

Mutations in RRM4 uncouple the splicing repression and RNA-binding activities of polypyrimidine tract binding protein

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ABSTRACT

The polypyrimidine tract binding protein (PTB, or hnRNP I) contains four RNA-binding domains of the ribonucleoprotein fold type (RRMs 1, 2, 3, and 4), and mediates the negative regulation of alternative splicing through sequence-specific binding to intronic splicing repressor elements. To assess the roles of individual RRM domains in splicing repression, a neural-specific splicing extract was used to screen for loss-of-function mutations that fail to switch splicing from the neural to nonneural pathway. These results show that three RRMs are sufficient for wild-type RNA binding and splicing repression activity, provided that RRM4 is intact. Surprisingly, the deletion of RRM4, or as few as 12 RRM4 residues, effectively uncouples these functions. Such an uncoupling phenotype is unique to RRM4, and suggests a possible regulatory role for this domain either in mediating specific RNA contacts, and/or contacts with putative splicing corepressors. Evidence of a role for RRM4 in anchoring PTB binding adjacent to the branch site is shown by mobility shift and RNA footprinting assays.

Keywords: alternative splicing; neural-specific nuclear extract; PTB; RRM domain

INTRODUCTION

Alternative RNA splicing is of major importance in amplifying proteomic diversity from animal genomes (Black, 2000). In the nervous system, numerous alternatively spliced mRNAs are translated into their protein counterparts where various isoforms play roles in learning and memory, cell communication, and neural development (Grabowski & Black, 2001). The frequent misregulation of alternative splicing in human diseases involving the nervous system and cancer underscores the importance of understanding molecular mechanisms of control.

Mammalian polypyrimidine tract binding protein (PTB, also called hnRNP I) is a potent regulator of tissue-specific alternative splicing (Valcarcel & Gebauer, 1997). PTB binds directly and with high affinity to intronic repressor elements (also called splicing silencers) composed of extended polypyrimidine tracts, in the vicinity

of neural and muscle-specific exons. These polypyrimidine tracts frequently contain multiple copies of the high affinity PTB binding motifs, UCUU, CUCUCU, and UUCUCU. Alternatively spliced pre-mRNA targets of PTB include the α and β tropomyosins (Mulligan et al., 1992; Singh et al., 1995; Perez et al., 1997a; Gooding et al., 1998), α actinin (Southby et al., 1999), *c-src* tyrosine kinase (Chan & Black, 1997), the GABA_A receptor γ 2 subunit (Ashiya & Grabowski, 1997), clathrin light chain B, and *N*-methyl-D aspartate receptor (NMDA) R1 exon 5 (Zhang et al., 1999). PTB also binds to intronic repressor elements in fibronectin (Norton, 1994), fibroblast growth factor receptor (FGFR) -1 (Jin et al., 2000), FGFR-2 (Carstens et al., 2000) pre-mRNAs, suggesting that these are additional regulatory targets.

Recent progress has been made in understanding how PTB functions as a negative regulator of alternative splicing. In the nervous system, PTB represses the neural (N1) exon of the *c-src* transcript by a mechanism that requires two pyrimidine-rich intronic regulatory regions, one in the N1 3' splice site and another in the downstream intron control sequence (DCS). Cooperative PTB binding to both of these regions is believed

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to sequester the N1 exon from spliceosome assembly by looping out the exon (Chou et al., 2000; Markovtsov et al., 2000). By a distinct mechanism, PTB represses the neural exon of the GABA_A receptor γ 2 pre-mRNA in conjunction with the 3' splice site intronic repressor region, which is both necessary and sufficient for splicing repression (Ashiya & Grabowski, 1997). In this mechanism, PTB blocks spliceosome assembly by the recognition of multiple pyrimidine-rich motifs surrounding the branch site.

The tissue-specific functions of PTB are believed to involve widespread repression of certain neural-specific splicing events in nonneural cells, or of certain muscle-specific exons outside of muscle tissue. Consistent with these models, PTB is widely expressed in mammalian cells, but lower levels are found in mature brain and skeletal muscle (Markovtsov et al., 2000; Lilleväli et al., 2001). Furthermore, biochemical studies demonstrate that tissue-specific exon inclusion is highly sensitive to changes in PTB concentration (Ashiya & Grabowski, 1997; Chan & Black, 1997; Southby et al., 1999; Chou et al., 2000). Splicing extracts prepared from a neural cell line (Chan & Black, 1997) or rat brain tissue (Zhang et al., 1999) show a characteristic increase in neural splicing activity. These extracts contain a neural enriched PTB form (termed nPTB or brPTB), together with reduced levels of PTB. In vivo, nPTB has been shown to interact with and counteract the positive effects of the brain-specific splicing regulator, Nova-1 (Polydorides et al., 2000). In vitro, nPTB has been shown to function as a splicing repressor, but is judged to be less potent than PTB (Markovtsov et al., 2000).

In addition to its role in the regulation of alternative splicing, PTB has been shown to interact functionally with polyadenylation control sequences (Lou et al., 1996, 1999; Moreira et al., 1998), and internal ribosome entry sites of picornaviruses (Belsham & Sonenberg, 1996). PTB has been shown to bind to several mRNA 3' untranslated regions where it is implicated in viral transcription, or mRNA stability (Irwin et al., 1997; Gontarek et al., 1999). In *Xenopus* oocytes, a role for PTB in the localization of Vg1 mRNA has been demonstrated (Cote et al., 1999). How PTB functions in these diverse cellular events is poorly understood.

What structural features of PTB are essential for its potent splicing repression activity? PTB contains four RNA-binding domains of the ribonucleoprotein fold type (RRMs 1, 2, 3, and 4), and its expression is diversified by alternative splicing of the central linker region separating the second and third RRM domains. The size of the central linker region increases for the three human isoforms in order of PTB1 < PTB2 < PTB4. Effects of domain deletions on the RNA-binding activity of PTB have been reported; however, in these previous studies the effects of the mutations were determined for RNA binding, but not for splicing repression (Perez et al., 1997b; Oh et al., 1998). In this study, we compare the

effects of engineered mutations on the RNA binding and splicing repressor functions of PTB using the GABA_A receptor γ 2 pre-mRNA as a model substrate. To assess effects on splicing repression activity we take advantage of a novel splicing extract derived from rat cerebellum to screen for loss-of-function mutations in PTB. Here we show that the splicing repression, but not RNA binding, activity of PTB is particularly sensitive to lesions in RRM4, indicating that not all RRMs play equivalent roles in the regulation of alternative splicing.

RESULTS

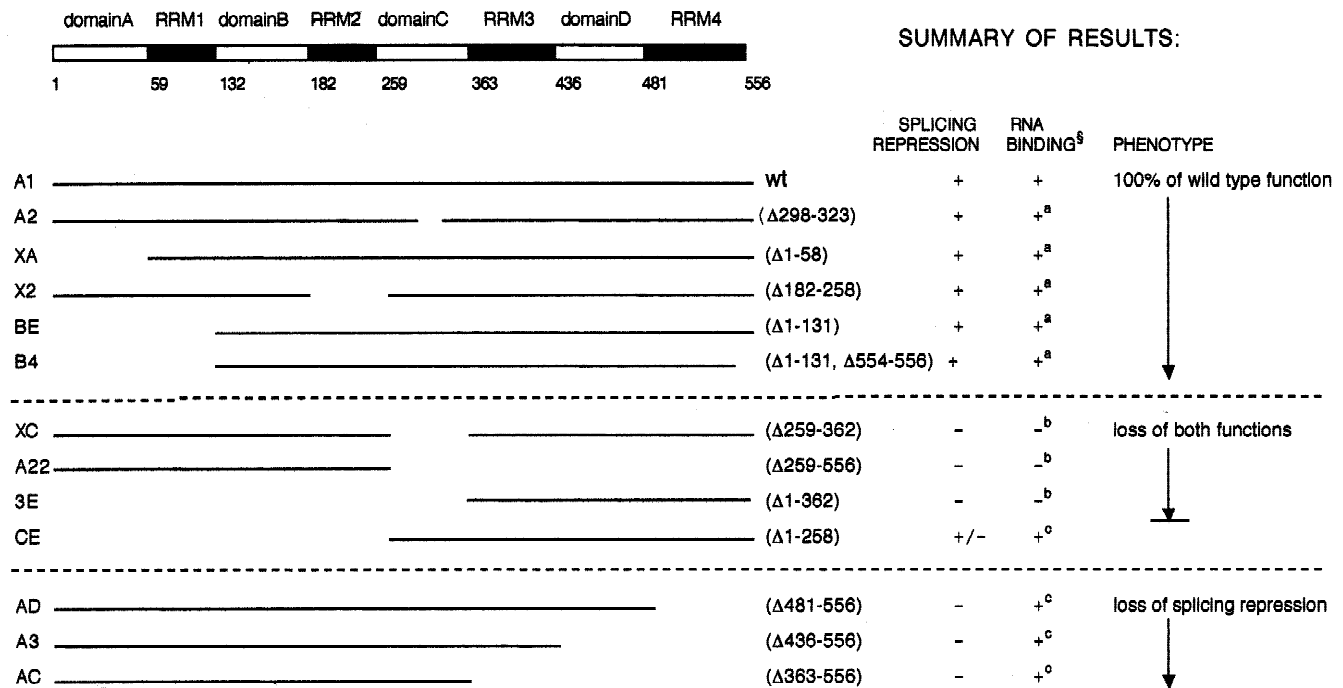
Splicing repression and RNA-binding activities of rat PTB are uncoupled by the deletion of RRM4

To determine how individual protein domains contribute to the splicing repression activity of rat PTB, a collection of domain variants was generated (Fig. 1), and subjected to functional assays (Fig. 2). Each variant was generated in the context of the rat PTB isoform (termed PTB A1 in this study), which is equivalent to largest human isoform, PTB4.

To test for splicing repression activity we took advantage of a physiologically relevant neural splicing extract developed from 28-day rat cerebellum. The rat cerebellum nuclear extract, termed C28, promotes neural-specific splicing and is an improved version of a total rat brain nuclear extract reported previously (Zhang et al., 1999). In contrast to the HeLa extract, the C28 extract supports neural splicing without the addition of exogenous RNA competitors, as is shown for the GABA_A receptor γ 2 pre-mRNA substrate, rG γ 25Xhol (Fig. 2A, lane 3). Differences in the levels of PTB and nPTB isoforms in the two extracts are apparent by western blotting (Fig. 2A, inset), although additional activities may contribute to the neural behavior of the C28 extract. The lower splicing efficiency of the C28 extract may be explained by the quantity of postmitotic neurons in the cerebellum tissue used for extract preparation.

The in vitro splicing assay, which tests the ability of each PTB variant to switch alternative splicing from the neural (exon selection) to the nonneural (exon skipping) pathway is shown (Fig. 2B). The effect of each PTB variant on splicing was inferred from three or more independent experiments. RNA-binding activity is assessed for the same PTB variants using a gel mobility shift assay; representative results are shown (Fig. 2C). The RNA substrate used for the binding experiments, rG γ 57Bam, contains the intronic repressor region of the GABA_A receptor γ 2 pre-mRNA, which is schematically indicated as the shaded bar. Relative to the wild-type protein (PTB A1), some mutants show a substantial loss of RNA binding (PTB 3E), or retain nearly wild-type RNA binding (PTB AC).

Rat PTB domain variants



PTB A1, large wild-type rat isoform (equivalent of human PTB4);

A2, small wild-type isoform (equivalent of human PTB1);

amino acid residues deleted relative to PTB A1, parentheses

§, RNA gel shift substrate, rG γ 57Bam

a, K_d within 2 fold of wild-type value

b, K_d greater than 10 fold of wild-type value

c, K_d within 3 fold of wild-type value

FIGURE 1. Collection of rat PTB domain variants and summary of results. The domain structure of the largest rat PTB isoform, A1 (accession number X74565), is represented schematically at the top with four RNA-binding domains, RRM1, 2, 3, and 4, (black boxes) and interdomain regions A–D. Amino acid positions are indicated as previously described (Ghetti et al., 1992). Line drawings indicate the amino acid sequence content of each PTB variant. The name of each protein variant is indicated at left. Positions of deleted residues are indicated in parentheses (right). PTB A2 (accession number X60789), which is the smallest rat isoform, lacks a 25 amino acid segment in the central linker region between RRMs 2 and 3. The behavior of each PTB variant in splicing repression and RNA binding assays is summarized at right.

The phenotypes of this initial set of mutants fall into three general categories (Fig. 1). One group of mutants retains wild-type function in spite of the deletion of one or more regions involving domain A, RRM1, RRM2, and three residues at the carboxy terminus. The sequences contained in the B4 mutant, for example, are sufficient under these conditions for full splicing repression and RNA-binding activities.

Deletions that destroy both splicing repression and RNA-binding activities involve the deletion of domain C, which is the central region of the protein between RRMs 2 and 3 (variants XC, A22, and 3E). A comparison of variants 3E and CE shows that good RNA-binding and splicing repression activities track together with the presence of domain C (Figs. 1 and 2). For PTB

variants XC, A22, and 3E, the defect in RNA binding is significant as shown by more than a 10-fold increase in the apparent equilibrium dissociation constant, K_d . Thus, the central region of PTB is important for splicing repression under these conditions, most likely as a consequence of its general requirement for RNA binding.

The most interesting group of PTB variants (AD, A3, and AC), which have in common a deletion of RRM4, show a selective loss of splicing repression activity. These variants contain domain C and exhibit substantial RNA-binding activity for the intronic repressor RNA, rG γ 57Bam, as shown by an apparent K_d within three-fold of the wild-type value (Fig. 1). Thus, under these assay conditions, RRM4 is critical for the splicing repression activity of PTB.

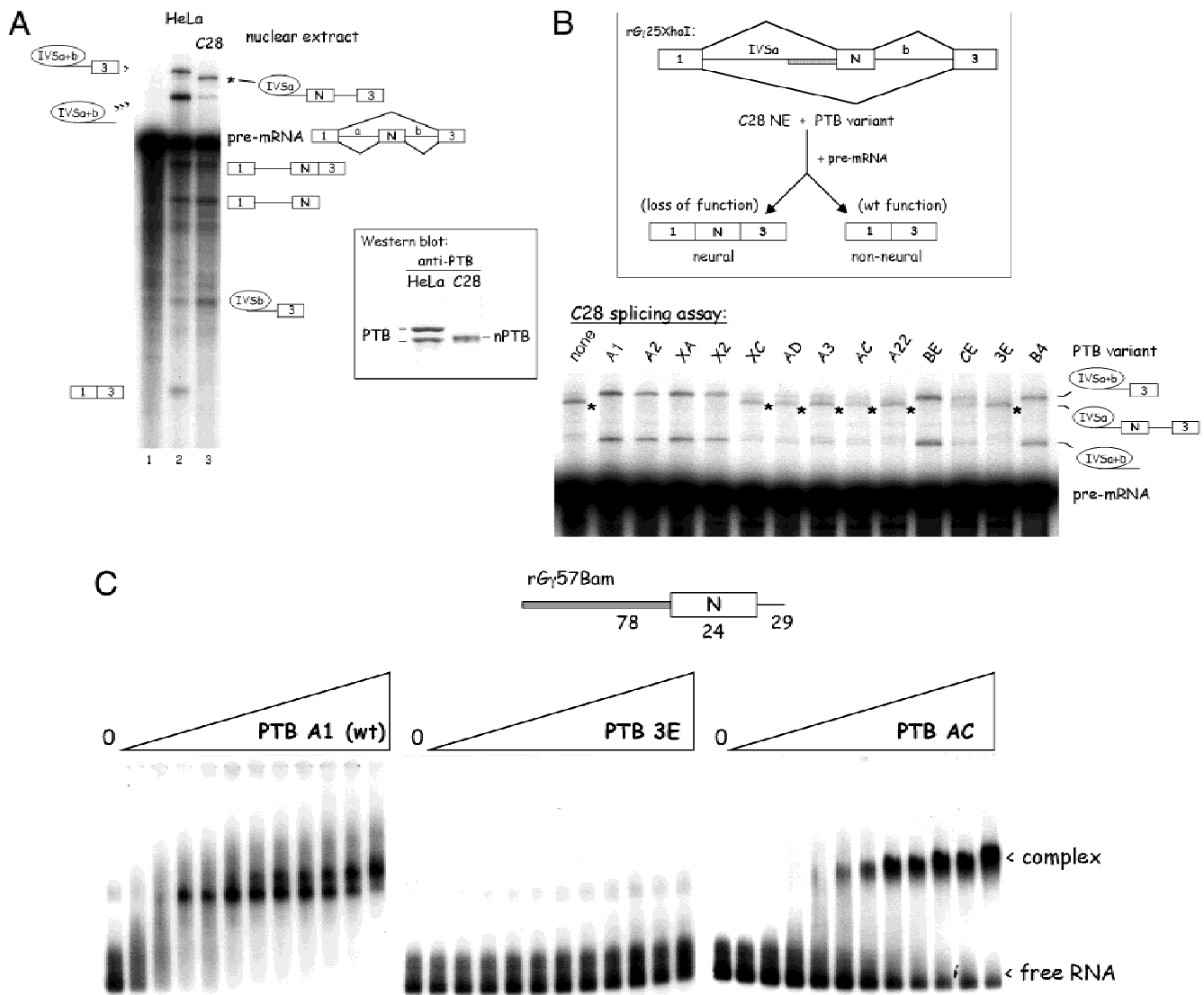


FIGURE 2. Splicing repression and RNA-binding activities of PTB variants. **A:** Biochemical properties of the C28 splicing extract. One-hour splicing reactions contain the GABA_A receptor γ 2 subunit pre-mRNA and HeLa nuclear extract (lane 2), or C28 nuclear extract (lane 3). Lane 1: unspliced pre-mRNA. The IVS_a lariet-3' exon RNA intermediate is diagnostic for the neuron-specific splicing pathway (asterisk). RNA products and intermediates of splicing reactions are separated in a 4% polyacrylamide/7 M urea gel. Inset: Western blot analysis of HeLa and C28 splicing extracts probed with a PTB-specific polyclonal antibody. **B:** C28 splicing assay: Test of PTB variants. Schematic of splicing repression assay and predicted results (top). Neutral splicing extract (C28 NE) is preequilibrated with 20 pmol of recombinant PTB variant prior to addition of pre-mRNA substrate, rG γ 25XhoI. Predicted results: Neutral mutations are expected to show wild-type PTB function by switching splicing to the nonneural pathway, whereas loss-of-function mutations should fail to switch. The extended polypyrimidine tract, which is the major PTB-binding region in the pre-mRNA, is indicated by the shaded bar in IVS_a. N: Neural exon. Representative results of splicing assay are shown (bottom). PTB variant present in the splicing reaction is indicated at the top of the gel. Lane none: control reaction without recombinant protein. **C:** Mobility gel shift analysis of RNA-binding activity. The RNA-binding activity of each PTB variant of Figure 1 was monitored by gel mobility shift analysis for the substrate rG γ 57Bam. A diagram of rG γ 57Bam is shown with RNA lengths indicated in nucleotides (top). Equilibrium dissociation constants, K_d , are estimated from the concentration of protein resulting in a 1:1 mixture of complex and free RNA. For each panel shown, left lane is a control reaction assembled without PTB protein (lane 0); wedge represents protein concentrations in μ M: 0.04, 0.08, 0.12, 0.16, 0.20, 0.24, 0.28, 0.32, 0.36, 0.40, 0.60. Complexes and free RNA are resolved on Tris-glycine gels as described (Konarska, 1989).

RRM4 is required for splicing repression activity in vivo

To verify the effect of RRM4 in vivo, protein expression plasmids containing wild-type PTB or RRM4 deletion

mutants were cotransfected together with splicing reporter constructs containing two unrelated neural pre-mRNAs. For these neural pre-mRNAs, alternative splicing would be expected to show a decrease in exon selection and an increase in exon skipping when

coexpressed with PTB variants that are active for splicing repression. To better visualize the effects of PTB, splicing reporters with strong neural exons were chosen for these experiments. The GABA_A receptor γ 2 construct contains an engineered 5' splice site mutation that improves exon inclusion due to enhanced base complementarity to U1 snRNA, whereas the NMDA exon 5 splicing reporter contains a naturally strong neural exon, which is active for splicing in non-neuronal cells.

Spliced mRNA products are detected by a reverse transcriptase polymerase chain reaction assay using primers specific for the flanking exons. Transfection assays with mouse C2C12 cells are shown in the experiment of Figure 3, although a variety of other non-neuronal and neuronal cell lines were tested with similar effects. These results show that in comparison to the

mock transfected control, wild-type PTB (A1) induces the switch to exon skipping for both γ 2 and NMDA splicing reporter constructs (Fig. 3, lanes 1–6). In contrast, RRM4 variants, AD and E510, show little or no effect on alternative splicing (Fig. 3, lanes 7–12). In comparison to the complete RRM4 deletion mutant, AD, the E510 variant retains the first 31 residues of RRM4 and exhibits a similar loss of repression activity *in vitro* (see below, Figs. 4 and 5). The expression of these protein variants was confirmed in nuclear extracts prepared from the transfected cells using western blot analysis, and the levels of mutant and wild-type proteins found to be comparable (Fig. 3). Thus, these results demonstrate that RRM4 is critical for the negative regulation of distinct alternative splicing events, *in vivo*, in agreement with the results of the *in vitro* experiments shown above.

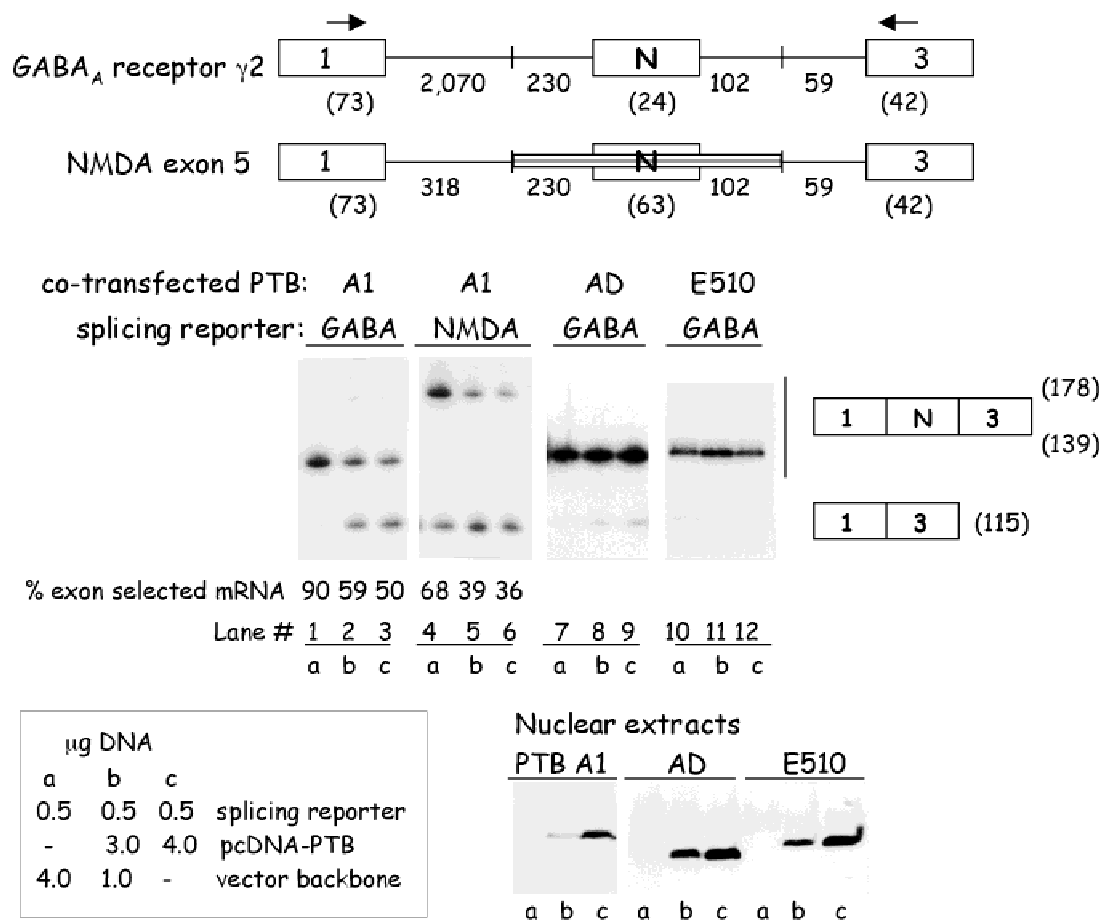


FIGURE 3. RRM4 is critical for the splicing repression activity of PTB *in vivo*. The pcDNA4 PTB expression plasmid was coexpressed in mouse C2C12 myoblasts with splicing reporter constructs at a ratio of 6/1 or 8/1 (inset). Control reactions were mock transfected with pcDNA4 vector backbone (lanes 1, 4, 7, 10). PTB variants include, A1 (wild type), AD, and E510. Splicing reporter constructs contain the neural exon of the GABA_A receptor γ 2 (GABA), or exon 5 of the NMDA R1 receptor (NMDA exon 5). NMDA exon 5 is a chimeric splicing reporter with NMDA sequences indicated by shaded bar; flanking sequences are derived from GABA_A receptor γ 2 (derived from rNM25; Zhang et al., 1999). RNA lengths are indicated below each schematic in nucleotides. Total RNA from the transfected samples was assayed by reverse transcription polymerase chain reaction with primers flanking the neural exon (arrows). Amplified segments (base pairs) are indicated in parentheses. Exogenous PTB protein expression was assayed from parallel, transfected samples by western blot analysis with a monoclonal antibody specific for the Xpress tag. This antibody does not cross-react with endogenous PTB.

PTB RRM4 variants

	490	500	510	520	530	540	550	SPLICING REPRESSION	K_d	FILTER BINDING
A1 (wt)	LHLSNIPPSVSEDDLKSLFSSNGGVVKGKFFQKDRKMALIQMGVVEEAVQALIELHNHDLGENHHLRVSF	SKSTI	+++	7 nM [‡]	19 nM [§]					
AD	-----	-----	-----	-----	-----	-----	-----	-	12 nM [‡]	12 nM [§]
E510	LHLSNIPPSVSEDDLKSLFSSNGGVVKGKFF-----	-----	-----	-----	-----	-----	-----	-	-	-
E530	LHLSNIPPSVSEDDLKSLFSSNGGVVKGKFFQKDRKMALIQMGVVEEAVQ-----	-----	-----	-----	-----	-----	-----	-	-	-
E530-1	LHLSNIPPSVSEDDLKSLFSSNGGVVKGKFFQKDRKMALIQMGVVEEAVQ-----	-----	-----	-----	-----	-----	-----	STI	-	-
E530-2	LHLSNIPPSVSEDDLKSLFSSNGGVVKGKFFQKDRKMALIQMGVVEEAVQ-----	-----	-----	-----	-----	-----	-----	SKSTI	-	-
E540-2	LHLSNIPPSVSEDDLKSLFSSNGGVVKGKFFQKDRKMALIQMGVVEEAVQALIELHNHDL-----	-----	-----	-----	-----	-----	-----	STI	12 nM [‡]	18 nM [§]
E540-1	LHLSNIPPSVSEDDLKSLFSSNGGVVKGKFFQKDRKMALIQMGVVEEAVQALIELHNHDL-----	-----	-----	-----	-----	-----	-----	SKSTI	10 nM [‡]	15 nM [§]

RNA substrate for filter binding assay: rG γ 75Bam(+) ; rG γ 25XhoI, (S)

FIGURE 4. PTB RRM4 variants and summary of results. Amino acid sequence of each RRM4 variant is indicated below the sequence of wild-type RRM4. Dashes indicate missing residues. Splicing repression and RNA-binding activities are summarized at right. Apparent equilibrium dissociation constants were determined for the full-length pre-mRNA, rG γ 25XhoI, and for the intronic repressor, rG γ 75Bam, by nitrocellulose filter binding assays. Residues required for splicing repression activity are indicated (black bars).

A functional RRM4 domain is required for splicing repression activity and for PTB binding adjacent to the RNA branch site

Additional mutations within RRM4 were generated for further study (Fig. 4). In this group of PTB variants, E540-2 and E540-1 are of particular interest, because these proteins show substantially different splicing repression activities, but differ in sequence by two residues at the C-terminus (S552, K553). In the C28 splicing assay, variant E540-1 behaves like the wild-type protein in switching splicing from the neural to the non-neural pathway, whereas E540-2 has no effect indicative of a loss of function (Fig. 5A, lanes 5, 6). These results are in good agreement with the results of the HeLa splicing switch assay, which shows that E540-1 has substantial activity, whereas E540-2 is inactive (Fig. 5A, lanes 16, 17). PTB variants with larger deletions within RRM4, such as E510, E530, E530-1, and E530-2 behave like PTB AD in that they exhibit a selective loss of splicing repression activity (Fig. 5A, lanes 12–15). Thus, we conclude that the 12 residues, 532–541 and 552–553, are required for a functionally active RRM4 domain. It is curious that the residues in between are non-essential, as the fourth β strand of the $\beta\alpha\beta\beta\alpha\beta$ motif is contained within this region (Conte et al., 2000).

Surprisingly, the RNA-binding activity of the RRM4 variants is essentially the same as wild-type PTB as measured by nitrocellulose filter binding assays with the full-length splicing substrate, rG γ 25XhoI, or with an RNA substrate, rG γ 75Bam, which contains the complete intronic repressor region (Fig. 5). The rG γ 75Bam substrate contains a short 5' extension, but is otherwise identical to the rG γ 57Bam substrate tested above. The

apparent K_d values of the wild-type and RRM4 variants are in the 10–20 nM range as determined by triplicate measurements.

To probe more closely for possible RNA binding defects, we tested the ability of the RRM4 variants to bind a truncated RNA substrate, rG γ 74Bam, which is missing the intronic repressor region upstream of the branch site. The location of the branch site of the GABA_A receptor γ 2 pre-mRNA was determined in a previous study (Ashiya & Grabowski, 1997). Similar to the wild-type protein, all of the PTB variants tested in the experiment show efficient binding to the full-length intronic repressor RNA (Fig. 5B, lanes 1–7). In contrast, the PTB AD and E540-2 variants fail to bind the truncated intronic repressor (Fig. 5B, lanes 8, 12, 13). This defect in binding appears to be specific for RRM4 and not a simple length effect, as wild-type PTB as well as the RRM1 and RRM2 deletion mutants bind efficiently to the truncated RNA (Fig. 5B, lanes 9–11). These results suggest that in the absence of a functional RRM4, the remaining RRMs, 1–3, are sufficient to rescue binding to the intronic repressor region, but this effect requires sequences upstream of the branch site. Furthermore, these results suggest that RRM4 is an important determinant for PTB binding to the RNA downstream of the branch site. The RRM4 variant complexes formed with the full-length intronic repressor substrate are judged to be specific based on competition experiments. That is, when the RNA-binding reactions of Figure 5B, lanes 2–7, are repeated in the presence of 100-fold molar excess of self competitor, complex formation is abolished, whereas equivalent levels of a nonspecific RNA of the same length have no effect (data not shown).

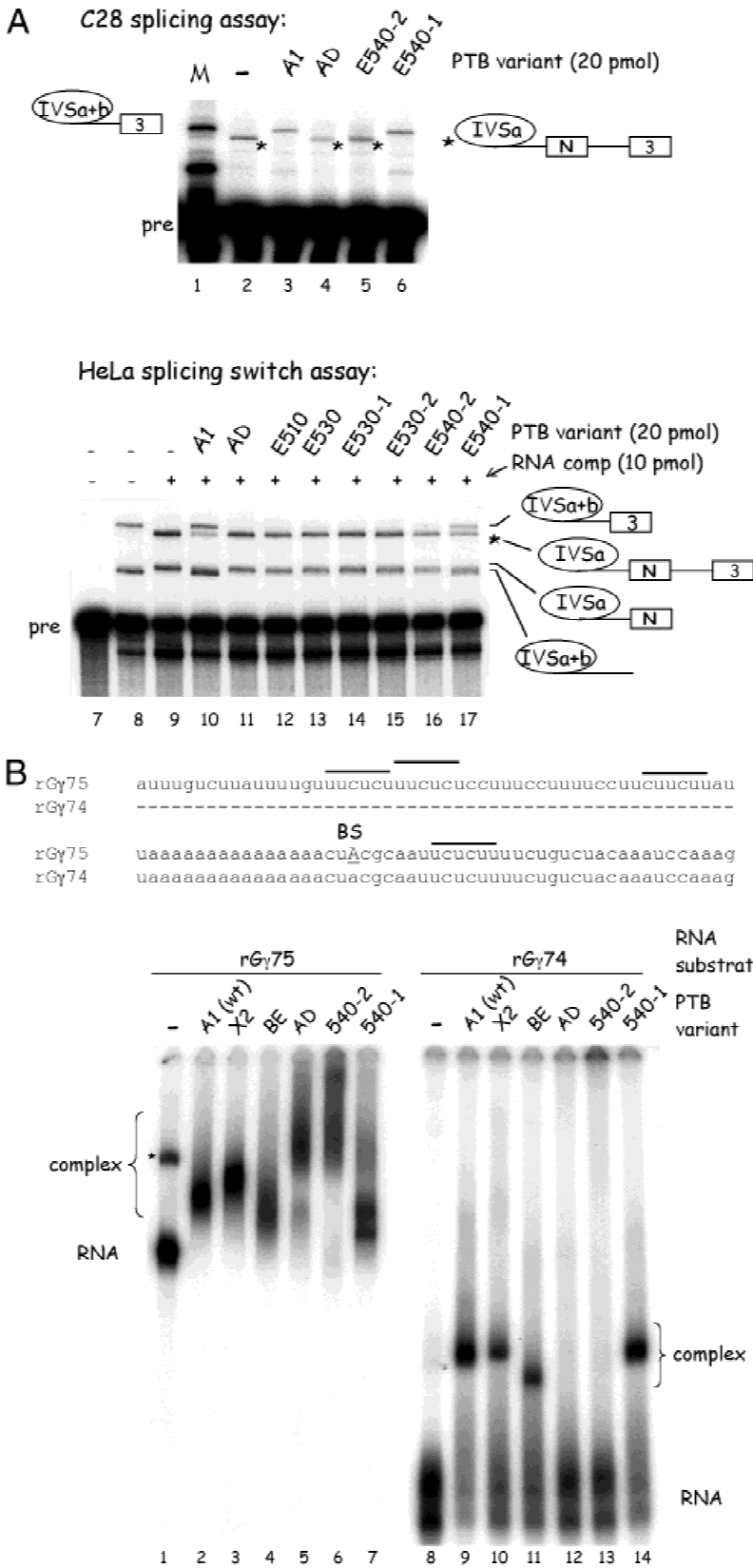


FIGURE 5. Splicing repression and RNA-binding activity of PTB RRM4 variants. **A:** The full-length pre-mRNA rG_γ25Xhol is used to test PTB variants in the C28 splicing assay (lanes 1–6), or in the HeLa splicing switch reaction (lanes 7–17). Lane M: HeLa splicing reaction. The HeLa splicing switch assay is performed as described (Ashiya & Grabowski, 1997) by equilibrating the nuclear extract with 10 pmol of RNA competitor ± recombinant PTB protein followed by addition of pre-mRNA substrate. In the absence of recombinant PTB, splicing switches to the neuronal pathway (lane 9). Under these conditions, the addition of 20 pmol of recombinant PTB (wild type) switches splicing to the nonneuronal pathway (lane 10). **B:** Top: Sequence of wild-type (rG_γ75), and truncated (rG_γ74) intronic repressor region (only intron nucleotides are shown). Dashes: missing nucleotides. Branch site (BS) and PTB binding motifs (overlines) are indicated. Bottom: RNA–protein complexes and free RNA are resolved as in Figure 2C. A portion of the rG_γ75 RNA substrate migrates aberrantly in the gel (lane 1, asterisk).

Interestingly, differential binding to the truncated intronic repressor correlates well with the splicing repression activity of E540-1, which is active, and with E540-2,

which is inactive (Fig. 5B, lanes 13, 14). Thus, the presence of the S552 and K553 residues in the E540-1 variant correlates with the rescue of both splicing re-

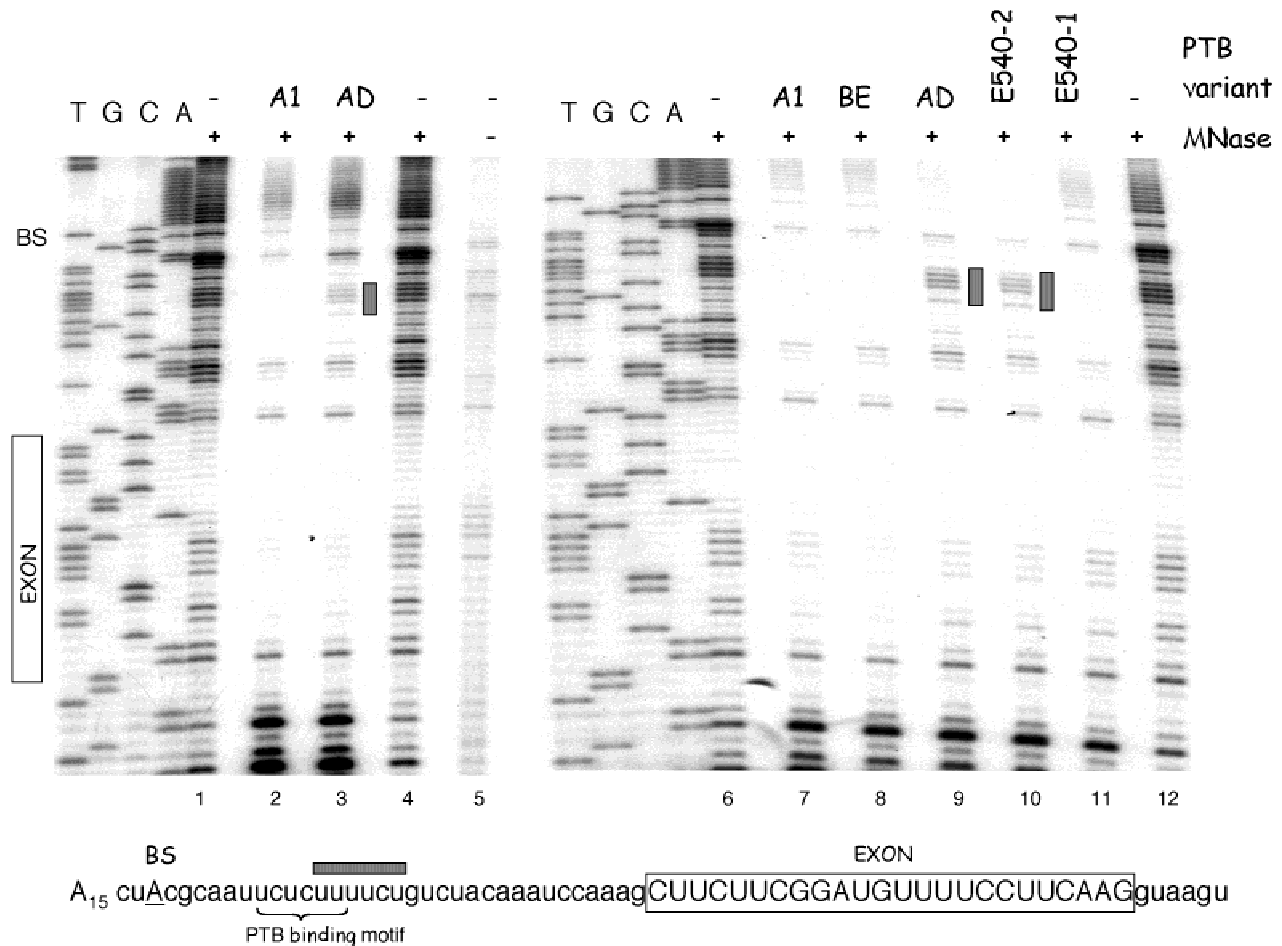


FIGURE 6. Micrococcal nuclease footprinting with wild-type PTB and variants. RNA substrate rG γ 75 was assembled into complexes with PTB wild-type (A1) or variant as indicated (top) followed by micrococcal nuclease treatment (MNase). Nuclease cleavages are detected by primer extension from a 5' end labeled primer positioned in the downstream intron. Sequencing ladder is generated with the labeled primer from plasmid DNA containing the rG γ 75 insert (lanes T, G, C, A). Nucleotides showing differential micrococcal nuclease protection are indicated by the shaded bar.

pression activity and binding adjacent to the RNA branch site.

To examine more directly the involvement of RRM4 in the binding of PTB downstream of the branch site, a micrococcal nuclease footprinting assay was employed. Equilibrium PTB binding reactions were assembled as above with the full-length intronic repressor RNA, rG γ 75Bam, followed by partial micrococcal nuclease treatment. Cleavage positions were determined relative to a sequencing ladder using a primer extension assay. This experiment shows a uracil-rich region located between the branch site and 3' splice site, which is reproducibly protected by wild-type PTB but not by the RRM4 deletion mutant, AD (Fig. 6, lanes 1–5). The protected region coincides with a PTB-binding motif and is indicated as the shaded bar in Figure 6. Detection of footprinted residues in this experiment is limited to uracil and adenosine residues due to the base cleavage preference of micrococcal nuclease.

In contrast to PTB AD, the RRM1 deletion mutant (BE) protects this region like the wild-type protein (Fig. 6,

lanes 7, 8). The RRM2 deletion mutant similarly protects this region in the assay (data not shown). Interestingly, the PTB E540-2 and E540-1 variants show a difference in this region of the footprint (Fig. 6, lanes 10, 11). That is, the presence of the S552 and K553 residues, which restores the splicing repression activity of E540-1, also restores the protection of the pyrimidine-rich region between the branch site and 3' splice site. Thus, the protection of the PTB-binding motif closest to the branch site correlates with splicing repression activity and a functional RRM4 domain.

DISCUSSION

PTB RRM4 plays a critical role in the negative regulation of alternative splicing

Here we show that mutations in RRM4 largely uncouple the splicing repression and RNA-binding functions of PTB, suggesting a unique role for RRM4 either in mediating specific RNA contacts, and/or contacts with

putative splicing corepressors. Such an uncoupling phenotype for an alternative splicing regulator is a novel feature of the present study. RRM4 appears to play a particularly important role in the splicing repression function of PTB, as an RRM4 deletion is not rescued by the remaining RRMs (1–3). Moreover, an RRM1 or RRM2 deletion is functionally neutral with respect to splicing repression activity.

Twelve RRM4 residues critical for the splicing repression function of PTB are identified (Fig. 4, black bars). To assay the effects of PTB mutations, we developed a tissue-specific splicing extract that supports the neural-specific pathway (exon inclusion), without a biochemical depletion step to remove PTB from the extract. The neural extract contains nPTB, but levels of PTB isoforms are quite low. Loss-of-function mutations are recognized in this assay as PTB variants that, in contrast to the wild-type protein, are unable to switch splicing to the nonneural pathway (exon skipping). The assay is also informative about regions of the protein that are nonessential for splicing repression. Although the results of the present study show that PTB variants lacking the amino terminal domains, domain A, RRM 1, or RRM2 are fully active in the splicing switch assay, these regions are important, *in vivo*, for other functions. Previous studies have shown that the nuclear localization of PTB requires the first 55 residues (domain A in the present study; Huang et al., 1997; Perez et al., 1997b), and protein dimerization requires RRMs 1 and 2 (Perez et al., 1997b; Oh et al., 1998).

RRM4 is functionally distinct from the central region of the protein domain C, which contains an alanine-rich region consistent with its role as a flexible linker between RRMs 2 and 3. Whereas the loss of RNA binding resulting from the deletion of domain C is closely linked to the loss of splicing repression activity, RRM4 mutations largely uncouple these two functions. One interpretation of the RNA-binding behavior of the five domain C variants tested here is that RRM2, RRM3, or both actually extend beyond their predicted boundaries into domain C. This interpretation would account for the global defects in RNA binding observed upon deletion of domain C. It would be of interest to test this idea using structural approaches; however, information is lacking on this point. A recent NMR study of PTB reports the structural characteristics of the region containing RRMs 3 and 4; however, the central linker region is absent from the studied proteins (Conte et al., 2000).

In a previous study, the human PTB isoforms PTB1, PTB2, and PTB4, which differ in the length of the central linker region, are reported to have differential effects on a muscle-specific exon of α -tropomyosin, although these isoforms have indistinguishable effects on the inclusion of a smooth muscle-specific exon found in α -actinin pre-mRNA (Wollerton et al., 2001). In comparison, we have not observed any significant difference in the function of the corresponding rat PTB

isoforms in the present study (rat PTB A1 corresponds to human PTB4; rat PTB A2 corresponds to human PTB1). The possibility that other neural pre-mRNAs are differentially affected cannot be ruled out, however. The physiological importance of PTB isoforms is implicated from experiments documenting alterations in the ratio of human PTB isoforms in metastatic, compared to nonmetastatic rat prostate epithelial cancer cell lines (Wagner et al., 1999). It has been speculated that these changes might induce irregularities in alternative splicing.

What is the biochemical role of RRM4 in the repression of neural-specific splicing?

The uncoupling phenotype of the RRM4 variants is not explained by a global RNA-binding defect, as these proteins exhibit apparent K_d values in the 10–20 nM range, equivalent to the wild-type protein for binding to the GABA_A receptor intronic repressor element. Only when the intronic repressor region upstream of the branch site is truncated is a defect in RNA-binding activity uncovered. This effect is specific for RRM4 mutants and cannot be explained by a simple length effect, as deletion mutants lacking either RRM1 or RRM2 show no significant defect in binding to the truncated RNA (Fig. 5B). The architecture of the intronic repressor is interesting in this regard. Whereas a cluster of PTB binding motifs is located upstream, a single motif is located immediately downstream of the branch site.

The results of the RNA-binding experiments are consistent with a model in which the assembly of a PTB–RNA complex involves the binding of RRMs 1–3 to pyrimidine motifs upstream of the branch site, anchored by an additional interaction of RRM4 with the motif immediately downstream of the branch site (Fig. 7). This would explain why RRM1 or RRM2 deletion variants bind efficiently to the truncated RNA, as these variants contain RRM4. Furthermore, these results are consistent with the idea that in the absence of a functional RRM4, RRMs 1–3 rescue protein binding to the complete intronic repressor region through contacts with the motifs upstream of the branch site. This would explain why the RRM4 mutants bind to the full-length, but not to the truncated, intronic repressor. One prediction of this model, which is tested here, is that the RNA region downstream of the branch site should be differentially protected from ribonuclease digestion by the presence or absence of RRM4. Evidence for such differential protection is shown here using a micrococcal nuclease footprinting assay. In contrast, protection of the PTB binding motifs upstream of the branch site is not affected by RRM4 (W. Liu & P.J. Grabowski, unpubl). We speculate that a repressive PTB binding topology might be achieved in a variety of ways depending on both the quaternary structure of PTB and the arrangement of intronic repressor elements in the target

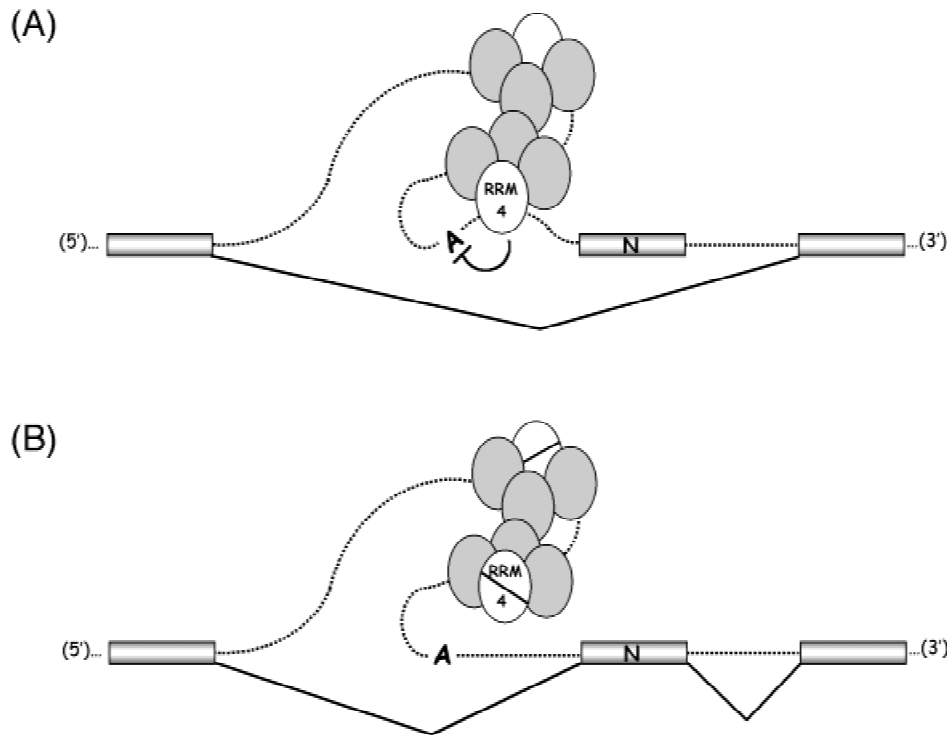


FIGURE 7. Model for PTB RRM4 function in the negative regulation of alternative splicing. **A:** PTB RRMs 1–4 (ovals) bind to the GABA_A receptor γ 2 intronic repressor region in a manner that isolates the branch site of the neural exon. RRM4 is important in promoting PTB interactions downstream of the branch site. **B:** RRM4 loss-of-function mutations (slash) uncouple the RNA binding and splicing repression functions of PTB by failure to inactivate the branch site, even though RRMs 1–3 (shaded ovals) retain binding to the intronic repressor region upstream of the branch site. Dashed lines: introns; boxes: exons. Splicing events are indicated as solid lines. PTB is modeled as a dimer, but the stoichiometry and topological arrangement of subunits is speculative. PTB function may be regulated by cell-specific mechanisms that enhance or antagonize the functions of RRM4.

pre-mRNA substrate. PTB is reported to exist as a dimer; however, its RNA-binding affinity is not affected by oligomerization (Perez et al., 1997b). How the quaternary structure of PTB influences its various functions in splicing, translation, polyadenylation, and RNA localization is not well understood.

The sensitivity of RRM4 function to residues S552 and K553 near the C terminus of the domain is striking. Upon deletion of these residues, splicing repression activity as well as RNA binding and footprinting downstream of the branch site are lost, whereas the presence of S552 and K553 rescues these activities. Interestingly, S552 and K553 lie just outside the fourth β strand of the $\beta\alpha\beta\beta\alpha\beta$ motif and these residues are invariant in all PTB, nPTB, and regulator of differentiation (ROD1; Yamamoto et al., 1999) isoforms identified (Fig. 8). The results shown here are consistent with the NMR study of PTB RRMs 3 and 4, which shows that the five amino acids extending just beyond the fourth β strand of RRM4 lie close to the RNA-binding surface (Conte et al., 2000). The results of this and the previous study are consistent with the idea that S552 and K553 contribute directly to RNA recognition by RRM4.

A role for RRM4 in the regulation of PTB function?

The uncoupling phenotype of the RRM4 variants might be explained largely or in part by additional factors in the splicing extract that modulate RRM4 function. That is, in the context of purified PTB, lesions in RRM4 have little or no effect on binding to the intact intronic repressor element, whereas in the splicing extract, its role in splicing repression activity is critical. Additional factors in nonneural extracts (putative corepressors) might stabilize the interaction of RRM4 with the RNA substrate, or neural-specific factors might destabilize PTB or nPTB by interfering with the function of RRM4. Moreover, differences in the spatial and temporal expression patterns of PTB and nPTB in the mammalian CNS may also modulate their functional properties. Evidence for a neural extract-specific process that dissociates PTB from an intronic repressor region has recently been demonstrated in a study of the neuron-specific *c-src* pre-mRNA (Chou et al., 2000). PTB has been shown to engage in direct interactions with intronic repressor elements flanking the *c-src* N1 exon, and these contacts are lost in neuronal extracts in the presence of ATP.



FIGURE 8. Alignment of PTB RRM4 sequences. RRM4 protein sequences from PTB, nPTB, and regulator of differentiation (ROD1) are aligned with differences from rat PTB highlighted in red. GenBank accession numbers are (from top to bottom): AF211191, T20381, AF091370, AB023966, AF176085, AJ010585, P26599, Q29099, X74565, and P17225. The *Caenorhabditis elegans*, human, pig, and mouse PTB are from protein databases, and the remaining sequences are from nucleotide databases. Regions shown in this study to be required for splicing repression activity are boxed (bottom).

The ATP dependence of the dissociation suggests the possible involvement of RNA helicase activity, which might alter the structure of the PTB–RNA complex to trigger PTB release. It will be of interest to determine if RRM4 plays a functional role in conferring protection against mechanisms involving RNP release or disassembly. Relevant to this possibility, a recent study demonstrates the ATP-dependent release of the U1A protein from its RNA ligand by a DEXH/D RNA helicase (Janowski et al., 2001).

MATERIALS AND METHODS

Plasmid construction and preparation of recombinant proteins

Recombinant proteins with an amino terminal 6xHis tag were prepared as follows. DNA fragments were generated by PCR using wild-type rat PTB cDNA (PTB A1) as template and inserted into the *Hind*III site of bacterial expression vector pQE11 (Qiagen). The *Xho*I site of the original vector was destroyed by Klenow treatment and ligation prior to inserting the PTB fragments. To generate proteins with internal deletions, the upstream fragment was inserted into the *Hind*III site, followed by insertion of the downstream fragment into the *Xho*I site at the 3' end of the first insert. Colonies were screened for protein expression followed by DNA sequencing. Proteins were grown in *Escherichia coli* strain XL1-blue to a density of 0.6 O.D. and induced with 2 mM IPTG. Cells were lysed under denaturing conditions, 1.5 mM imidazol, 50 mM Na₂HPO₄, 20 mM Tris-HCl, pH 8.0, 100 mM NaCl, 6 M guanidine HCl, 2 mM β-mercaptoethanol. His-tagged recombinant proteins were affinity purified on Talon columns (Clontech), and dialyzed into 0.5× D buffer (10% glycerol, 50 mM KCl, 10 mM HEPES, pH 7.6, 0.1 mM EDTA, 0.25 mM DTT) or 10 mM Tris, pH 7.5.

PTB mammalian expression vectors were constructed as fusion proteins with a 6xHis and X-Press epitope tag at the amino terminus. DNA fragments were generated by PCR from the pQE11 parent plasmids and inserted into the *Bam*HI site of the pcDNA4/HisMax vector (Invitrogen).

C28 nuclear extract preparation and in vitro splicing assays

C28 nuclear extracts were prepared from two dozen, 28-day Sprague–Dawley rats (Harlan or Zivic Miller). Freshly dissected rat cerebellum tissue was minced and transferred to two tubes containing 22.5 mL of ice-cold homogenization buffer (10 mM HEPES, pH 7.6, 15 mM KCl, 1 mM EDTA, 2.2 M sucrose, 5% glycerol, 0.15 mM spermine, 0.5 mM spermidine, 0.5 mM DTT). Tissue was homogenized three to five times at maximum speed in a Con-Torque (Eberback). Homogenate was layered on top of 10 mL cushion buffer (10 mM HEPES, pH 7.6, 15 mM KCl, 1 mM EDTA, 2.0 M sucrose, 10% glycerol, 0.15 mM spermine, 0.5 mM spermidine) and centrifuged at 27,000 rpm (SW28 rotor) for 1 h at 4 °C. The supernatant was removed and the nuclear pellet rinsed twice in 0.5 mL of Buffer C (20 mM HEPES, pH 7.6, 20 mM KCl, 0.2 mM EDTA, 0.5 mM DTT, 25% glycerol, 1.5 mM MgCl₂). The pellet was resuspended in 0.6 mL of Buffer C containing 0.23 M KCl (final concentration) and incubated on ice for 30 min, followed by centrifugation at 14,000 rpm, for 10 min. Proteinase inhibitors (10 μg/mL benzamide, 1.5 μg/mL aprotinin, 15 μg/mL bovine trypsin inhibitor, and 0.5 μg/mL leupeptin) were present in all solutions. Supernatant was removed, divided into small aliquots, flash frozen on dry ice, and stored at –80 °C. RNA substrate preparation and in vitro splicing assays were carried out as described (Zhang et al., 1999).

Transient expression assays

C2C12 cells were grown in DMEM, 10% (v/v) fetal bovine serum. For each transfection, 1.0 × 10⁶ cells were seeded on 60-mm plates to achieve 60–80% confluency. The pcDNA PTB expression vector was cotransfected with splicing reporter plasmid at ratios as indicated in Figure 3. Plasmids were mixed with 300 μL Opti-Mem followed by the addition of an equal volume of Opti-Mem mixed with 23 μL Lipofectamine (GibcoBRL). After 30 min incubation at room temperature, 2.4 mL of Opti-Mem were added and the mixture dispensed to one plate of cells. Cells were washed with serum-free DMEM immediately before applying the DNA-Liposome com-

plex to the plate. Total RNA was isolated with RNazol (Biotecx) 48 h after transfection, followed by treatment with RQ1 DNase (Promega). Total RNA purified from each transfection, 1 μ g, was reverse transcribed in a 20- μ L reaction containing 400 ng hexanucleotide random primers, 0.5 mM dNTPs, 80 U M-MLV reverse transcriptase (Promega), and 10 mM DTT. Reactions were incubated at 37 °C for 1 h, followed by 75 °C for 10 min to terminate the reactions. Each PCR reaction, 10 μ L total volume, was performed with 1/20th of the reverse transcription reaction, 0.2 μ M primers, 0.2 mM dNTPs, 2 mM MgCl₂, 2 U Taq polymerase and 1 μ Ci [α -³²P]dCTP. Cycling parameters included: denaturation at 94 °C for 1 min, annealing at 60 °C for 1 min, and elongation at 72 °C for 1 min for 30 cycles, followed by a final step at 72 °C for 10 min. A portion of each reaction, 1 μ L, was resolved on 6% polyacrylamide/5 M urea gels. Data were quantified directly from the gel using a FUJI phosphorimager system.

Western blot analysis

To detect the nuclear expression of transfected PTB, cells were harvested with a rubber policeman and washed with ice cold 1 \times PBS. Nuclear extracts were prepared according to the procedure of Marzluff and Huang (1985). Nuclear extract, 250 μ g, was separated by SDS-PAGE on 6% polyacrylamide gels, followed by electroblotting to immobilon-P membrane. Membranes were blocked for 60 min in 1 \times PBS, 0.05% Tween 20, and 5% nonfat dry milk. After a 1-h incubation with primary antibody diluted in blocking buffer (1:5,000 Anti-Xpress McAb antibody; Invitrogen) at room temperature, the blot was incubated for 1 h in the presence of secondary antibody conjugated to horseradish peroxidase diluted in blocking buffer, 1:20,000 anti-mouse IgG (Fab)₂ fragment. Bands were visualized with NEN chemiluminescent substrate.

RNA binding and micrococcal nuclease footprinting assays

All RNA-binding reactions were carried out for 1 h at 30 °C. Nitrocellulose filter binding assays were carried out essentially as described previously (Hall & Stump, 1992). Briefly, the ³²P-labeled RNA substrate, 50 pM, was incubated with protein, 1 to 800 nM, in 50 μ L binding buffer (50 or 250 mM KCl, 10 mM HEPES, pH 7.6, 10% glycerol, 5 mM MgCl₂, 0.1 mM EDTA, 0.02% NP40, 100 μ g/mL *E. coli* tRNA, 5 mM DTT). For each KCl concentration, the binding reactions were performed in triplicate. Reactions were diluted into 450 μ L of binding buffer containing 0.56 mg/mL heparin and filtered through 25 mm BA85 filters backed by a DE81 filters in a Millipore 1225 vacuum manifold. Filters were washed six times with 1 mL binding buffer without tRNA. For each protein concentration tested, the cpm retained on each dried filter was measured and used to calculate the fraction of bound RNA as BA85 cpm/(BA85 cpm + DE81 cpm). K_d values were determined with the program Kaleidagraph (Synergy Software).

Gel mobility shift experiments were carried out with 6 nM RNA substrate (~50,000 cpm) and protein between 0.4 and 0.6 μ M, final concentration in a 25 μ L solution containing Buffer M (50 mM KCl, 10 mM HEPES, pH 7.6, 10% glycerol, 2 mM EDTA, 0.025% NP40, 500 μ g/mL tRNA, and 1 mM DTT). Heparin, 0.4 mg/mL final concentration, was added to

each mixture and incubated at 30 °C for 10 min. Loading dye, 3 μ L, was added and 8 μ L were loaded onto a 5% polyacrylamide (80:1 acrylamide:bisacrylamide), 50 mM Tris-glycine gel. Electrophoresis was carried out at 200 V for 3.5 h at 4 °C.

Micrococcal nuclease footprinting was performed in 50 μ L reactions containing 50 pmol protein and 50 ng of RNA substrate assembled in Buffer M. After binding, reactions were treated with 0.4 mg/mL heparin for 10 min at 30 °C. Cleavage was initiated by the addition of 0.25 U micrococcal nuclease and 1 mM CaCl₂ (final concentration). Cleavage reactions were incubated for 10 min at 30 °C. The amount of micrococcal nuclease used for the assay and the incubation time was determined by pilot titration and time course experiments (not shown). Reactions were terminated by the addition of 4 mM EDTA, followed by proteinase K treatment, phenol/chloroform extraction, and ethanol precipitation. Primer extension reactions were carried out as follows. Half of each cleavage reaction in 5 μ L of water was incubated with 2 μ L 10 \times first strand buffer, 1 μ L 0.1 M DTT, 1 μ L 0.3 μ M ³²P-labeled primer, 0.5 μ L 5 mM deoxynucleoside triphosphates, and 0.5 μ L Superscript II (Stratagene). Primer extensions were performed for 30 min at 48 °C. Ethanol precipitated reactions were dissolved in 6 μ L sequencing loading dyes and resolved on an 8% polyacrylamide/7 M urea sequencing gel for 1.5 h at 80 W.

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