RNA (1997), 3:996–1015. Cambridge University Press. Printed in the USA.

A neuron-specific splicing switch mediated by an array of pre-mRNA repressor sites: Evidence of a regulatory role for the polypyrimidine tract binding protein and a brain-specific PTB counterpart

MONA ASHIYA and PAULA J. GRABOWSKI

Howard Hughes Medical Institute and Department of Biological Sciences, University of Pittsburgh, Pittsburgh, Pennsylvania 15260, USA

ABSTRACT

Tissue- and stage-specific alternative splicing events are widespread in mammals, yet the factors and mechanisms that direct these important posttranscriptional events are poorly understood. In this study, we focus on the 24-nt exon of the $GABA_A$ receptor $\gamma 2$ pre-mRNA, which is subject to neuron-specific and developmental splicing regulation in the rat cerebellum. Here we show biochemical evidence for a mechanism that directs the selective repression of the neuron-specific exon in non-neuronal splicing extracts derived from HeLa cells. Key evidence includes the discovery that the pathway of $\gamma 2$ pre-mRNA splicing switches from exon skipping to exon selection in splicing reactions with a short RNA competitor containing the 3′ splice site region upstream of the 24-nt exon. In this assay, exon selection results from the coordinate activation of both flanking introns. A detailed dissection of this pre-mRNA region shows that it contains four repressor sites clustered around the branch site and extending into the 24-nt exon. These repressor sites are pyrimidine rich and bind avidly to the polypyrimidine tract binding protein (PTB) in HeLa nuclear extracts as determined by UV crosslinking/competition assays. Repression of the exon selection pathway is closely associated with the appearance of a specific RNA–protein complex, indicative of an inhibitor complex, that assembles on the repressor array. Upon the switch to the exon selection pathway, a substantial decrease in the inhibitor complex and a reciprocal increase in spliceosome complex A is observed. Excess recombinant PTB squelches the splicing switch and reestablishes exon skipping as the predominant splicing pathway. Extracts prepared from rat brain nuclei show reduced levels of conventional PTB compared to other splicing factors. Nonetheless, the rat brain nuclear extracts contain an activity that assembles an analogous inhibitor complex efficiently. We report a 59-kDa protein, p59, which has an electrophoretic mobility distinct from HeLa and rat kidney PTB, and which behaves in RNA binding assays as if it is the PTB counterpart in rat brain. Evidence that rat brain p59 is structurally related to PTB stems from western blot and immunoprecipitation analysis with a monoclonal antibody specific for the hnRNP I isoform of PTB. A model describing how the repressor array directs coordinate splicing regulation of flanking introns in the context of overlapping positive regulatory elements is discussed. The sequence, (5′) UUCUCU (3′), in a pyrimidine context is associated with one class of intron splicing repressor sites that binds PTB in a variety of pre-mRNAs that are regulated by tissue-specific programs.

Keywords: alternative splicing; hnRNP I; PTB; splicing regulation; splicing repression

INTRODUCTION

The regulation of alternative pre-mRNA splicing by tissue-specific and developmental programs is a characteristic feature of gene control employed by higher eukaryotes. Yet the identities of the molecular machineries and how these regulators modulate complex splicing decisions are largely unknown. In theory, the selective expression of splicing regulatory machineries in some tissues relative to others could account for the appearance of unique spliced mRNAs.

Previous studies have identified ubiquitous RNA binding proteins of the SR and hnRNP families as mediators of splicing decisions (Fu, 1995; Manley & Tacke,

Reprint requests to: Paula J. Grabowski, Howard Hughes Medical Institute and Department of Biological Sciences, University of Pittsburgh, Fifth Avenue and Ruskin Street, Pittsburgh, Pennsylvania 15260, USA; e-mail: pag4+@pitt.edu.

1996). The relative level of ASF/SF2 and hnRNP A1, for example, modulates the choice between proximal and distal alternative 5′ splice sites in a variety of pre-mRNAs in HeLa nuclear extracts and in cell lines (Mayeda & Krainer, 1992; Caceres et al., 1994). Although multiple SR proteins exhibit similar effects on splice site utilization or alternative splicing, differential effects have also been observed (Fu et al., 1992; Fu, 1993; Ramchatesingh et al., 1995; Screaton et al., 1995; Wang & Manley, 1995). Furthermore, the relative levels of some SR and hnRNP proteins have been shown to vary in different tissues, or cell lines, suggestive of the idea that these differences might contribute to splicing regulation (Zahler et al., 1993; Kamma et al., 1995). The extent to which tissue-specific splicing events are modulated by changes in the relative levels of general splicing factors, compared to the selective expression of designated splicing regulators, is a major unresolved question.

A striking variety of RNA binding proteins that fall outside of the SR protein family have been shown to modulate splicing decisions in response to specific RNA signals in the target pre-mRNA. P-element somatic inhibitor (PSI) functions to repress the splicing of the third intron of *Drosophila* P element transposase pre-mRNA in response to specific RNA signals in the second exon (Siebel et al., 1995). PSI functions together with hrp48 and U1 snRNP to mediate splicing repression in the context of a multicomponent complex (Siebel et al., 1992). *Drosophila Transformer* (*Tra*) is involved in the positive regulation of 3′ splice site selection in *doublesex* pre-mRNA, in response to prototypical exon enhancer RNA signals and in the context of a multicomponent complex containing SR proteins (Tian & Maniatis, 1992, 1993). *Sex-Lethal* (*Sxl*), another regulator of the sex determination pathway of *Drosophila*, functions as a splicing repressor. *Sxl* binds to specific polypyrimidine tracts in *transformer* pre-mRNA and in its own transcript, resulting in 3′ splice site inhibition due to the absence of a required effector domain in the *sxl* protein (Horabin & Schedl, 1993; Valcarcel et al., 1993). Even the single splicing regulatory event known to occur in *Saccharomyces cerevisiae* is dependent on Mer1, a meiosis-specific RNA binding protein. Mer1 positively regulates the splicing of the Mer2 pre-mRNA in response to specific sequences in the exon, apparently by relieving a defect in U1 snRNP binding to the 5′ splice site (Nandabalan & Roeder, 1995).

The 60-kDa polypyrimidine tract binding protein (PTB) is emerging as a potent splicing repressor and alternative splicing modulator in mammalian systems. PTB was identified initially as a protein involved in 3′ splice site recognition, and later as a structural component of hnRNP and spliceosome complexes, although a requirement for PTB in splicing has not been demonstrated (Garcia-Blanco et al., 1989; Gil et al., 1991; Patton et al., 1991; Ghetti et al., 1992). Nonetheless, a growing body of evidence shows that PTB binds to negative regulatory elements in 3′ splice site regions of α- and β-tropomyosin pre-mRNAs (Guo et al., 1991; Mullen et al., 1991; Patton et al., 1991; Mulligan et al., 1992; Gooding et al., 1994). PTB recognizes distinct polypyrimidine tract sequences, compared to *sxl* and U2AF^{65}, and has been shown to function as a sequence-specific repressor of 3′ splice site activity (Singh et al., 1995).

PTB is an atypical member of the large family of RNA binding proteins containing RNA recognition motifs, because the four RNA binding domains in the protein conform to general structural features but differ from the canonical RNP1 and RNP2 sequences (Ghetti et al., 1992; Dreyfuss et al., 1993). Sequence information from PTB cDNAs indicates that hnRNP I and three additional PTB isoforms are generated by alternative splicing (Gil et al., 1991; Patton et al., 1991; Ghetti et al., 1992).

From the collective evidence, PTB behaves in the nucleus as a remarkably versatile and multifunctional RNA binding protein, acting as a structural component of hnRNP complexes, and playing the role of a splicing repressor/alternative splicing modulator for certain pre-mRNAs. To what extent PTB represses, or modulates alternative splicing events is poorly understood, and additional functions of PTB cannot be excluded. Recent evidence indicates that PTB has at least two additional roles in the cell as a sequence-specific RNA binding protein. In one case, PTB binds to an intron enhancer element in calcitonin CGRP pre-mRNA, where it is involved in a mechanism that activates polyadenylation and terminal exon selection (Lou et al., 1996). Furthermore, PTB recognizes multiple regions of an RNA stem loop structure characteristic of internal ribosome entry sites in picornavirus RNAs and their associated pyrimidine tracts (Belsham & Sonenberg, 1996). Functional evidence demonstrating a requirement for PTB in one internal translation initiation event, presumably occurring in the cytosol, is derived from in vitro depletion/reconstitution assays (Kaminski et al., 1995).

Here we report evidence for the involvement of PTB as a *trans*-acting repressor of a neuron-specific splicing event in non-neuronal nuclear extracts. Although neuron-specific splicing events abound in mammalian systems, the nature of the regulatory molecules and mechanisms represent significant gaps. Alternative splicing of the 24-nt exon of the γ2 subunit of the $GABA_A$ receptor, γ2 pre-mRNA, is subject to cell-specific and developmental regulation in defined neurons of the rat cerebellum (Wang & Grabowski, 1996). In this previous study, γ2 pre-mRNA behaves as if it is coordinately regulated with several other neuron-specific exons in cerebellum tissue during postnatal development. However, resolution at the single neuron level reveals significant differences even in the

extent and timing of the γ2 pre-mRNA splicing event in cerebellar Purkinje and Granule neurons, indicative of cell-specific differences in the splicing regulatory machinery. Recently, we have identified two novel RNA elements that are essential for selection of the 24-nt exon, based upon the expression of γ2 mini genes in a cerebellar cell line (Zhang et al., 1996). The essential RNA elements comprise a noncanonical 9-nt sequence between the 3′ splice site/polypyrimidine tract and AG dinucleotide, as well as a 3-nt segment within the 24-nt exon.

Stemming from recent efforts to devise an in vitro-regulated splicing assay for the identification of the activities that recognize these positive regulatory elements, we now document biochemical evidence for the involvement of PTB in a complex mechanism responsible for the repression of neuron-specific exon selection. An array of repressor sites surrounding the branch site, adjacent to the 24-nt exon, is a key feature of this alternative splicing event due to its ability to bind PTB selectively at multiple sites and assemble a specific inhibitory complex. We propose that the involvement of PTB in the inhibition of neuron-specific exon selection, which is documented here in HeLa cell nuclear extracts, is representative of similar events that occur in non-neuronal cells of mammalian brain in which γ2 pre-mRNA is spliced by predominant exon skipping (Bovolin et al., 1992). Interestingly, nuclear extracts prepared from rat brain nuclei assemble an analogous inhibitory complex on the repressor array, but contain significantly less of the standard PTB doublet. Nonetheless, a protein, p59, with an electrophoretic mobility distinct from the HeLa and rat kidney PTB, appears to be the predominant form of PTB in rat brain based upon its RNA binding specificity and reactivity with a monoclonal antibody specific for hnRNP I.

RESULTS

Switch to the neuron-specific splicing pathway is triggered, in vitro, by a short RNA competitor containing an inhibitory intron sequence

In a recent study, cell and substrate-specific splicing regulation of γ2 pre-mRNA was recapitulated in a cerebellar cell line, and essential RNA elements defined for the minimal substrate, rGγ25 (Zhang et al., 1996). To address the biochemical mechanism of regulation, we have used the essentially identical rGγ25 *Xho* I substrate as a starting point for in vitro studies (Fig. 1C). In a HeLa nuclear extract, rGγ25 *Xho* I undergoes efficient splicing by exon-skipping, whereas little or no splicing is observed between the regulated exon and either its upstream or downstream exon partner (Fig. 1A, lane 2). These results are consistent with the hypothesis that HeLa extracts lack neuronal factors that positively regulate exon selection. Alternatively, HeLa extracts might contain a characteristic inhibitory activity that represses exon selection in non-neuronal cell types.

The involvement of splicing inhibition in the γ2 mechanism was indicated initially by the observation that the neuron-specific exon is spliced to its downstream partner when the 3′ splice site region adjacent to the regulated exon is replaced by another weak 3′ splice site. For example, intron b, the intron b lariat 3′ exon intermediate, and the linear product of the intron b splicing event are reproducibly increased for rGγ83 *Xho* I, compared to rGγ25 *Xho* I (Fig. 1A, lanes 5 and 6). These results suggested that a sequence involved in splicing inhibition might be contained within the 3′ splice site region of the γ2 intron a. To test this idea, we constructed the 57B RNA competitor with the 3′ splice site region of intron a, the 24-nt exon, and a short segment of the downstream intron. When the 57B RNA competitor is added to the splicing reaction at a 50-fold molar excess compared to the ^{32}P-labeled substrate RNA, a switch from exon skipping to exon selection results (Fig. 1A, lane 3). The alternate splicing pathways are shown schematically (Fig. 1B). In particular, the lariat intron–3′ exon intermediates for the splicing of intron a first, and intron b first, and the intron b product accumulate at the expense of the intermediate and products for the exon skipping pathway. The asterisk denotes the lariat intron–3′ exon intermediate for the first step of intron a splicing. Whereas excised intron a is masked by pre-mRNA in the experiment of Figure 1A, it is clearly resolved on 5.5% polyacrylamide/7 M urea gels. A similar splicing switch is observed for the 63B RNA competitor, which lacks the essential 9-nt element defined previously (lane 4). The predominant RNA signal responsible for inducing the splicing switch is located just upstream of the branch site in intron a (see below, Fig. 3).

The results shown above are consistent with the hypothesis that the RNA competitors sequester, by mass action, one or more factors required for splicing repression in a manner that is independent of the essential 9-nt element. In this assay, a 1-h splicing reaction is chosen to maximize lariat RNA intermediates, rather than the exon-selected mRNA product. The identities of the mRNA products and intermediates are inferred from the kinetics and ATP dependence of their appearance during the splicing reaction, their electrophoretic mobility on 4% and 7% polyacrylamide/7 M urea gels, the effect of incubation in an S100 extract under debranching conditions, and by comparison to the splicing of RNA variants, including an artificially generated transcript containing the 5′ exon, intron a, followed by the fused middle and 3′ exons (not shown). In addition, the accuracy of splicing was demonstrated for the exon-skipped mRNA product by reverse transcription,

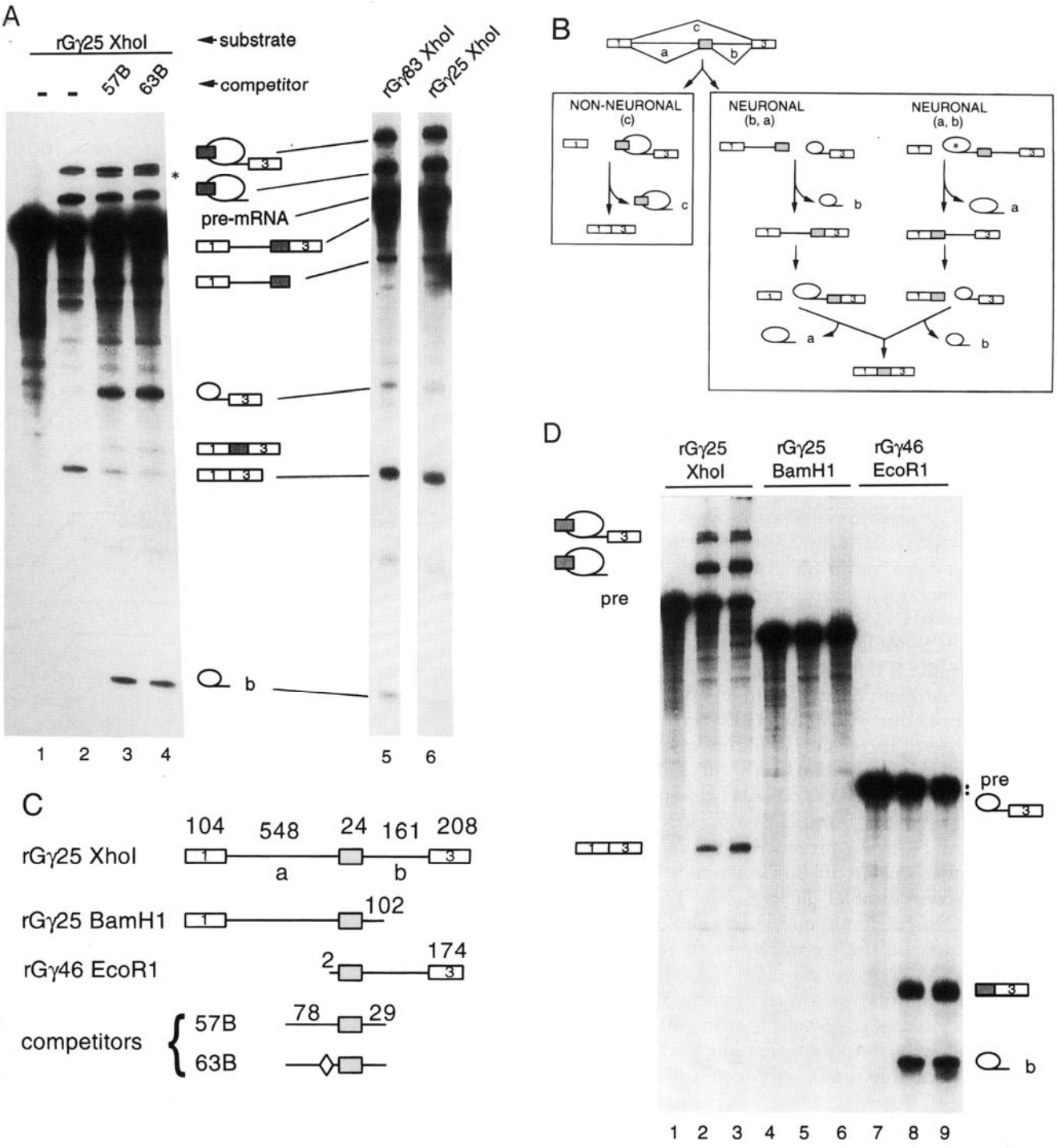

FIGURE 1. An alternative splicing switch induced by a short RNA competitor. **A:** HeLa nuclear extract was incubated with ^{32}P-labeled pre-mRNA substrate under splicing conditions for 0 (lane 1), 60 min (lanes 2, 3, 4), or 120 min (lanes 5, 6). Unlabeled RNA competitors, at 50-fold molar excess over substrate RNA, were preincubated with the nuclear extract for 5 min on ice before addition of the substrate (lanes 3, 4). Schematic of intermediate and product RNAs are shown at right. Lariat intron–3′ exon intermediate (asterísk, *) (see Fig. 1B). Samples were separated by electrophoresis in 4% polyacrylamide/7 M urea gels, followed by autoradiography of the dried gel. Substrate rGγ83 is identical to rGγ25 except that the region between −227 and −3 nucleotides upstream of the intron a 3′ splice site is replaced with the equivalent region of rat preprotachykinin intron 3 (Zhang et al., 1996). **B:** Schematic of the neuronal and non-neuronal splicing pathways. Exons and introns are denoted by boxes and lines, respectively. The alternative exon is shaded. **C:** Schematic of RNA substrates and competitors. Exon and intron lengths are indicated in nucleotides. The 63B RNA competitor is identical to 57B, except for deletion of the 9-nt essential element separating the polypyrimidine tract from the AG dinucleotide (diamond). **D:** Splicing reactions with the indicated substrate RNAs were incubated for 0 (lanes 1, 4, 7), 60 (lanes 2, 5, 8), and 120 min (lanes 3, 6, 9) with HeLa nuclear extract and the RNA products resolved on a 4% polyacrylamide/7 M urea gel. RNA intermediates and products are indicated schematically.

PCR amplification, and sequencing of the cloned products. The accuracy of the exon-selected RNA product was determined by primer extension analysis.

When introns a and b are tested individually in the context of the two exon substrates, rGγ25 *Bam*H 1 and rGγ46 *Eco*R 1, it is evident that intron a splicing is extremely inefficient, whereas intron b splicing is highly active (Fig. 1D, lanes 4–9). Thus, there is no intrinsic block to the splicing of intron b upon the deletion of intron a sequences. A schematic of the RNA substrates is shown (Fig. 1C). Only two nucleotides of intron a are present in the rGγ46 *Eco*R 1 substrate. These results suggest that, for the wild-type substrate, rGγ25 *Xho* I, a sequence located in intron a might be responsible for splicing inhibition of both introns a and b.

PTB binds selectively to an intron a RNA sequence associated with the inhibition of intron a and b splicing

To identify candidate proteins involved in the mechanism of inhibition of intron a and b splicing in the HeLa extract, we employed a UV crosslinking/competition scheme. Reactions are incubated under binding conditions, followed by UV crosslinking, ribonuclease digestion, and SDS-PAGE analysis. In the absence of RNA competitor, the 57B RNA substrate crosslinks predominantly to a protein doublet with an apparent molecular weight of approximately 60 kDa (Fig. 2, lane 3). In the presence of increasing amounts of the 57A RNA competitor, which contains only the 3′ region of intron a, crosslinking to the 60-kDa protein doublet decreases substantially, indicative of specific binding (lanes 4 and 5). In the presence of the 74B competitor, there is only a small reduction in the crosslinking of the 60-kDa protein doublet (lanes 6 and 7). These results show that the predominant binding site of the 60-kDa protein must be the pyrimidine-rich region that is present in 57A RNA, but absent in 74B RNA (Fig. 2, bottom). The molecular weight and pyrimidine-rich binding characteristics suggested that the 60-kDa protein doublet might be the polypyrimidine tract binding protein, PTB. For this reason, we tested the ability of a polyclonal PTB-specific antibody obtained from J. Patton (Vanderbilt University) to immunoprecipitate the 60-kDa protein doublet from UV crosslinking reactions similar to that shown in Figure 2, lane 3. As expected, the 60-kDa protein doublet was immunoprecipitated selectively with the anti-PTB antibody (data not shown; see also Fig. 9D).

To further assess the binding characteristics of the intron a 3′ splice site region, phosphorimager analysis was used to estimate the change in protein crosslinking upon the addition of RNA competitor. At the maximum level of 57A RNA competitor, there is a fourfold reduction in PTB binding, compared to a twofold reduction in U2AF^{65} binding (Fig. 2, lanes 3 and 5). Thus,

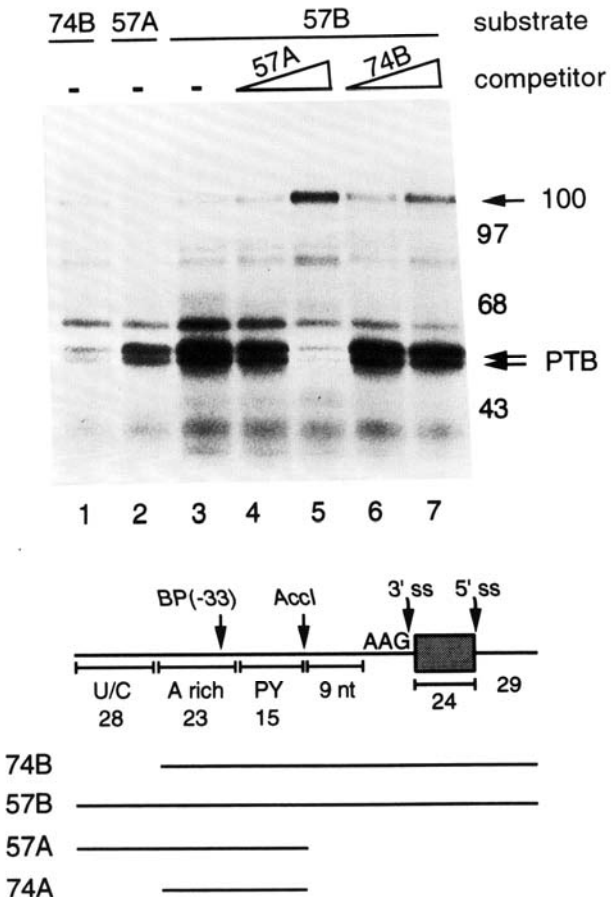

FIGURE 2. Selective binding of PTB to the polypyrimidine tract upstream of the branch site in intron a. Schematic of the region flanking the regulated 24-nt exon is shown below with the salient sequence features indicated: U/C, a 28-nt pyrimidine-rich stretch; A-rich, a 23-nt A-rich element including the branch site; BP, branch point present 33 nt upstream of the 3′ splice junction; PY, 15-nt long polypyrimidine tract; 9 nt, essential regulatory element separating the polypyrimidine tract from the AG dinucleotide. 5′ and 3′ splice sites (5′ ss; 3′ ss). Sequences contained in the RNA substrates and competitors are indicated by the lines at bottom. Reactions shown contain RNA substrate and HeLa nuclear extract incubated in the absence (lanes 1, 2, 3) or presence of the indicated competitor (lanes 4, 5, 6, 7) for 5 min on ice immediately prior to UV treatment. RNA competitor is present in the indicated reactions at 2- and 12-fold molar excess compared to substrate RNA (increase indicated by wedge). Following UV irradiation and RNase digestion, proteins are resolved on a 12.5% polyacrylamide gel. Molecular weight markers are indicated at right. Crosslinking of the polypyrimidine tract binding protein, PTB, is indicated by the double arrow. Identification of PTB is provided by immunoprecipitation. U2AF^{65} is visualized as the protein immediately above the PTB doublet. A protein of apparent molecular weight 100 kDa, which shows a reproducible increase in UV crosslinking upon addition of 57A RNA competitor, is indicated.

the 66-nt 3′ splice site region of intron a (57A) binds preferentially to PTB.

In agreement with the above results, the 57A RNA competitor induces the switch to the exon selection pathway, whereas the RNA competitor, 74A, has little or no effect on splicing of rGγ25 *Xho* I (Fig. 3, lanes 1–3). These results are also consistent with previous reports that establish a role for PTB as a splicing repressor in

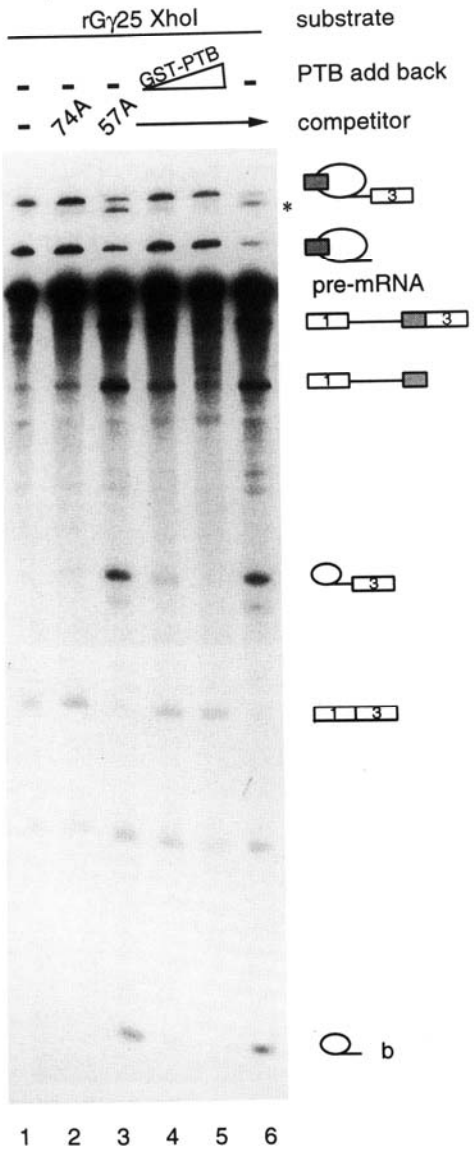

FIGURE 3. GST-PTB suppresses the splicing switch induced by the 57A RNA competitor. Splicing reactions containing rGγ25 *Xho* I substrate were incubated for 60 min in HeLa nuclear extract in the presence and absence of RNA competitor and GST-PTB as specified, and the RNA products were resolved on a 4% polyacrylamide/7 M urea gel. GST-PTB was purified from 1 L of *Escherichia coli* strain XA90 by sonication and affinity selection on glutathione-agarose exactly as described previously (Lin & Green, 1991). RNA competitor, 9 pmol, is present at a 75-fold molar excess compared to RNA substrate, 0.12 pmol, for reactions shown in lanes 2–6. GST-PTB is added to reactions at 0 (lane 3), 10 (lane 4), and 15 (lane 5) pmol. Intermediates and product RNAs are indicated at right. A mock reaction, containing 13 pmol of bovine serum albumin added instead of PTB, is shown (lane 6). Asterisk (*) denotes the lariat intron–3′ exon intermediate resulting from the first step of intron a splicing (see Fig. 1B).

other systems. Under the same conditions, the 57A RNA competitor has no effect on the splicing of a control RNA substrate derived from adenovirus (data not shown). The 57A RNA competitor contains a 28-nt, pyrimidine-rich sequence upstream of the intron a branch site region that is lacking in the 74A competitor. Otherwise, these two RNAs are identical. A convenient *Acc* I restriction site located between the 3′ splice site/polypyrimidine tract and 9-nt element represents the 3′ end of both RNA competitors. The location of the branch site in intron a was mapped to position −33 relative to the 24-nt exon by primer extension analysis of debranched and mock-treated reactions (Fig. 2, bottom; and data not shown). The RNA substrate used for the branch site mapping experiments is active for intron a splicing, and contains the 5′ exon, intron a, followed by the fused middle and 3′ exons.

Further evidence that the 57A RNA competitor induces the splicing switch by activating intron a and b splicing is derived from a quantitative analysis of the in vitro splicing results (Table 1). Whereas the product and intermediate RNAs of the exon-skipping pathway decrease, there is a corresponding increase in the intermediate and product RNAs of the exon selection pathway. Although the splicing of introns a and b are induced coordinately by the 57A RNA competitor, intron b splicing shows the most pronounced effect.

PTB is responsible for the inhibition of intron a and b splicing

To test directly the role of PTB as a *trans*-acting repressor of the splicing of introns a and b, we purified the recombinant protein, GST-PTB, and asked if its addition to splicing reactions in excess would squelch the splicing switch induced by the 57A RNA competitor. GST-PTB, which was obtained from M. Garcia-Blanco (Duke University), has a structure in which glutathione-

TABLE 1. Relative change in RNA intermediates and products upon addition of the 57A RNA competitor.[a]

	Lane 2	Lane 3	Fold change
Exon skipping			
	386.4	155.2	2.5× decrease
	515.4	225.2	2.3× decrease
	185.5	71.5	2.6× decrease
Exon selection			
	102.2	181.3	1.8× increase
	101.4	389.2	3.8× increase
Intron b	40.3	271.9	6.7× increase
	277.9	661.4	2.4× increase

[a]Data were quantified by phosphorimager analysis of the bands visualized in lanes 2 and 3 of Figure 3. Columns 2 and 3 represent raw scores for the RNA species diagrammed at left. Far right column provides an estimate of the fold change in columns 2 and 3, and is representative of multiple trials. Bands in high background regions of the gel were not quantified.

S-transferase (GST) is fused to the amino terminus of human PTB. If the addition of the 57A RNA competitor induces exon selection by shifting the equilibrium from the free to the RNA competitor-bound form of PTB, we would expect the addition of excess GST-PTB to favor the free form of the protein and thereby suppress the splicing switch. The experiment was performed by preincubating the nuclear extract in order with the 57A RNA competitor, followed by GST-PTB and RNA substrate with a subsequent incubation under splicing conditions for 1 h. RNA competitor is present at a 75-fold molar excess, and PTB is added at 0, 83, or 125-fold molar excess, compared to RNA substrate (Fig. 3, lanes 3, 4, and 5, respectively). These results show that the 57A RNA competitor-induced splicing switch is suppressed in a dose-dependent manner by the addition of GST-PTB. Control reactions show no effect on the splicing of rGγ25 *Xho* I when purified GST protein alone is added back at 83 and 125 molar excess compared to RNA substrate (data not shown). Furthermore, when GST-PTB is added at 83- or 125-fold molar excess to splicing reactions containing control RNAs, rGγ46 *Eco*R I, or adenovirus L1–L2, there is no effect on splicing (data not shown). Collectively these results show that PTB is involved in a mechanism that represses splicing of the γ2 neuron-specific exon in a HeLa nuclear extract in response to an inhibitory sequence located in the 3′ splice site region of intron a.

Reciprocal assembly of an inhibitor complex and spliceosome complex A on the intron a repressor region in HeLa and rat brain nuclear extracts

A gel mobility shift approach was used to test the possibility that splicing inhibition is mediated through the formation of a specific RNA–protein complex. Radiolabeled 57B RNA was incubated in a HeLa nuclear extract at 4 °C, in the presence and absence of the 57A RNA competitor, and the complexes were resolved on Tris-glycine gels. Whereas a slowly migrating complex is assembled on the RNA substrate in the absence of RNA competitor, this complex is reduced significantly in the presence of the 57A RNA (Fig. 4, top, lanes 1 and 2). Furthermore, complex assembly does not require ATP (lanes 5 and 6). The shifted complexes are destabilized when half of these reactions are resolved at room temperature (Fig. 4, bottom). The RNA competitor, 74A, which lacks the pyrimidine tract upstream of the branch site, has little or no effect on reducing the level of the shifted complex (data not shown).

Compared to the inhibitor complex, spliceosome complex A shows the opposite behavior during assembly in a HeLa nuclear extract followed by separation in Tris-glycine gels at room temperature. Complex A is assembled efficiently at 30 °C in the presence, but not

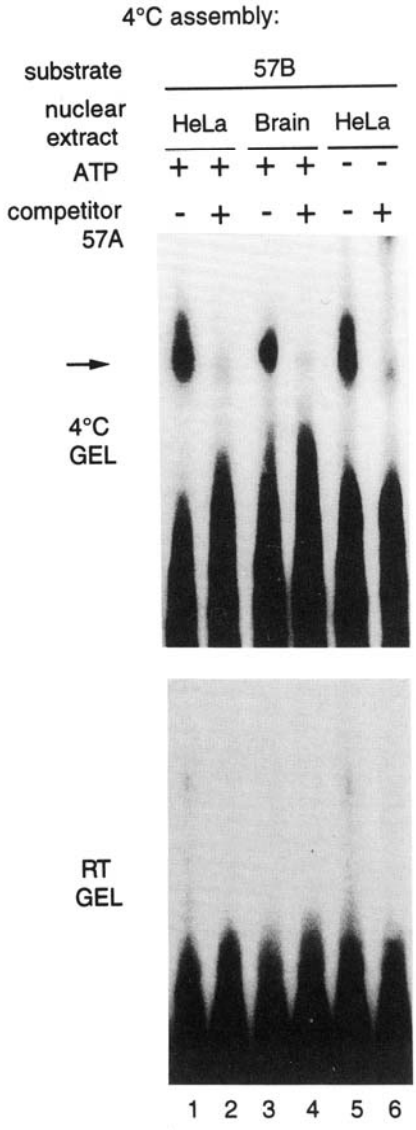

FIGURE 4. Evidence for a specific inhibitor complex that assembles on the intron a repressor region in HeLa and rat brain nuclear extracts. Substrate RNA was incubated for 5 min on ice with HeLa or rat brain nuclear extract, followed by separation of the assembled complexes on 3.75% native gels run at 4 °C (top) or room temperature, RT (bottom). RNA competitor, present at a 55-fold molar excess over substrate RNA, was preincubated with nuclear extract for 5 min on ice prior to addition of substrate RNA (lanes 2, 4, 6). Essentially identical results are obtained when the complete reactions are incubated at 30 °C for 5–15 min without ATP (data not shown). RNA–protein complexes are indicated by the arrow. The HeLa and rat brain nuclear extracts were adjusted to the same protein concentration for each binding reaction.

the absence, of the 57A RNA competitor (Fig. 5, lanes 1 and 2). Evidence that the shifted complex observed in the experiment of Figure 5 is complex A includes the requirement for ATP, as well as the 5′ end of U2 snRNA (lane 10). In contrast to 57A, the 74A RNA competitor, which lacks the polypyrimidine tract upstream of the branch site in intron a, has only a small effect in promoting the assembly of complex A on the 57B RNA substrate (lane 3). It is important to note that com-

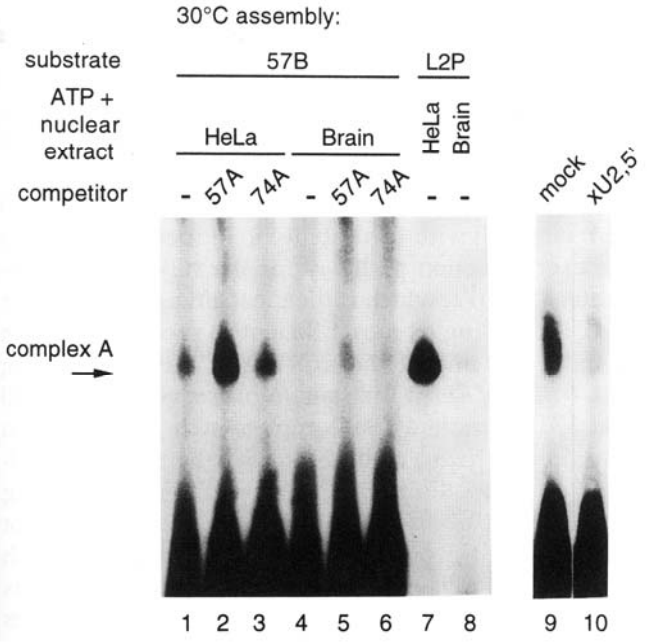

FIGURE 5. RNA competitor-induced switch to the exon selection pathway is closely associated with the assembly of U2 snRNP and the intron a branch site region. Mobility shift analysis in native gels was used to assess the efficiency of U2 snRNP complex assembly in the presence and absence of the 57A RNA competitor. Splicing reactions containing RNA substrate and HeLa or rat brain nuclear extract were incubated at 30 °C for 5 min with ATP to assemble U2 snRNP (complex A). RNA competitor, which is present at 55-fold molar excess compared to substrate RNA, was preincubated with HeLa (lanes 2, 3), or rat brain (lanes 5, 6), nuclear extract for 5 min on ice prior to the addition of substrate RNA. Model RNA substrate, L2P (lanes 7, 8), assembles complex A efficiently, as demonstrated previously (Wang et al., 1995). Control reactions are identical to that shown in lane 2, except that the nuclear extract was mock treated (lane 9), or subjected to quantitative U2 snRNP cleavage, as described (Wang et al., 1995). Samples were resolved on a 3.75% native gel run at room temperature.

plex A cannot be contaminated with the inhibitor complex because the latter, but not the former, is destabilized in Tris-glycine gels resolved at room temperature. These results are consistent with a model in which PTB is involved in promoting the assembly of an inhibitor complex on intron a, at an early step in spliceosome assembly that precludes the formation of complex A.

Because γ2 pre-mRNA is subject to splicing regulation in rat brain tissue, it is of interest to ask similar questions in a more biologically relevant system. For this reason, we prepared nuclear extracts from rat brain tissue by purification of nuclei through a 2 M sucrose cushion (see Materials and Methods). The purified brain nuclei are then extracted with KCl similar to the method of Dignam et al. (1983). The rat brain nuclear extracts contain spliceosomal snRNAs, but lack sufficient activity to splice on their own. When the rat brain nuclear extract is incubated with radiolabeled 57B RNA substrate, similar levels of an inhibitor complex are observed in the absence, but not the presence, of the 57A RNA competitor (Fig. 4 top, lanes 3 and 4). The characteristics of mobility, ATP-independence, and instability in Tris-glycine gels separated at room temperature are similar to that observed in the HeLa nuclear extract (compare lanes 1 and 3). In addition, the reciprocal behavior of complex A, compared to the inhibitor complex, is also observed in the rat brain nuclear extract. Complex A increases in the presence of the 57A, but not 74A, RNA competitor (Fig. 5, lanes 4–6). Control reactions performed under standard conditions show that the rat brain nuclear extract promotes complex A formation for a prototypical 3′ splice site, L2P RNA, but at much reduced efficiency compared to the HeLa extract (lanes 7 and 8). The same RNA competitors tested above have no effect on complex A assembly with the L2P substrate (data not shown). Experiments that probe the level of PTB, and activities that bind the intron a repressor sequences in the rat brain nuclear extract are presented below (Fig. 9).

Mechanism of inhibition: Repression of intron b splicing is largely dependent upon competition with the intron c splicing event

To investigate the extent to which the splicing of introns a and b is coordinately or independently repressed, we tested the effect of intron a sequences on the splicing of intron b in the absence of the competing intron c splicing event. For this purpose, we constructed an RNA substrate in which the 5′ exon and adjacent 5′ splice site is deleted, but the intron a 3′ splice site region is retained, rGγ81 *Xho* I (Fig. 6, bottom). When the splicing of rGγ81 *Xho* I is compared to rGγ46 *Eco*R I RNA, which contains only the last two nucleotides of intron a, both substrates are found to exhibit strong splicing activity, even in the absence of RNA competitor (Fig. 6, lanes 2 and 6). In the presence of the 57A RNA competitor, rGγ81 *Xho* I RNA shows a modest increase in splicing activity, whereas no effect is observed for rGγ46 *Eco*R I RNA, which lacks the intron a 3′ splice site (lanes 7 and 8, compared to lanes 3 and 4). Furthermore, when GST-PTB is added to splicing reactions lacking RNA competitor, a modest level of inhibition is observed for rGγ81 *Xho* I RNA, but not rGγ46 *Eco*R I RNA, at the highest level of GST-PTB added (data not shown). Together, these results indicate that intron a sequences are required to repress intron b splicing, but this inhibitory effect is largely dependent upon competition with the intron c splicing event.

PTB modulates exon selection from an array of repressor sites within intron a

To more carefully localize the PTB binding site(s) in the 57A RNA competitor, we constructed four mini RNAs spanning the 3′ splice site region of intron a,

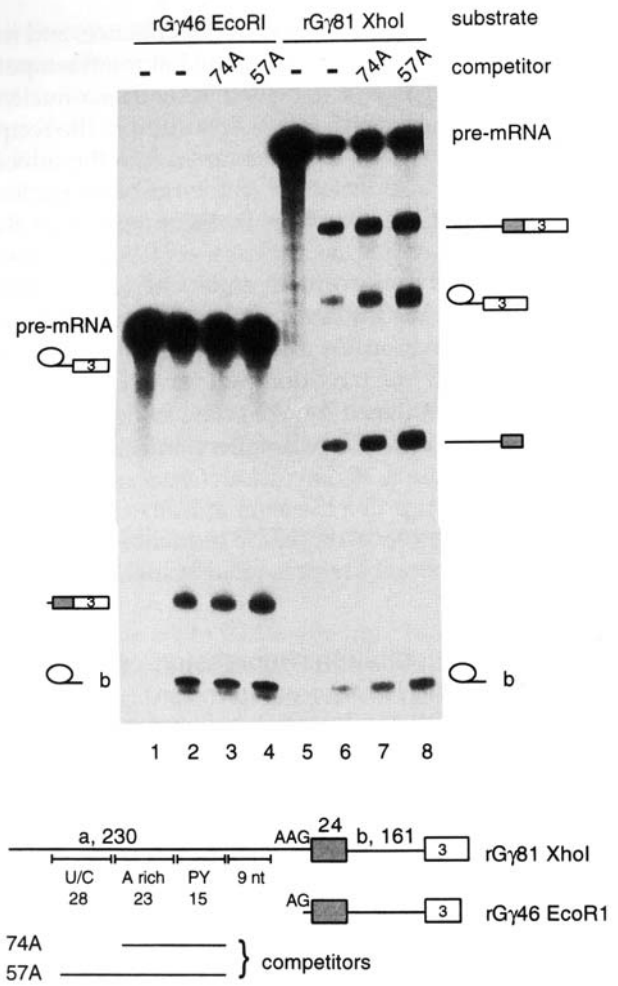

FIGURE 6. Repression of intron b splicing is mediated by the intron a repressor region, and is largely dependent upon competition with the 5′ splice site of intron a. Splicing reactions containing RNA substrate were incubated for 60 min at 30 °C with HeLa nuclear extract. RNA competitor, at 75-fold molar excess, was preincubated with HeLa nuclear extract as indicated for 5 min on ice prior to the addition of RNA substrate. Schematic of the RNA substrates and competitors (bottom). Intron (lines) and exon (boxes) lengths are indicated in nucleotides. Brackets denote features of the intron a repressor region as described above (Fig. 2). RNA intermediates and products of the reactions are labeled at left and right.

and tested their ability to bind PTB in the HeLa nuclear extract (Fig. 7A, bottom). RNA 171 competes efficiently for the binding of PTB to the radiolabeled 57B RNA substrate (Fig. 7A, lane 4). In comparison to PTB, RNA 171 competes poorly for binding to U2AF^{65}, which migrates just slower than PTB on these gels (lane 4). RNA 171 contains two copies of a 19-nt pyrimidine-rich sequence, which is located immediately upstream of an adenosine-rich region containing the branch site. Two additional pyrimidine-rich RNAs, 170 and 172, compete preferentially for PTB compared to U2AF^{65} binding (compare lane 1 to lanes 3 and 5). Of the RNAs tested, the RNA 171 region clearly represents the most efficient PTB binding site. In contrast to the pyrimidine-rich RNAs, RNA 173, which contains four copies of the 9-nt essential RNA element, fails to compete for PTB binding (lane 6). Together these results indicate that there are, at minimum, three PTB binding sites in the 3′ splice site region of intron a, represented by the sequences contained in the 170, 171, and 172 RNA competitors (see below, Fig. 10).

The efficiencies with which the mini RNAs bind PTB correlate well with their ability to induce the switch to the exon selection pathway for the rGγ25 *Xho* I substrate. RNA 171, which is the most efficient competitor for PTB binding, is most efficient in promoting intron a and b splicing at the expense of intron c splicing (Fig. 7B, lane 4). At the same molar excess, RNAs 170 and 172 promote a less complete switch to the exon selection pathway, coincident with their moderate effects as competitors for PTB in the UV crosslinking assay (lanes 3 and 5). Finally, RNA 173, which does not compete for PTB binding, does not promote the switch to exon selection (lane 6). Similar to the experiments shown above, the exon-selected mRNA product does not accumulate appreciably during the 1-h reaction time.

Differential binding of PTB in the vicinity of competing 5′ splice sites: Evidence for a repressor site within the neuron-specific exon that overlaps with a positive regulatory element

Because intron b splicing inhibition is largely dependent upon competition with the intron c splicing event, we next looked for PTB binding sites in close proximity to these two 5′ splice sites. The intron b and c splicing events can be viewed as a competition of these two 5′ splice sites for a common 3′ splice site (Fig. 10, top). PTB may be involved in a mechanism that disfavors the intron b splicing event, for example, by decreasing the rate of U1 snRNP binding to the 5′ splice site of intron b, relative to that of intron c. If the former is true, we would expect to see differential binding of PTB in the vicinity of these two sites. For this reason, RNA competitors spanning the neuron-specific exon and a short region of intron b were constructed and their ability to compete for PTB binding assessed (Fig. 8). Crosslinking of PTB to the radiolabeled substrate 57B RNA is diminished in the presence of the 175 and 177 RNA competitors, both of which contain the neuron-specific exon sequence (lanes 2 and 4). Deletion of the 5′ half of the neuron-specific exon, RNA 176, results in a loss of competition, indicating that this pyrimidine-rich region of the exon functions as a PTB binding site (lane 3). In contrast, RNA 178, which encompasses an analogous region of the 5′ exon and a portion of the adjoining intron a, fails to compete efficiently for PTB crosslinking (lane 5). These results show that PTB binds preferentially to the neuron-specific exon in a region that overlaps with a positive

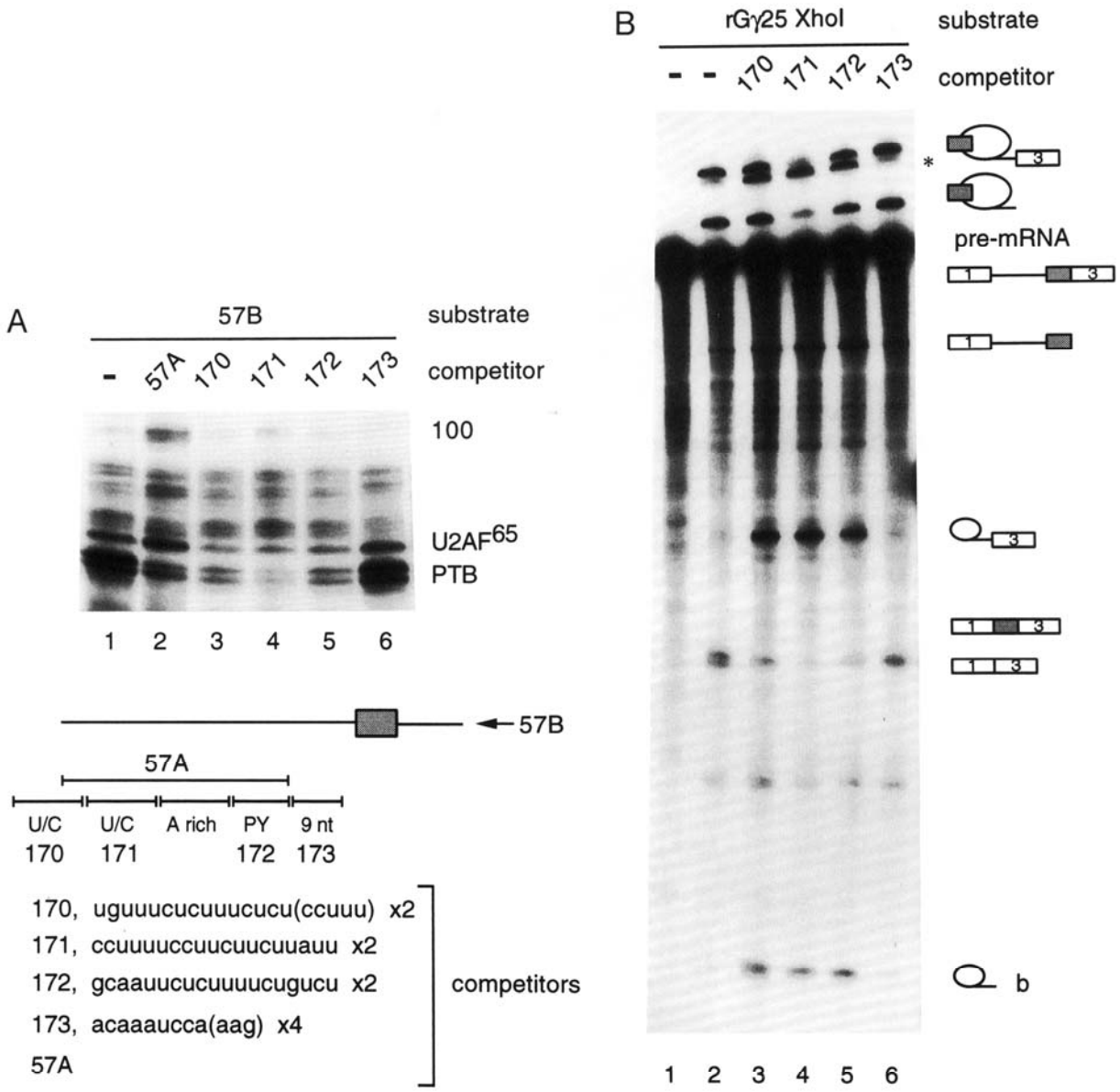

FIGURE 7. Multiple PTB binding sites flanking the branch site region of intron a function to coordinately repress the splicing of introns a and b: Evidence for an RNA repressor array. Mini RNAs spanning the 3′ splice site region of intron a were used as competitors in the UV crosslinking/competition (A) and RNA splicing (B) assays in order to dissect the location of additional PTB binding sites. **A:** The 57B RNA substrate was incubated with HeLa nuclear extract for 5 min at 30 °C in the absence (lane 1) or presence (lanes 2, 3, 4, 5, 6) of unlabeled RNA competitor before UV crosslinking. RNA competitor present at 12-fold molar excess over substrate RNA for 57A, and 30-fold molar excess for the remaining RNA competitors. Otherwise, experimental conditions are as described in the legend to Figure 2. PTB, U2AF^{65}, and 100-kDa proteins are indicated. Sequences are present in two (170, 171, 172) or four (173) copies, whereas sequences in parentheses are present in one copy in the mini RNAs. **B:** Splicing reactions containing RNA substrate were incubated at 30 °C for 0 (lane 1) and 60 min (lanes 2–6) with HeLa nuclear extract and the products resolved on a 4% polyacrylamide/7 M urea gel. Unlabeled RNA competitor was preincubated for 5 min on ice with the nuclear extract in reactions as indicated at a 250-fold molar excess over substrate RNA. RNA intermediates and products of the reactions are indicated. Asterisk signifies the lariat intermediate resulting from the first step of intron a splicing.

regulatory element defined previously (see below, Fig. 10). The presence of a positive element might account for the observation that the 175 RNA competitor is inhibitory when added to in vitro splicing reactions containing γ2 pre-mRNA, in contrast to the effect of mini RNAs containing portions of the intron a repressor array.

Evidence for a reduced level of conventional PTB in rat brain nuclear extracts and a 59-kDa brain-specific PTB counterpart

Given the fact that γ2 pre-mRNA is subject to splicing regulation in rat brain, it is relevant to characterize PTB and any activity that recognizes the RNA repres-

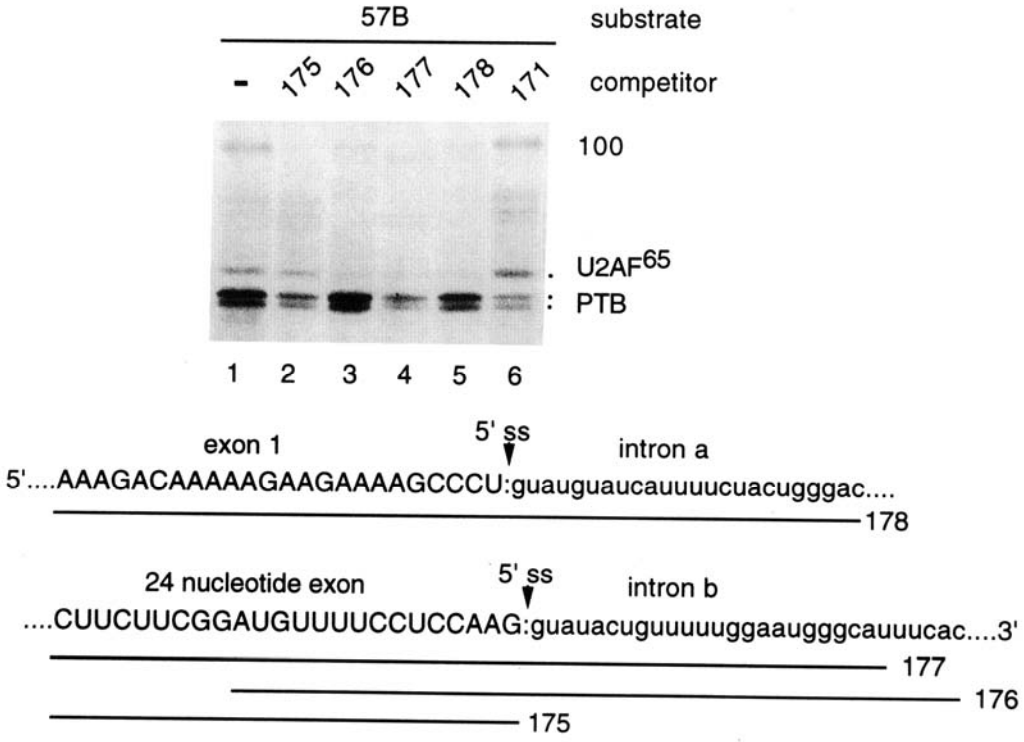

FIGURE 8. Differential PTB binding to competing 5′ splice site regions. UV crosslinking/competition assay was used to assess the relative binding of PTB to the intron a and intron b 5′ splice site regions. Experimental conditions are the same as described for Figure 2, except that the RNA competitors are present at 30-fold molar excess. The sequence of the intron a and intron b 5′ splice site region is shown (bottom); upper-case letters (exon); lower-case letters (intron). The sequence contained in each RNA competitor is underscored.

sor array in rat brain nuclei. An in vitro-regulated splicing assay utilizing the rat brain nuclear extract is currently under development, and, consequently, will not be presented in this paper. It is evident, however, that the rat brain nuclear extract contains an activity that efficiently assembles a specific complex on the repressor array indicative of an inhibitor complex (Fig. 4, lanes 3 and 4). Furthermore, complex A assembly in the rat brain nuclear extract is completely blocked under conditions permissive for the assembly of the inhibitor complex, whereas, a low level of complex A accumulates in the presence of the 57A RNA competitor (Fig. 5, lanes 4 and 5).

It is reported that a lower level of PTB mRNA is expressed in rat brain, compared to other tissues, as determined by northern blot analysis (Patton et al., 1991). Thus, in light of this published study and the experiments shown above, it is of interest to ask directly how much PTB protein is present in the rat brain nuclear extracts, compared to other splicing factors. Tissue-specific differences in PTB levels might contribute to the mechanism of splicing regulation. To address this question directly, we prepared nuclear extracts from rat brain, rat kidney, and HeLa cells for western blot analysis. At equal levels of protein loaded, there is a significant reduction in the level of the 60-kDa PTB doublet in the rat brain nuclear extract, compared to the analogous rat kidney and HeLa nuclear extracts (Fig. 9A, center panel). The reduced level of PTB in the rat brain nuclear extract cannot be explained by degradation, or loss, of the protein sample because PTB-associated splicing factor (PSF), with an apparent molecular weight of 100 kDa, is intact in the same lane (Fig. 9A, top panel). Furthermore, a duplicate blot demonstrates similar levels of the splicing factor U2AF^{65}, in all three protein samples (Fig. 9A, bottom panel). The antibody used to detect PTB in the experiment of Figure 9A is a rabbit polyclonal antiserum raised against human recombinant PTB (J. Patton, pers. comm.). It is established that the human and rat PTB genes are 97% identical at the protein level (Brunel et al., 1991).

If PTB is present at a characteristically lower level in the rat brain nuclear extracts, then what is the activity responsible for the efficient assembly of the apparent inhibitor complex observed in the experiment of Figure 4? To probe this question, we used the UV crosslinking/competition assay to ask if there is a protein in the rat brain nuclear extract that interacts with the RNA repressor array. Similar to the experiments shown above, the 57B RNA, which contains the entire repressor array, was used as the ^{32}P-labeled substrate for UV crosslinking in the presence and absence of specific RNA competitors (Fig. 9B). In the rat brain nuclear extract, a protein with an apparent molecular weight

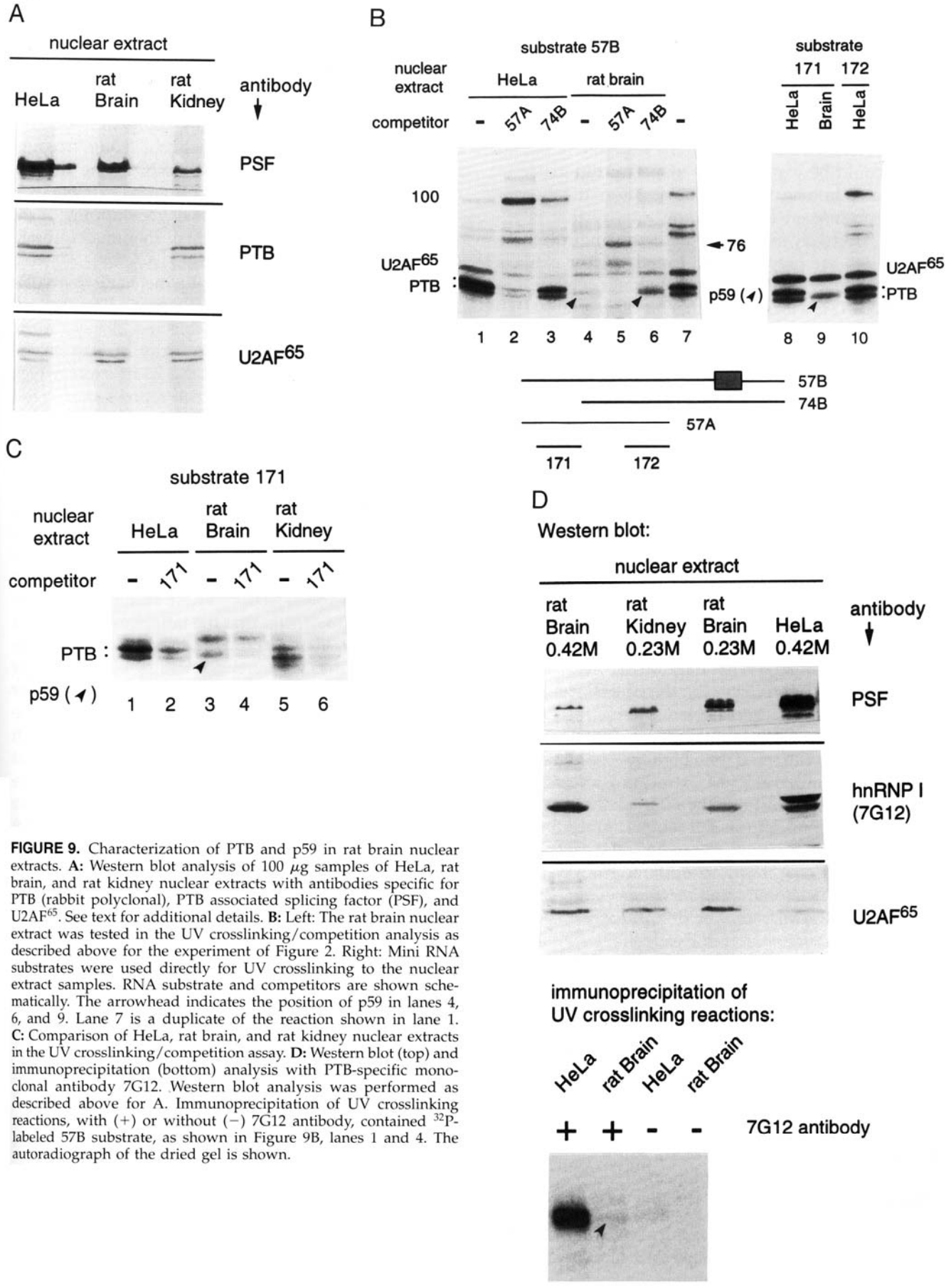

FIGURE 9. Characterization of PTB and p59 in rat brain nuclear extracts. **A:** Western blot analysis of 100 μg samples of HeLa, rat brain, and rat kidney nuclear extracts with antibodies specific for PTB (rabbit polyclonal), PTB associated splicing factor (PSF), and U2AF^{65}. See text for additional details. **B:** Left: The rat brain nuclear extract was tested in the UV crosslinking/competition analysis as described above for the experiment of Figure 2. Right: Mini RNA substrates were used directly for UV crosslinking to the nuclear extract samples. RNA substrate and competitors are shown schematically. The arrowhead indicates the position of p59 in lanes 4, 6, and 9. Lane 7 is a duplicate of the reaction shown in lane 1. **C:** Comparison of HeLa, rat brain, and rat kidney nuclear extracts in the UV crosslinking/competition assay. **D:** Western blot (top) and immunoprecipitation (bottom) analysis with PTB-specific monoclonal antibody 7G12. Western blot analysis was performed as described above for A. Immunoprecipitation of UV crosslinking reactions, with (+) or without (−) 7G12 antibody, contained ^{32}P-labeled 57B substrate, as shown in Figure 9B, lanes 1 and 4. The autoradiograph of the dried gel is shown.

of 59 kDa, p59, interacts specifically with the repressor array, because the crosslinking signal is eliminated with the 57A, but not 74B, RNA competitor (lanes 4–6). The p59 protein also interacts specifically with the mini RNA 171 competitor (Fig. 9C, lanes 3 and 4).

The difference in electrophoretic mobility of rat brain p59 and HeLa PTB observed in the experiment of Figure 9B could be explained by a species difference in the rat and human PTB isoforms. Alternatively, p59 could represent an RNA binding activity that is expressed selectively in brain tissue. To distinguish these possibilities, we prepared and tested nuclear extracts from rat kidney in the UV crosslinking/competition assay. RNA 171, which represents the high-affinity PTB binding site, was employed as the radiolabeled substrate RNA, as well as the unlabeled competitor. These results show that the rat kidney extract contains an RNA binding activity that selectively recognizes the RNA 171 site, and that comigrates with the characteristic 60-kDa PTB doublet observed in the HeLa reactions (Fig. 9C). Thus, the appearance of p59 in the rat brain nuclear extracts cannot be explained by a species-specific difference in PTB isoforms between human and rat.

To further explore the possibility that rat brain p59 is structurally related to PTB, we obtained monoclonal antibody 7G12, which is specific for the hnRNP I isoform of PTB (Ghetti et al., 1992). When the 7G12 antibody is used in a western blot analysis, a protein in the rat brain nuclear extract that migrates in the vicinity of p59 is clearly reactive, whereas the 60-kDa doublet is reactive in the HeLa and rat kidney nuclear extracts (Fig. 9D, western blot, right three lanes). When the rat brain nuclei are extracted with higher salt (0.42 M KCl, following the initial 0.23 M extraction), more of the p59 reactive band, but not the 60-kDa doublet, is released from the nuclei (far left lane).

If rat brain p59 is an isoform of PTB, we would expect the 7G12 antibody to immunoprecipitate this protein from UV crosslinking reactions containing the rat brain nuclear extract. For this purpose, reactions equivalent to those shown in Figure 9B, lanes 1 and 4, were immunoprecipitated with the 7G12 antibody. In agreement with this expectation, rat brain p59 and the HeLa 60-kDa doublet are immunoprecipitated (Fig. 9D, bottom). Thus, we conclude that p59 is the predominant form of PTB in rat brain nuclear extracts.

DISCUSSION

An array of pre-mRNA repressor sites directs the coordinate regulation of adjacent introns in a HeLa nuclear extract

Here we show biochemical evidence for a model in which PTB functions as a *trans*-acting repressor of a neuron-specific splicing event in HeLa nuclear extracts. In theory, the unique appearance, in neurons, of the γ2L spliced mRNA isoform, which retains the 24-nt exon, could be explained by the selective expression of a positive-acting splicing regulatory machinery in neurons but not non-neuronal cells. Alternatively, the expression of a ubiquitous splicing repressor, which is excluded or inactive in neuronal cells, could account for the neuron-specific spliced mRNA. The mechanism documented here in HeLa nuclear extracts may be representative of events that ensure skipping of the 24-nt exon in non-neuronal cells of mammalian brain (Bovolin et al., 1992).

In this study, novel features of a mechanism involving PTB as a modulator of an alternative pre-mRNA splicing event, in vitro, are established. A key feature of this mechanism involves an array of intronic repressor sites, just upstream of the 24-nt exon, which bind selectively to PTB and which repress exon selection in splicing reactions with radiolabeled pre-mRNA (Fig. 10). An additional PTB binding site in the 24-nt exon has been identified, which overlaps with a positive regulatory element defined previously (Zhang et al., 1996). When a short RNA competitor containing the 3′ splice site repressor array, 57A, is added in excess compared to the radiolabeled pre-mRNA, the splicing pathway switches from exclusive exon skipping to predominant exon selection. These results are consistent with the prediction that the latter conditions shift the binding equilibrium toward the RNA competitor-bound form of PTB, thereby releasing PTB from the four pre-mRNA repressor sites. Further evidence for the involvement of PTB in the mechanism of repression stems from experiments that are predicted to shift the binding equilibrium in the opposite direction. That is, exon skipping is re-established as the dominant splicing pathway when purified GST-PTB is added back to in vitro splicing reactions in excess of the 57A RNA competitor. Importantly, GST-PTB has no effect when added to splicing reactions with control pre-mRNAs that lack RNA repressor sites.

A limitation of this experimental approach is that a loss of function results from the sequestration, rather than depletion, of PTB. A more rigorous approach involving quantitative depletion of PTB from nuclear extracts is not successful in our hands using immobilized RNA 171 ligand, because a significant amount of the protein remains in the unbound fraction (M. Ashiya & P.J. Grabowski, unpubl.). Thus, we cannot rule out the involvement of other factors in addition to PTB that may play a role in splicing repression.

A second characteristic feature of the γ2 mechanism involves the coordinate activation of both flanking introns by the RNA competitor-induced splicing switch. That is, the RNA competitor activates both of the characteristic lariat intron–3′ exon intermediates of the intron a first, or intron b first (neuronal) splicing pathways (Fig. 1B, right columns). Thus, a strict sequential acti-

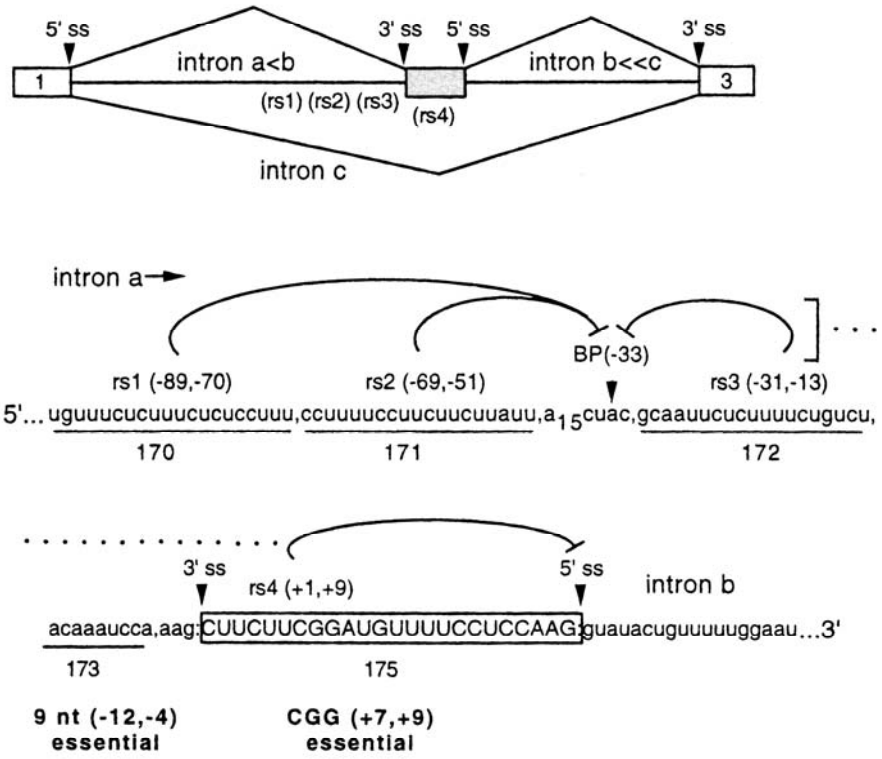

FIGURE 10. Arrangement of RNA regulatory elements in the γ2 pre-mRNA. Top: The model γ2 pre-mRNA substrate, rGγ25, with positions of the repressor sites (rs1–4) is shown schematically. The splicing hierarchy, in which intron c splicing predominates almost exclusively, is inferred from the in vitro splicing experiments shown in Figures 1, 3, and 7B. Bottom: Sequences and arrangement of repressor sites relative to the branch site (BP) and 24-nt exon (boxed). Positive elements identified previously are indicated in bold type. Commas in sequence represent individual repressor sites; within parentheses, commas signify nucleotide range. The involvement of rs1–3 in the repression of intron b splicing is symbolized by the dotted line.

vation mechanism can be ruled out, in which the activation of one splicing event is required to stimulate the second. Nonetheless, introns a and b differ in the sensitivity by which they are activated by the RNA competitor. At every concentration of the 57A RNA competitor tested so far, intron b is activated to a greater extent than intron a, as judged in each case by the proportion of excised lariat intron product compared to lariat intron–3′ exon intermediate. This differential sensitivity is most likely explained by the concentrated array of repressor sites surrounding the branch site of intron a. By comparison, the repressor array is located upstream, but not downstream, of the 5′ splice site adjacent to the 24-nt exon.

A model for neuron-specific splicing regulation mediated by overlapping positive and negative RNA elements

Collectively, the results shown here support a model in which interactions between the repressor array and PTB suppress intron a splicing by interfering with the base pairing of U2 snRNP at the branch site, which is embedded within the repressor array. We have shown that a specific RNA–protein complex assembles on the intron a repressor array, indicative of an inhibitor complex (Fig. 4). Under conditions permissive for the assembly of the inhibitor complex, very low levels of U2 snRNP complex formation (complex A) are observed. When the switch to the exon selection pathway is induced by the addition of RNA competitor, U2 snRNP complex formation increases substantially, whereas the inhibitor complex decreases (Fig. 5). Although our data demonstrate that PTB is required for the assembly of the inhibitor complex, and is a likely component of this complex, further studies will demand purification of the complex and its complete characterization.

The finding that the intron a branch site is embedded within the repressor array suggests that a mechanism has evolved to ensure strong repression of intron a splicing in non-neuronal cells. Previous work has established that the RNA duplex formed between the pre-mRNA branch site and the branchpoint complementary region of U2 snRNA is required for splicing (Parker et al., 1987; Wu & Manley, 1989; Zhuang & Weiner, 1989). Accessory factors that mediate this crucial base pairing event have been identified. The binding of U2AF to the 3′ splice site/polypyrimidine tract is required for proper U2 snRNP binding at the branch site (Ruskin et al., 1988). Moreover, it has been shown that the binding of U2 snRNP-associated protein complexes, SF3a and SF3b, just upstream of the branch site, are also required for the U2 snRNP binding event (Gozani et al., 1996). We note that the polypyrimidine tract located upstream of the γ2 intron a branch site is a particularly potent repressor site (rs2). Intimate protein contacts in the close vicinity of the branch site, some of which change during spliceosome assembly,

have also been reported (MacMillan et al., 1994; Staknis & Reed, 1994; Gaur et al., 1995). Based upon these lines of evidence, we predict that the activation of intron a splicing would require the complete disassembly of the inhibitor complex from the repressor array in order to clear the pre-mRNA for U2 snRNP binding. Of possible relevance to this model, we observe increased crosslinking of a 100-kDa protein to the 3′ splice site region of intron a upon the addition of the specific 57A RNA competitor (Fig. 2). The 100-kDa protein might be a factor that participates in branch site selection, which gains access to the pre-mRNA when PTB is released from the RNA repressor sites. Further work will be required to identify the 100-kDa protein and determine its functional significance.

In the case of intron b, 5′ splice site activity is modulated by a more subtle mechanism that is largely dependent upon the competing upstream 5′ splice site. That is, the strong splicing inhibition of intron b in the full-length substrate, rGγ25 *Xho* I, is largely relieved in a substrate, rGγ81 *Xho* I, which lacks the 5′ exon but retains the intron a repressor region (Fig. 6). Even when recombinant PTB is added in excess over endogenous levels, only partial intron b inhibition is observed for the rGγ81 *Xho* I substrate at the highest level of protein added (data not shown). Thus, a model in which the intron a repressor array envelopes the 5′ splice site of intron b in an inhibitory complex does not account fully for these results. Instead, a differential effect on the diffusion of U1 snRNP to competing 5′ splice sites is a more plausible model. We have looked carefully for PTB binding sites near the 5′ splice sites of intron a and b using the UV crosslinking/competition assay. These results show that, although PTB binds selectively to the 24-nt exon, little or no PTB binding is found in the analogous region of the 5′ splice site of intron a (Fig. 8). We propose that the proximity of the entire repressor array, including the most proximal PTB binding site, rs4, functions to decrease the rate of collision of U1 snRNP with the 5′ splice site of intron b, under conditions in which the repressor sites are fully occupied. This latter model can be viewed as an effect on three-dimensional diffusion, but may also involve the one-dimensional diffusion of U1 snRNP along the pre-mRNA chain. In the presence of RNA competitor, which sequesters PTB, splicing to the 5′ splice site of intron b is then favored due to the proximity of its 3′ splice site partner. The results shown here provide indirect support for such a model.

The results shown here do not exclude the possibility that other splicing factors may be required for the complete regulation of γ2 pre-mRNA splicing. The location of the recently identified positive regulatory elements, which overlap with the repressor array, is intriguing (Fig. 10). This arrangement of RNA signals suggests that exon selection may predominate in neurons because these cells express specialized factors that recognize the positive signals and engage them rapidly in spliceosome assembly with a commitment to neuron-specific exon selection. Once U2 snRNP is base paired to the intron a branch site, the repressor sites may be incapable of recognition by existing repressor molecules. Alternatively, neurons may express lower levels or less active forms of the inhibitory factors. The finding that the extent and developmental timing of γ2 splicing regulation differs in two types of cerebellar neurons invites additional speculation (Wang & Grabowski, 1996). Such an arrangement of overlapping positive and negative regulatory elements may allow each neuron the ability to tune the level of regulated spliced product according to developmental cues, or in response to electrical or chemical signals from other cells. Previous studies have demonstrated, at the level of protein function, the significance of site-specific phosphorylation of the γ2L (exon selected) subunit, as well as physiological differences of $GABA_A$ chloride channels in distinct cerebellar neurons (Puia et al., 1994; Harris et al., 1995). These studies provide a biological framework for the idea that pre-mRNA splicing regulation contributes to mechanisms that establish the intricate functional differences of heterogeneous $GABA_A$ receptors.

The sequence, (5′) UUCUCU (3′), in a pyrimidine context is a common feature of one class of intron splicing repressor sites

To evaluate the relationship of the intron repressor sites identified in this study to those reported in other systems, we performed a sequence alignment (Table 2). Sequences were selected for comparison based upon their location in an intron, and their demonstrated ability to bind PTB and repress splicing activity. Interestingly, five of the five pre-mRNAs contain one or more copies of a short consensus sequence, (5′) UUCUCU (3′), which is embedded in a pyrimidine context. In the case of fibronectin pre-mRNA, there are two hexanucleotide sequences with an imperfect match to the consensus. It is interesting that the sequences of five pre-mRNAs compared in Table 2 are derived from mammalian examples that are subject to tissue-specific splicing, even though this was not used as a criterion for their selection. When the γ2 repressor sites are used in a Blast N search of the available databases, no additional information is revealed because the >300 sequence matches are mostly from uncharacterized genomic regions.

We note that the (5′) UUCUCU (3′) sequence is not present in each of the repressor sites identified in this study, in particular, the most potent site, rs2. Thus, this sequence alone cannot be required strictly for PTB binding and splicing repression. The structural features of the rs2 region that confer its superior PTB binding and repressor function are not yet established. Based upon

TABLE 2. Sequence alignment of intron splicing repressor sites with an affinity for PTB.[a]

Site	Sequence	Reference
β-TM	CUUUGCUCUUCUCUUUUCUCcUCCUCCCCUCCUCCACUGUGCCA	Mulligan et al. (1992); Singh et al. (1995)
α-TM	.....UUUCUCUUUCUCUCUCCCUCCCUGUCUUUCCCUCU..	Mullen et al. (1991); Patton et al. (1991)
γ2, rs1,2	.....UGUUUCUCUUUCUCUCCUUUCCUUUUCCUUCUUCUUA..	This study
γ2, rs3	....GCAAUUCUCUUUUCUGUCU.....	This study
Fibronectin	UUAUUUUUUcCUCUUUUUUUGCUCUACUUUUCCUUUG.....	Norton (1994)
c-src	..CUCUCCUUCUCUCUGCUUCUCUCUCGCUGGCCCUU.....	Chan and Black (1995; pers. comm.)
γ2, rs4	.....CUUCUUCG....	This study
Consensus	.....UUCUCU.....UUCCUU...CUUCUUC.....	

[a]Intron sequences associated with splicing repression and with an affinity for PTB were selected from the literature and this study, and aligned using MacVector version 5.0 sequence software. Sequences are indicated in the 5′ to 3′ direction. Only the sequences near the consensus are shown. Sites with a match to one of three consensus sequences are underscored; mismatches are indicated in lower case.

the function and architecture of this pre-mRNA region, we favor the hypothesis that the array of γ2 repressor sites identified here represents a functional unit. For example, rs4 fails to repress splicing without the upstream repressor sites (rGγ46, Fig. 6).

The role of PTB shown here as a modulator of γ2 pre-mRNA splicing differs in several respects from results shown previously for mutually exclusive exons of the muscle-specific α- and β-tropomyosin pre-mRNAs. For β-tropomyosin, PTB binds selectively to pyrimidine-rich intron regions downstream of the branch site and near the skeletal muscle-specific exon 7, resulting in 3′ splice site inhibition (Mulligan et al., 1992; Singh et al., 1995). It has also been reported that the upstream pyrimidine-rich region is important for long-distance branch site selection, which suggests that this region may have multiple functions in splicing (Mulligan et al., 1992). For α-tropomyosin pre-mRNA, PTB binding appears to modulate alternative 3′ splice site selection by a similar mechanism. In this case, PTB blocks the binding of U2AF to the 3′ splice site/polypyrimidine tract because the addition of U2AF partially reverses the inhibitory effect induced by PTB (Lin & Patton, 1995). Although less extensively characterized, a pyrimidine-rich region in fibronectin pre-mRNA upstream of the EIIIB exon has been shown to bind PTB and to repress single intron splicing (Norton, 1994). Here we show that the arrangement of positive and negative elements in γ2 pre-mRNA is distinct from examples noted above. This arrangement also differs from the splicing repressor sites found upstream and downstream of the c-*src* N1 exon (Chan & Black, 1995).

Other examples of splicing regulation are mediated by distinct inhibitory RNA elements, but the sequences of these inhibitory elements bear no resemblance to those reported in Table 2. A sequence localized to the alternative K-SAM exon of the fibroblast growth factor receptor 2 pre-mRNA is associated with the repression of the K-SAM exon (Del Gatto & Breathnach, 1995; Del Gatto et al., 1996). Additional exonic inhibitory sequences have been defined in HIV1 tat and the SK exon 5 of the α-tropomyosin pre-mRNA (Graham et al., 1992; Amendt et al., 1994; Si et al., 1997). Furthermore, when a purine-rich exon enhancer is repositioned to the 3′ splice site region of an adenovirus pre-mRNA, splicing is inhibited and this effect is apparently mediated by SR proteins (Kanopka et al., 1996). Thus, the position of the regulatory element in the pre-mRNA may be paramount in determining whether it stimulates or inhibits splicing. Specific RNA secondary structures have also been shown to mediate splicing repression for chicken β-tropomyosin, adenovirus E1A and E3, immunoglobulin μ heavy chain, and human growth hormone pre-mRNAs (Chebli et al., 1989; Watakabe et al., 1989; Domenjoud et al., 1991; D'Orval et al., 1991; Libri et al., 1991; Estes et al., 1992). Finally, in the case of influenza virus NS1 pre-mRNA, splicing repression is associated with the assembly of aberrant spliceosomes and with a specific disruption of the required U6-U2 snRNP base pairing event (Nemeroff et al., 1992; Qiu et al., 1995). Thus, it appears that a variety of mechanisms have evolved to regulate specific pre-mRNA splicing events by a repression scheme.

p59, the predominant form of PTB in rat brain nuclear extracts

A novel aspect of the present study includes the finding of a protein, p59, which is abundant in rat brain, but not rat kidney or HeLa nuclear extracts, and which binds selectively to the RNA repressor array (Fig. 9). This protein is of interest because the rat brain nuclear extracts contain significantly lower levels of the conventional PTB doublet, and because these extracts assemble a specific RNA-protein complex efficiently, indicative of an inhibitor complex. Based upon its RNA binding characteristics and reactivity with the PTB-specific monoclonal antibody 7G12, it is likely that p59 is the predominant form of PTB in rat brain. p59 may be a specific isoform, or a covalently modified form of PTB. Exactly how the structure of rat brain p59 differs from other forms of PTB, and whether or not this structural form has functional consequences, remains to be established.

The involvement of other RNA binding proteins in neuron-specific splicing regulation have been demonstrated. Both hnRNP F and a novel splicing regulator, KSRP, have been identified based upon their association with specific RNA–protein complexes containing c-*src* pre-mRNA (Min et al., 1995, 1997). Although hnRNP F is ubiquitous, KSRP is more abundant in a neuronal, compared to a HeLa, nuclear extract. Several neuron-specific RNA binding proteins of the ELAV family have also been demonstrated to bind selectively to AU-rich 3′ untranslated mRNAs, indicating their possible roles in regulating posttranscriptional gene control (Levine et al., 1993; King et al., 1994). In addition, there is one report in which a disruption in the splicing of *Drosophila* Nrg^{180} correlates with mutations in ELAV (Koushika et al., 1996). In spite of these and many other advances in the field, the extent to which tissue-specific splicing events are dominated by differences in the levels of general splicing components, such as SR proteins, in contrast to the selective expression of tissue, or stage-specific RNA binding proteins, remains a major unresolved question.

MATERIALS AND METHODS

Construction of plasmids

Substrates used for splicing, UV crosslinking, and complex formation are derived from the parent substrate, rGγ25 (Zhang et al., 1996). Plasmid rGγ81 was generated by deleting the *Not* I–*Bgl* II segment from rGγ25, followed by treatment with Klenow enzyme (Promega) and ligation to reclose the plasmid. Plasmid rGγ46 was generated by PCR using rGγ25 as template with an upstream primer complementary to the regulated exon, including a *Hin*d III site at the end of the primer, together with a downstream primer complementary to the a region of the vector downstream of the insert. The PCR product was gel purified and inserted into a pBS vector (Stratagene) as a *Hin*d III and *Eco*R 1 fragment. RNA competitors, 57B and 74B, were generated by PCR using rGγ25 as template. RNA competitor, 63B, was generated in a similar manner using rGγ49 (Zhang et al., 1996) as template. PCR products were gel purified and cleaved with *Eco*R 1 and *Bam*H 1 at sites present at the ends of the primers and cloned into pBS. The identity of each clone was verified by DNA sequence analysis.

Transcription and in vitro splicing reactions

Templates for transcription were generated by digestion of the plasmid with *Xho* I, *Bam*H 1, or *Acc* I. Labeled substrate RNAs, rGγ25 *Xho* 1, rGγ25 *Bam*H 1, rGγ46 *Eco*R 1, rGγ81 *Xho* 1, rGγ83 *Xho* 1, L2P, 57B, 74B, and 57A were synthesized from linearized plasmid template present at 40 μg/mL in a 25-μL reaction containing 0.4 mM each of ATP, CTP, and GTP, 0.3 mM UTP, 2.5 μL of [^{32}P]UTP (NEN 007H), 0.5 mM diguanosine triphosphate and T3, or T7 RNA polymerase (Stratagene). Reactions were incubated at 37 °C for 30 min followed by digestion with DNase I (Promega) for 10 min at room temperature. Unincorporated nucleotides were removed by Sephadex G50 or G25 chromatography.

RNA competitors were transcribed in a 200-μL reaction volume in the absence of diguanosine triphosphate and [^{32}P]UTP. RNA concentrations were determined by absorbance at 260 nm after removal of unincorporated nucleotides, and the integrity of the RNAs was checked on a 15% polyacrylamide/7 M urea gel stained with ethidium bromide. RNA competitors, 170–178, were synthesized by T7 RNA polymerase as described by Milligan and Uhlenbeck (1989). Briefly, a template DNA strand, containing the complementary sequence of the desired RNA followed by a sequence complementary to the T7 RNA polymerase promoter, was synthesized for each RNA competitor (Bottom strand). Sequences of Bottom strand deoxyoligonucleotides (oligos) are available upon request. For each transcription reaction, a Bottom strand oligo was hybridized to a Top strand oligo containing the T7 RNA polymerase promoter sequence, (5′-TAATACGACTCACTATAG-3′), followed by transcription and workup as described above.

In vitro splicing reactions contained 44% (v/v) HeLa nuclear extract, 1 mM $MgCl_2$, 1.5 mM ATP, 5 mM creatine phosphate, 60 mM KCl, 20 mM Hepes, pH 7.4, 120,000 cpm of substrate RNA in a 25-μL reaction volume. Reactions were incubated at 30 °C for the indicated times followed by digestion with proteinase K (100 units/mL for 10 min at 30°C), phenol/chloroform extraction, and ethanol precipitation. In reactions containing RNA competitor, RNA competitor was preincubated with nuclear extract for 5 min on ice prior to addition of the labeled substrate RNA.

Nuclear extract preparation

HeLa nuclear extract was prepared as described by Dignam et al. (1983). Rat whole brain and kidney nuclear extract protocols were adapted from Sierra et al. (1993). Briefly, fresh tissue was obtained from six 28-day Sprague–Dawley rats (Zivic-Miller, Pennsylvania), rinsed in cold PBS, and minced. The minced tissue, in 30 mL of homogenization buffer cooled to −20 °C (10 mM Hepes, pH 7.6, 15 mM KCl, 0.15 mM spermine, 0.5 mM spermidine, 1 mM EDTA, 2.2 M sucrose, 5% glycerol, 0.5 mM DTT), was homogenized in a precooled, 55-mL Teflon-glass homogenizer at 400 rpm for 5–7 min. The homogenate was diluted to 50 mL with homogenization buffer, layered over a 10-mL sucrose cushion (homogenization buffer plus 2 M sucrose and 10% glycerol, cooled to −20 °C), and centrifuged in a SW28 rotor, at 27,000 rpm, for 1 h at 4 °C. The nuclear pellets were rinsed in Buffer C with 20 mM KCl (20 mM Hepes, pH 7.6, 25% glycerol, 1.5 mM $MgCl_2$, 0.2 mM EDTA, 0.5 mM DTT) briefly and resuspended in 0.5 mL Buffer C containing 0.23 M KCl. After a 30-min incubation on ice, the resuspended nuclei were spun at 16,000 × *g* for 10 min in a microcentrifuge and the supernatant dialyzed for 4 h against 2 L of Buffer D (20 mM Hepes, pH 7.6, 20% glycerol, 0.1 M KCl, 0.2 mM EDTA, 0.5 mM DTT) with one change of buffer. Nuclear extracts were flash frozen in liquid nitrogen and stored in aliquots at −80 °C. Protease inhibitors were added to Homogenization buffer, sucrose cushion, and Buffer C plus 0.23 M KCl.

UV crosslinking

Nuclear extracts were adjusted to 4 mg/mL protein concentration as measured by the standard Bradford assay. Splicing

reactions were assembled as described above with 36% (v/v) HeLa nuclear extract or rat brain nuclear extract, 300,000 cpm of ^{32}P-labeled substrate RNA and incubated for 5 min on ice or at 30 °C as indicated. RNA competitor was preincubated with nuclear extract for 5 min on ice prior to addition of labeled substrate RNA. Samples were then irradiated (in open 1.5-mL Eppendorf tubes) on ice with 1.2 J using a Stratalinker (Stratagene) at a distance of 6.5 cm from the UV source. Following irradiation, samples were digested with 1 mg/mL Ribonuclease IA (Pharmacia) for 20 min at 30 °C, electrophoresis sample buffer was added, the samples boiled for 5 min, and loaded on a 12.5% polyacrylamide gel. Gels were run at 30 mA for approximately 4.5 h, fixed in 45% methanol, 9% acetic acid for 1–12 h, and then dried. Crosslinked proteins were detected by autoradiography.

Mobility shift analysis

Scaled down splicing reactions were assembled (10 μL total volume) with 44% (v/v) HeLa or Brain nuclear extract (each at 4 mg/mL) and 30,000 cpm of ^{32}P-labeled substrate RNA and incubated for 5 min on ice or at 30 °C as indicated. Heparin was then added (2 mg/mL final concentration) followed by a 3-min incubation at 30 °C. Samples were placed on ice immediately following the heparin treatment and half the sample was loaded on a 3.75% polyacrylamide gel containing 50 mM Tris-Glycine, pH 8.8, and 10% glycerol, electrophoresed at 4 W at 4 °C, or room temperature for 3.5 h, and dried onto Whatman paper. Complexes were visualized by autoradiography. Assembly reactions containing RNA competitors were preincubated with nuclear extract for 5 min prior to addition of labeled substrate RNA.

Western blot and immunoprecipitation analysis

After separation on 12.5% SDS-polyacrylamide gels, proteins were transferred to Immobilon (Millipore) by electroblotting. Blocking was performed in 3% gelatin and primary antibodies were used at a dilution of 1:10,000 for PTB, 1:2,500 for PSF, 1:2,900 for PepA (U2AF^{65} specific), and 1:250 for 7G12. Goat anti-rabbit IgG conjugated to alkaline phosphatase was used as the secondary antibody at a dilution of 1:2,900. Anti-mouse IgG conjugated to alkaline phosphatase was used at a dilution of 1:3,000 for the 7G12 antibody. NBT and BCIP (Promega) were used for color development. For immunoprecipitation analysis, UV crosslinking reactions were incubated with 5 μL of the 7G12 antibody and 1.5 μL anti-mouse IgG, followed by precipitation with an equal volume of Protein A beads (Pharmacia) in 1× NET buffer: 50 mM Tris, pH 7.5, 150 mM NaCl, 5 mM EDTA. Immunoprecipitates were washed 4× in 1× NET + 0.5% NP40, boiled in SDS sample buffer, and resolved on 12.5% SDS-polyacrylamide gels.

NOTE ADDED IN PROOF

A related study by R.C. Chan and D.L. Black is in press, to appear in *Mol Cell Biol.*

ACKNOWLEDGMENTS

Supported by the Howard Hughes Medical Institute and a grant from the NIH to P.G. We are indebted to J. Patton for the polyclonal PTB and PSF antibodies, M. Garcia-Blanco for the GST-PTB plasmid, and G. Dreyfuss for the PTB monoclonal 7G12 antibody. We thank D.L. Black for providing information prior to publication; L. Zhang, R. Reed, F. Ding, and other members of the Grabowski laboratory for helpful discussions and a critical reading of the manuscript.

Received May 2, 1997; returned for revision June 3, 1997; revised manuscript received June 19, 1997

REFERENCES

Amendt BA, Hesslein D, Chang LJ, Stoltzfus CM. 1994. Presence of negative and positive *cis*-acting RNA splicing elements within and flanking the first tat coding exon of human immunodeficiency virus type 1. *Mol Cell Biol 14*:3960–3970.

Belsham GJ, Sonenberg N. 1996. RNA–protein interactions in regulation of picornavirus RNA translation. *Microbiol Rev 60*:499–511.

Bovolin P, Santi MR, Puia G, Costa E, Grayson D. 1992. Expression patterns of γ-aminobutyric acid type A receptor subunit mRNAs in primary cultures of granule neurons and astrocytes from neonatal rat cerebella. *Proc Natl Acad Sci USA 89*:9344–9348.

Brunel F, Alzari PM, Ferrara P, Zakin MM. 1991. Cloning and sequencing of PYBP, a pyrimidine-rich specific single strand DNA-binding protein. *Nucleic Acids Res 19*:5237–5245.

Caceres JF, Stamm S, Helfman DM, Krainer AR. 1994. Regulation of alternative splicing in vivo by overexpression of antagonistic splicing factors. *Science 265*:1706–1709.

Chan RC, Black DL. 1995. Conserved intron elements repress splicing of a neuron-specific c-src exon in vitro. *Mol Cell Biol 15*:6377–6385.

Chebli K, Gattoni R, Schmitt P, Hildwein G, Stevenin J. 1989. The 216-nucleotide intron of the E1A pre-mRNA contains a hairpin structure that permits utilization of unusually distant branch acceptors. *Mol Cell Biol 9*:4852–4861.

Del Gatto F, Breathnach R. 1995. Exon and intron sequences, respectively, repress and activate splicing of a fibroblast growth factor receptor 2 alternative exon. *Mol Cell Biol 15*:4825–4834.

Del Gatto F, Gesnel MC, Breathnach R. 1996. The exon sequence TAGG can inhibit splicing. *Nucleic Acids Res 24*:2017–2021.

Dignam JD, Lebovitz RM, Roeder RG. 1983. Accurate transcription initiation by RNA polymerase II in a soluble extract from isolated mammalian nuclei. *Nucleic Acids Res 11*:1475–1489.

Domenjoud L, Gallinaro H, Kister L, Meyer S, Jacob M. 1991. Identification of a specific exon sequence that is a major determinant in the selection between a natural and a cryptic 5′ splice site. *Mol Cell Biol 11*:4581–4590.

D'Orval BC, Carafa YD, Sirand-Pugnet P, Gallego M, Brody E, Marie J. 1991. RNA secondary structure repression of a muscle-specific exon in HeLa cell nuclear extracts. *Science 252*:1823–1828.

Dreyfuss G, Matunis MJ, Pinol-Roma S, Burd CG. 1993. hnRNP proteins and the biogenesis of mRNA. *Annu Rev Biochem 62*:289–321.

Estes PA, Cooke NE, Liebhaber SA. 1992. A native RNA secondary structure controls alternative splice-site selection and generates two human growth hormone isoforms. *J Biol Chem 267*:14902–14908.

Fu XD. 1993. Specific commitment of different pre-mRNAs to splicing by single SR proteins. *Nature 365*:82–85.

Fu XD. 1995. The superfamily of arginine/serine-rich splicing factors. *RNA 1*:663–680.

Fu XD, Mayeda A, Maniatis T, Krainer AR. 1992. General splicing factors SF2 and SC35 have equivalent activities in vitro, and both affect alternative 5′ and 3′ splice site selection. *Proc Natl Acad Sci USA 89*:11224–11228.

Garcia-Blanco MA, Jamison SF, Sharp PA. 1989. Identification and purification of a 62,000-dalton protein that binds specifically to the polypyrimidine tract of introns. *Genes & Dev 3*:1874–1886.

Gaur RK, Valcarcel J, Green MR. 1995. Sequential recognition of the pre-mRNA branch point by U2AF^{65} and a novel spliceosome-associated 28-kDa protein. *RNA 1*:407–417.

Ghetti A, Pinol-Roma S, Michael WM, Morandi C, Dreyfuss G. 1992. hnRNP I, the polypyrimidine tract-binding protein: Distinct nuclear localization and association with hnRNAs. *Nucleic Acids Res 20*:3671–3678.

Gil A, Sharp PA, Jamison SF, Garcia-Blanco MA. 1991. Characterization of cDNAs encoding the polypyrimidine tract-binding protein. *Genes & Dev 5*:1224–1236.

Gooding C, Roberts GC, Moreau G, Nadal-Ginard B, Smith CWJ. 1994. Smooth muscle-specific switching of α-tropomyosin mutually exclusive exon selection by specific inhibition of the strong default exon. *EMBO J 13*:3861–3872.

Gozani O, Feld R, Reed R. 1996. Evidence that sequence-independent binding of highly conserved U2 snRNP proteins upstream of the branch site is required for assembly of spliceosomal complex A. *Genes & Dev 10*:233–243.

Graham IR, Hamshere M, Eperon IC. 1992. Alternative splicing of a human α-tropomyosin muscle-specific exon: Identification of determining sequences. *Mol Cell Biol 12*:3872–3882.

Guo W, Mulligan GJ, Wormsley S, Helfman DM. 1991. Alternative splicing of β-tropomyosin pre-mRNA: Cis-acting elements and cellular factors that block the use of a skeletal muscle exon in nonmuscle cells. *Genes & Dev 5*:2096–2107.

Harris RA, McQuilkin SJ, Paylor R, Abeliovich A, Tonegawa S, Wehner JM. 1995. Mutant mice lacking the γ isoform of protein kinase C show decreased behavioral actions of ethanol and altered function of γ-aminobutyrate type A receptors. *Proc Natl Acad Sci USA 92*:3658–3662.

Horabin JI, Schedl P. 1993. Sex-lethal autoregulation requires multiple *cis*-acting elements upstream and downstream of the male exon and appears to depend largely on controlling the use of the male exon 5′ splice site. *Mol Cell Biol 13*:7734–7746.

Kaminski A, Hunt SL, Patton JG, Jackson RJ. 1995. Direct evidence that polypyrimidine tract binding protein (PTB) is essential for internal initiation of translation of encephalomyocarditis virus RNA. *RNA 1*:924–938.

Kamma H, Portman DS, Dreyfus G. 1995. Cell type-specific expression of hnRNP proteins. *Exp Cell Res 221*:187–196.

Kanopka A, Muhlemann O, Akusjarvi G. 1996. Inhibition by SR proteins of splicing of a regulated adenovirus pre-mRNA. *Nature 381*:535–538.

King PH, Levine TD, Fremeau RT Jr, Keene JD. 1994. Mammalian homologs of *Drosophila* ELAV localized to a neuronal subset can bind in vitro to the 3′ UTR of mRNA encoding the Id transcriptional repressor. *J Neurosci 14*:1943–1952.

Koushika SP, Lisbin MJ, White K. 1996. ELAV, a *Drosophila* neuron-specific protein, mediates the generation of an alternatively spliced neural protein isoform. *Curr Biol 6*:1634–1641.

Levine TD, Gao F, King PH, Andrews LG, Keene JD. 1993. Hel-N1: An autoimmune RNA-binding protein with specificity for 3′ uridylate-rich untranslated regions of growth factor mRNAs. *Mol Cell Biol 13*:3494–3504.

Libri D, Piseri A, Fiszman MY. 1991. Tissue-specific splicing in vivo of the β-tropomyosin gene: Dependence on an RNA secondary structure. *Science 252*:1842–1845.

Lin CH, Patton JG. 1995. Regulation of alternative 3′ splice site selection by constitutive splicing factors. *RNA 1*:234–245.

Lin YS, Green MR. 1991. Mechanism of action of an acidic transcriptional activator in vitro. *Cell 64*:971–981.

Lou H, Gagel RF, Berget SM. 1996. An intron enhancer recognized by splicing factors activates polyadenylation. *Genes & Dev 10*:208–219.

MacMillan AM, Query CC, Allerson CR, Chen S, Verdine GL, Sharp PA. 1994. Dynamic association of proteins with the pre-mRNA branch region. *Genes & Dev 8*:3008–3020.

Manley JL, Tacke R. 1996. SR proteins and splicing control. *Genes & Dev 10*:1569–1579.

Mayeda A, Krainer AR. 1992. Regulation of alternative pre-mRNA splicing by hnRNP A1 and splicing factor SF2. *Cell 68*:365–375.

Milligan JF, Uhlenbeck OC. 1989. Synthesis of small RNAs using T7 RNA polymerase. *Methods Enzymol 180*:51–62.

Min H, Chan RC, Black DL. 1995. The generally expressed hnRNP F is involved in a neural-specific pre-mRNA splicing event. *Genes & Dev 9*:2659–2671.

Min H, Turck CW, Nikolic JM, Black DL. 1997. A new regulatory protein, KSRP, mediates exon inclusion through an intronic splicing enhancer. *Genes & Dev 11*:1023–1036.

Mullen MP, Smith CWJ, Patton JG, Nadal-Ginard B. 1991. α-Tropomyosin mutually exclusive exon selection: Competition between branchpoint/polypyrimidine tracts determines default exon choice. *Genes & Dev 5*:642–655.

Mulligan GJ, Guo W, Wormsley S, Helfman DM. 1992. Polypyrimidine tract binding protein interacts with sequences involved in alternative splicing of β-tropomyosin pre-mRNA. *J Biol Chem 267*:25480–25487.

Nandabalan K, Roeder GS. 1995. Binding of a cell-type-specific RNA splicing factor to its target regulatory sequence. *Mol Cell Biol 15*:1953–1960.

Nemeroff ME, Utans U, Kramer A, Krug RM. 1992. Identification of *cis*-acting intron and exon regions in influenza virus NS1 mRNA that inhibit splicing and cause the formation of aberrantly sedimenting presplicing complexes. *Mol Cell Biol 12*:962–970.

Norton PA. 1994. Polypyrimidine tract sequences direct selection of alternative branch sites and influence protein binding. *Nucleic Acids Res 22*:3854–3860.

Parker R, Siliciano PG, Guthrie C. 1987. Recognition of the TACT AAC box during mRNA splicing in yeast involves base pairing to the U2-like snRNA. *Cell 49*:229–239.

Patton JG, Mayer SA, Tempst P, Nadal-Ginard B. 1991. Characterization and molecular cloning of polypyrimidine tract-binding protein: A component of a complex necessary for pre-mRNA splicing. *Genes & Dev 5*:1237–1251.

Puia G, Costa E, Vicini S. 1994. Functional diversity of GABA-activated Cl^- currents in Purkinje versus Granule neurons in rat cerebellar slices. *Neuron 12*:117–126.

Qiu Y, Nemeroff M, Krug RM. 1995. The influenza virus NS1 protein binds to a specific region in human U6 snRNA and inhibits U6-U2 and U6-U4 snRNA interactions during splicing. *RNA 1*:304–316.

Ramchatesingh J, Zahler AM, Neugebauer KM, Roth MB, Cooper TA. 1995. A subset of SR proteins activates splicing of the cardiac troponin T alternative exon by direct interactions with an exonic enhancer. *Mol Cell Biol 15*:4898–4907.

Ruskin B, Zamore PD, Green MR. 1988. A factor, U2AF, is required for U2 snRNP binding and splicing complex assembly. *Cell 52*:207–219.

Screaton GR, Caceres JF, Mayeda A, Bell MV, Plebanski M, Jackson DG, Bell JI, Krainer AR. 1995. Identification and characterization of three members of the human SR family of pre-mRNA splicing factors. *EMBO J 14*:4336–4349.

Si Zh, Amendt BA, Stoltzfus CM. 1997. Splicing efficiency of human immunodeficiency virus type 1 tat RNA is determined by both a suboptimal 3′ splice site and a 10 nucleotide exon splicing silencer element located within tat exon 2. *Nucleic Acids Res 25*:861–867.

Siebel CW, Admon A, Rio DC. 1995. Soma-specific expression and cloning of PSI, a negative regulator of P element pre-mRNA splicing. *Genes & Dev 9*:269–283.

Siebel CW, Fresco LD, Rio DC. 1992. The mechanism of somatic inhibition of *Drosophila* P-element pre-mRNA splicing: Multiprotein complexes at an exon pseudo-5′ splice site control U1 snRNP binding. *Genes & Dev 6*:1386–1401.

Sierra F, Tian JM, Schibler U. 1993. In vitro transcription with nuclear extracts from differentiated tissues. In: Hames BD, Higgins SJ, eds. *Gene transcription—A practical approach*. New York: Oxford University Press. pp 125–152.

Singh R, Valcarcel J, Green MR. 1995. Distinct binding specificities and functions of higher eukaryotic polypyrimidine tract-binding proteins. *Science 268*:1173–1176.

Staknis D, Reed R. 1994. Direct interactions between pre-mRNA and six U2 small nuclear ribonucleoproteins during spliceosome assembly. *Mol Cell Biol 14*:2994–3005.

Tian M, Maniatis T. 1992. Positive control of pre-mRNA splicing in vitro. *Science 256*:237–240.

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

Valcarcel J, Singh R, Zamore PD, Green MR. 1993. The protein sex-lethal antagonizes the splicing factor U2AF to regulate alternative splicing of transformer pre-mRNA. *Nature 362*:171–175.

Wang J, Manley JL. 1995. Overexpression of the SR proteins ASF/SF2 and SC35 influences alternative splicing in vivo in diverse ways. *RNA 1*:335–346.

Wang Z, Grabowski PJ. 1996. Cell- and stage-specific splicing events resolved in specialized neurons of the rat cerebellum. *RNA 2*:1241–1253.

Wang Z, Hoffmann HM, Grabowski PJ. 1995. Intrinsic U2AF binding is modulated by exon enhancer signals in parallel with changes in splicing activity. *RNA 1*:21–35.

Watakabe A, Inoue K, Sakamoto H, Shimura Y. 1989. A secondary structure at the 3′ splice site affects the in vitro splicing reaction of mouse immunoglobulin μ chain pre-mRNAs. *Nucleic Acids Res 17*:8159–8169.

Wu J, Manley JL. 1989. Mammalian pre-mRNA branch site selection by U2 snRNP involves base pairing. *Genes & Dev 3*:1553–1561.

Zahler AM, Neugebauer KM, Lane WS, Roth MB. 1993. Distinct functions of SR proteins in alternative pre-mRNA splicing. *Science 260*:219–222.

Zhang L, Ashiya M, Sherman TG, Grabowski PJ. 1996. Essential nucleotides direct neuron-specific splicing of γ2 pre-mRNA. *RNA 2*:682–698.

Zhuang Y, Weiner AM. 1989. A compensatory base change in human U2 snRNA can suppress a branch site mutation. *Genes & Dev 3*:1545–1552.