Negative feedback regulation among SR splicing factors encoded by *Rbp1* and *Rbp1-like* in *Drosophila*

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SR proteins constitute a widely conserved family of splicing regulators. Negative autoregulation of SR proteins has been proposed to exert homeostatic control on the splicing environment, but few examples have been studied and the role of isoforms that lack the RS domain is unclear. We show that genes *Rbp1* and *Rbp1-like*, which encode *Drosophila* homologs of mammalian SRp20, negatively autoregulate and crossregulate at the level of alternative 3' splice site selection. This adjusts the relative expression of isoforms with either an RS domain or unrelated C-terminal domains (ALT) that are rich in serine and threonine. The effects of RBP1-ALT on splicing of *doublesex* and *Rbp1-like* are opposite to those of RBP1-RS and RBP1L-RS. RBP1-ALT and -RS exert opposing negative feedback on the ALT/RS ratio. However, RBP1-ALT inhibits the expression of RBP1-RS while stimulating that of RBP1L-RS. This asymmetry may contribute to changes in the RBP1-RS/RBP1L-RS ratio that are observed during development. These results provide the first example of a feedback-regulated SR protein network with evidence of an active homeostatic role for alternative isoforms.

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Introduction

Removal of introns by splicing of pre-mRNA is essential for the expression of most metazoan protein-coding genes. An important consequence is the opportunity for alternative splicing, which expands the structural and functional diversity of the proteome. Many cases of alternative splicing are regulated during development or in response to physiological signals, with diverse quantitative and qualitative effects on gene function (reviewed by Black, 2000, 2003; Graveley, 2001). Dysregulation of alternative splicing has been associated with a number of inherited diseases and cancer (reviewed by Faustino and Cooper, 2003), underscoring the

importance of understanding the mechanisms that normally control splicing.

Factors implicated in regulation of alternative splicing include the widely conserved family of SR proteins (Zahler *et al*, 1992; Fu, 1995), whose members are characterized by a modular architecture consisting of an N-terminal domain with one or two distinctive RRM-type RNA-binding motifs (the RBD), a glycine-rich region and a C-terminal region with repeating arginine/serine dipeptides (the RS domain). The RS domain plays a role in protein–protein interactions and its phosphorylation can influence RNA binding, nucleo-cytoplasmic shuttling, recruitment to nascent RNA and splicing activation (reviewed by Misteli, 1999; Tacke and Manley, 1999; Graveley, 2000; Black, 2003). RS domains of SR proteins also contact the branchpoint and the 5' splice site during spliceosome assembly (Shen and Green, 2004; Shen *et al*, 2004). Generally, SR proteins can replace one another for an essential splicing function *in vitro*, but they can exhibit different concentration-dependent effects on alternative splicing of specific substrates in cell transfection and *in vitro* assays (reviewed by Fu, 1995; Tacke and Manley, 1999). Variations in the levels and activities of particular SR proteins have been observed in different tissues, at different times during development and in response to mitogens, suggesting that SR proteins serve a broad role as concentration-dependent regulators of alternative splicing (reviewed in Fu, 1995; Sreanion *et al*, 1995; Manley and Tacke, 1996). This implies a need to control the levels of individual SR proteins relative to each other and to antagonists during development. Accordingly, severe developmental disruptions are observed when SR proteins B52 or dASF/SF2 are overexpressed in transgenic *Drosophila* (Kraus and Lis, 1994; Allemand *et al*, 2001). Alternatively spliced mRNAs that encode truncated variants have been isolated for several SR proteins, suggesting that these proteins may autoregulate to maintain homeostatic control of the splicing environment (Ge and Manley, 1991; Kim *et al*, 1992; Sreanion *et al*, 1995; Lejeune *et al*, 2001).

There is some experimental evidence for negative autoregulation by SR proteins in vertebrate cells. Expression of human ASF/SF2 in chicken B-cell line DT40 is accompanied by reduction of endogenous ASF/SF2 mRNA through an undetermined mechanism (Wang *et al*, 1996). Overexpression of SC35 in HeLa cells alters the processing of its own transcripts to produce unstable mRNAs that are degraded by the nonsense-mediated decay pathway (Sureau *et al*, 2001). In murine B-cell lymphoma line K46, overexpression of SRp20 changes the splicing of its own transcripts by enhancing the inclusion of a cassette exon that incorporates an early stop codon, leading to expression of a protein variant that lacks the RS domain (Jumaa and Nielsen, 1997). Alternative isoforms with reduced or absent RS domains have also been identified for mammalian ASF/SF2,

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9G8, Srp40 and SRp55 (Ge and Manley, 1991; Popielarz *et al.*, 1995; Sreaton *et al.*, 1995) and for *Drosophila* SC35, RBP1 and B52 (Kim *et al.*, 1992; FlyBase Consortium, 2003), but their regulation has not been investigated. The significance of RS-lacking isoforms for homeostatic control is unclear, however, because deletion of the RS domain does not necessarily eliminate nuclear or subnuclear localization, concentration-dependent effects on alternative splice site selection or even splicing activation at constitutive or enhancer-dependent sites (Caceres and Krainer, 1993; Zuo and Manley, 1993; Caceres *et al.*, 1997; Zhu and Krainer, 2000). Thus, the RS-lacking isoforms of different SR proteins could be activators of splicing for a subset of target sites, competitive antagonists at some sites or nonfunctional proteins.

An additional complication associated with concentration-dependent roles in regulation of alternative splicing is that feedback control must be integrated with the mechanisms that specify the appropriate levels for each SR protein in different cell types or developmental stages. The genetics and developmental biology of *Drosophila* should provide excellent opportunities for investigating these issues, but surprisingly little is known about the functions or regulation of SR proteins in this organism. Among the seven SR protein genes of *Drosophila*, mutant effects have been reported only for B52 (Ring and Lis, 1994; Peng and Mount, 1995; Hoffman and Lis, 2000). Even in this case, it has been difficult to identify loss-of-function effects on constitutive or regulated splicing, apparently because of partial functional overlap with other SR proteins (Ring and Lis, 1994; Hoffman and Lis, 2000). In addition, SR protein genes could be refractory to forward and reverse methods of genetic analysis if feedback and cross-regulatory mechanisms can compensate for reductions in dosage or function.

We are addressing these questions with the closely related genes *Rbp1* and *Rbp1-like*, which encode *Drosophila* homologs of mammalian SRp20 (Kim *et al.*, 1992; Mount and Salz, 2000). Their RS-containing products (RBP1-RS and RBP1L-RS, respectively) are 90% identical, but they differ by three amino-acid substitutions within the RBD and by the extension in RBP1L-RS of the glycine- and arginine-rich region between the RBD and RS domains (Supplementary data 1). The biological functions of RBP1L-RS have not been investigated, but RBP1-RS is proposed to play a role in regulating the splicing of *doublesex* (*dsx*), which encodes alternative isoforms of a transcription factor that promote either male or female development (Burtis and Baker, 1989; Coschigano and Wensink, 1993). Overexpression of RBP1-RS in *Drosophila* SL2 cells shifts alternative splicing of *dsx* reporter constructs from the male to the female mode (Heinrichs and Baker, 1995), and RBP1-RS is recruited to *dsx* splicing regulatory elements in *Drosophila* Kc cell extracts supplemented with TRA and TRA2 (Lynch and Maniatis, 1996). Hence, variations in the level of RBP1-RS could potentially destabilize somatic sexual differentiation.

We report that RBP1-RS and RBP1L-RS have equivalent effects on splicing of endogenous *dsx*, *tra* and *tra2* transcripts and that these effects are distinct from those of their closest relative in *Drosophila*, the SR protein 9G8. Using gain-of-function and loss-of-function strategies to analyze expression of the endogenous genes and reporter constructs, we show that *Rbp1* and *Rbp1-like* negatively autoregulate and cross-regulate each other at the level of alternative splicing. For

both genes, this feedback adjusts the ratio of alternative mRNAs that encode protein variants with either the standard RS domains or unrelated C-terminal domains (ALT) that are rich in serine and threonine. The RBP1-ALT isoform has functional properties unlike those of RBP1-RS or of a truncated protein lacking either C-terminal domain. RBP1-ALT contributes to homeostatic control by exerting effects on the *dsx* and *Rbp1-like* RNAs that are opposite to those of either RBP1-RS or RBP1L-RS. RBP1-ALT also exerts opposing feedback control on the ALT/RS ratio by suppressing its own production and stimulating that of RBP1L-RS. These results provide the first example of a feedback-regulated SR protein network with evidence of an active homeostatic role for an alternative isoform.

Results

RBP1-RS and RBP1L-RS have similar effects on splicing of *dsx*, *tra* and *tra-2*

Previously, overexpression of RBP1-RS in *Drosophila* SL2 cells was shown to increase the relative frequency of female splicing for transcripts from a transfected partial *dsx* reporter construct (Heinrichs and Baker, 1995). In the present work, we wished to compare the effects of RBP1-RS and RBP1L-RS on splicing of the normal endogenous *dsx* transcripts. We overexpressed each protein in SL2 cells by stable transformation with the corresponding cDNA under control of the inducible metallothionein promoter, and we used semiquantitative RT-PCR to examine the effect on splicing of endogenous *dsx* transcripts. Control cells were stably transfected with the parental vector and were induced with cupric sulfate under the same conditions. Figure 1B shows that induction led to strong overexpression of RBP1-RS and RBP1L-RS proteins from the transfected constructs. Phosphatase treatment confirmed that the two bands observed for RBP1-RS correspond to the hyperphosphorylated forms described by Du *et al.* (1998) (not shown). The lower mobility form was not observed with RBP1L-RS, suggesting that this results from phosphorylation at one or more of the positions that differ between the two proteins; nevertheless, the results described below show that RBP1-RS and RBP1L-RS have equivalent effects on all tested RNAs.

Overexpression of RBP1-RS or RBP1L-RS increased the female/male ratio of endogenous *dsx* mRNAs by approximately four-fold (Figure 1C). This effect was comparable to that observed previously with RBP1-RS using *dsx* reporter constructs in the absence of cotransfected *tra* or *tra2* (Heinrichs and Baker, 1995, 1997; quantitative data in the latter study indicate a 5.8-fold increase in the female/male ratio). Overexpression of RBP1-RS or RBP1L-RS also increased the total level of processed mRNA from the endogenous *dsx* gene (Figure 1C). This enhancement of *dsx* mRNA expression is consistent with the finding that mutation of RBP1-binding sites within the female exon of *dsx* reporter constructs increased the accumulation of unprocessed transcripts (Heinrichs and Baker, 1995). Overexpression of RBP1-RS or RBP1L-RS also stimulated production of a novel alternatively spliced form from the endogenous *dsx* gene ('*dsx-C*' in Figure 1C). Sequencing of the amplicons revealed that *dsx-C* results from use of an alternative 3' splice site located 73 nucleotides upstream of the site used in the Male isoform (Figure 1A). Translation of *dsx-C* RNA would produce a

Figure 1 Effects of RBP1-RS, RBP1L-RS and 9G8 on splicing of *dsx* RNA. (A) Gene structure and alternative splicing patterns of *dsx*. F: female 3' splice site; M: male 3' splice site; C: novel 3' splice site. Arrows show the locations of PCR primers. (B) Immunoblot analysis of RBP1 and RBP1L protein expression in control and overexpressing cells. (C) RT-PCR analysis of endogenous *dsx* RNAs in SL2 cells overexpressing RBP1-RS, RBP1L-RS or 9G8. rp49 is a coamplified internal standard. The graph shows levels of alternative *dsx* mRNAs relative to rp49 in control and experimental cells. The average and standard error for four experiments are shown in each case. * $P < 0.05$; ** $P < 0.01$. (D) RT-PCR analysis of endogenous *tra* and *tra2* RNAs in SL2 cells overexpressing RBP1-RS, RBP1L-RS or 9G8.

protein with the same DNA-binding domain, but with a novel C-terminal region of 18 amino acids. However, since we have not observed *dsx*-C in flies or SL2 cells under normal conditions, this variant may represent activation of a cryptic 3' splice site by high levels of RBP1-RS/RBP1L-RS.

Since female splicing of *dsx* during normal development requires TRA and TRA2, we also examined the expression of the corresponding mRNAs. We found that *tra* is spliced predominantly in the non-sex-specific (nonfunctional) mode in the control SL2 cells (Figure 1D; a small amount of female *tra* can be detected with greater amplification). This agrees with previous reports (Heinrichs and Baker, 1995) and with the low proportion of female *dsx* isoforms. Unexpectedly, overexpression of RBP1-RS or RBP1L-RS stimulated the splicing of *tra* in the female mode (Figure 1D) and also altered the splicing of *tra2*, causing an increase in the relative amount of type B and C isoforms (Figure 1D). TRA2 A and B function redundantly to promote female splicing of *dsx*, and TRA2 C exhibits a similar but weaker activity (Mattox *et al.*, 1996). The increased expression of female TRA may be

expected to enhance female splicing of *dsx* and to potentiate the effect of overexpressed RBP1-RS and RBP1L-RS. It was not essential, however, because the same changes in splicing of endogenous *dsx* were observed without obvious feminization of *tra* splicing when a truncated RBP1 protein lacking the RS domain (RBP1-delRS) was overexpressed under identical conditions (described below; Figure 6).

We also observed feminization of *dsx* splicing in the absence of obvious changes in *tra* or *tra2* splicing upon overexpression of *Drosophila* 9G8 (Figure 1C and D). 9G8 is the SR protein most closely related to RBP1-RS/RBP1L-RS in the RBD (66% identity). In addition to not altering *tra* or *tra2* splicing, overexpression of 9G8 did not induce *dsx*-C or increase the total level of spliced *dsx* mRNA significantly (Figure 1C). We cannot be certain whether these results reflect qualitative or quantitative differences between 9G8 and RBP1/RBP1L, because the absence of a suitable antibody for *Drosophila* 9G8 precludes a comparison of protein expression levels. However, the differential effects of RBP1-RS/RBP1L-RS were maintained over a wide range of overexpression (using 25–300 μ M cupric sulfate; not shown), suggesting that 9G8 is not simply a weaker version of these proteins. In contrast, the effects of the truncated protein RBP1-delRS, which were similar to those of RBP1-RS, disappeared at low levels of induction. Previous studies have found that a more distantly related *Drosophila* SR protein, B52, does not induce female splicing of *dsx* (Heinrichs and Baker, 1995, 1997). Thus, our results confirm that RBP1-RS and RBP1L-RS are functionally equivalent with respect to splicing of *dsx*, *tra* and *tra2* and indicate that they differ from other SR proteins in these activities.

Negative feedback regulation by RBP1-RS and RBP1L-RS

To test whether the expression of *Rbp1* and *Rbp1-like* is stabilized by negative feedback, we began by examining the levels of RBP1-RS and RBP1L-RS mRNA in adult flies heterozygous for deletions of either gene. *Df(1)C246* (cytological breakpoints 11D-E;12A01-02) deletes *Rbp1-like* (located at 11F1). Despite the 50% reduction of gene dosage, adults heterozygous for this deficiency have unchanged levels of mRNA encoding RBP1L-RS compared to wild type (Figure 2).

Figure 2 Levels of *Rbp1*-RS and *Rbp1L*-RS mRNAs are unaffected by a 50% reduction of gene dosage. The upper left panel shows RT-PCR analysis of mRNA in adult females heterozygous for *Df(1)C246* or *Df(3R)cu*. The ratios of *Rbp1*-RS or *Rbp1L*-RS to rp49 are plotted in the right panel, showing the average of three replicates and standard error. The lower left panel shows that PCR can distinguish a 50% reduction of input cDNA for either mRNA.

Similarly, *Df(3R)cu* (cytological breakpoints 86C1-2;86D8) deletes *Rbp1* (located at 86C6), but adults heterozygous for this deficiency have unchanged levels of mRNA encoding RBP1-RS (Figure 2). Figure 2 shows that a 50% reduction in amplicon signal could be detected for RBP1-RS and RBP1L-RS when half the amount of wild-type cDNA was used to program the PCR assay. These observations suggested that mRNAs for RBP1-RS and RBP1L-RS are maintained at constant levels by feedback regulation.

Negative feedback was confirmed with overexpression and knockdown experiments. First, we overexpressed RBP1-RS or RBP1L-RS in *Drosophila* SL2 cells as described above. The effects on endogenous RBP1-RS and RBP1L-RS mRNAs were analyzed by RT-PCR using reverse primers targeting the 3' untranslated regions, which are absent in the cDNA constructs. Figure 3A shows that overexpression of either RBP1-RS or RBP1L-RS reduced the levels of endogenous mRNAs for both proteins (~70% reduction, $N=3$, $P<0.001$), indicating that both genes are subject to negative regulation in response to either protein. In contrast, overexpression of either RBP1-RS or RBP1L-RS did not produce statistically significant changes in mRNAs encoding SR proteins 9G8, SC35, ASF/SF2 or B52 (Figure 3A). The absence of an effect on 9G8 is particularly interesting, since this is the SR protein most closely related to RBP1-RS/RBP1L-RS. Overexpression of 9G8 caused at best a slight decrease in endogenous RBP1-RS and RBP1L-RS mRNAs (Figure 3A; ~20% reduction but only marginally significant, with $P=0.07$ for RBP1L-RS). In contrast, endogenous 9G8 mRNA itself was reduced strongly upon overexpression of 9G8 protein (Figure 3A; 80% reduction, $N=3$, $P<0.01$). Thus, 9G8 and RBP1/RBP1L constitute distinct feedback circuits, although they may be coupled weakly.

We confirmed these results by using RNAi to knock down expression of *Rbp1* or *Rbp1-like* in SL2 cells. In this case, we could also analyze the effect on expression of each protein by immunoblotting. As predicted from the overexpression results, treatment with double-stranded RNA (dsRNA) against *Rbp1* reduced RBP1-RS mRNA and protein levels and caused a compensating increase in RBP1L-RS mRNA and protein

(Figure 3B and C). Treatment with dsRNA against *Rbp1-like* reduced RBP1L-RS mRNA and protein, as expected, but had little or no detectable effect on RBP1-RS mRNA or protein (Figure 3B and C). This apparently asymmetric behavior can be explained by the different contribution of RBP1-RS and RBP1L-RS to the combined pool of both proteins. Since RBP1L-RS makes up a minor proportion of the pool in SL2 cells (Figure 3C, control lane), a strong reduction of this protein is only expected to cause a small fractional increase in RBP1-RS mRNA or protein. In contrast, the difference in contribution to the pool does not matter when overexpressing RBP1L-RS, as demonstrated by the consequent strong reduction of RBP1-RS (Figure 3A).

To assess the relevance of these results to the developing embryo, we knocked down expression of *Rbp1* or *Rbp1-like* by microinjection of dsRNA at the syncytial blastoderm stage. The effects of RNAi were weaker in embryos (Figure 3D) than in SL2 cells, possibly because the feedback and crossregulation prevented strong reduction of target expression over the shorter experimental time course. Nevertheless, injection of dsRNA against *Rbp1* caused the predicted increase in RBP1L-RS mRNA (Figure 3D), although the confidence level was lower in embryos ($P<0.1$) than in SL2 cells. As in SL2 cells, we did not expect that knockdown of *Rbp1-like* would produce a significant increase in RBP1-RS mRNA (Figure 3D), because RBP1L-RS also makes up a minor proportion of the protein pool in embryos (Figure 3E).

Together, these results show that *Rbp1* and *Rbp1-like* are under mutual negative feedback control. However, other regulatory inputs must exist to establish different ratios of RBP1-RS and RBP1L-RS during development, since the proportion of the two proteins differs between embryos and adults (Figure 3E).

Feedback regulation of *Rbp1* and *Rbp1-like* occurs at the level of alternative splicing

Kim *et al* (1992) reported detection of polyadenylated but unspliced RNAs from *Rbp1* that would encode a protein truncated just before the RS domain. However, we have not observed differences in the accumulation of unspliced RNAs

Figure 3 Feedback and crossregulation of *Rbp1* and *Rbp1-like*. (A, upper and lower panels) RT-PCR analysis of endogenous mRNAs for SR proteins in SL2 cells overexpressing RBP1-RS, RBP1L-RS or 9G8. (B) RT-PCR analysis of mRNA levels following RNAi-mediated knockdown of *Rbp1* or *Rbp1-like* in SL2 cells. (C) Immunoblot analysis of RBP1-RS and RBP1L-RS after RNAi in SL2 cells. The lower panel shows γ -tubulin as a loading control. (D) RT-PCR analysis of mRNA levels following RNAi knockdown of *Rbp1* or *Rbp1-like* in embryos. (E) Immunoblot of RBP1-RS and RBP1L-RS in normal *Drosophila* embryos and adults. Graphs in panels B and D show the ratios of Rbp1-RS and Rbp1L-RS to rp49 mRNA, plotting the mean for three experiments and standard errors. * $P<0.1$; ** $P<0.05$; *** $P<0.01$.

from *Rbp1* or *Rbp1-like* that might account for the feedback-induced changes in accumulation of the RS-encoding mRNAs (not shown). EST clones from the *Drosophila* Genome Project (FlyBase Consortium, 2003) suggested that *Rbp1* also expresses an alternative mRNA resulting from use of a proximal 3' splice site located 754 nt upstream of the previously identified end of intron 2 (Figure 4A). This mRNA would encode a protein with an alternative C-terminal domain (which we designate ALT) in place of the RS domain (Figure 4B). This ALT domain is largely uncharged but it is rich in serine and threonine residues. Inspection of the *Rbp1-like* genomic sequence revealed the potential for a similar alternatively spliced form using a proximal 3' splice site located 2580 nt upstream of the previously identified intron end (Figure 4A). The resulting ALT region of *Rbp1-like* would also be rich in serine and threonine, but it would contain a hydrophobic stretch and a potential PEST sequence (PEST-find score: +9.09; <http://www.at.emblnet.org/emblnet/tools/bio/PESTfind/>). These alternative splice sites in *Rbp1* and *Rbp1-like* are conserved among *Drosophila* species with

available sequence for the relevant regions (Supplementary data 2). The open reading frames diverge among more distantly related species but maintain their general character and several highly conserved features (Supplementary data 3). We confirmed that the alternative splice forms for both *Rbp1* and *Rbp1-like* are expressed under normal conditions in SL2 cells (Figure 4C) and in male and female flies (not shown). The ALT isoforms constitute about 20% of the total (Figure 4C).

Figure 4D shows that overexpression of RBP1-RS or RBP1L-RS reduced the proportion of mRNAs encoding the RS isoforms (~80% reduction in RS levels; $N=3$, $P<0.01$); and increased the proportion encoding the ALT variants (~2-fold increase in ALT levels; $N=3$, $P<0.05$) from each gene. Under these conditions, two RT-PCR amplicons were observed that correspond to the ALT isoform of *Rbp1* (Figure 4D). Sequencing showed that they differ by the retention of intron 1; this makes no difference to the protein product because exon 1 is noncoding. Thus, overexpression of either RBP1-RS or RBP1L-RS shifts the ratio of splice forms for either transcript in favor of the ALT isoform.

We tested whether this regulation occurs at the level of alternative splice site selection itself by examining the RNA lariats produced by the first catalytic step of the splicing reaction. If overexpression of the RS forms causes a shift in 3' splice site selection to favor the proximal site (ALT), the production of lariats generated by use of this site should increase and the production of lariats generated by use of the distal site (RS) should decrease. To facilitate this analysis, we stabilized lariats in SL2 cells by using RNAi to knock down the expression of lariat debranching enzyme (DBR). This was carried out by transient transfection with a DNA construct that expresses dsRNA targeting the *Drosophila* DBR transcripts. Transcription of the RNAi construct was under control of the metallothionein promoter in plasmid pMT and was induced with copper sulfate at the same time as overexpression of RBP1-RS or RBP1L-RS. Analysis of endogenous lariats was performed by RT-PCR as diagrammed in Figure 5A and B. Figure 5C shows that the lariat representing use of the distal 3' splice site (RS) in *Rbp1* was detected in control cells, but it was absent when either RBP1-RS or RBP1L-RS was overexpressed. In contrast, the lariat corresponding to use of the proximal 3' splice site (ALT) increased when either RBP1-RS or RBP1L-RS was overexpressed. Both lariat amplicons were sequenced to confirm their identity and to locate the branchpoints. For the RS lariat, the branchpoint adenosine (AAGUAAU) was found 32 nt upstream from the distal 3' splice site. For the ALT lariat, the branchpoint adenosine (GUUAAAU) was found 24 nt upstream from the proximal 3' splice site. Thus, regulation of the RBP1 RS/ALT mRNA ratio occurs at the level of alternative 3' splice site selection. A decrease in the lariat for the RS isoform of *Rbp1-like* was also observed when either RBP1-RS or RBP1L-RS was overexpressed (Figure 5C). The branchpoint in this lariat (AAUCUAAU) was found 118 nt upstream of the distal 3' splice site. Although this is unusually far upstream, the region between this branchpoint and the 3' splice site is very rich in pyrimidines and contains no intervening AG dinucleotide. We could not detect the ALT lariat for *Rbp1-like*, possibly because secondary structure interfered with the RT-PCR reactions. In this case also, however, the results with the RS lariat are consistent with regulation of splicing as in *Rbp1*.

Figure 4 Alternative splicing of *Rbp1* and *Rbp1-like* transcripts. (A) Diagrams of *Rbp1* and *Rbp1-like* showing relative locations of alternative 3' splice sites, start and stop codons (not to scale). The coding regions for the RBD, RS and ALT domains are labeled. Arrows show the relative positions of PCR primers used to detect the endogenous mRNAs. (B) Amino-acid sequences of the RS and ALT domains of RBP1 and RBP1L. The putative PEST domain of RBP1L-ALT is shaded. (C) RT-PCR analysis of endogenous *Rbp1* and *Rbp1L* isoforms in control SL2 cells; *Rbp1* mRNAs were amplified with a single primer set for 27 cycles; *Rbp1L*-RS and *Rbp1L*-Alt were amplified with separate primer sets for 30 cycles. (D) RT-PCR analysis of endogenous RS and ALT isoforms in SL2 cells that overexpress RBP1-RS or RBP1L-RS. The endogenous ALT and RS isoforms were amplified with separate primer sets in both cases; amplification and loading were adjusted to obtain comparable signals for all isoforms from control cells.

Figure 5 Lariat analysis of 3' splice site selection. (A) Diagram of relevant alternative splicing events in *Rbp1* and *Rbp1-like*. Arrows show relative position and orientation of primers for lariat analysis. (B) Strategy used for RT-PCR analysis of lariats. In the cDNA product, the F and B priming sites become convergent across the branch junction (bp). (C) RT-PCR analysis of *Rbp1* and *Rbp1-like* lariats in SL2 cells after knockdown of debranching enzyme expression.

Additional evidence consistent with regulation at the level of alternative splicing comes from the analysis of reporter constructs, which shows that feedback regulation of the RS isoform levels requires the intron within the coding regions of *Rbp1* and *Rbp1-like* but not the native promoters nor the 5' or 3' untranslated regions (Supplementary data 4).

Contribution of RBP1-ALT to homeostasis and regulation

If the lack of an RS domain renders the ALT isoforms nonfunctional, alternative splicing of *Rbp1/Rbp1-like* could exert homeostatic control simply by adjusting the production of functional versus nonfunctional isoforms. However, the ALT isoforms could also contribute to homeostatic control if they antagonize the effects of the RS forms. The behavior of RBP1-ALT conforms to this second hypothesis, and it exhibits features that indicate distinct active roles for the ALT domain.

To investigate the function of RBP1-ALT, we generated a stably transfected SL2 cell line that overexpresses this protein from a cDNA under control of the metallothionein promoter, as described above for RBP1-RS. As a control, we generated an equivalent line to overexpress a truncated RBP1 protein (RBP1-delRS) that ends at the same point where the RS or ALT domains would normally begin (Figure 6A). Consistent with the presence of PEST-like sequences in the ALT domain, the protein levels achieved upon overexpression of RBP1-ALT were lower than for RBP1-RS or RBP1-delRS, and smaller fragments that probably result from proteolysis were ob-

served (Figure 6B). Nevertheless, overexpression of RBP1-ALT had strong effects on splicing, which were qualitatively different from those of RBP1-RS or RBP1-delRS under identical conditions. In the first place, overexpression of RBP1-ALT in SL2 cells severely reduced expression of the male and female *dsx* isoforms (Figure 6C); this effect was opposite to that of RBP1-RS, RBP1L-RS, or even RBP1-delRS, all of which stimulated production of the three *dsx* isoforms (Figures 1C and 6C). Furthermore, RBP1-ALT did not cause a shift in splicing of *tra* or *tra2* (unlike the RS forms), whereas RBP1-delRS shifted the splicing of *tra2* like the RS form (Figure 6C). RBP1-ALT also opposed the effects of RBP1-RS and RBP1L-RS and exerted negative feedback by reducing the level of its own endogenous mRNA and increasing the level of RBP1L-RS (Figure 6D). However, overexpression of RBP1-ALT reduced the expression of mRNA for RBP1-RS (Figure 6D); this effect was not the opposite of RBP1-RS or RBP1L-RS, either of which also reduced the expression of both RS mRNAs (Figure 3D). Thus, RBP1-ALT is not simply a passive antagonist of the RS forms with respect to 3' splice site choice, and it discriminates among targets in a different way.

Analysis of changes in pre-mRNA levels confirmed that RBP1-ALT reduces expression of both RBP1 isoforms by reducing splicing efficiency (Figure 6E). In contrast, a change in *dsx* pre-mRNA levels was not evident, suggesting that reduction of *dsx* isoforms may be due to changes in export or stability; however, results described above (Figure 1C) indicate that *dsx* is not spliced efficiently in control cells, so that a further reduction of splicing may not produce an obvious change in pre-mRNA levels.

Our results with RBP1-delRS (Figure 6C) appear to contradict earlier reports, which indicated that this truncated protein has no effect on splicing of *dsx* constructs (Heinrichs and Baker, 1997). This discrepancy is probably due to differences in level of overexpression, because the effects of RBP1-delRS were weaker when the protein was induced to lower levels using 75 μ M cupric sulfate (not shown). That RBP1-delRS is a partially weakened form of RBP1-RS is also supported by the presence of RS dipeptides within the retained Gly-rich region and by the observation that addition of one more RS dipeptide to the truncated protein is sufficient to restore nearly full effects on *dsx* splicing (Heinrichs and Baker, 1995).

Discussion

We have presented evidence for a regulatory network involving the SRp20 homologs of *Drosophila*, encoded by *Rbp1* and *Rbp1-like*. This network includes effects on alternative splicing of transcripts from sex determination genes and mechanisms for feedback and crossregulation that adjust the combined levels of RS versus ALT isoforms encoded by *Rbp1* and *Rbp1-like*, thus exerting homeostatic control on the system (summarized in Figure 7). We have also discovered strong autoregulation by 9G8, which has partially overlapping effects on the sex determination RNAs. Thus, RBP1/RBP1L and 9G8 appear to constitute two weakly coupled but distinct regulatory circuits.

Regulation of alternative splicing in the sex determination pathway

Although RBP1, RBP1L and 9G8 are probably involved in the processing of mRNAs with diverse developmental functions,

Figure 6 Functional analysis of the RBP1-ALT protein. (A) Structures of RBP1-RS, RBP1-ALT and mutant RBP1-delRS. (B) Immunoblot analysis of SL2 cells overexpressing RBP1-ALT or RBP1-delRS. The antibody was raised against the RRM exon and detects both proteins as well as RBP1-RS and RBP1L-RS. The blot was also probed with an antibody against hrp48 as a quantitative standard. The bands corresponding to RBP1-RS and RBP1L-RS were identified by overexpression of the corresponding cDNAs (Figure 1B) and knockdown of the mRNAs (Figure 3C). (C) RT-PCR analysis of *dsx*, *tra* and *tra2* mRNA isoforms in SL2 cells overexpressing RBP1-ALT or RBP1-delRS. (D) RT-PCR analysis of *Rbp1* and *Rbp1L*-like mRNAs in cells overexpressing RBP1-ALT or RBP1-delRS. The endogenous RS and ALT isoforms of both genes were amplified with separate primers; cycle number and loading were adjusted to equalize the signals from control cells. (E) RT-PCR analysis of pre-mRNA levels for *Rbp1* and *dsx*. Each experiment of panels B–E was replicated at least three times and similar results were obtained.

a premise of our investigation was that homeostatic regulation of these SR proteins would at least be important to avoid destabilization of sex determination. This was based on previous studies in transfected cells and cell extracts that had implicated RBP1 as a non-sex-specific factor that cooperates with TRA to regulate splicing of *dsx* RNA (Heinrichs and Baker, 1995; Lynch and Maniatis, 1996) and which had shown similar capabilities for human 9G8 (Lynch and Maniatis, 1996). Normally, sex-specific alternative splicing of *dsx* RNA is determined by the presence or absence of TRA protein, whose expression is also restricted to females as a consequence of alternative splicing controlled by SXL protein. *Drosophila* SL2 cells have low levels of functional TRA protein because their endogenous *tra* RNAs are spliced primarily in the male mode. Accordingly, *dsx* RNA in SL2 cells is also spliced primarily in the male mode. Previous studies had shown that overexpression of RBP1-RS in SL2 cells, with or without cotransfected TRA, could increase female splicing of a transfected *dsx* reporter construct, and it was suggested that high levels of RBP1-RS could bypass the requirement for TRA or potentiate the effect of low TRA concentration (Heinrichs and Baker, 1995). Our present study shows that overexpression of RBP1-RS or RBP1L-RS shifts the splicing of *tra* RNA itself toward the female mode. The increased expression of female TRA may be expected to

Figure 7 Regulatory circuit showing homeostatic regulation of *Rbp1* and *Rbp1*-like. Horizontal arrows indicate alternative splicing patterns. Curved arrows indicate stimulation and bars indicate inhibition. Effects on *tra* and *tra2* splicing are not shown (see text).

enhance female splicing of *dsx* and also to potentiate the effect of overexpressed RBP1-RS and RBP1L-RS; this has been demonstrated previously in cotransfection experiments

(Heinrichs and Baker, 1995). However, our results indicate that feminization of TRA expression is not essential for the shift in *dsx* splicing, because overexpression of a related SR protein (9G8) or of a truncated RBP1 protein lacking the RS domain (RBP1-delRS) also induced female splicing of endogenous *dsx* but did not produce an obvious increase in female splicing of *tra* (Figures 1 and 6). We also observed that overexpression of RBP1-RS or RBP1L-RS changed the alternative splicing of *tra2*, although all of the isoforms produced can support female splicing of *dsx* (Mattox *et al*, 1996).

Our study does not address which of these effects are direct consequences of RBP1-RS and RBP1L-RS overexpression, but previous work supports a role for direct interactions between RBP1-RS and *dsx* regulatory elements (Heinrichs and Baker, 1995; Lynch and Maniatis, 1996). Human 9G8 (and a protein similar in size to *Drosophila* 9G8) have also been shown to interact with *dsx* regulatory elements (Lynch and Maniatis, 1996). In addition, the feminization of *tra* splicing by overexpression of RBP1-RS/RBP1L-RS was not a consequence of feminization of *Sxl* splicing, because *Sxl* was spliced in the male mode both in control cells and in cells that overexpressed RBP1-RS (not shown). Similarly, the changes in *tra2* splicing were observed in the absence of changes in *tra* when Rbp1-delRS was overexpressed (Figure 6). Regardless of which effects are direct, however, the premise of our study is validated with respect to the expression of at least three genes involved in sex determination.

As noted above, overexpression of 9G8 feminized the splicing of *dsx* RNA without changing the splicing of *tra*. This is consistent with the observation that addition of TRA2 alone is sufficient to recruit human 9G8 to *dsx* regulatory elements in HeLa cell extracts, and that human 9G8 can activate splicing through the *dsx* regulatory elements in such extracts (Lynch and Maniatis, 1996). Although the same study found that in *Drosophila* Kc extracts RBP1 was recruited to the *dsx* repeats instead of 9G8, the relative levels of these two proteins in Kc cells are not known. The effects of 9G8 in our study differed from those of RBP1-RS and RBP1L-RS in additional respects: overexpression of 9G8 did not affect the splicing of *tra2*, it did not increase the total level of *dsx* mRNA and it did not induce the *dsx*-C isoform. These differences may be meaningful for normal development of different cell types, since RBP1 is expressed ubiquitously (Kim *et al*, 1992) whereas 9G8 is strongly enriched in the nervous system and in specific regions of the imaginal disks (Vorbruggen *et al*, 2000).

Autoregulation of Rbp1 and Rbp1-like

Our results show that alternative splicing of *Rbp1* and *Rbp1-like* pre-mRNAs is regulated by negative feedback to adjust the RS/ALT isoform ratio. The ALT isoforms produced in both cases still have the RBD and glycine-rich region in addition to novel C-terminal domains. We have not investigated the functional properties of RBP1L-ALT; consistent with the presence of a PEST sequence in its ALT domain, this protein appears to be very unstable and we have not detected endogenous expression. A band that may correspond to endogenous RBP1-ALT is observed in Western blots (Figures 1B and 6B) but its identity is not certain because RBP1-ALT is expected to migrate very close to unphosphorylated RBP1-RS and antibodies that react only with RBP1-ALT

are not available. RBP1-ALT expressed from exogenous constructs does accumulate to detectable levels (although it appears to be less stable than RBP1-RS) and it exhibits novel functional properties that are distinct from those of the RS isoforms or those of a truncated RBP1 protein (RBP1-delRS) lacking either the RS or ALT domains. First, RBP1-ALT does not affect splicing of *tra* or *tra2*. More significantly, RBP1-ALT has effects on expression of *dsx* and *Rbp1-like* that are opposite to those of RBP1-RS and RBP1L-RS. Therefore, RBP1-ALT can contribute actively to homeostatic control of *Rbp1/Rbp1-like* function. Nevertheless, RBP1-ALT does not behave like a strict antagonist of the RS forms, because it shares with them the ability to suppress the expression of RBP1-RS. The complex behavior of RBP1-ALT probably reflects differential, context-dependent interactions with other factors.

In contrast to RBP1-ALT, RBP1-delRS has the same qualitative effects on *dsx* and *tra2* as do RBP1-RS and RBP1L-RS (although titration of overexpression levels indicates that RBP1-delRS is less potent), it does not suppress RBP1-ALT, and it has no effect on *Rbp1-like*. These differences in behavior suggest that the serine/threonine-rich domain of RBP1-ALT plays an active role in splicing regulation, possibly by mediating protein-protein interactions with other regulators or components of the splicing machinery. Additional observations argue that the effects of RBP1-ALT are not due merely to reduction of RBP1-RS. First, overexpression of RBP1-ALT did not eliminate RBP1-RS completely in our experiments and it increased the expression of RBP1L-RS mRNA and protein, so that the combined RS levels were similar to or higher than controls (Figure 6); since the data presented in this paper show that RBP1L-RS is functionally equivalent to RBP1-RS with respect to all known effects, this indicates that RBP1-ALT must affect *dsx* independently of the reduction of RBP1-RS. Second, RBP1-delRS reduces RBP1-RS without increasing RBP1L-RS, but it affects *dsx* and *tra2* in the same way as the RS isoforms (Figure 6).

The self-repression by RBP1-ALT should contribute to feedback control of the RS/ALT ratio, but because RBP1-ALT also represses production of RBP1-RS while stimulating RBP1L-RS, changes in the level or activity of RBP1-ALT could shift expression between the two RS forms. Although we do not know which of these effects are direct, the interplay may contribute to the developmental differences that we have observed in the ratio between RBP1-RS and RBP1L-RS proteins (Figure 3). We expect that additional regulatory inputs exist in this circuit beyond those identified in Figure 7; for example, none of the known inputs promote the formation of RBP1-RS.

Feedback regulation of splicing among SR proteins

Autoregulation at the level of splicing provides direct coupling between the activity of SR proteins and the operation of feedback control. Negative autoregulation by alternative splicing to produce structurally distinct proteins has been proposed for mammalian SRp20 (Jumaa and Nielsen, 1997), but functional studies of the truncated RS-lacking isoform thus produced have not been reported, and it is not known whether this isoform contributes to homeostatic control. Alternative isoforms ASF-2 and ASF-3 act as competitive inhibitors of ASF/SF2 *in vitro* (Zuo and Manley, 1993), but their regulation has not been described. Unlike RBP1-ALT, the

altered properties of ASF-2 and -3 with respect to splice site selection result from partial deletion of the second RRM and not from substitution of the RS (Zuo and Manley, 1993). In *Drosophila*, negative autoregulation at the level of splicing has been documented for SR-related protein TRA2, which encodes an alternative isoform lacking an N-terminal arginine/serine-rich region; this deleted variant is less active with respect to regulation of known target RNAs but it is still partially redundant with the full-length isoform (Mattox *et al.*, 1996). Unlike RBP1-ALT, this alternative TRA2 isoform does not seem to play an antagonistic role in autoregulation (Mattox *et al.*, 1996). Thus, *Rbp1/Rbp1-like* provide the first documentation of autoregulation and homeostatic control by SR proteins involving the production of alternative isoforms with distinct, actively antagonistic functions. The gene for *Drosophila* SR protein B52 also encodes alternatively spliced isoforms in which the RS domain is replaced by a serine-rich domain (FlyBase Consortium, 2003); it will be interesting to determine the functional relationship between the isoforms in this case and whether their ratio is also controlled by feedback regulation.

In summary, our results provide novel insights into the control of an important class of splicing regulators. In addition, the elucidation of feedback regulation and functional relationships among *Rbp1*, *Rbp1-like* and *9G8* will aid the investigation of SR protein roles in *Drosophila* development.

Materials and methods

RNA extraction and RT-PCR analysis

RNA from adults, embryos and SL2 cells was extracted using the RNeasy Mini kit (Qiagen, CA). Reverse transcription was performed with Superscript-II (Invitrogen, CA) and oligo(dT) primers on 1 µg RNA. One-tenth of the reverse transcription product was used for PCR in a 25 µl reaction, using primers described in Supplementary Table I (Supplementary data 5). PCR conditions were 3 min at 95°C, followed by 21 cycles (Rbp1-RS), 27 cycles (Rbp1-ALT), 29 cycles (Rbp1L-RS and ALT), 27 cycles (9G8), 18 cycles (rp49) or 40 cycles (dsx, using primer E2F) each consisting of 30 s at 95°C, 30 s at 55°C and 1 min at 72°C, followed by a final extension for 7 min. In some experiments, cycle numbers were adjusted as indicated in text to equalize signals or visualize less abundant products. The *dsx* PCR was followed by nesting a 1:160 dilution of the reaction with primer dsxE3F for 15 cycles. A 10 µl portion of each reaction was run on a 2% agarose gel and stained with GelStar (Cambrex Bio Science). Amplimers were quantitated from digital images using Kodak 1D Analysis software. Data, normalized to *rp49* levels, are presented as mean ± s.e.m. The significance of differences between control and experimental samples was analyzed using Student's *t*-test for unpaired samples.

cDNA cloning and expression in SL2 cells

cDNAs for overexpression in SL2 cells were cloned between the *EcoRI* and *XhoI* sites of vector pMT-V5-His-A (Invitrogen, CA).

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Primers used to amplify cDNA (Supplementary data 6; Supplementary Table II) spanned the start and stop codons of the respective genes and had *EcoRI* or *XhoI* restriction sites designed into their 5' ends. Amplification of cDNA was carried out using Platinum Taq-HIFI (Invitrogen, CA). SL2 cells were stably transfected using the calcium phosphate method, with pcopneo as the selected plasmid (Rio and Rubin, 1985). cDNA expression was induced with 300 µM cupric sulfate for 48 h.

RNAi in SL2 cells and embryos

dsRNAs against *Rbp1* and *Rbp1-like* were prepared by *in vitro* transcription from PCR products with T7 promoters on both ends of the amplimers, using the Megascript RNAi kit (Ambion, TX). The PCR primers are described in Supplementary Table III (Supplementary data 5). A 20 µg portion of dsRNA was applied to SL2 cells every 48 h and the cells were harvested after 120 h. Embryos were injected with a 5 µM solution of dsRNA in 5 mM KCl and 0.1 mM NaPO₄ injection buffer after dechorionation using 50% bleach.

Immunoblot analysis

Total protein was extracted from SL2 cells using PARIS cell disruption buffer (Ambion, TX) and quantitated using the Bio-Rad dye-binding assay. Equal amounts of protein per sample were electrophoresed on a 15% SDS-polyacrylamide gel at 100 V and transferred onto PVDF at 100 V for 45 min. Immunoblotting was performed by standard procedures (Harlow and Lane, 1988). Membrane blocking and incubations with primary and secondary antibodies were for 1 h each. The rabbit polyclonal antibody against RBP1/RBP1L was prepared by immunization with a GST-RBP1L-RS fusion protein purified from *Escherichia coli*. Specific antibodies were affinity-purified on a GST-RBP1L-Sepharose column after passage through a GST column. The affinity-purified antibody was used at a 1:200 dilution. The secondary antibody was goat anti-rabbit IgG-HRP conjugate (Bio-Rad Laboratories, CA). Blots were developed using the ECL Plus Detection System (Amersham Biosciences) and imaged on a Storm 840 Scanner (Molecular Dynamics).

RT-PCR analysis of lariats

Reverse transcription of total RNA was performed with random hexamer primers. Primers for PCR are described in Supplementary Table IV (Supplementary data 5). PCR was performed for 30 cycles as above, followed by nesting 1:160 dilutions for 20 cycles. PCR products were analyzed on 2% agarose gels in TBE buffer. Amplimers were gel-purified and sequenced at the University of Pittsburgh Core facilities.

Supplementary data

Supplementary data are available at *The EMBO Journal* Online.

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