgenes to rescue male fertility

Table IV. Male fertility in transgenic lines

RS domain	Number	Rescue of male fertility
N-FLAG lines:		
None (ΔRS)	31	0/31
TRA-2 RS2	19	13/19
dU2AF^{50}	20	10/20
SC35	23	2/23
dSRp55 (full length)	14	1/14
dSRp55 (fragment A)	18	0/18
dSRp55 (fragment B)	15	0/15
RS2$_{carb}$	15	9/15
lacZ fusion lines:		
none (ΔRS)	14	0/14
TRA-2 RS2	10	0/10
dU2AF^{50}	9	0/9
dSRp55	20	0/20

in *tra-2* mutant males. Table IV summarizes the results obtained for all transgenic N-FLAG lines, testing three to five males from each line. These results show that TRA-2/dU2AF^{50} RS and TRA-2/RS$_{carb}$ frequently restored fertility whereas TRA-2/SC35 RS and TRA-2/dSRp55 RS did so only in a few lines. Comparison of fertile lines from various N-FLAG constructs indicated that they differed in the frequency at which males were fertile whereas the number of progeny was the same for all fertile males (data not shown). A large number of unfertilized eggs was observed in all vials with infertile males, indicating that the males had mated successfully. These results suggest that there are large differences in the ability of various RS fusions to perform male germline functions.

One of the targets of the TRA-2 protein in the male germline is the *tra-2* pre-mRNA itself. As a part of autoregulation, TRA-2 functions to repress splicing of the M1 intron. Accordingly, the appearance of M1-containing *tra-2* RNA is indicative of the presence of functional TRA-2 protein able to regulate M1 splicing. In order to examine TRA-2 autoregulation in the transgenic lines RT–PCR was performed on RNA from *XY; tra-2^{B}* mutant males carrying the various transgenes (Figure 5). Since the endogenous *tra-2^{B}* allele results from a nonsense mutation it still produces RNA. Our assay detects transcripts from both the endogenous gene and the transgene. Figure 5, lane 1, shows products from the two RNA forms present in the wild type. In *tra-2* mutants, the upper band representing RNA with the retained M1 intron is almost completely missing. The very low residual amount derives from unregulated residual retained intron in *tra-2* mRNA precursors found in the soma. TRA-2ΔRS constructs were not capable of providing autoregulatory function (Figure 5, lanes 13 and 15). Addition of the RS domain of dU2AF^{50} (Figure 5, lanes 17 and 19) restored splicing repression in one of the two lines examined. The TRA-2/dSRp55 RS (Figure 5, lanes 25 and 27) trangene also partially restored splicing repression in at least one of the lines tested. TRA-2/SC35 RS (Figure 5, lanes 29 and 31) did not produce retained M1 intron significantly above background in either line tested. Although there is clear line-to-line variation in autoregulatory function, these results are roughly consistent with the effects of the transgenes on male fertility and suggest that some but not all RS domain substitutions function in the male germline.

Effects of RS domains on subnuclear localization of TRA-2 in primary spermatocytes

In previous studies it has been shown that *Drosophila* TRA-2 and other RS domain proteins localize in nuclear speckles when expressed in COS-1 and other mammalian cells. Our epitope-tagged versions of the TRA-2 protein allowed us to observe the distribution of the protein in fly cells and to examine the effect of changes in the RS domain on the localization of the protein. In primary spermatocytes, we found that the tagged TRA-2 proteins were highly localized to specific sites within the nucleus. These sites did not appear to be distributed randomly, but rather appeared to be associated with chromosomes in a pattern that changes dynamically as primary spermatocytes mature and bulk transcription ceases (B.Dauwalder and W.Mattox, in preparation). Figure 6A shows a schematic of the organization of chromosomes in primary spermatocytes. The two major autosomes and the X-chromosome are clearly distinguishable and situated close to the periphery of the nucleus (Cenci *et al*., 1994). Figure 6B and C shows the distribution of the wild-type TRA-2 protein in growth stage primary spermatocytes. The protein is highly localized at sites associated with chromosomes that appear to differ from the speckles seen in mammalian localization studies. In contrast, TRA-2ΔRS protein is confined to the nucleus but fails to localize subnuclearly (Figure 6D and E). This suggests that RS2 is essential for subnuclear localization. We therefore examined whether the RS domains of other proteins were capable of restoring subnuclear localization. When substituted for RS2, the dSRp55 RS domain does not confer normal subnuclear localization, the protein is distributed homogeneously in the nucleus and rarely did we observe a slight concentration around the chromosomes (Figure 6F and G). Similar results were obtained when the C-*lacZ* version of TRA-2/dSRp55 was tested (data not shown). In contrast to the above, the dU2AF^{50} and SC35 RS domains (Figure 6H–K) as well as RScarb (Figure 6L and M) restored the wild-type pattern of TRA-2 localization in all lines examined. We conclude from these experiments that RS domains can differ substantially in their ability to confer subnuclear localization.

To examine whether the localization of the TRA-2 wild-type and the TRA-2/dU2AF^{50} proteins is identical, we generated flies simultaneously carrying both the N-FLAG TRA-2 transgene and the TRA-2/dU2AF^{50} β-gal fusion transgene, and performed double stainings on spermatocytes. Figure 6N and O shows such a double staining experiment on spermatocytes. The two proteins co-localize, suggesting that the TRA-2/dU2AF^{50} pattern is like that of the full-length TRA-2 protein. If the RS domain of dU2AF^{50} is itself sufficient to confer this pattern we might expect that the dU2AF^{50} protein localizes in a similar way. However, when we stained testes with an antibody against *Drosophila* dU2AF^{50} (kindly provided by D.Rudner and D.Rio), we observed no subnuclear localization but rather homogeneous distribution in the nucleus (Figure 6P and Q). This is clearly different from the localization we observe in TRA-2/dU2AF^{50} RS flies. Thus, the dU2AF^{50} RS domain when fused to the TRA-2ΔRS protein restores localization to the TRA-2-specific and not the dU2AF^{50}-specific pattern, suggesting that although a functional RS domain is necessary for

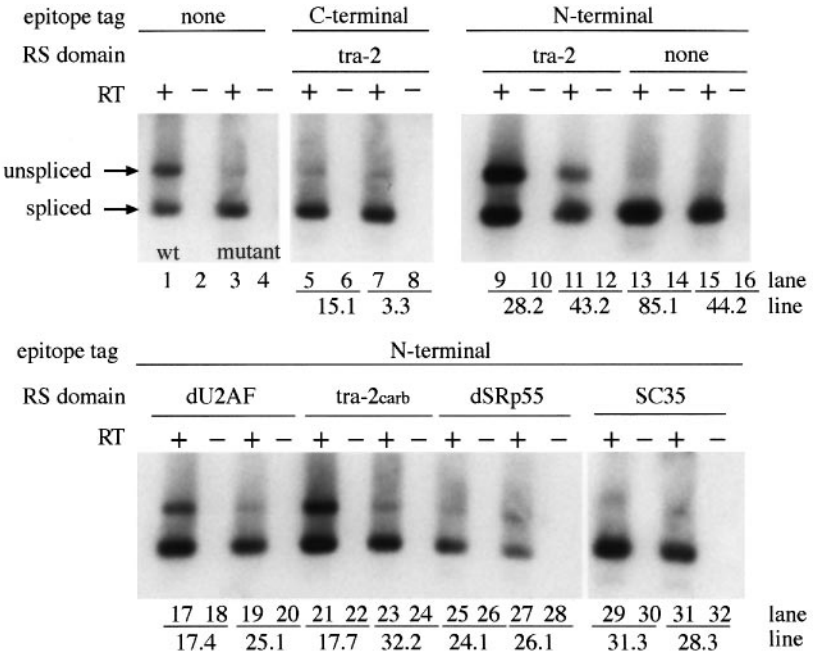

Fig. 5. Alternative splicing of the *tra-2* M1 intron in the germline of transgenic males. RT–PCR was performed to amplify the M1 intron and adjacent sequences and products were visualized by Southern blot analysis. PCR fragments originating from *tra-2* RNAs with spliced or retained (unspliced) M1 intron sequences are indicated. The identity of the RS domains replacing the TRA-2 RS2 domain are indicated. Wild-type (wt) and *tra-2* (mutant) controls are shown. Proteins were epitope tagged at the C-terminus with β-gal or at the N-terminus with FLAG. The transgenic lines assayed are indicated by their line number. RT–PCRs were performed either in the presence (+) or absence (–) of reverse transcriptase (RT).

TRA-2 protein localization, it is not alone sufficient for protein specific localization. This implies that other regions of the protein must also be required.

The inability of dSRp55 fusions to substitute for RS2 is not due simply to its length

It is possible that the differences in function observed above for different RS domains are related to their length. The RS domain of dU2AF[50] functions well and is the shortest of the foreign RS domains we have tested (31 aa compared with 69 aa in RS2), while that of dSRp55 functions poorly and is the largest of the domains tested (135 aa). We therefore generated additional fusions tagged with the N-terminal FLAG epitope to test whether shorter segments of dSRp55 were able to functionally substitute for RS2. Two non-overlapping subregions of sizes like the RS domains of dU2AF[50] and RS2 itself were tested. One of these subregions (fragment A) encompasses the first 69 aa of the RS domain of dSRp55 (residues 198–266) while the other subregion (fragment B) spans a 31 aa region downstream of fragment A (residues 268–298). As with other fusions, a number of independent transgene insertions were identified and tested for function in a *tra-2* mutant background. Somatic sexual phenotype (Tables II and III), male fertility (Table IV), *dsx* splicing (Figure 3, lanes 21–24) and subnuclear localization (Figure 7) of both the fragment A and B protein fusions were similar to that observed with the original full-length TRA-2/dSRp55 fusion lines. These results demonstrate that RS regions of similar size deriving from SRp55 and other SR proteins differ in their ability to functionally replace RS2. This suggests that it is the specific sequences of these RS regions, rather than their size, that are responsible for the differences in function.

Discussion

RS domains from other splicing factors differ in their ability to functionally replace TRA-2 RS2

RS domains are found in a large number of splicing factors and have been shown to be necessary for protein–protein interactions that facilitate formation of both spliceosomal and regulatory complexes (Wu and Maniatis, 1993; Amrein *et al*., 1994; Kohtz *et al*., 1994). While it is probable that interactions between SR proteins are highly specific *in vivo*, the contributions of RS domains to the functional specificity of individual SR proteins has remained unclear. To address this issue we have examined how replacement or deletion of an RS domain in the splicing regulator TRA-2 affects its defined genetic functions in transgenic fly strains.

There are two RS domains within the TRA-2 protein (Amrein *et al*., 1988; Goralski *et al*., 1989). RS1 is located near the N-terminus and RS2 near the C-terminus. In previous genetic studies we have shown that RS1 is essential for functions carried out by TRA2 in the male germline, but is less important for functions in somatic tissues (Mattox *et al*., 1996). In agreement with earlier studies (Amrein *et al*., 1994), we find that a deletion completely removing RS2 but not the upstream linker region almost completely abolishes TRA-2 somatic functions (*dsx* and *fru* splicing, and female sexual differentiation of cuticular structures) and eliminates all functions tested in the male germline (male fertility, autoregulation of TRA-2 splicing and subnuclear localization of TRA-2).

For somatic functions of TRA-2, the RS domains from several other SR proteins were able to at least partly replace RS2. This strongly indicates that, at least for some

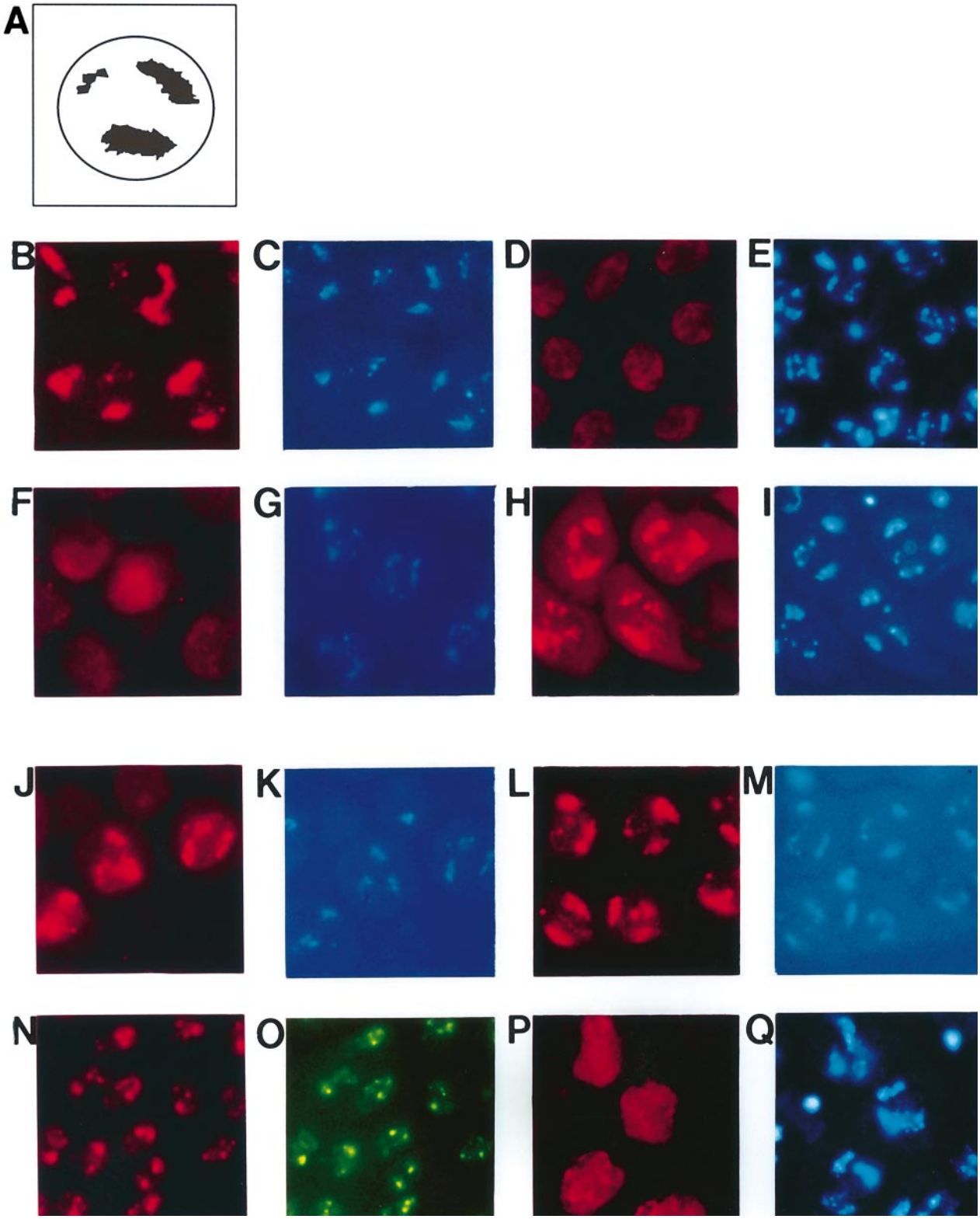

Fig. 6. Subnuclear distribution of fusion proteins in primary spermatocytes. (**A**) A schematic of a primary spermatocyte nucleus, showing the three chromosome bivalents. (B–M) N-FLAG tagged proteins are detected with anti FLAG antibody (red), chromosomes are stained with Hoechst 33258 (blue). (**B** and **C**) Wild-type TRA-2; (**D** and **E**) TRA-2ΔRS; (**F** and **G**) TRA-2/dSRp55 RS; (**H** and **I**) TRA-2/dU2AF^{50} RS; (**J** and **K**) TRA-2/SC35 RS; (**L** and **M**) TRA-2 RS$_{carb}$. (**N** and **O**) Double staining of flies carrying both N-FLAG-TRA-2 [red, (N)] and TRA-2/dU2AF^{50}-*lacZ* [green, detected with anti-β-gal antibody, (O)]. (**P** and **Q**) Localization of endogenous dU2AF^{50}, detected with anti-dU2AF^{50} antibody (red), chromosomes are stained with Hoechst. All transgenes are in a wild-type genetic background. The magnification in all panels is 630×, except for (N) and (O), where it is 400×.

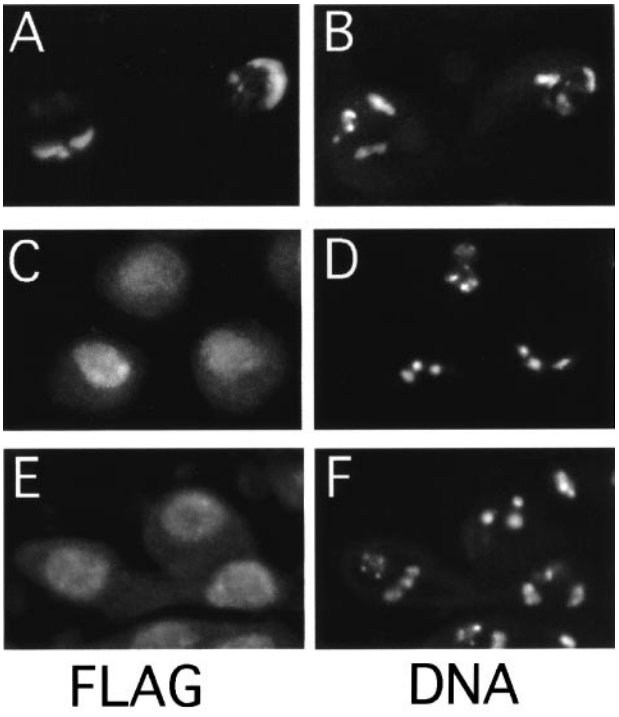

Fig. 7. Subnuclear distribution of fusions between TRA-2 and subsegments of the dSRp55 RS domain. Immunostaining of TRA-2 with anti-FLAG antibody (**A**, **C** and **E**) and DNA with Hoechst 33258 (**B**, **D** and **F**) is shown. The cells in (A) and (B) are from N-FLAG-TRA-2 males, (C) and (D) from N-FLAG TRA-2/Fragment A, and (E) and (F) from N-FLAG TRA-2/Fragment B.

functions, the RS2 domain performs a generic role that can be fulfilled by other Arg–Ser rich sequences. However, it is important to note that RS domains were not equivalent in their ability to replace RS2. Variations were observed in somatic tissues, and more pronounced differences in function were found in the male germline where TRA-2 is required for the completion of normal spermatogenesis and male fertility. For instance, we found that fusion genes carrying the SC35 and dSRp55 RS domains functioned poorly in the germline as reflected by the fact that mutant males carrying these transgenes were mostly sterile. In contrast, substitution of the dU2AF^{50} RS domain for RS2, resulted in a protein that functioned normally by all criteria examined. These results suggest that the TRA-2 RS2 domain and the dU2AF^{50} RS domain share at least one functional capability that the SC35 and dSRp55 RS domains lack.

Studies on the RS domain of the human U2AF large subunit (U2AF^{65}) have suggested that the specific arrangement of positively charged residues within it is responsible for its ability to facilitate base pairing between U2 snRNA and the branchpoint when U2AF is bound to the nearby polypyrimidine tract (Lee *et al.*, 1993; Valcarcel *et al.*, 1996). The TRA-2 RS2 domain has yet to be tested for an RNA annealing activity, but has been shown to facilitate protein–protein interactions with SR proteins that assemble into the *dsx* splicing enhancer complex (Wu and Maniatis, 1993; Amrein *et al.*, 1994). Although it has been suggested that the RS domain of the U2AF large subunit is functionally distinct from that of other SR proteins (Valcarcel *et al.*, 1996), our findings indicate that, within the context of the TRA-2 protein, the U2AF^{50} RS domain has the

Fig. 8. Amino acid region of similarity in the RS domains of TRA-2 and dU2AF^{50}. Alignment is shown comparing sequences from the redundant segments of the TRA-2 RS2 domain and from dU2AF^{50} RS. Amino acid identities are shaded black, gray boxes indicate conservative substitutions.

capability of carrying out functions similar to those of TRA-2 RS2.

At this point the specific features of various RS domains giving rise to functional differences and similarities are difficult to define in terms of primary sequence. Size of the RS domain alone does not seem to be a critical factor since RS domains of identical size were observed to differ significantly in function (i.e. dU2AF and dSRp55 fragment B). Because all the RS domains tested have arrays of repeating RS dipeptides, we believe it is most likely that functional specificity arises from sequences interdigitated between these arrays. One clue to the identity of these sequences comes from the observation that elements sufficient to confer functionality are duplicated within the RS2 domain itself. We have shown here that the conserved C-terminal 26 aa of this domain are able to functionally substitute for the entire RS2 region. It has previously been shown (Amrein *et al.*, 1994) that protein produced from a transgene in which this same region is deleted is almost fully functional. Thus, at least two functionally redundant sequences exist within RS2 that are each sufficient to perform its essential functions. One of these sequences is located between amino acids 193 and 235, and the other between amino acids 238 and 264. Comparison of these regions for repeated sequences that might correspond to such a functional motif revealed a 12–13 aa region of similarity (Figure 8). Interestingly, the dU2AF^{50} RS domain contains a sequence that is also similar to part of this repeat while the dSRp55 RS domain does not contain similar sequences. Thus, there is a correlation between the presence of this loosely-defined sequence and the functionality of fusion proteins that have been tested.

Somatic and germline functions of TRA-2 can be separated genetically

The requirement for a specific RS domain in TRA-2 appears to be more dramatic in the male germline than in the somatic tissues. This suggests that the functions of RS2 may differ in these two cell types. The SR proteins, or other factors, that interact with TRA-2 in the male germline are not known but several pieces of evidence suggest that the way TRA-2 functions in the germline differs from that which has been described in the regulation of *dsx* and *fru* splicing in the soma. Not only do the target pre-mRNAs affected by TRA-2 differ in these cells, but in addition it is known that the TRA protein, an important cofactor of TRA-2 in the soma, is not expressed in males (McKeown *et al.*, 1987). Thus, TRA-2 must act in a TRA-independent manner in the male germline. Here we have found that fusion of additional protein sequences to the C-terminus of TRA-2 (without deletion of RS2), causes a strong disruption of germline function but has little, if any, effect on function of the protein in the soma.

This demonstrates that the TRA-2 germline and somatic functions can be separated from each other genetically and further suggests that the mechanisms by which this factor affects RNA processing differs in these two tissue types.

RS2 is required for discrete subnuclear localization of TRA-2 in primary spermatocytes

When *Drosophila* splicing factors are expressed in mammalian cells, they behave much like native mammalian splicing factors, localizing to discrete nuclear speckles associated with interchromatin granules in a pattern identical to that of many mammalian splicing factors (Li and Bingham, 1991; Hedley *et al*., 1995). In the case of the *Drosophila* splicing regulator *suppressor of white apricot* (SWAP) it was demonstrated that localization to speckles in COS cells was dependent upon an RS domain in SWAP that could be replaced by an RS domain from the TRA protein (Li and Bingham, 1991). Thus RS domains have been implicated in the subnuclear localization of *Drosophila* splicing factors when they are expressed in mammalian cells.

Studies on the nuclear distribution of splicing factors in *Drosophila* cells, however, have not in general revealed distribution patterns like that of the nuclear speckles seen in mammalian cells (Bopp *et al*., 1991; Kraus and Lis, 1994; Yannoni and White, 1997). In the polytene nuclei of larval salivary gland cells, several *Drosophila* splicing factors have been shown to localize at discrete chromosomal sites where they appear to be associated with nascent pre-mRNAs (Bopp *et al*., 1991; Champlin *et al*., 1991; Amero *et al*., 1992; Zu *et al*., 1996; M.E.McGuffin and W.Mattox, unpublished results).

In the course of these studies we have examined the distribution of TRA-2 within the nuclei of growth stage primary spermatocytes and found that it appears to be localized in association with partially condensed premeiotic chromosomes. Although the precise nature of these localized sites has yet to be established, we believe that they are likely to correspond to the association of the TRA-2 protein with a subset of nascent pre-mRNAs that are being transcribed in these cells. Localization of TRA-2 to these sites depends upon RS2. A deletion removing this domain results in protein that is diffusely distributed in the nuclei of spermatocytes. Given that RS2 has been implicated in both protein–protein and RNA–protein interactions (Amrein *et al*., 1994), it seems likely that TRA-2 protein missing this region cannot assemble into specific complexes that normally form at localized sites.

RS domains from other proteins varied in their ability to restore appropriate subnuclear localization to the fusion proteins. Again the most notable difference observed was between the RS domains of dU2AF^{50} and dSRp55. The dU2AF^{50} RS domain conferred localization like that of the full-length TRA2 protein, while protein with sequences from the dSRp55 RS domain was distributed in a diffuse pattern with only traces of localization in a subset of cells. These results indicate that the ability of TRA-2 to associate with specific sites depends on sequences in the RS2 domain. The ability of individual fusion proteins to localize was well correlated with male fertility, and thus it is possible that the inability of fusions proteins to function is a consequence of their failure to localize. However, in strains carrying the TRA-2/SC35 transgene the fusion protein localized normally, but failed to rescue male fertility in mutants. This latter result suggests that localization is not the only germline function affected by the RS domain.

With respect to the specificity of RS domain function, it is interesting to note that the N-terminal RS1 region is an invariant feature of the TRA-2 fusion proteins visualized here. Because deletion of RS2 results in diffuse nuclear localization in these strains, RS1 is not by itself sufficient to direct TRA-2 to the subnuclear sites. Instead RS1 appears to be necessary for nuclear/cytoplasmic localization of TRA-2 in primary spermatocytes (B.Dauwalder and W.Mattox, unpublished material) and contains within it sequences that are similar to a nuclear localization signal identified in the TRA protein (Hedley *et al*., 1995).

While specific RS domains are needed for correct subnuclear localization of TRA-2, RS2 alone is not likely to be sufficient. The RS domain of dU2AF^{50} supports subnuclear localization of TRA-2 to appropriate sites when substituted for RS2, but does not direct similar localization within the context of the dU2AF^{50} protein itself which is distributed diffusely in the nucleus. We suggest that sequences in the TRA-2 protein that are outside the RS2 domain must also play a necessary role in determining the subnuclear localization pattern.

RS domains and functional specificity of SR proteins

The results presented here suggest that the TRA-2 RS2 domain performs specific functions that are replaced by only a subset of the RS domains tested. Functional specificity as observed in RS2 may not be a universal characteristic of RS domains in other SR proteins. In support of this, several RS domains that have been subjected to similar analysis both *in vivo* and *in vitro*, appeared to function generically in that they could be functionally substituted by either artificially generated RS rich sequences or the RS domains of other splicing factors (Chandler *et al*., 1997b). In contrast, it has recently been demonstrated that the RS domains of several mammalian SR proteins differ in their ability to target the protein to the nucleus and to nuclear speckles and it has been suggested that the determinants for localization differ between proteins with two or only one RRM (Caceres *et al*., 1997). The observation that some RS domains perform specific functions might explain the fact that these regions in some cases exhibit striking evolutionary conservation. For example, the RS domain of human SC35 is 98% identical to that of chicken SC35 (Fu and Maniatis, 1992; Vellard *et al*., 1992). Likewise, the RS domains of human and rat *TRA-2* β are 100% identical (Matsuo *et al*., 1995; Dauwalder *et al*., 1996). The conserved sequences in these domains include not only the arrays of RS dipeptides, but also other amino acid residues that are interspersed throughout the RS domain. These observations are consistent with our results on TRA-2 RS2 and further support the idea that the RS domains of some proteins perform specific functions *in vivo* that differ from those of other SR proteins.

Materials and methods

Generation of fusion constructs

Protein fusion constructs were generated in the context of a 3.9 kb *Eco*RI genomic fragment containing the entire *tra-2* transcribed region

and all of the flanking sequences necessary for its expression (Goralski *et al*., 1989). For C-terminal FLAG epitope tags, a plasmid, pTRA2ΔRS, missing the RS2 domain, was first generated by oligonucleotide directed mutagenesis. This replaced sequences from amino acid 193 to C-terminus with sequences coding for the FLAG epitope (GACTACAAGGACG-ATGACGATAAGGGC) and a TAA stopcodon, flanked by *Bam*HI and *Xba*I sites. The RS domains of various SR proteins were then PCR-amplified and inserted as *Bam*HI or *Bam*HI–*Bgl*II restriction fragments immediately upstream of the FLAG sequences in pTRA2ΔRS. The wild-type control (pTRA2 C-FLAG) was made by oligonucleotide directed mutagenesis (Zoller and Smith, 1987) to introduce a *Bam*HI site, the FLAG epitope, a stop codon and an *Xba*I site in place of the natural stop codon. For all C-FLAG constructs the fragment from the 5′ *Eco*RI site to the newly created *Xba*I site was then introduced into pCASPA (Mattox *et al*., 1990) which contains the *tra-2* poly(A) signal and downstream flanking sequences. C-terminal *lacZ* fusion constructs were generated by introduction of the same *Eco*RI–*Xba*I fragments (see above) into pCaZPA (Mattox *et al*., 1990).

N-terminal FLAG fusion constructs were generated similarly but the FLAG epitope was introduced 5 aa downstream from the start codon of the $TRA2^{226}$ isoform. The TRA-2ΔRS and the RS fusion proteins were created in analogous fashion to the C-FLAG constructs except that no epitope tag sequences were added at the C-terminal fusion point and the entire region 3′ of the natural stop codon site was retained (as opposed to the more limited 3′ UTR sequences present in pCaSPA). The entire mutagenized 3.9 kb fragment was then introduced into the pCaSPeR P-element transformation vector. The RS domains and protein fusion sites in all constructs were confirmed by DNA sequencing.

RS domains were amplified from cDNAs coding for dSRp55, SC35 and TRA. The $dU2AF^{50}$ RS domain was amplifed from *Drosophila* CS genomic DNA. The regions amplified were from amino acids 198–333, 198–266 and 268–298 in dSRp55, 117–206 in SC35, 19–119 in TRA and 10–41 in $dU2AF^{50}$. The primers used were:

tra RS-1: CCGGGATCCAGGTCTCGATCCCGGCGAGA;
tra RS-2: GGAAGATCTTCGTTCACTGCTGCGACTTC;
dSRp55 RS-1: CCGGGATCCAGCGGACGTGGACGCTCCCG;
dSRp55 RS-2: GGAAGATCTCGAGGCCGAACGGGAGCGTG;
dSRp55 RS-3: GGAAGATCTGGACTTGGACACGTCACGCG;
dSRp55 RS-4: CCGGGATCCTCAAAGTCCCACTCCCGCAC;
SC35 RS-1: CCGGGATCCCGCCGGAGCCGCAGCCCTAG;
SC35 RS-2: CGCGGATCCCGATCGCGACCTGGATTTGG;
$dU2AF^{50}$ RS-1: CCGGGATCCCGCGAGAGACGCCGACATCG;
$dU2AF^{50}$ RS-2: GGAAGATCTCGGCTTGCGCCTCGAGTTCC;
tra-2 RScarb-1: CCGGGATCCAGTCGCAACCGTTACACTCG;
tra-2 RScarb-2: GGAAGATCTATAGCGCGATGAAGTTCGAC.

Generation of transgenic fly lines and crosses

Transgenic flies were obtained by P-element mediated germline transformation of w^{1118}/B^sY; $tra\text{-}2^B/CyO$ embryos. The $tra\text{-}2^B$ allele is a nonsense muation in exon 5 (Mattox *et al.*, 1991). Transformants were identified by increased eye pigmentation conferred by the w^+ gene which was included in the P transposon. All phenotypic and molecular results presented were obtained using flies that carry one copy of the respective transposons.

For the *fru* RT–PCR experiments the transposons were introduced into the strain w^{1118}/B^sY; *Bl-tra-2/CyO*. Flies were then crossed to w^{1118}/B^sY; *Df(2R) trix/CyO* (Mattox *et al.*, 1991) flies and the *tra-2* heteroallelic transgenic progeny identified by the absence of *CyO* and presence of the w^+ marker.

Male fertility was tested by culturing a single male with three w^{1118} virgins. Control crosses with wild-type males or mutant *tra-2* males were set up simultaneously. All fertile males produced adult progeny.

RNA isolation and low cycle RT–PCR

Drosophila RNA was isolated and *dsx* RT–PCR was performed as described (Dauwalder *et al*., 1996). RT–PCR to detect splicing of the M1 intron from *tra-2* was carried out in essentially the same way except that cDNA was produced using an oligo dT(12–18 nt) primer and PCR amplifications were carried out with 1 mM $MgCl_2$ with an annealing step of 60°C. Amplification primers derived from *tra-2* exon 3 (5′-CTCAGCCGATTCAGCTGGTGCTCTTG-3′) and the exon 5/6 junction (5′- CGCTGTGT/TTGTGCGTCAATCA-3′). Amplified fragments were electrophoresed, blotted and detected with a ^{32}P-labeled fragment extending from exon 3 to exon 4.

RT–PCR on *fru* RNA was carried out using random primers for cDNA synthesis and amplifications were done with 1.5 mM $MgCl_2$ using the primers described by Ryner *et al*. (1996). Male/common region and female-specific region hybridization probes were PCR amplified, using the same sex specific primers used for RT–PCR, in combination with a downstream sex-specific primer. The downstream primers used were: *fru* female antisense: 5′-CGCGCCAGTTGGTGGGGATTTG-3′ and *fru* male antisense: 5′-GGGGATGCGGGCGGAAGCGGA-3′. The PCR fragments were labeled with ^{32}P following the protocol of Mertz and Rashtchian (1994).

Immunohistochemistry

Flies were dissected in Testis Dissection Buffer (TDB) [0.183 M KCl, 47 mM NaCl, 10 mM Tris–HCl pH 6.8; (Bonaccorsi *et al*., 1988)]. Testes were placed on a microscope slide in a drop of TDB and covered with a silanized coverslip. They were then gently squashed until cells started flowing out of the testes. The slides were placed onto a Kimwipe with the coverslip side down and superfluous liquid allowed to drain. Slides were then dropped into liquid nitrogen. Fixation was done according to Pisano *et al*. (1993). Slides were washed three times in phosphate-buffered saline (PBS) for 15 min, in PBX (PBS + 0.2% Triton X-100) for 15 min and blocked in 1% bovine serum albumin (BSA) in PBX (BBX) for 20 min. Primary antibody was added in PBX + 2% normal goat serum, the slide covered with a coverslip and incubated at 4°C overnight in a humid chamber. The slides were washed three times in PBX for 15 min, incubated with the secondary antibody (in PBX + 10% normal goat serum) for 5 h at room temperature (RT) and washed three times in PBX. For double staining, the slides were again blocked for 20 min in BBX before application of the second primary antibody. DNA was stained with bis-Benzimide (Hoechst No. 33258, 0.5 μg/ml in PBX). The slides were then rinsed in 1× PBS and mounted in Aqua poly/mount (Polysciences) and viewed using a Zeiss Axiophot Microscope.

Antibodies. M2-anti-FLAG (Kodak) was used at dilutions ranging from 1:400 to 1:800 and detected with TR- or FITC-coupled goat anti-mouse IgG at 1:200 or 1:100 dilution (Jackson Laboratories). Affinity-purified anti-$dU2AF^{50}$ was a gift from D.Rudner and D.Rio, and was used at 1:500 dilution and detected with TR-coupled goat anti-mouse IgG at 1:200 dilution (Jackson Laboratories). Rabbit anti-β-gal (Cappel) was used at 1:500 to 1:700 and detected by TR- or FITC-coupled goat anti-rabbit IgG (Jackson Laboratories) at 1:500 dilution. All antibodies (except for anti $dU2AF^{50}$) were pre-absorbed against *Drosophila* w^{1118} embryos before use.

Acknowledgements

We thank Mitzi Kuroda, Tom Cooper and the members of the Mattox laboratory, especially Dawn S.Chandler, for helpful discussions and comments. We are grateful to M.Roth and Jo Yeakley for providing plasmid DNAs, and to D.Rudner and D.Rio for the $dU2AF^{50}$ antibody. This work was supported by research grants from the American Cancer Society (NP876), the National Institutes of Health (GM50825) and by a Pew Scholars Award to W.M. Sequencing was done by the DNA Core Sequencing Facility, which is supported by NIH grant CA16672.

References

Amero,S.A., Raychaudhuri,G., Cass,C.L., van Venrooij,W.J., Habets, W.J., Krainer,A.R. and Beyer,A.L. (1992) Independent deposition of heterogeneous nuclear ribonucleoproteins and small nuclear ribonucleoprotein particles at sites of transcription. *Proc. Natl Acad. Sci. USA*, **89**, 8409–8413.

Amrein,H., Gorman,M. and Nöthiger,R. (1988) The sex-determining gene *tra-2* of *Drosophila* encodes a putative RNA binding protein. *Cell*, **55**, 1025–1035.

Amrein,H., Maniatis,T. and Nöthiger,R. (1990) Alternatively spliced transcripts of the sex-determining gene *tra-2* of *Drosophila* encode functional proteins of different size. *EMBO J.*, **9**, 3619–3629.

Amrein,H., Hedley,M.L. and Maniatis,T. (1994) The role of specific protein–RNA and protein–protein interactions in positive and negative control of pre-mRNA splicing by *Transformer 2*. *Cell*, **76**, 735–746.

Birney,E., Kumar,S. and Krainer,A.R. (1993) Analysis of the RNA-recognition motif and RS and RGG domains: conservation in metazoan pre-mRNA splicing factors. *Nucleic Acids Res.*, **21**, 5803–5816.

Bonaccorsi,S., Pisano,C., Puoti,F. and Gatti,M. (1988) Y chromosome loops in *Drosophila melanogaster*. *Genetics*, **120**, 1015–1034.

Bopp,D., Bell,L.R., Cline,T.W. and Schedl,P. (1991) Developmental distribution of female-specific *Sex-lethal* proteins in *Drosophila melanogaster*. *Genes Dev.*, **5**, 403–415.

Caceres,J.F., Misteli,T., Screaton,G.R., Spector,D.L. and Krainer,A.R. (1997) Role of the modular domains of SR proteins in subnuclear localization and alternative splicing specificity. *J. Cell Biol.*, **138**, 225–238.

Cenci,G., Bonaccorsi,S., Pisano,C., Verni,F. and Gatti,M. (1994) Chromatin and microtubule organization during premeiotic, meiotic and early postmeiotic stages of *Drosophila melanogaster* spermatogenesis. *J. Cell Sci.*, **107**, 3521–3534.

Champlin,D.T., Frasch,M., Saumweber,H. and Lis,J.T. (1991) Characterization of a *Drosophila* protein associated with boundaries of transcriptionally active chromatin. *Genes Dev.*, **5**, 1611–1621.

Chandler,D., McGuffin,M.E., Piskur,J., Yao,J., Baker,B.S. and Mattox,W. (1997a) Evolutionary conservation of regulatory strategies for the sex determination factor *transformer-2*. *Mol. Cell. Biol.*, **17**, 2908–2919.

Chandler,S.D., Mayeda,A., Yeakley,J.M., Krainer,A.R. and Fu,X.D. (1997b) RNA splicing specificity determined by the coordinated action of RNA recognition motifs in SR proteins. *Proc. Natl Acad. Sci. USA*, **94**, 3596–3601.

Dauwalder,B., Amaya-Manzanares,F. and Mattox,W. (1996) A human homologue of the *Drosophila* sex determination factor *transformer-2* has conserved splicing regulatory functions. *Proc. Natl Acad. Sci. USA*, **93**, 9004–9009.

Fu,X.D. (1995) The superfamily of arginine/serine-rich splicing factors. *RNA*, **1**, 663–680.

Fu,X.D. and Maniatis,T. (1992) Isolation of a complementary DNA that encodes the mammalian splicing factor SC35. *Science*, **256**, 535–538.

Goralski,T.J., Edstrom,J.E. and Baker,B.S. (1989) The sex determination locus *transformer-2* of *Drosophila* encodes a polypeptide with similarity to RNA binding proteins. *Cell*, **56**, 1011–1018.

Hazelrigg,T. and Tu,C. (1994) Sex-specific processing of the *Drosophila exuperantia* transcript is regulated in male germ cells by the *tra-2* gene. *Proc. Natl Acad. Sci. USA*, **91**, 10752–10756.

Hedley,M.L., Amrein,H. and Maniatis,T. (1995) An amino acid sequence motif sufficient for subnuclear localization of an arginine/serine-rich splicing factor. *Proc. Natl Acad. Sci. USA*, **92**, 11524–11528.

Heinrichs,V. and Baker,B.S. (1995) The *Drosophila* SR protein RBP1 contributes to the regulation of *doublesex* alternative splicing by recognizing RBP1 RNA target sequences. *EMBO J.*, **14**, 3987–4000.

Heinrichs,V., Ryner,L.C. and Baker,B.S. (1998) Regulation of sex-specific selection of *fruitless* 5′ splice sites by *transformer* and *transformer-2*. *Mol. Cell. Biol.*, **18**, 450–458.

Ito,H., Fujitani,K., Usui,K., Shimizunishikawa,K., Tanaka,S. and Yamamoto,D. (1996) Sexual orientation in *Drosophila* is altered by the *satori* mutation in the sex-determination gene *fruitless* that encodes a zinc finger protein with a BTB domain. *Proc. Natl Acad. Sci. USA*, **93**, 9687–9692.

Kohtz,J.D., Jamison,S.F., Will,C.L., Zuo,P., Lührmann,R., Garcia-Blanco,M.A. and Manley,J.L. (1994) Protein–protein interactions and 5′-splice-site recognition in mammalian mRNA precursors. *Nature*, **368**, 119–124.

Kraus,M.E. and Lis,J.T. (1994) The concentration of B52, an essential splicing factor and regulator of splice site choice *in vitro*, is critical for *Drosophila* development. *Mol. Cell. Biol.*, **14**, 5360–5370.

Lee,C., Zamore,P.D., Green,M.R. and Hurwitz,J. (1993) RNA annealing activity is intrinsically associated with U2AF. *J. Biol. Chem.*, **18**, 13472–13478.

Li,H. and Bingham,P.M. (1991) Arginine/serine-rich domains of the *su(w^a)* and *tra* RNA processing regulators target proteins to a subnuclear compartment implicated in splicing. *Cell*, **67**, 335–342.

Lynch,K.W. and Maniatis,T. (1995) Synergistic interactions between two distinct elements of a regulated splicing enhancer. *Genes Dev.*, **9**, 284–93.

Lynch,K.W. and Maniatis,T. (1996) Assembly of specific SR protein complexes on distinct regulatory elements of the *Drosophila doublesex* splicing enhancer. *Genes Dev.*, **10**, 2089–2101.

Madigan,S.J., Edeen,P., Esnayra,J. and McKeown,M. (1996) *att*, a target for regulation by *tra2* in the testes of *Drosophila melanogaster*, encodes alternative RNAs and alternative proteins. *Mol. Cell. Biol.*, **16**, 4222–4230.

Manley,J.L. and Tacke,R. (1996) SR proteins and splicing control. *Genes Dev.*, **10**, 1569–1579.

Matsuo,N., Ogawa,S., Imai,Y., Takagi,T., Tohyama,M., Stern,D. and Wanaka,A. (1995) Cloning of a novel RNA binding polypeptide (RA301) induced by hypoxia/reoxygenation. *J. Biol. Chem*, **270**, 28216–28222.

Mattox,W. and Baker,B.S. (1991) Autoregulation of the splicing of transcripts from the *transformer-2* gene of *Drosophila*. *Genes Dev.*, **5**, 786–796.

Mattox,W., Palmer,M.J. and Baker,B.S. (1990) Alternative splicing of the sex determination gene *transformer-2* is sex-specific in the germ line but not in the soma. *Genes Dev.*, **4**, 789–805.

Mattox,W., McGuffin,M.E. and Baker,B.S. (1996) A negative feedback mechanism revealed by functional analysis of the alternative isoforms of the *Drosophila* splicing regulator *transformer-2*. *Genetics*, **143**, 303–314.

McKeown,M., Belote,J.M. and Baker,B.S. (1987) A molecular analysis of *transformer*, a gene in *Drosophila melanogaster* that controls female sexual differentiation. *Cell*, **48**, 489–499.

Mertz,L.M. and Rashtchian,A. (1994) Nucleotide imbalance and polymerase chain reaction: Effects on DNA amplification and synthesis of high specific activity radiolabeled DNA probes. *Anal. Biochem.*, **221**, 160–165.

Pisano,C., Bonaccorsi,S. and Gatti,M. (1993) The kl-3 loop of the Y chromosome of *Drosophila melanogaster* binds a tektin-like protein. *Genetics*, **133**, 569–579.

Ruskin,B., Zamore,P.D. and Green,M.R. (1988) A factor, U2AF, is required for U2 snRNP binding and splicing complex assembly. *Cell*, **52**, 207–219.

Ryner,L.C., Goodwin,S.F., Castrillon,D.H., Anand,A., Villella,A., Baker,B.S., Hall,J.C., Taylor,B.J. and Wasserman,S.A. (1996) Control of male sexual behavior and sexual orientation in *Drosophila* by the *fruitless* gene. *Cell*, **87**, 1079–1089.

Staknis,D. and Reed,R. (1994) SR proteins promote the first specific recognition of pre-mRNA and are present together with the U1 small nuclear ribonucleoprotein particle in a general splicing enhancer complex. *Mol. Cell. Biol.*, **14**, 7670–7682.

Tian,M. and Maniatis,T. (1993) A splicing enhancer complex controls alternative splicing of *doublesex* pre-mRNA. *Cell*, **74**, 105–114.

Valcarcel,J., Gaur,R.K., Singh,R. and Green,M.R. (1996) Interaction Of U2AF^{65} RS region with pre-mRNA of branch point and promotion base pairing with U2 snRNA. *Science*, **273**, 1706–1709.

Vellard,M., Sureau,A., Soret,J., Martinerie,C. and Perbal,B. (1992) A potential splicing factor is encoded by the opposite strand of the *trans*-spliced c-myb exon. *Proc. Natl Acad. Sci. USA*, **89**, 2511–2515.

Watanabe,T.K. (1975) A new sex transforming gene on the second chromosome of *Drosophila melanogaster*. *Jpn J. Genet.*, **50**, 269–271.

Wu,J.Y. and Maniatis,T. (1993) Specific interactions between proteins implicated in splice site selection and regulated alternative splicing. *Cell*, **75**, 1061–1070.

Yannoni,Y.M. and White,K. (1997) Association of the neuron-specific RNA binding domain-containing protein ELAV with the coiled body in *Drosophila* neurons. *Chromosoma*, **105**, 332–341.

Zahler,A.M., Lane,W.S., Stolk,J.A. and Roth,M.B. (1992) SR proteins: a conserved family of pre-mRNA splicing factors. *Genes Dev.*, **6**, 837–847.

Zoller,M.J. and Smith,M. (1987) Oligonucleotide-directed mutagenesis: a simple method using two oligonucleotide primers and a single-stranded DNA template. *Methods Enzymol.*, **154**, 329–350.

Zu,K., Sikes,M.L., Haynes,S.R. and Beyer,A.L. (1996) Altered levels of the *Drosophila* HRB87F/hrp36 hnRNP protein have limited effects on alternative splicing *in vivo*. *Mol. Biol. Cell*, **7**, 1059–1073.

Zuo,P. and Maniatis,T. (1996) The splicing factor U2AF^{35} mediates critical protein–protein interactions in constitutive and enhancer-dependent splicing. *Genes Dev.*, **10**, 1356–1368.

Received April 9, 1998; revised August 13, 1998; accepted August 14, 1998