Regulation of tissue-specific P-element pre-mRNA splicing requires the RNA-binding protein PSI

Christian W. Siebel,¹ Roland Kanaar, and Donald C. Rio

Department of Molecular and Cell Biology, Division of Genetics and Division of Biochemistry and Molecular Biology, University of California, Berkeley, Berkeley, California 94720 USA

Binding of a multiprotein complex to a 5' exon inhibitory element appears to repress splicing of the *Drosophila* P-element third intron (IVS3) in the soma. We have purified 97- and 50-kD proteins that interact specifically with the inhibitory element using RNA affinity chromatography. Antibodies specific for the 97-kD protein relieve inhibition of IVS3 splicing in somatic extracts, providing direct evidence that inhibition requires this protein, P-element somatic inhibitor (PSI). We identify the 50-kD protein as hrp48, a protein similar to the mammalian splicing factor hnRNP A1, and show that hrp48 recognizes specific nucleotides in a pseudo-5' splice site within the inhibitory element. The results indicate that PSI is an alternative splicing factor that regulates tissue-specific splicing, probably through interactions with generally expressed factors such as hrp48.

[**Key Words:** Alternative splicing; P elements; PSI; hrp48; RNA binding proteins; purification]

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Premessenger RNA (pre-mRNA) splicing, the process that removes noncoding intron sequences, is required to produce mature eukaryotic mRNA. Regulation of splicing and splice site selection provide an important means to control gene expression and generate protein diversity (Green 1991; Maniatis 1991; Rio 1993). Although the precise mechanism of splicing catalysis remains enigmatic, the basic splicing reaction is well understood. A number of proteins and small nuclear ribonucleoprotein (snRNP) particles assemble on the pre-mRNA to generate the spliceosome, which catalyzes two transesterification reactions: (1) 5' splice site cleavage and branch formation, and (2) exon ligation, which is concomitant with intron release (Guthrie 1991; Moore et al. 1993). How the spliceosome recognizes the conserved sequences that mark the branch site and splice sites is not well understood. For example, although 5' splice site recognition involves binding of U1 snRNP, it is unclear how the accurate site is selected and alternative or cryptic sites are ignored. Experiments that address splice site selection during alternative pre-mRNA splicing should help to answer such questions of specificity and thus illuminate mechanisms that govern an important control point in gene expression.

Germ line-specific splicing of the *Drosophila* P element third intron (IVS3) has provided one paradigm for

identifying splicing regulators and characterizing their mechanism of action. An inhibitory activity represses IVS3 splicing in the soma (Siebel and Rio 1990; Chain et al. 1991), apparently by blocking U1 snRNP binding to the accurate 5' splice site and stabilizing U1 snRNP binding to an inactive pseudo-5' splice site (Siebel et al. 1992). Mutations in a 5' exon sequence element that includes two pseudo-5' splice sites activate IVS3 splicing in somatic cells (Chain et al. 1991; P. Seshiah, C.W. Siebel and D.C. Rio, unpubl.) and extracts (Siebel et al. 1992), demonstrating that the inhibitory activity functions by recognizing this element. Candidate components of the inhibitory activity have been identified by noting a correlation between specific binding to this inhibitory element and inhibition of IVS3 splicing (Siebel and Rio 1990; Chain et al. 1991; Siebel et al. 1992). For example, a 97-kD protein in somatic extracts specifically cross-links to the inhibitory element (Siebel and Rio 1990). In addition, native gel electrophoresis detects four protein complexes that specifically bind to a pseudo-5' splice site within the inhibitory element (Siebel et al. 1992). These complexes contain the 97-kD protein as well as the RNA-binding proteins of 50, 65, and 45 kD. Two of these complexes (numbered 3 and 4) generate a modification-interference footprint at the pseudo-5' splice site regulatory sequence, suggesting that these complexes are functionally relevant. Notably, addition of 5' exon RNA fragments, but not mutant RNA that fails to bind the complexes, activates IVS3 splicing, suggesting that 5' exon RNA titrates inhibitory proteins

¹Present address: Department of Biochemistry and Biophysics, University of California, San Francisco, San Francisco, California 94143-0448 USA.

away from the splicing substrate. Moreover, mutations in the pseudo-5' splice site that disrupt complex formation also relieve inhibition of IVS3 splicing, suggesting that at least one of these four proteins plays a central role in repressing IVS3 splicing (Siebel et al. 1992).

Despite this strong correlation between the inhibitory activity and the binding of these proteins to the inhibitory element, it has remained unclear whether some of the proteins function directly in somatic inhibition, or bind fortuitously to the inhibitory element. Determining the function of these proteins in somatic inhibition requires protein purification and biochemical characterization of the individual proteins.

Using conventional and RNA affinity chromatography, we purified the activities that generate complexes 3 and 4 and characterized them in RNA-binding and splicing inhibition assays. Consistent with previous results, these activities are comprised of the 97- and 50-kD proteins, which we identified as the *Drosophila* heterogeneous nuclear ribonucleoprotein (hnRNP), hrp48. The recombinant 97-kD protein binds specifically to the IVS3 5' exon, and hrp48 recognizes the pseudo-5' splice site. Most important, antibodies against the 97-kD protein specifically relieve inhibition of IVS3 splicing in somatic extracts, providing direct evidence that the 97-kD protein is necessary for somatic inhibition. Given these biochemical data and the observation that the 97-kD protein is expressed specifically in the soma (C. Siebel, A. Admon, and D. Rio, in prep.), we have named the 97-kD protein PSI, for P element somatic inhibitor. Taken together, our results suggest that PSI is a soma-specific alternative splicing factor that functions with general factors including hrp48 to regulate germ line-specific gene expression.

Results

Purification of proteins that recognize the pseudo-5' splice site regulatory sequence using RNA-affinity chromatography

Figure 1 and Table 1 outline the scheme used to purify the RNA-binding proteins that form native gel complexes 3 and 4, the two complexes that generate a modification-interference footprint at a pseudo-5' splice site within the inhibitory element. The first steps were designed to remove endogenous nucleic acids and ribonucleases that could hinder RNA affinity chromatography. Q-Sepharose anion exchange chromatography also separated the binding activities of complexes 3 and 4, which failed to bind to this resin, from the binding activity of complex 2 (Fig. 1). Consistent with previous results (Siebel et al. 1992), the 65- and 45-kD RNA-binding proteins cofractionated with the complex 2 binding activity (data not shown).

The complex 3 and 4 binding activities were further purified five- to sixfold by chromatography on blue trisacryl, an immobilized dye that has been used to purify nucleotide- and nucleic acid-binding proteins (Stell-

Figure 1. Purification flow chart. (For details, see Materials and methods.)

wagen 1990). The complex 3 and 4 binding activities primarily eluted at 0.6 M KCl ("B.6 fraction"; data not shown).

To optimize purification during RNA affinity chromatography, we sought to use the most stringent binding conditions that would still permit formation of complexes 3 and 4. We tested various binding conditions using the B.6 fraction in the native gel assay (Fig. 2). Formation of complexes 3 and 4 on 5' exon RNA was extremely stable, occurring efficiently in 0.75 M KCl, 10 mM MgCl₂, 25 mg/ml of heparin, or a 1000-fold excess (wt/wt) of nonspecific RNA (Fig. 2, lanes 6,10,14,15). Complex formation did not require ATP (cf. lanes 2 and 19) and was specific because mutations in the pseudo-5' splice sites prevented formation of complexes 3 and 4 [lane 20].

The B.6 fraction was loaded onto an IVS3 5' exon RNA affinity column in the presence of 0.5 M KCl and 10 mg/ml of heparin. Bound material was eluted by increasing the KCl concentration in steps. This procedure separated the two binding activities (Fig. 3A) and purified them

Table 1. Purification and yield

Fraction	Volume (ml)	Protein		Activity		Yield (%)	Purification
		conc. (mg/ml)	total (mg)	specific (U/mg)	total (units)		
Nuclear extract	2.66	11.1	29.5	180	5310	100	1.0
Ultracentrifugation	5.80	4.30	25.1	286	7179	135	1.6
Q-Sepharose	3.90	1.56	6.09	720	4420	83	4.1
Blue trisacryl	1.50	0.41	0.62	4,060	2500	47	22
RNA affinity	1.90	0.006	0.012	183,000	2200	41	1000

One particular analytical-scale purification is outlined here. One unit was defined as the activity that bound half of the RNA in the native gel assay. The number for the yields and fold purification are only approximate. This purification has been scaled up >10-fold with similar results (data not shown).

~50-fold (Table 1). The peak fractions, which generated complexes 3 and 4, contain only a few polypeptides (Fig. 3B, lanes 9,10). Interestingly, predominant doublets at 97 and 50 kD comigrated with the previously described 97- and 50-kD proteins that can be cross-linked to the IVS3 5' exon (Siebel and Rio 1990; data not shown). On the basis of their fractionation, cross-linking (data not shown), and immunoreactive properties (see below), the polypeptides in each doublet may represent highly related proteins that differ because of post-translational

modifications or alternative pre-mRNA processing. Fractions that contained only the 50-kD protein as the most

Figure 2. RNA-binding stability of the complexes formed on the IVS3 5' exon. To determine suitably stringent binding conditions for the RNA affinity column, the binding activity of the B.6 fraction was tested in the native gel electrophoresis assay, varying the following conditions: (1) volume of the fraction assayed (μ l FX), (2) KCl concentration ([KCl]), (3) $MgCl_2$ concentration ([Mg]), (4) heparin concentration, and (5) amount of total yeast RNA competitor ([RNA]). All reactions contained 3.7 μ l of the B.6 fraction except where noted otherwise. Reactions were performed at 20°C without ATP except those in lane 3, which was incubated on ice, and lane 19, which was incubated in the presence of ATP. The reaction in lane 20 was identical to that in lane 2 except that it contained a mutant 5' exon RNA with the pseudo-5' splice sites disrupted (U Δ 11 F1,F2; Siebel et al. 1992) in place of wild-type 5' exon RNA. The positions of free RNA and complexes 3 and 4 are indicated (right).

Figure 3. Purification of the 97-kD (PSI) and 50-kD (hrp48) RNA-binding proteins by RNA affinity chromatography. (A) Native gel assay of fractions eluted from the RNA affinity column. The flowthrough (FT) and odd-numbered fractions (FX) as well as the B.6 fraction (B.6P; from the blue trisacryl column) were assayed. The positions of free RNA and complexes 3 and 4 are indicated (right). (B) SDS-PAGE. Proteins from the odd-numbered fractions were resolved by electrophoresis through an SDS-7.5% polyacrylamide gel and stained with silver. The positions of the 97- and 50-kD protein doublets, most prominent in the peak fractions (lanes 9,10), are indicated at right. The positions of molecular mass standards are indicated in kD at left.

prominent common protein still assembled a large amount of complex 3 (Fig. 3A,B, FX 25–31), suggesting that binding of the 50-kD protein alone may generate this complex.

The 50-kD protein is the hnRNP protein hrp48

As a first attempt to identify the purified proteins, we performed immunoblotting experiments. We tested a collection of five monoclonal antibodies against specific *Drosophila* hnRNPs as well as a number of antibodies against members of the SR protein family, that influence 5' splice site selection (Materials and methods). Only antibodies against hrp48, a *Drosophila* hnRNP, reacted strongly with the most purified fractions, recognizing a 50-kD doublet (Fig. 4B, lanes 11,12). We conclude that the 50-kD RNA-binding protein is hrp48 because (1) the immunoreactive species and the 50-kD protein copurified (Fig. 1 and 4B) and comigrated in SDS-PAGE (Fig. 4A,B), and (2) amino acid sequence from peptides derived from the purified 50-kD protein matched the hrp48 amino acid sequence (data not shown).

PSI and hrp48 bind specifically to the IVS3 5' exon

To facilitate the biochemical analysis of PSI and hrp48, we overexpressed them from cDNAs (C. Siebel, A. Admon, and D. Rio, in prep.; E.L. Matunis et al. 1992) in *Escherichia coli*. The recombinant proteins (rPSI), tagged at the amino terminus with six histidines, were purified to near homogeneity using nickel affinity chromatography (Fig. 5A and 6A). Immunoblotting experiments revealed that unlike *Drosophila* PSI (dPSI), rPSI migrated as a single species (Fig. 5B; cf. Fig. 3B with Fig. 5A). Moreover, PSI generated by in vitro translation is a single species that comigrates with the fastest-migrating dPSI band (C. Siebel, A. Admon, and D. Rio, in prep.) whereas PSI produced from a cDNA using a baculovirus expression vector appears as multiple bands (M.D. Adams and D.C. Rio, unpubl.). Taken together, these results suggest

that the different dPSI species result from post-translational modifications.

Native gel electrophoresis experiments indicated that rPSI binds specifically to the IVS3 5' exon (Fig. 5C). Binding was observed even with the lowest amount of protein tested, 0.015 μ g (lane 8). The rPSI–RNA complex (Fig. 5C, lanes 8,9) comigrated with the *Drosophila* complex 4 (lane 7), suggesting that complex 4 may contain only PSI and that rPSI binds 5' exon RNA similarly to dPSI. The additional complexes that appeared with higher concentrations of rPSI (lanes 9,10) may result from changes in RNA conformation or in the number of rPSI proteins bound per RNA or may represent a less-specific binding mode of PSI. In contrast, no binding to an unrelated RNA (PL2) was observed, even with the highest amount of protein tested, 1.5 μ g (lane 5). In similar experiments, rPSI failed to bind to two other RNAs, including one that contains a consensus 5' splice site sequence (–5' SS and +5' SS; Siebel et al. 1992; data not shown). Recombinant PSI did bind to 5' exon RNA carrying mutations in the pseudo-5' splice sites (U Δ 11–F1,2; Siebel et al. 1992), suggesting that PSI recognizes nucleotides within the IVS3 inhibitory element but outside of the pseudo-5' splice sites (Fig. 6C; data not shown). Other RNA-binding assays, including binding of PSI translated in vitro to biotinylated RNAs, support these conclusions (data not shown).

Recombinant hrp48 (rhrp48) also bound to the IVS3 5' exon, forming two RNA–protein complexes observed by native gel electrophoresis (Fig. 6B, lane 4–7). A 200-fold molar excess of tRNA failed to compete for formation of either complex (lane 7), indicating that rhrp48 binding to the 5' exon was specific. Furthermore, rhrp48 failed to bind to 5' exon RNA that carried mutations only in the pseudo-5' splice sites (lanes 8–10), suggesting that hrp48 recognizes the pseudo-5' splice sites and confirming the specificity of binding. The slowest migrating complex comigrated with complex 3 formed by *Drosophila* protein fractions (cf. lanes 4–7 with lane 3), confirming the hypothesis that hrp48 alone forms complex 3 (see Fig. 3) and suggesting that at least a subfraction of the rhrp48

Figure 4. Immunoblotting identifies the 50-kD RNA-binding protein as hrp48, a *Drosophila* hnRNP protein. (A) SDS-PAGE. RNA affinity fraction 19 (R19) was electrophoresed through a gel in parallel to the one displayed in B. Silver staining revealed the positions of the 50- and 97-kD proteins, indicated at left. The positions of molecular mass markers are indicated in smaller type. (B) Immunoblot. Samples from the indicated column fractions were resolved by electrophoresis through an SDS–10% polyacrylamide gel, transferred to nitrocellulose, and probed with an anti-hrp48 monoclonal antibody. Equal volumes of each fraction were loaded for each column. (NE) Nuclear extract; (Q) Q–Sepharose fractions (the number after the letter indicates the salt concentration at which the fraction eluted); (B) blue trisacryl fractions; (R) RNA affinity fractions (the numbers refer to the fraction number; cf. Fig. 3); (FT) flowthrough.

Figure 5. Purified PSI expressed in bacteria binds specifically to the IVS3 5' exon. (A) Purified recombinant PSI was visualized by Coomassie brilliant blue staining following SDS-PAGE. The positions of rPSI and molecular mass standards are indicated to the right and left, respectively. The small amount of proteins that bound to the Ni-NTA agarose column and migrated near the 66-kD marker likely are carboxy-terminal deletions of rPSI. (B) Immunoblot analysis using affinity-purified antibodies against PSI. Crude *Drosophila* somatic cell nuclear extract (Kc, lane 1) and purified rPSI (lane 2) were resolved by electrophoresis through an SDS–8.5% polyacrylamide gel, transferred to nitrocellulose, and probed with anti-PSI antibodies. The positions of molecular mass standards are indicated in-kD at right. (C) RNA-binding of purified rPSI assayed by native gel electrophoresis. The indicated amounts of rPSI were assayed for binding to the IVS3 5' exon or an unrelated control RNA fragment of similar length (PL2; Siebel and Rio 1990). The flowthrough fraction (FT) from the RNA affinity column was also assayed to mark the position of complex 4. This fraction also generates a small amount of complex 3. The positions of free and bound RNA are indicated; free PL2 RNA migrates as two distinct bands probably because two RNA structures are resolved.

protein binds 5' exon RNA similarly to the *Drosophila* protein. Although the fastest migrating complex represents specific binding, it was observed only with hrp48 and not the *Drosophila* protein fractions (cf. lanes 4–7 with lane 3). The bacterially produced protein may be able to bind differently from the *Drosophila* protein, perhaps because a subfraction of the recombinant protein is not properly folded or modified.

To define precisely the nucleotides required for RNA-binding, we performed chemical modification-binding interference experiments. IVS3 5' exon RNA was chemically modified at purines or pyrimidines and then incubated with the indicated protein samples (Fig. 6C). RNA from RNA–protein complexes, as well as free RNA, was purified from a native gel and cleaved with aniline at the chemically modified nucleotides. Under-representation of RNA fragments in the ladder, observed by comparing bound RNA with free RNA (lanes 4,7,10,13), defined nucleotides important for protein binding. As determined previously (Siebel et al. 1992), *Drosophila* somatic extract contains RNA-binding proteins that specifically recognized nucleotides in the F2 pseudo-5' splice site as well as nucleotides upstream from this site (lanes 5,11). Binding of purified hrp48 required most of the same pseudo-5' splice site nucleotides (lanes 6,12), suggesting that hrp48 alone can recognize this part of the inhibitory

element. In contrast, single nucleotide modifications were not sufficient to disrupt binding of PSI; rPSI failed to generate a strong modification–interference pattern (lanes 2,8) as did the dPSI that is present (data not shown) in the RNA affinity column flowthrough fraction (lanes 3,9). These and other results are consistent with the hypothesis that additional factors or protein modifications affect the RNA-binding specificity of PSI (see Discussion). Alternatively, the chemical modifications that were introduced may have failed to monitor the functional groups contacted by PSI.

PSI is necessary for inhibition of IVS3 splicing

As a first step in examining whether PSI or hrp48 is necessary for somatic inhibition in vitro, we generated antibodies against each of these proteins. Polyclonal anti-PSI antibodies were affinity purified from mouse ascites fluid by binding to a rPSI column. These antibodies specifically recognize only PSI on immunoblots of crude nuclear extract (C. Siebel, A. Admon, and D. Rio, in prep.). Antibodies that flowed through the column, and thus did not recognize PSI, were purified by binding to an anti-mouse IgG column. These antibodies, which were from the same batch of ascites fluid and thus highly related to the anti-PSI antibodies, served as a control.

4,7; 6- to 12-fold activation). Longer exposures revealed that anti-PSI antibodies also stimulated the appearance of the mRNA product, which is released from the spliceosome and susceptible to ribonuclease degradation (data not shown; e.g., see Lamond et al. 1987; Barabino et al. 1990; Maschhoff and Padgett 1992). In contrast, the

addition of antibody buffer (lanes 1,2,5) or control antibody (lanes 3,6) failed to relieve the block to IVS3 splicing,

demonstrating that activation did not reflect salt or nonspecific antibody effects. These results demonstrate

Figure 7. (See following page for legend.)

that anti-PSI antibodies relieve inhibition of IVS3 splicing; therefore we conclude that somatic inhibition in vitro requires PSI.

We observed similar results repeatedly, using a number of wild-type or modified IVS3 splicing substrates. Anti-PSI antibodies activated splicing of the unmodified IVS3 substrate, although a number of cryptic splicing events from the pseudo-5' splice sites were also activated (data not shown). To minimize cryptic splicing, we also examined modified IVS3 substrates in which the branchpoint was altered to resemble the consensus sequence more closely (Fig. 7B) or in which the pseudo-5' splice sites within the intron were removed (Fig. 7C). Again, only anti-PSI antibodies (Fig. 7C, lane 3), but not control antibodies (lane 2) or antibody buffer (lane 1), activated accurate IVS3 splicing.

Experiments with other introns revealed that activation of splicing by anti-PSI antibodies was not a general effect. The adenovirus L1-2 intron provides a good control because, similar to IVS3 splicing, splicing of the adenovirus L1-2 intron is weak in *Drosophila* somatic extract and slightly inhibited in the somatic inhibition assay (Siebel and Rio 1990). However, unlike IVS3 splicing, adenovirus L1-2 splicing was not activated by anti-PSI antibodies (Fig. 7D, cf. lanes 2 and 3). These antibodies also had no effect on splicing of the *Drosophila fushi tarazu* (*ftz*) intron (Fig. 7E).

In addition to these experiments, we have attempted to immunodeplete PSI from somatic splicing extracts. However, a subfraction of PSI resists depletion (data not shown), perhaps because the presence of PSI in complexes masks its interaction with antibodies. Likewise, we performed similar immunodepletion and antibody addition experiments with affinity-purified anti-hrp48 antibodies. In contrast to the anti-PSI antibodies, the anti-hrp48 antibodies inhibited general splicing (data not shown), suggesting that hrp48 may be a general splicing factor. This antibody-induced block in splicing prevented us from using this assay to examine directly whether somatic inhibition requires hrp48.

Discussion

Studies of alternative splicing factors should help to illuminate how the use of splice sites is specifically selected and controlled, yet few have been purified and characterized biochemically. As a paradigm for regulated alternative splicing, we have studied the soma-specific inhibition of P-element IVS3 splicing. Previous experiments identified candidate alternative splicing factors by establishing a strong correlation between protein binding to the IVS3 5' exon inhibitory element and inhibition of IVS3 splicing in *Drosophila* somatic extracts (Siebel and Rio 1990; Siebel et al. 1992). Here, we have exploited the specific and highly stable binding of two of these proteins to the inhibitory element to purify them from somatic extracts. Notably, one of the proteins, a 97-kD RNA-binding protein, is required to inhibit IVS3 splicing in vitro. For this reason and because expression of this protein is limited to the soma (C. Siebel, A. Admon, and D. Rio, in prep.), we have named this protein PSI. We have identified the second protein as hrp48, a previously identified hnRNP protein (E.L. Matunis et al. 1992; M.J. Matunis et al. 1992). Purified PSI binds specifically to the IVS3 5' exon inhibitory element within 55 nucleotides of the 5' splice site. Purified hrp48 recognizes a pseudo-5' splice site contained within this same regulatory region. These and other results indicate that hrp48 may function in general splicing by recognizing 5' splice sites. Together with the soma-specific expression of PSI (C. Siebel, A. Admon, and D. Rio, in prep.), our biochemical results indicate that PSI is a tissue-specific alternative splicing factor that may function by altering the composition, positioning, or activities of general complexes that include proteins such as hrp48.

RNA affinity chromatography and the purification of alternative splicing factors

Binding of PSI and hrp48 to the IVS3 5' exon inhibitory element was resistant to treatment with high concentra-

Figure 7. Anti-PSI antibodies specifically relieve inhibition of IVS3 splicing in somatic cell extracts. The anti-PSI and control antibodies were used at a concentration of 0.13 mg/ml. (A) (Top) The IVS3 splicing substrates; (bottom) the nucleotide changes, marked with asterisks, in the – down substrate. Open and shaded boxes denote exons and IVS3, respectively. Black and hatched boxes mark the accurate 5' splice site (5'SS) and the pseudo-5' splice sites (F1, F2, and D1–D4), respectively. The wild-type substrate, with the *ftz* branchpoint (F-BP) substituting for the IVS3 BP, was used in B. The – down substrate was used in C. Both the – down and F-BP changes were made to reduce cryptic splicing reactions. (B) IVS3 splicing in vitro. Standard in vitro splicing reactions using *Drosophila* somatic cell extract were performed in the presence of the indicated amounts of affinity-purified anti-PSI antibodies (lanes 4,7), antibody buffer (lanes 1,2,5), or control antibody (lanes 3,6). Lane 8 displays a reaction using human splicing extract to mark the positions, noted at right, of the accurate intermediates and products. Cryptic splicing reactions generated additional, previously characterized bands (Siebel et al. 1992). The positions of size standards are shown at left. Using a PhosphorImager to measure the amount of radioactivity in each band and defining splicing as the ratio of accurate products and intermediates to pre-mRNA in repeated experiments, we have found that the anti-PSI antibody activates IVS3 splicing four- to eightfold. Unlike the mRNA, the lariats (intron and intron–3' exon) resist degradation by ribonucleases in the *Drosophila* extracts, and thus their activation appears even higher, at 6- to 12-fold. The radioactivity spread immediately above the pre-mRNA seemed to result from an ATP-dependent polymerization reaction (Siebel and Rio 1990). The experiments shown in C–E were performed in parallel with the experiment described in B, using the same batch of extract and 3.0 μ l of antibody. (C) IVS3 – down splicing in vitro. IVS3 – down is an IVS3 substrate in which the pseudo-5' splice sites downstream of the accurate 5' splice sites were removed to minimize cryptic splicing. These changes have little, if any, effect on IVS3 splicing (Siebel et al. 1992). A lariat from a cryptic splicing reaction is observed between the accurate lariats in lane 3. (D) Adenovirus L1-2 intron splicing in vitro. (E) *ftz* intron splicing in vitro.

tions of salt, nonspecific RNA, and the polyanion heparin. This stability of binding allowed us to use RNA affinity chromatography with the IVS3 5' exon in a purification scheme that yielded a nearly homogeneous preparation of both proteins; this step was particularly important, providing at least a 50-fold purification. Similar RNA affinity steps have been used to purify ribosomal proteins, translation initiation regulators, and protein components of RNase P (Burrell and Horowitz 1977; Ulbrich and Wool 1978; Vioque and Altman 1986; Rouault et al. 1989; Neupert et al. 1990), and immobilized poly(U) has been used to purify the splicing factors U2AF (Zamore and Green 1989) and PSF (Patton et al. 1993). Our results extend these studies by showing that RNA affinity chromatography with specific RNA sequences can be used to purify microgram amounts of certain alternative splicing factors.

Somatic inhibition requires PSI

Anti-PSI antibodies relieved the block to P-element IVS3 splicing in *Drosophila* somatic cell extracts, indicating that PSI inhibits IVS3 splicing. We conclude that splicing activation results from the anti-PSI antibodies disrupting PSI function directly, rather than affecting splicing nonspecifically, for a number of reasons. First, the affinity-purified anti-PSI antibodies only recognize PSI on immunoblots (C. Siebel, A. Admon, and D. Rio, in prep.). Second, only the anti-PSI antibodies, but not antibody buffer nor highly related control antibodies, activated IVS3 splicing. Third, anti-PSI antibodies failed to activate splicing of the *ftz* intron or the inefficiently spliced (in *Drosophila* extract) adenovirus intron, suggesting that splicing activation was specific for IVS3. Fourth, although it is evident that antibodies can inhibit splicing in vitro nonspecifically, we are unaware of any report, either published or anecdotal, of antibodies activating splicing nonspecifically. Taken together, these results represent direct biochemical evidence that regulation of IVS3 splicing requires PSI.

Relative to the level of IVS3 splicing in somatic extracts in the absence of anti-PSI antibody, the level of splicing after the addition of anti-PSI antibodies is significantly elevated. The extent of this activation in *Drosophila* extracts appears at least as high as that observed after mutations in the 5' exon inhibitory element (Siebel et al. 1992). Nevertheless, the overall efficiency of the activated splicing reaction is low. This low level of splicing efficiency may be biologically relevant. For example, even in the *Drosophila* germ line in vivo, an unknown percentage of mature P-element mRNA appears to retain unspliced IVS3 (Misra et al. 1993). That is, IVS3 splicing appears to be inherently inefficient, even in vivo in the absence of somatic inhibitors. Similarly, IVS3 splicing is also inefficient in human extracts that lack *Drosophila* somatic inhibitors (Tseng et al. 1991; Siebel et al. 1992). A somatic inhibitor such as PSI may serve to reduce IVS3 splicing from a moderate level to zero.

Somatic inhibition may require additional factors

Purified recombinant PSI bound specifically to IVS3 5' exon RNA within a genetically (Chain et al. 1991) and biochemically (Siebel and Rio 1990; Siebel et al. 1992) defined regulatory region, suggesting that this interaction mediates the inhibitory effects of PSI on IVS3 splicing. Within this region, two pseudo-5' splice sites play an important role in inhibiting IVS3 splicing; however, modification–interference and splicing experiments also point to nucleotides outside of the pseudo-5' splice sites as necessary for complete inhibition (Siebel et al. 1992). Purified PSI bound specifically to 5' exon RNA that was wild type or carried mutations in the pseudo-5' splice sites, suggesting that PSI at least in part recognizes these nucleotides outside of the pseudo-5' splice sites.

Relative to PSI in crude fractions, purified PSI appears to bind RNA differently, suggesting that additional factors or protein modifications affect RNA-binding. Native gel electrophoresis experiments showed that the B.6 fraction, which contains PSI, generates an RNA–protein complex that specifically forms on wild-type 5' exon RNA but fails to form on 5' exon RNA carrying mutations in the pseudo-5' splice sites. In contrast, recombinant PSI bound to both wild-type and mutant 5' exon RNA. Such binding differences were not restricted to the recombinant protein. For example, there appeared to be different functional isoforms of *Drosophila* PSI that were fractionated on the RNA affinity column; although approximately half of the PSI bound to the RNA affinity column, the remainder flowed through and bound RNA, like recombinant PSI in the native gel assay (data not shown). The B.6 fraction may contain additional factors that influence PSI recognition of the 5' exon. Alternatively, protein modifications, such as phosphorylation, may affect RNA binding. Consistent with this explanation, phosphorylation of certain splicing factors and RNA-binding proteins alters their activity (Cobianchi et al. 1993; Mayrand et al. 1993; Tazi et al. 1993), and we detected a number of PSI isoforms that likely result from differences in post-translational modifications.

Although PSI is required to inhibit IVS3 splicing, we have been unable to reconstitute somatic inhibition in human splicing extracts (which splice IVS3) using the most purified fractions. This result may reflect technical limitations inherent to the purification scheme. For example, the high salt and low protein concentrations of the purified RNA affinity fractions restrict the amount of protein that can be added to splicing reactions. The presence of heparin, a splicing inhibitor, also prevents testing the inhibitory activity of some of these fractions. However, as our current and past results (Siebel et al. 1992) suggest, reconstituting the inhibitory activity probably requires that a number of factors interact in the correct stoichiometry to form a multiprotein complex on the IVS3 5' exon. In addition to PSI, these factors may include hrp48 and U1 snRNP (Siebel et al. 1992). The 65- and 40-kD RNA-binding proteins, which bind to the 5' exon but were separated from hrp48 and PSI at an early purification step, may also be needed. Moreover, post-

translational modifications may affect the inhibitory activity. Therefore, reconstitution of somatic inhibition will apparently require a number of protein factors, purified from a eukaryotic source to ensure proper protein modification, in addition to U1 snRNP.

The role of hrp48

hrp48 was first identified by immunopurification of *Drosophila* hnRNP particles as a protein that binds to the majority of nascent transcripts (M.J. Matunis et al. 1992). Sequence analysis of cDNAs encoding hrp48 (E.L. Matunis et al. 1992) revealed that like a number of hnRNP proteins, the amino-terminal half of hrp48 possesses two ribonucleoprotein consensus sequence domains [RNP-CS, also known as the RNA recognition motif, (RRM), or the RNA-binding domain (RBD)] and is thus referred to as a 2× RBD protein (for review, see Dreyfuss et al. 1993). A number of general and alternative splicing factors also carry at least one copy of the RNP-CS domain, which is necessary for RNA-binding (Zamore et al. 1992; Cáceres and Krainer 1993; Zuo and Manley 1993). In addition, some 2× RBD proteins, including hrp48, have a glycine-rich carboxy-terminal domain that may mediate RNA-binding, RNA-RNA annealing, or protein-protein interactions (Dreyfuss et al. 1993).

Protein sequence comparisons and RNA-binding experiments point to the intriguing possibility that hrp48 is the *Drosophila* functional homolog of mammalian hnRNP A1, a protein that stimulates splicing from upstream 5' splice sites (Mayeda and Krainer 1992) and facilitates RNA-RNA annealing (Kumar and Wilson 1990; Pontius and Berg 1990; Buvoli et al. 1992; Munroe and Dong 1992). Experiments in which bound RNA molecules were selected and amplified from a pool of random RNA oligonucleotides reveal that the highest affinity binding site of hnRNP A1 carries two juxtaposed copies of the core sequence UAGG(U/G)(U/A) (Burd and Dreyfuss 1994). Strikingly, this RNA sequence closely resembles the regulatory sequence in the IVS3 5' exon where UAGGUU is part of one pseudo-5' splice site and the related sequence UAAGUA is part of the second adjacent pseudo-5' splice site (Siebel et al. 1992). Thus, hrp48, which requires nucleotides in both of these sites for binding, appears to have RNA-binding sequence requirements similar to those for hnRNP A1. Moreover, in a search for the *Drosophila* homolog for hnRNP A1, a *Drosophila* protein of ~50-kD cross-linked specifically to the optimal hnRNP A1-binding site; conceivably this protein is hrp48 (C. Burd and G. Dreyfuss, pers. comm.).

The relationship between hrp48 and hnRNP A1 is particularly appealing given that a protein complex containing hrp48 appears to mediate somatic inhibition by shifting U1 snRNP binding from the accurate 5' splice site to an upstream inactive pseudo-5' splice site (Siebel et al. 1992). Like this activity, increasing concentrations of hnRNP A1 shift splicing from downstream to upstream 5' splice sites (Mayeda and Krainer 1992). In addition, hnRNP A1 stimulates RNA-RNA annealing (Kumar and

Wilson 1990; Pontius and Berg 1990; Buvoli et al. 1992; Munroe and Dong 1992), suggesting that hrp48 could also function by stabilizing U1 snRNA base-pairing at the inactive pseudo-5' splice site.

Interactions between PSI and hrp48

Two simple models could explain the roles of PSI and hrp48 in somatic inhibition. In the first model, hrp48 is an essential splicing factor, and PSI blocks hrp48 activity. In the second model, PSI and hrp48 function together as required components of the inhibitory activity. Thus, the models differ depending on whether hrp48 is the target for or a component of the inhibitory activity. We favor the second model because the highly specific binding of purified hrp48 to the pseudo-5' splice site regulatory sequence suggests a direct role of hrp48 in somatic inhibition. Interactions between hrp48 and PSI, either direct or through other factors, may affect the binding specificity of PSI. These interactions could explain why PSI in partially purified fractions (also enriched for hrp48) but not purified PSI, differentiates between 5' exon RNA with wild-type versus mutant pseudo-5' splice sites.

A role for hrp48 in somatic inhibition does not exclude a role in general splicing. hrp48 may be an essential splicing factor that is recruited to function as a component of the inhibitory activity. Consistent with this proposed function of hrp48 in splicing, affinity-purified anti-hrp48 antibodies specifically inhibited general splicing. However, proof that hrp48 functions as a general splicing factor awaits further biochemical or genetic analysis. Similar to this model for hrp48 function, U1 snRNP is a general splicing factor that appears to play a role in somatic inhibition by binding to a pseudo-5' splice site (Siebel et al. 1992). A common theme that has emerged from alternative splicing studies is that differentially expressed alternative splicing factors may function by altering the activities of general splicing factors. In this light, PSI is a specifically expressed alternative splicing factor that may function by altering the activities of the general splicing factors hrp48 and U1 snRNP.

A number of other hnRNP proteins appear to affect splicing or bind to specific transcripts (for review, see Dreyfuss et al. 1993). For example, hnRNP A1 can alter 5' splice site selection (Mayeda and Krainer 1992). Immunodepletion and RNA-binding experiments suggest that the hnRNP A/B and C proteins, as well as the polypyrimidine tract-binding protein (PTB, identical to hnRNP I; Bennett et al. 1992a), play a role in splicing or increase splicing efficiency (Choi et al. 1986; Sierakowska et al. 1986; Gil et al. 1991; Patton et al. 1991). Moreover, all transcripts do not bind the same set of hnRNP proteins (Matunis et al. 1993; Mayrand and Pederson 1990; Bennett et al. 1992a, b), and hnRNP complexes appear to assemble at unique positions on specific pre-mRNAs (Osheim et al. 1985). This property makes hnRNP proteins ideal candidates for factors that influence alternative splice site selection. Immunopurification of *Drosophila* hnRNP particles reveals the presence

of seven major polypeptides, including hrp48, that associate with one another through RNA binding (M.J. Matunis et al. 1992). This purification also detects a number of minor polypeptides, suggesting that these proteins exist in a lower ratio per complex or associate with only a subset of hnRNP particles. It is tempting to speculate that PSI may be one such transcript-specific RNA-binding protein that regulates alternative splicing by controlling the assembly, positioning or activity of hnRNP complexes.

Materials and methods

Purification of PSI and hrp48

All manipulations were performed at 4°C. Protein concentrations were estimated using a modified Bradford reagent assay (Pierce) with BSA as a standard. To *Drosophila* Kc cell nuclear extract (9–28 ml, 11 mg/ml) 3 M KCl was added (Rio 1988) to 0.34 M final concentration, and the mixture was centrifuged for 30 min at 70,000 rpm in a 70.1 Ti rotor (Beckman). Buffer D (Dignam et al. 1983) without KCl was added to the supernatant to lower the KCl concentration to 0.15 M, and the extract was loaded onto a Q-Sepharose (Sigma; 11–15 mg protein/ml resin) column at a flow rate of 4–5 ml/cm² per hour. Elution of proteins was monitored by measuring UV absorbance at 280 nm. After washing the column with buffer D containing 0.1 M KCl (D0.1 M), proteins were eluted by successively adding D0.25, D0.5, and D1.0 M. The peak of the flowthrough fraction (Q.1P) was loaded onto a blue trisacryl column (LKB/IBF; 4–6.5 ml) at a flow rate of 5–6 ml/cm² per hour. Unbound (flowthrough) material was recycled onto the column, and proteins were eluted with D0.35, D0.6, and D1.0. Heparin and NP-40 were added to final concentrations of 8 mg/ml and 0.05%, respectively, to the 0.6M fraction. This mixture was loaded at a flow rate determined by gravity onto a 0.4-ml RNA affinity column that had been equilibrated in D0.5 M containing 8 mg/ml of heparin and 0.05% NP-40 (D0.5 M+). The flowthrough material was recycled three times, and the column was washed with three column volumes (CVs) of D0.5 M+. Fractions (100–200 µl) were collected. Proteins were eluted by successively adding two CVs of D0.75 M (no heparin, with NP-40), two CVs of D1.1 M (with NP-40) and three CVs of D2.0 M (with NP-40). The intensity of silver staining provided a rough and simple method to estimate protein concentrations. Also, assuming one protein molecule bound per RNA molecule, a minimum amount of protein could be estimated by determining the amount required to completely convert free RNA to bound RNA in the native gel assay.

Synthesis of the RNA affinity column

An 88-nucleotide DNA oligonucleotide complementary to IVS3 5' exon sequences was used to synthesize RNA (Milligan and Uhlenbeck 1989) for the column (Neupert et al. 1990). The RNA sequence was identical to UΔ11+ (Siebel et al. 1992) except that a 30-nucleotide poly(A) tail replaced the last (3') 3 nucleotides of UΔ11+. A standard 400-µl reaction, including a trace label of 30 µCi of [α -³²P]UTP (NEN/DuPont, 800 Ci/mmol), yielded ~21 µg of RNA after purification on a denaturing gel. The RNA was hybridized to a 380-µl poly(U)-Sepharose column (Sigma) poured in a 10-ml Econocolumn (Bio-Rad). Approximately 0.72 nmole of RNA (80%) hybridized to the poly(U)-Sepharose.

Purification of recombinant hrp48 and PSI from *Escherichia coli*

A cDNA encoding hrp48 (E.L. Matunis et al. 1992) was subcloned into the *Escherichia coli* expression vector pRSETA (Invitrogen). The fusion protein (hrp48) derived from this vector contains a 36-amino-acid amino-terminal tag that includes six consecutive histidine residues to allow affinity purification using Ni-NTA agarose (Qiagen). hrp48 expression was induced in the *E. coli* strain BL21 (DE3) pLysS as described (Studier et al. 1990). hrp48 was extracted from inclusion bodies in buffer containing 50 mM Tris-HCl (pH 8.0), 5 mM β -mercaptoethanol, 100 mM KCl, 0.1 mM phenylmethylsulfonyl fluoride (PMSF), 0.05% NP-40, 10% glycerol, and 8 M urea (buffer A at pH 8.0) (Sambrook et al. 1989). The mixture was loaded onto a Ni-NTA agarose column. The column was washed with buffer A (pH 6.3). hrp48 was eluted with buffer A (pH 5.9). The urea concentration was gradually reduced to 0.5 M by dialysis. Protein aliquots were stored at –85°C in 50 mM Tris-HCl (pH 7.5), 50 mM KCl, 10 mM DTT, 1 mM EDTA, 10% glycerol, and 0.05% NP-40. Upon sequencing the plasmid construct that expressed hrp48, we found that the following 108 nucleotides need to be added between positions 877 and 878 in the published sequence: 5'-ACCAGGACCCAGGGACCACCACCGCAGTG-GTCCGAACCTACGCTGGACCTCAGCAGACGCAGGGCTACGGCGGATACGACATGTATACTCGACGTCGACCGGAGCTCC-3' (coding strand). The corrected sequence predicts a protein composed of 422 amino acids as opposed to 386. We have communicated this change to the investigators who published the hrp48 cDNA sequence (E.L. Matunis et al. 1992).

Antibodies against gel-purified hrp48 were generated in rabbits and affinity purified on a hrp48-agarose column, essentially as described (Harlow and Lane 1988).

rPSI was expressed as described for hrp48. The cell pellet from a 500-ml culture was resuspended in 15 ml of extraction buffer [50 mM Tris (pH 8.0), 5 mM β -mercaptoethanol, 0.1 mg/ml of PMSF, 50 µg/ml of leupeptin, 4 µg/ml of pepstatin, 2 µg/ml of aprotinin]. NaCl was added to a final concentration of 1 M. The cells were lysed by sonication (Branson Sonifier 450, setting 2) in 5- to 10-sec bursts separated by cooling on ice. All of the remaining steps were performed at 4°C. The suspension was centrifuged for 30 min at 65,000 rpm in a 70 Ti rotor. The supernatant was mixed with 8 ml of Q-Sepharose (Pharmacia) for 1.5 hr. The slurry was placed in a column (2.5 cm diam.), the extract was allowed to flow through by gravity, and the column was washed with 15 ml of extraction buffer containing 1 M NaCl. The extract was loaded onto a 1-ml (5×0.5 cm) Ni-NTA agarose column. The column was washed with buffer A [20 mM Tris (pH 8.0), 100 mM KCl, 10% glycerol, 5 mM β -mercaptoethanol, 0.5 mM PMSF, 50 µg/ml of leupeptin, 4 µg/ml of pepstatin, 2 µg/ml of aprotinin] and then with buffer A containing 20 mM imidazole. rPSI was eluted with buffer A containing 80 mM imidazole.

Native gel electrophoresis

Native gel electrophoresis was performed essentially as described (Siebel et al. 1992), except that voltages between 140 and 210 V were used. UΔ11+ RNA [60,000 cpm (5×10⁵ cpm/pmol)] was incubated for 30 min at 20°C in a 10 µl reaction containing 0.5–7 µl of the fraction to be tested (in buffer D plus the KCl concentration of the particular fraction) plus 10 mg/ml of heparin (Sigma). The KCl concentration varied depending on the fraction but was always kept below 0.75 M (see Fig. 2).

Protein immunoblotting and silver staining

Proteins were resolved by SDS-PAGE (Laemmli 1970) and stained with silver (Switzer et al. 1979). Immunoblotting experiments (Towbin et al. 1979) used a goat anti-mouse immunoglobulin G and M secondary antibody conjugated to alkaline phosphatase (Boehringer Mannheim). Except for anti-hrp48 antibody, the four other monoclonal antibodies against *Drosophila* hnRNPs (M.J. Matunis et al. 1992) failed to react with proteins in the RNA affinity column fractions (data not shown). One other antibody, against the messenger RNP protein mrp53, reacted with a minor protein of ~50 kD in these fractions, suggesting that one of the three bands that migrates immediately above hrp48 (see Fig. 3B) is mrp53 (data not shown). In addition, although they did cross-react with *Drosophila* proteins in nuclear extracts, the following antibodies did not recognize proteins in the RNA affinity column fractions: anti-SF2 (Krainer et al. 1990); anti-SC-35 (Fu and Maniatis 1990); anti-SRp55 (Roth et al. 1991); and anti-B52 (Champlin et al. 1991) [data not shown].

Splicing in vitro

Splicing assays in *Drosophila* somatic extract or human HeLa extract were performed (Siebel and Rio 1990; Siebel et al. 1992) for 3 hr in the presence of anti-PSI antibodies or, as controls, in the same amount of antibody buffer or control antibodies (C. Siebel, A. Admon, and D. Rio, in prep.). The wild-type substrate was described previously, except that to minimize cryptic splicing reactions, the branch point-polypyrimidine nucleotides 3005–3036 (Siebel et al. 1992) were replaced by the corresponding region from the *ftz* intron: 5'-AGCTAACCCATTTT-TCTTTTGCTTATGCTTA-3'.

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Regulation of tissue-specific P-element pre-mRNA splicing requires the RNA-binding protein PSI.

C W Siebel, R Kanaar and D C Rio

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