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Coordinate repression of a trio of neuron-specific splicing events by the splicing regulator PTB

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ABSTRACT

In this study, we demonstrate the ability of the polypyrimidine tract binding protein PTB to function as a coordinator of splicing regulation for a trio of neuron-specific exons that are subject to developmental splicing changes in the rat cerebellum. Three neuron-specific exons that show positive regulation are derived from the $GABA_A$ receptor $\gamma 2$ subunit 24 nucleotide exon, clathrin light chain B exon EN, and *N*-methyl-D-aspartate receptor NR1 subunit exon 5 pre-mRNAs. The functional activity of splicing repressor signals located in the 3′ splice site regions adjacent to the neural exons is shown using an alternative splicing switch assay, in which these short RNA sequences function *in trans* to switch splicing to the neural pathway in HeLa splicing reactions. Parallel UV crosslinking/competition assays demonstrate selective binding of PTB in comparison to substantially lower binding at adjacent, nonneural 3′ splice sites. Substantially lower PTB binding and splicing switch activity is also observed for the 3′ splice site of NMDA exon 21, which is subject to negative regulation in cerebellum tissue in the same time frame. In splicing active neural extracts, the balance of control shifts to positive regulation, and this shift correlates with a PTB status that is predominantly the neural form. In this context, the addition of recombinant PTB is sufficient to switch splicing to the nonneural pathway. The neural extracts also reveal specific binding of the CUG triplet repeat binding protein to a subset of regulatory 3′ splice site regions. These interactions may interfere with PTB function or modulate splicing levels in a substrate-specific manner within neural tissue. Together these results strengthen the evidence that PTB is a splicing regulator with multiple targets and demonstrate its ability to discriminate among neural and nonneural substrates. Thus, a variety of mechanisms that counterbalance the splicing repressor function of PTB in neural tissue are capable of mediating developmental splicing control. Altered expression of PTB isoforms during cerebellar development, as documented by Western blot analysis, is proposed to be a contributing mechanism.

Keywords: alternative splicing; clathrin light chain B; CUG triplet repeat binding; developmental regulation; $GABA_A$ receptor $\gamma 2$ subunit; *N*-methyl-D-aspartate receptor NR1 subunit protein

INTRODUCTION

Regulated alternative splicing events in neurons contribute changes to protein function that have profound consequences for cell physiology and development. Neurons exemplify the extraordinary combinatorial variations in alternative splicing due, most likely, to the complexity of neuronal cell types in brain (Black, 1998). The molecular basis of the neural specificity of splicing, however, is not understood. One current paradigm postulates that RNA signals with opposing functions are clustered near the neural exon to allow for a shift to positive regulation (exon selection) in neuronal cell types and a shift to negative regulation (exon skipping) in nonneuronal cell types (Grabowski, 1998). Supporting evidence for a balance of control mechanism comes from in vitro experiments that map positive and negative RNA signals in close proximity to the neural exons of the $\gamma 2$ subunit of the $GABA_A$ receptor ($\gamma 2$) and *c-src* pre-mRNAs. Although the physiological relevance of such a mechanism has not been formally tested, the observed variations in the extent of splicing regulation, or tuning, in different neurons of the rat cerebellum (Wang & Grabowski, 1996), or in different neuronal cell lines (Wei et al., 1997), agrees well with this idea.

The importance of negative regulation in alternative splicing pathways that give rise to the nonneural mRNAs of *c-src* and $\gamma 2$ is emphasized by the common involvement of the polypyrimidine tract binding protein PTB. For $\gamma 2$, multiple PTB binding sites are clustered around the branch site of the neural exon, and an additional site is found in the neural exon superimposed on an

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essential positive element (Ashiya & Grabowski, 1997). For *c-src*, PTB binding sites are found in the polypyrimidine tract of the upstream intron and in the downstream intron enhancer, which is critical for splicing regulation (Chan & Black, 1997). When PTB is sequestered on an RNA competitor in HeLa splicing extracts, there is a resulting activation of neural splicing events, *in trans*, for related pre-mRNAs. Furthermore, there is good agreement between the activation of the neural splicing pathway and the ability of PTB to bind the negative elements contained in the RNA competitor. Since HeLa cells are not neuronal, a likely explanation is that PTB, or an activity containing PTB, functions as a splicing repressor that acts on these neural splicing events. If splicing repression dominates in nonneuronal cells, the prediction then follows that in neuronal cells, splicing derepression mechanisms should be an important feature of some splicing regulatory pathways. PTB alone is not likely to account for the neural specificity of splicing, however. There is compelling evidence that PTB acts to repress α and β tropomyosin splicing events, which are subject to regulation in muscle (Mullen et al., 1991; Patton et al., 1991; Mulligan et al., 1992; Gooding et al., 1998). Similar to the case in neurons, however, a mechanistic explanation for the source of the muscle specificity of these regulated splicing events is obscure.

It is possible for RNA sequences with positive functionality (splicing enhancers) to counteract the splicing repressor effects of PTB. The basic function of RNA splicing enhancers is to promote early spliceosome assembly events on intrinsically weak splice sites. A prominent class of such enhancers is found within exons, and contains purine rich sequences that bind to one or more SR splicing proteins. The specific roles of SR proteins as positive regulators of exon selection and 5′ splice site choice are described in several recent reviews (Fu, 1995; Manley & Tacke, 1996). Intron enhancer sequences fulfill a similar function in promoting exon selection and/or polyadenylation, even though these sequences are diverse in sequence and distinct from exon enhancers. Intron enhancer sequences contain important regulatory signals for neural splicing events of *c-src*, calcitonin/CGRP, and agrin (Min et al., 1995; Lou et al., 1996; Wei et al., 1997). For γ2 pre-mRNA, the shift to positive regulation may involve neuron-specific factors that recognize the essential RNA elements defined previously within the 3′ splice site region and neural exon (Zhang et al., 1996).

An alternative explanation is that the shift to positive regulation for γ2 pre-mRNA may arise in neural cells from lower levels or less active forms of PTB, or by mechanisms that otherwise interfere with the splicing repressor functions of PTB in neural cells. Western blot analysis indicates that there are lower levels of the 60-kDa PTB doublet in rat brain, compared to rat kidney nuclear extracts (Ashiya & Grabowski, 1997). Furthermore, the existence of a neuron-specific PTB counterpart is described in nuclear extracts prepared from rat brain tissue and from a neural (WERI-1) cell line (Ashiya & Grabowski, 1997; Chan & Black, 1997). The p59/PTB form identified in rat brain nuclei has an RNA binding behavior similar to that of HeLa PTB, but its electrophoretic mobility is a single band of an apparent molecular weight of 59 kDa, in contrast to the characteristic doublet of 60 kDa. These previous studies raise a variety of questions that remain unanswered. The relationship of rat p59/PTB to the known human isoforms of PTB is not known, nor is it understood how p59/PTB is related to the neural form identified in WERI-1 extracts. In addition, to what extent does PTB mediate splicing regulation of other neural pre-mRNAs? If mechanisms exist to counterbalance the effects of PTB in rat brain tissue, these effects might have widespread consequences for neural splicing pathways.

Related questions arise from the observation that a variety of neural splicing events in brain are developmentally regulated, as well as modulated in a cell- or region-specific manner. Exons 5 and 21 of the *N*-methyl-D-aspartate receptor NR1 subunit (NMDA), for example, are neuron-specific, but significant developmental and region specific variations in splicing throughout brain are shown by in situ hybridization approaches (Zukin & Bennett, 1995). Developmental and regional variations are also observed for the neural γ2 splicing event in rat brain (Whiting et al., 1990; Zhang et al., 1996). Neuron-specific expression of clathrin light chain B (CLCB), with variations in expression among neuronal cell types, has also been shown throughout brain tissue (Wong et al., 1990; Stamm et al., 1992). These and related observations fuel speculation that the regulatory RNA signals in these pre-mRNAs must have considerable information to act as sensors of developmental and/or cell-specific cues.

In the present study, we investigate the involvement of PTB in the regulation of γ2, CLCB, and NMDA exon 5 (NMDA E5) neuron-specific splicing events, which are subject to developmental splicing regulation in rat cerebellum tissue during postnatal development (Wang & Grabowski, 1996). These positive splicing changes are documented in vivo between postnatal day 14 (P14) and 28 (P28). In contrast, NMDA E21 exhibits negative regulation during the same time frame. We use these neural pre-mRNAs as a framework to test the prediction that the regulatory activity of PTB depends upon selective recognition of target RNA sequences and to assess possible models that account for coordinate splicing changes during development. In addition, we develop splicing competent neural extracts to directly test the repressor activity of PTB and to identify RNA binding activities that recognize the neural 3′ splice site regions. The manner in which PTB discriminates among these neural RNA targets together with in vivo changes in PTB expression lend

support to models in which release with time from PTB mediated repression contributes to developmental splicing control.

RESULTS

A trio of neural exons subject to negative control in HeLa splicing extracts by PTB and repressor sites clustered at the branchpoint/3′ splice site

An alternative splicing switch assay was used previously to identify RNA signals in γ2 pre-mRNA that operate in concert with PTB to repress the neural, but not nonneural, pathway of splicing (Ashiya & Grabowski, 1997). In this assay, a radiolabeled γ2 pre-mRNA substrate, rGγ25, is spliced by the nonneural pathway (exon skipping) in HeLa splicing extracts, whereas a substantial switch to the neural pathway (exon selection) is induced when the extract is preincubated with the RNA competitor 57A, which contains the 3′ splice site region of the neural exon (Fig. 1B, lanes 2 and 3). Because the RNA competitor switches the splicing pathway but does not affect general splicing activity, it is likely that these effects are mediated, *in trans*, by the selective binding of the splicing repressor PTB, which is known to bind avidly to 57A RNA.

To assess the ability of PTB to impose negative regulation in the neural splicing events of CLCB and NMDA E5, RNA substrates similar to rGγ25 were constructed that contain the neural exon and flanking intron regions of clathrin light chain B exon EN (rCL25) and NMDA E5 (rNM25) (Fig. 1A). In addition, RNA competitors corresponding to the 3′ splice site region of each neural exon were constructed and tested in the splicing switch assay.

These experiments show that the rGγ25, rCL25, and rNM25 substrates splice by exon skipping in the HeLa splicing extract in the absence of added RNA competitor (Fig. 1B, lanes 2, 7, and 12). In all three cases, it is evident that the neural pathway is blocked by the inability to splice intron a. Splicing of intron a is substantially activated, however, when the reactions are performed in the presence of the cognate RNA competitor containing the 3′ splice site adjacent to the neural exon (Fig. 1B, lanes 3, 9, and 15, see asterisk). The lariat intron b-exon 3 intermediate is also diagnostic for activation of the neural pathway under these conditions.

The behavior of the noncognate RNA competitors is complex, but reproducible at a range of RNA concentrations. Although these RNAs induce a shift to the neural pathway, they are less effective than the cognate RNA competitors. In addition, the noncognate RNA competitors do not have uniform effects with the entire set of splicing substrates. For example, the 57A RNA competitor effectively induces intron a splicing for rGγ25 and rNM25, but only partially for rCL25. These results are in agreement with the relative number of UCUU(C) PTB consensus motifs, but the effects of other 3′ splice site binding factors cannot be ruled out (Fig. 1D).

According to the current model, γ2 pre-mRNA is subject to negative regulation by a mechanism in which multiple RNA repressor signals surrounding the branch site of the neural exon engage in PTB binding at the expense of U2 snRNP assembly. A prediction of this model is that in order to assist the splicing machinery in distinguishing neural from nonneural exons, these signals should be differentially concentrated in the 3′ splice site region of the neural exon, compared to adjacent 3′ splice sites in the same pre-mRNA. To test this prediction, RNA competitor Gγd1, which contains the 3′ splice site region of γ2 pre-mRNA downstream of the neural exon, was constructed and tested in the splicing switch assay (Fig. 1A). These results show that Gγd1 RNA has little or no effect on the splicing pathway under the same conditions tested for the proficient 57A RNA competitor (Fig. 1C, lane 3). Similarly, RNA competitors derived from the natural 3′ splice site regions downstream of the EN exon of CLCB and exon 5 of NMDA fail to switch splicing to the neural pathway (Fig. 1C, lanes 7, 10). Similar results are obtained when a substrate similar to rCL25 but containing sequences entirely derived from CLCB is tested in the assay (data not shown).

It is an assumption that the splicing switch assay provides a comparison of the ability of PTB to bind an RNA competitor relative to that of the available 3′ splice sites within the pre-mRNA substrate. Given that the rGγ25, rCL25, and rNM25 substrates contain common first and last exon/intron regions, the RNA competitors are expected to switch splicing to the neural pathway if their splicing repressor binding activity is similar to or greater than that of the neural 3′ splice site region. Since the downstream RNA competitors neither switch the splicing pathway nor diminish general splicing activity, we conclude that the ability of these RNAs to bind splicing repressors is lower than that of the neural 3′ splice site regions.

Based on the results of the splicing switch assay and their pyrimidine-rich sequence context, PTB would be expected to bind preferentially to the neural 3′ splice sites of γ2, CLCB, and NMDA E5. This expectation was tested using a UV crosslinking/competition approach with HeLa splicing extract. Each of these RNAs competes for PTB binding to the γ2, CLCB, and NMDA E5 RNA fragments, although the cognate RNA competitors are the most effective (Fig. 2, lanes 1–12). In contrast, the downstream RNA competitors Gγd1, CLd1, and NMd1 are ineffective competitors for PTB binding (Fig. 2, lanes 13, 14 and data not shown). Thus, there is good agreement in the ability of the neural RNA competitors, 57A, EN3, and E51, to bind the 60-kDa PTB doublet and switch splicing to the neural pathway.

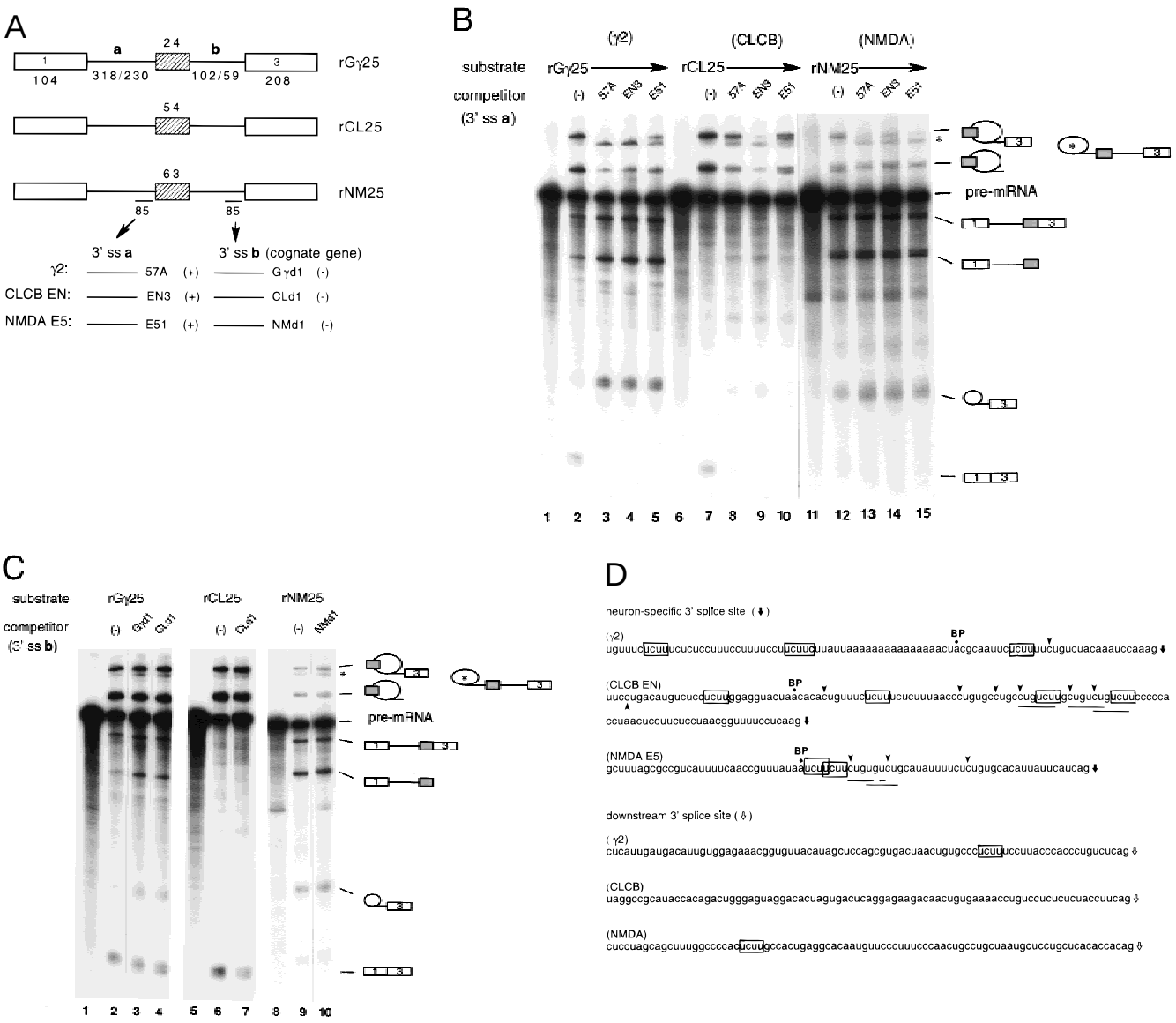


FIGURE 1. Splicing repressor sites are clustered near the branchpoint/3′ splice sites of a trio of neural exons. **A**, Top: diagram of pre-mRNAs used for in vitro splicing reactions. Three exon substrates are derived from the γ2 subunit of the $GABA_A$ receptor (rGγ25), the EN region of clathrin light chain B (rCL25), and the exon 5 region of the NMDA receptor NR1 subunit (rNM25). For rCL25 and rNM25, the neural exon, 230 nt of upstream intron, and 102 nt of downstream intron are inserted in place of the neural exon fragment of rGγ25. All sequences are derived from the rat. Sizes of exon (boxes), and intron (lines) fragments are indicated in nucleotides. The unique sizes of the neural exons (crosshatched) are indicated above each substrate. Bottom: RNA competitors contain approximately 85 nt encompassing the 3′ splice site of the γ2 (57A), CLCB (EN3), and NMDA (E51) neural exons. Downstream 3′ splice regions (85 nt) are derived by PCR from genomic clones of γ2 (Gγd1), CLCB (CLd1), or NMDA (NMd1), which contain the immediate downstream intron/exon region. Relative effects on cognate pre-mRNAs in the splicing switch assay are indicated at right of each RNA competitor. **B**: A splicing switch induced by neural 3′ splice site regions of γ2, CLCB, and NMDA E5. HeLa splicing reactions contain ^{32}P-labeled γ2 (0 min, lane 1; 60 min, lanes 2–5), CLCB (0 min, lane 6; 60 min, lanes 7–10), NMDA (0 min, lane 11; 60 min, lanes 12–15) pre-mRNAs. Unlabeled RNA competitors are present in reactions at 75× molar excess of substrate RNA as indicated at top. Right: Schematic of intermediate and product RNAs. Lariat intron a: 3′ exon intermediate (*). Samples are resolved on 4% polyacrylamide/7M urea gels. **C**: Adjacent, nonneural 3′ splice site regions fail to induce the switch to the neural pathway. HeLa splicing reactions performed as in **B** without (lanes −), or with competitors (75× molar excess) as indicated at top. **D**: Diverse, pyrimidine-rich sequences of 3′ splice site regions adjacent to neural exons of γ2, CLCB, and NMDA E5 (top). Filled arrows indicate position of neural 3′ splice sites. Downstream, nonneural 3′ splice sites are shown at bottom (open arrows). Branchpoint positions mapped in this and a previous study (Ashiya & Grabowski, 1997) are indicated for the neuron-specific 3′ splice sites (BP). The location of branchpoints adjacent to the neural exons of rCL25 and rNM25 pre-mRNAs were determined by primer extension of debranched and mock debranched RNA (data not shown). RNA template for the primer extension reactions was obtained from HeLa splicing reactions adjusted to conditions in which the neural pathway is activated by addition of competitor RNA. Core PTB binding motifs are indicated in boxes; thick lines. Arrowheads indicate positions of CUG sequences in the neural 3′ splice site sequences. CUGUCU sequences are underscored.

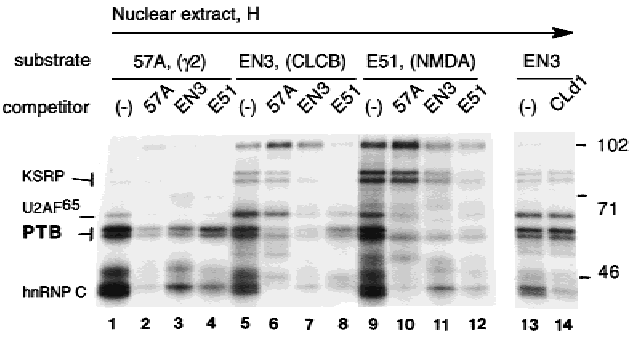


FIGURE 2. Selective binding of PTB is a common feature of the neural 3′ splice site regions of γ2, CLCB exon EN, and NMDA E5. HeLa binding reactions (H) contain indicated RNA substrates and competitors (top). Lanes −: Reactions without RNA competitor. RNA competitor is present in indicated reactions at 12× molar excess. Following UV treatment and RNase digestion, proteins are resolved by SDS-PAGE on a 12.5% polyacrylamide gel. Protein size standards (in kiloDaltons) are indicated at right. Identity of known proteins is shown at left.

As an additional comparison, we tested the ability of PTB to bind the 3′ splice site of NMDA E21, since this neural splicing event is negatively regulated during cerebellar development in the same time frame that γ2, CLCB, and NMDA E5 neural splicing events are positively regulated. For this purpose, the natural 3′ splice site regions adjacent to NMDA E21 (NM21), and its downstream partner (NM22) were tested in the UV crosslinking/competition assay. Clearly, PTB shows little or no crosslinking to these RNA substrates under conditions in which binding to 57A RNA is evident (Fig. 3A, lanes 1–4). In contrast to the neural 3′ splice sites discussed previously, the sequences of the NM21 and NM22 RNAs have more G residues, fewer pyrimidines, and are lacking in candidate PTB binding motifs (Fig. 3C). Furthermore, when tested in the splicing switch assay, NM21 RNA is found to be completely inactive (Fig. 3B, lanes 4, 5). Thus, among the eight 3′ splice sites tested previously, PTB binding is selective for the trio of neural 3′ splice sites that are subject to positive regulation during development.

A neural splicing extract shifts the balance of control to positive regulation under conditions in which the PTB status is predominantly p59/PTB

The PTB counterpart p59/PTB was previously identified in extracts prepared from rat brain nuclei and found to bind specifically to the RNA repressor sites in γ2 pre-mRNA (Ashiya & Grabowski, 1997). In the previous study, functional splicing studies in the presence of p59/PTB were not possible because the neural extracts were not splicing active. In this study, the neural extracts are made splicing competent by a three- to four-fold concentration step. When the neural extracts are used for in vitro splicing reactions, the neural pathway of splicing is activated without the addition of RNA competitor, as illustrated for substrate rCL25 (Fig. 4, lanes 4, 5). The extent of activation of the neural pathway is comparable to that observed in the HeLa extract in the presence of the cognate RNA competitor, EN3 (lane 2).

It is possible that the neural splicing pathway is promoted in these extracts because of an altered PTB content, and/or because of the presence of neuron-specific activities. To assess the PTB content of the neural extract, Western blot analysis was performed using a PTB monoclonal antibody, 7G12, and a chicken polyclonal antibody raised against the recombinant protein, GST-PTB1. The chicken polyclonal antibody recognizes rat p59/PTB as well as the 60-kD PTB doublet, whereas the 7G12 monoclonal antibody displays a preference for p59/PTB (data not shown). Similar to the results reported previously, p59/PTB is enriched in the neural splicing extract (extract B, Fig. 5). Antibodies used to assess the neural content of the extract show substantial enrichment for neural RNA binding proteins of the Elav-related Hu family, as well as Nova-1 (Fig. 5). Evidence for the neural expression of some members of the Hu protein family (HuD, HuC, and Hel-N1) and Nova-1 is shown elsewhere (King et al., 1994; Buckanovich et al., 1996).

Because these extracts promote the splicing of the neural pathway and contain predominantly p59/PTB, we asked if the addition of purified recombinant PTB1 is sufficient to induce the switch to the nonneural pathway (exon skipping). An advantage of the neural extract is that, unlike the HeLa extract, it does not require the addition of RNA competitor to activate neural splicing. To test this, the neural extract was preincubated for 5 min on ice with 9 pmol of either glutathione S transferase (GST) alone or GST-PTB1, followed by the addition of 1 pmol RNA substrate, rGγ25 or rCL25. These results show that GST-PTB1, but not GST, represses intron a and intron b splicing substantially, but does not impair exon skipping (Fig. 6, lanes 3–12). Thus, PTB1 has a dominant effect in the neural extract and its effects selectively repress the neural splicing pathway.

CUG triplet repeat binding protein interacts with a subset of neural 3′ splice site regions in the neural splicing extract

To identify RNA binding activities in the neural extract that interact with the 3′ splice site regions of 57A, EN3, and E51 RNAs, a UV crosslinking/competition approach was used. Although these RNA substrates show complex binding behavior, a prominent group of proteins in the 50-kDa region bind specifically to the CLCB (EN3) and the NMDA (E51) substrates (Fig. 7, lanes 7–10, 13–16). Furthermore, p50 binding is extract dependent, since binding reactions containing HeLa nuclear

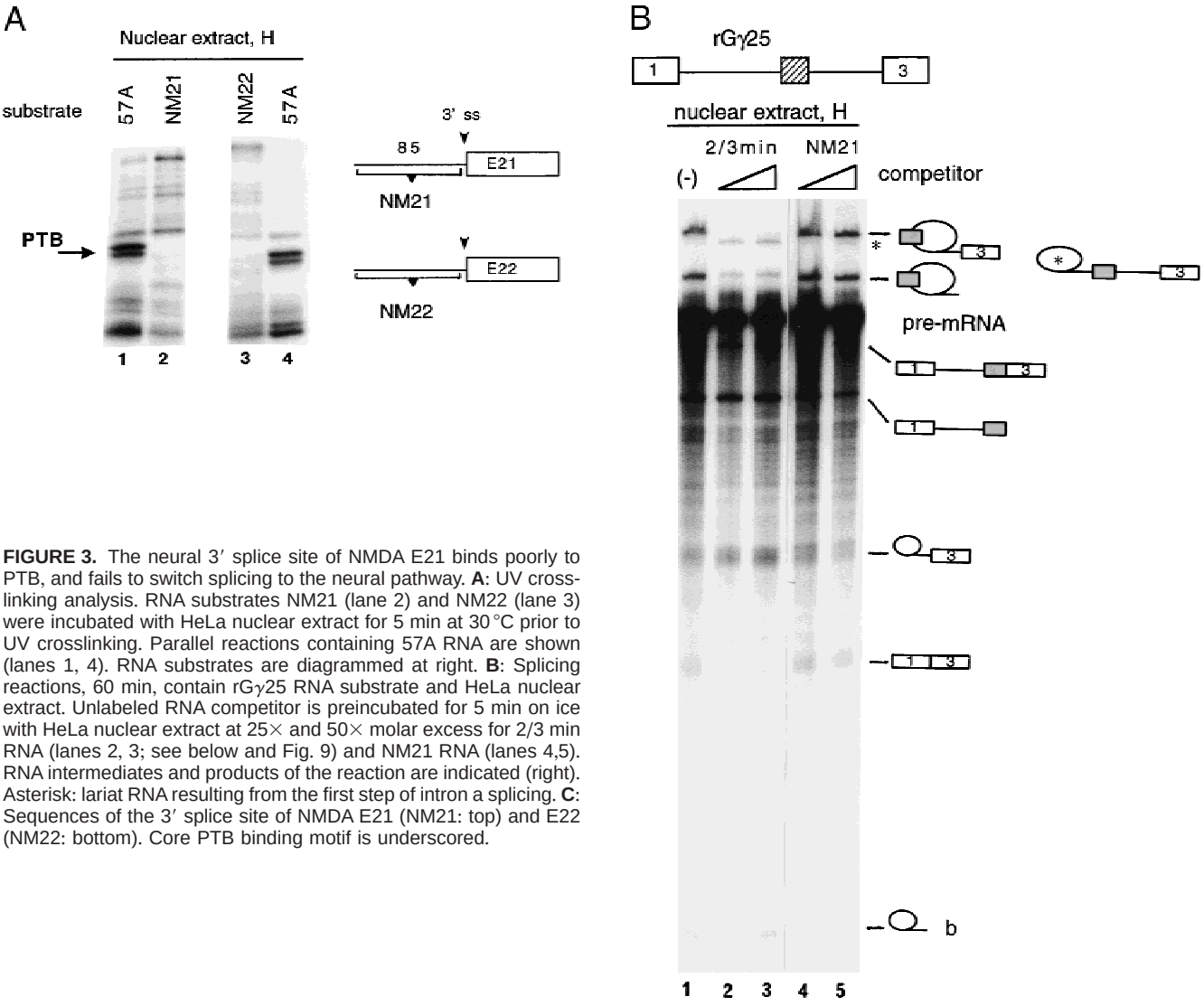


FIGURE 3. The neural 3′ splice site of NMDA E21 binds poorly to PTB, and fails to switch splicing to the neural pathway. **A**: UV crosslinking analysis. RNA substrates NM21 (lane 2) and NM22 (lane 3) were incubated with HeLa nuclear extract for 5 min at 30 °C prior to UV crosslinking. Parallel reactions containing 57A RNA are shown (lanes 1, 4). RNA substrates are diagrammed at right. **B**: Splicing reactions, 60 min, contain rGγ25 RNA substrate and HeLa nuclear extract. Unlabeled RNA competitor is preincubated for 5 min on ice with HeLa nuclear extract at 25× and 50× molar excess for 2/3 min RNA (lanes 2, 3; see below and Fig. 9) and NM21 RNA (lanes 4,5). RNA intermediates and products of the reaction are indicated (right). Asterisk: lariat RNA resulting from the first step of intron a splicing. **C**: Sequences of the 3′ splice site of NMDA E21 (NM21: top) and E22 (NM22: bottom). Core PTB binding motif is underscored.

C

neuron-specific 3' splice site (NMDA E21) (⬇)

gccuggcagcggcccaaggagcaggcaagggcagguguggccgccacgcuuagcugucuaaucgcuuauacauauucauuuuag⬇

downstream 3' splice site (⇩)

ggccagggcagcacugggcgcucagggcugggaucccugguggccggcggggccagcagguauugauuguugguucuuauuuauag ⇩

extract show reduced crosslinking to proteins in the 50-kDa size range (Fig. 7, lanes 6, 12). The p50 proteins show little or no binding to the γ2 substrate, 57A (Fig. 7, lanes 2–5).

As a step toward identifying p50, deletion mutagenesis was used to map the RNA binding site using the neural extract. These results show that EN3, EN9, and 2/3 RNAs have similar activities in binding p50, whereas fragments outside of the 2/3 region exhibit reduced binding (Fig. 7B). Furthermore, in this set of mutants, any changes in the 5′ half of the 2/3 RNA sequence result in reduction of p50 binding activity (2/3A, 2/3B), whereas changes in the 3′ half have little or no effect (2/3C). There is little or no effect on the binding of p50 by a control RNA competitor with an unrelated sequence (Fig. 7, lane 17).

These results show that the CLCB binding site for p50 resides in a sequence, which contains three copies of CUGUCU in an 18-nt region. The NMDA substrate contains a similar sequence with multiple CUG

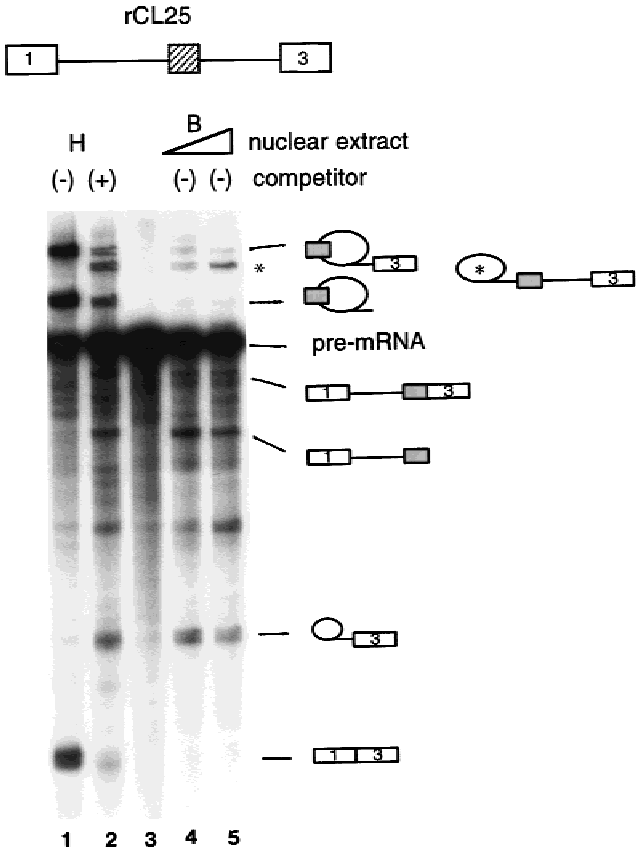


FIGURE 4. A splicing-competent neural extract derived from rat brain nuclei shifts the balance of control to the neural pathway. Splicing reactions, 60 min, contain rCL25 RNA substrate and rat brain (B) (lanes 4, 5), or control, HeLa (H) nuclear extract (lanes 1, 2). Increasing amounts of rat brain nuclear extract are indicated by wedge. Reactions contain extract B at 3 (lane 4), or 4 (lane 5) mg/mL protein concentration as determined by Bradford assay. EN3 RNA competitor is present in splicing reaction of lane 2 to provide a reference for the neural pathway of splicing. RNA substrate incubated for 0 min in extract B at 3 mg/mL (lane 3).

triplets (Fig. 1D, arrowheads). Based on its molecular weight, and the observation that both the CLCB and NMDA binding sites contain multiple CUG triplets, we considered that p50 might be related to the CUG triplet repeat binding protein CUG-BP, reported previously (Timchenko et al., 1996). CUG-BP is identical to the hnRNP protein hNAB50, and two major isoforms are known, CUG-BP1 and CUG-BP2, which migrate as a doublet at 50 kDa. To test the possibility that p50 is CUG-BP, biotinylated EN3 and E51 RNAs were assembled in the neural extract and affinity selected on streptavidin–agarose. After extensive washes of the selected material, the proteins were eluted and subjected to Western blot analysis with the monoclonal antibody mAb 3B1, which is specific for CUG-BP. These results clearly show interaction of CUG-BP with CLCB and NMDA E5 RNA substrates, but not with a control sample containing 57A RNA (Fig. 8).

Adjoining RNA binding sites and enrichment of CUG-BP in the neural extract contribute to differential binding of PTB and CUG-BP

When the minimal 2/3 RNA sequence is reiterated, (2/3 min RNA), and tested for protein binding activity in the extracts, this RNA is found to reproduce the extract-dependent binding effects observed with the longer RNA sequences (Fig. 9). The minimal sequence is highlighted in its natural context in the 3′ splice site of CLCB EN RNA (Fig. 1D, underlines). Interestingly, 2/3 min RNA binds preferentially to the 60-kDa PTB doublet in the HeLa nuclear extract, in contrast to the neural extract where CUG-BP binding predominates.

The sequence of 2/3 min RNA must contribute to the differential binding effect, because this sequence contains CUG triplets as well as the UCUU core PTB binding motif (see Fig. 9, top). PTB is not capable of

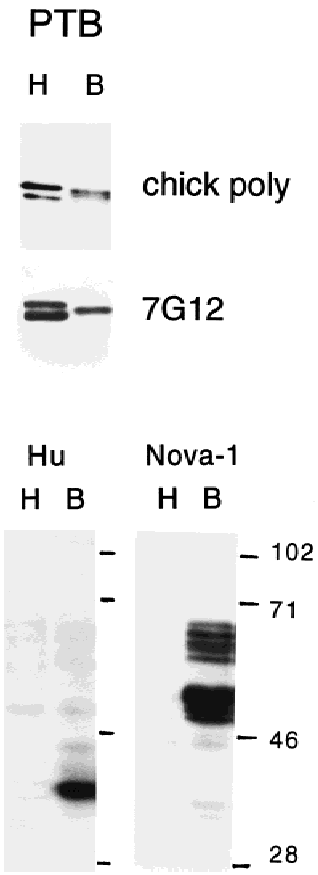


FIGURE 5. Western blot analysis shows an enrichment in p59/PTB and neuron-specific RNA binding proteins. Protein samples, 12 μg, containing HeLa (H) or rat brain (B) nuclear extract are resolved by SDS-PAGE, electroblotted onto Immobilon P membrane, and probed with specific antibodies as indicated at the top of each panel. PTB is detected by a chicken polyclonal antibody (chick poly) at a dilution of 1:6,000 or by the monoclonal 7G12 at 1:1,500. The chicken polyclonal antibody was generated against the human GST-PTB1 recombinant protein. Patient antisera specific for the Hu or Nova-1 antigen is used at 1:2,000 or 1:3,000, respectively.

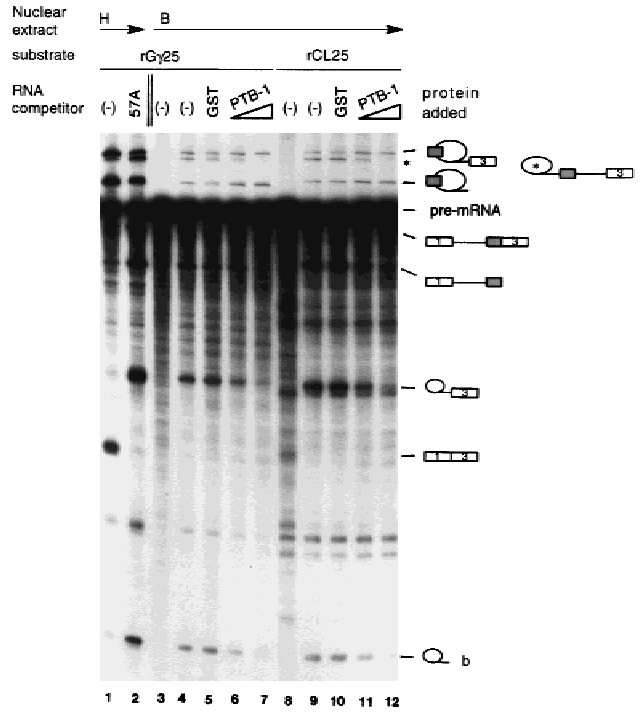


FIGURE 6. PTB is sufficient to repress the neural pathway of splicing. Splicing reactions, 60 min, were carried out in neural extract (B) with rGγ25 or rCL25 RNA substrates as indicated at top. Recombinant GST-PTB1 was present in reactions at 4 (lanes 6, 11), or 9 pmol (lanes 7, 12). Control reactions contain GST alone at 9 pmol (lanes 5, 10), or no addition lanes (−). Lanes 3, 8: Unreacted pre-mRNA. Parallel reactions with HeLa nuclear extract, 60 min, show the switch to the neural pattern of splicing in the absence (lane 1) and in the presence of 50× molar excess of RNA competitor 57A (lane 2). RNA samples are resolved on 4% polyacrylamide/7 M urea gels.

recognizing CUG triplets, because in similar experiments using a variant of 2/3 min RNA that contains only CUG triplets, we observe binding of CUG-BP, but not PTB in these extracts (data not shown). A functional test using the splicing switch assay is also informative. When tested in HeLa splicing reactions, the 2/3 min RNA switches splicing to the neural pathway, demonstrating that the interaction with PTB is functional (Fig. 3B, lanes 1–3).

If PTB and CUG-BP bind to the same region of the CLCB EN 3′ splice site, the relative levels of each protein may contribute to the differential binding observed in the nuclear extracts. The expression of CUG-BP has been detected as a predominantly nuclear protein in a variety of cell lines using the monoclonal antibody mAb 3B1, suggesting a ubiquitous distribution (Timchenko et al., 1996). To determine if the levels of CUG-BP could contribute to differential binding, these protein levels were compared in a Western blot analysis using the antibody mAb 3B1. These results show that CUG-BP is enriched in the neural splicing extracts (Fig. 8, bottom panel). Thus, the relative increase of CUG-BP in the neural extract as well as the difference in PTB levels are likely to account for the differential binding effects observed.

Complex changes in PTB expression during postnatal cerebellar development

To address the physiological relevance of a model in which the developmental activation of neural splicing might be directed by mechanisms that counteract PTB function, we asked if any changes in PTB expression occur in the rat cerebellum during postnatal development. For this purpose, nuclear extracts were prepared from rat cerebellum at postnatal day 7 (P7), P14, and P28 to compare PTB levels to the developmental changes reported previously for γ2, CLCB, and NMDA E5 splicing events.

Equal amounts of the nuclear extracts from each time point were analyzed by Western blot analysis with two PTB-specific antibodies (Fig. 10). A control blot is stained with imido black to show total protein (Fig. 10, lanes 12–14). In this experiment, the time of electrophoresis was extended to better separate the PTB isoforms. These results clearly show a decrease in the fastest migrating PTB isoform in this time frame as detected by the chicken polyclonal antibody, which recognizes rat p59/PTB as well as the 60-kD doublet (Fig. 10, lanes 6–8). Furthermore, the expression of p59/PTB shows a net increase with time as detected by the 7G12 antibody, which preferentially recognizes p59/PTB (Fig. 10, lanes 2–4). In the latter case, the p59/PTB form appears at day 7 as a doublet, which is converted to the more slowly migrating form by day 28. These experiments do not determine whether the changes observed represent covalent protein modifications, nor do they distinguish alterations in steady state protein levels from changes in nuclear versus cytoplasmic distributions. Nonetheless, the finding that obvious changes in PTB expression occur in parallel with (P14–P28) and just prior to (P7) developmental changes in splicing regulation is indicative of a role for PTB in these effects. For comparison, the monoclonal antibody mAb 3B1 is used to test for changes in CUG-BP expression. These results show a net increase in CUG-BP levels during postnatal cerebellar development.

DISCUSSION

Mechanisms that counterbalance the splicing repressor function of PTB are capable of mediating the coordinate regulation of a trio of neural splicing events during development

In this study we show that PTB mediated splicing repression in nonneuronal extracts is a common feature of a diverse trio of neural splicing events, including γ2, CLCB, and NMDA E5. In support of this conclusion, the 3′ splice site regions, approximately 85 nt long, adjacent to these neural exons bind selectively to PTB and function *in trans* to switch splicing to the neural pathway in agreement with a repressive function intrinsic to

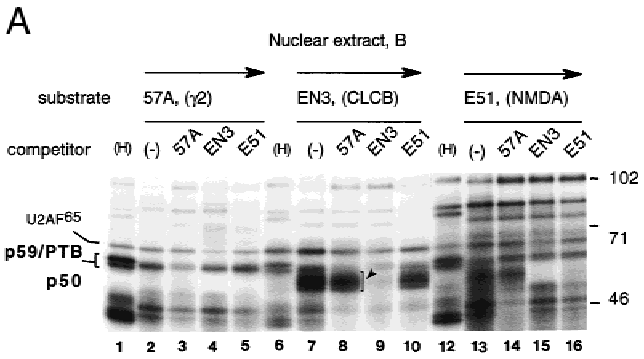
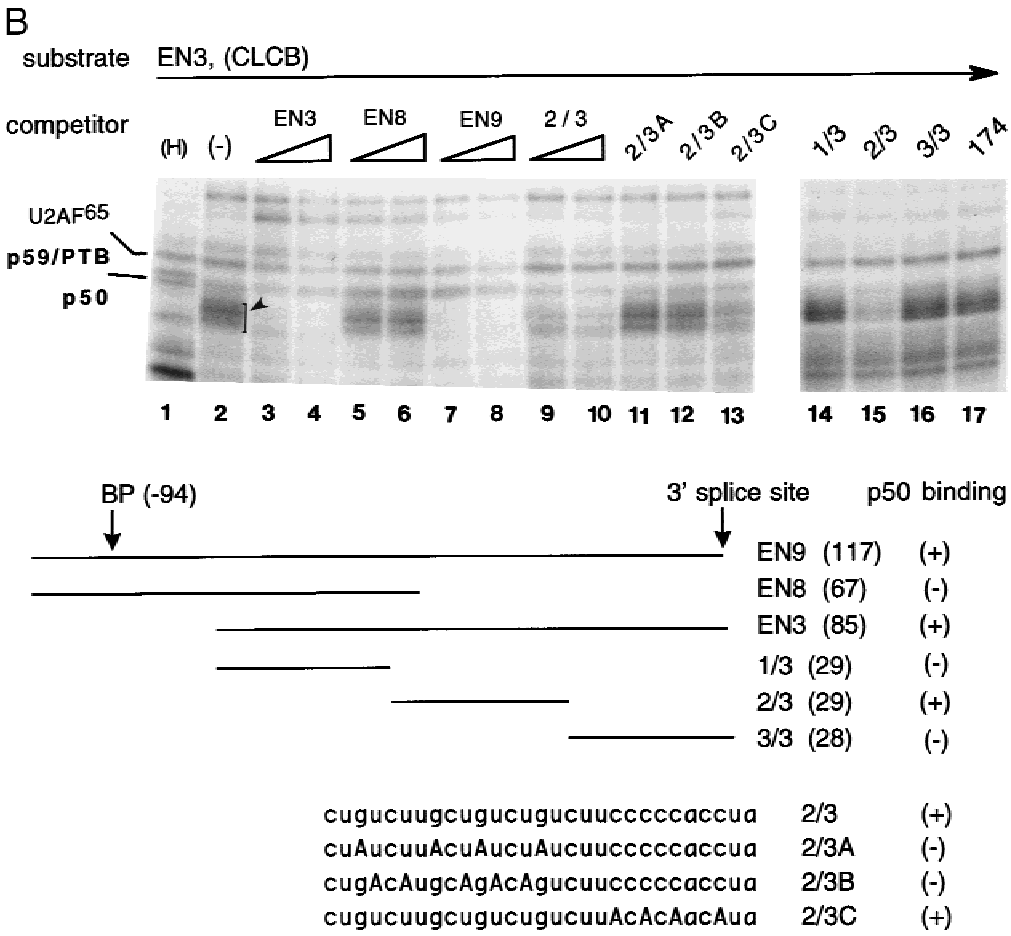


FIGURE 7. Fifty-kiloDalton proteins interact with a subset of neural 3′ splice site regions in a neural extract-dependent manner: identification of RNA binding site. **A**: RNA substrates contain the neural 3′ splice site regions of γ2, CLCB, and NMDA E5 pre-mRNAs (top). The neural extract (B) is preincubated in the presence of 12× molar excess of RNA competitor as indicated, or without competitor (lanes −), followed by addition of RNA substrate and UV crosslinking. Parallel reactions contain HeLa nuclear extract with 57A (lane 1), EN3 (lane 6), or E51 (lane 12). Arrowhead indicates position of p50 proteins. Protein molecular weight standards are shown (right). **B**: UV crosslinking analysis of deletion and substitution substrates is performed in the neural extract as in (**A**) with substrate EN3 RNA, together with RNA competitors as indicated (top). RNA competitors are present in reactions at 12× (lanes 4, 6, 8, 10, 11–17), or 6× (lanes 3, 5, 7, 9) molar excess. Control reactions contain HeLa (lane 1) or neural (lane 2) extract without RNA competitor. Position of p50 is designated by arrowhead. Sequence content of RNA competitors relative to EN9 RNA is diagrammed (middle), with RNA lengths indicated in nucleotides at right. Sequences of parent and variant 2/3 RNAs are shown at bottom. Effect of a control RNA competitor with an unrelated sequence, RNA 174, is shown (lane 17). Relative p50 binding activity is summarized (far right). BP: branchpoint.



these RNA regions. PTB was originally identified as a protein that binds to unregulated 3′ splice sites derived from the adenovirus major late transcription unit and hypothesized to function as a required splicing factor (Garcia-Blanco et al., 1989). This hypothesis has been revised as a result of accumulating evidence that PTB functions as a splicing repressor in neural and muscle systems, together with a lack of demonstrated requirement for a role in general splicing (Valcárcel & Gebauer, 1997).

Here evidence of the splicing regulatory function of PTB is extended to show that the discrimination of PTB among neural 3′ splice sites agrees well with the mode of developmental splicing regulation observed in neural tissue. Three neural exons that are positively regulated during development in the rat cerebellum, γ2,

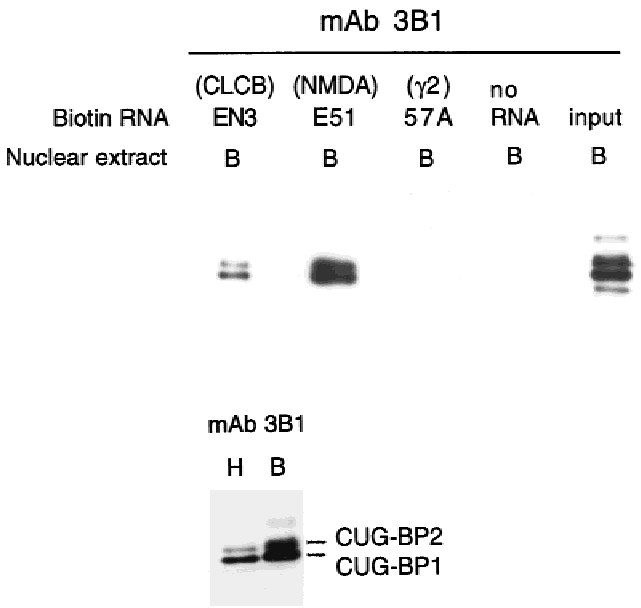


FIGURE 8. CUG-BP is identified as p50 RNA binding activity and is enriched in the neural splicing extract. Top: Affinity selection and Western blot analysis. RNA substrates EN3, E51, and 57A are transcribed with biotin UTP and used as substrates for binding in the neural extract (B) as indicated. 57A RNA lacks the p50 binding site and is expected to show reduced p50 binding. Control reaction performed without added RNA (lane no RNA). A reaction containing EN3 substrate RNA prior to affinity selection is shown at right (lane input). After incubation at 30 °C for 5 min, binding reactions are selected on streptavidin-agarose. Samples are eluted in sample prep buffer and resolved by SDS-PAGE on a 12.5% acrylamide gel, followed by transfer to Immobilon P membrane. Blots are developed with primary antibody mAb 3B1 (1:1,000) with detection by anti-mouse IgG-HRP. Bottom: Equal amounts (24 μg) of HeLa (H) and rat brain nuclear extract are detected by Western blot analysis with mAb 3B1.

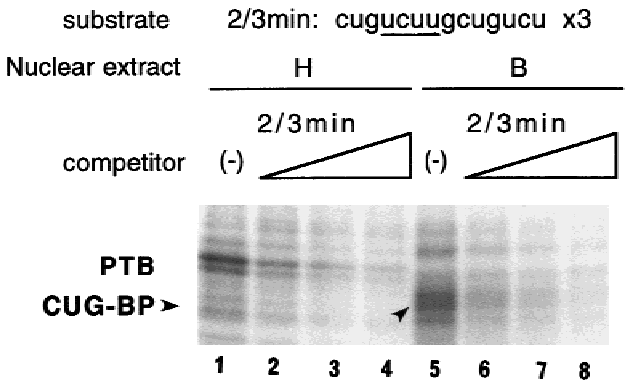


FIGURE 9. Differential protein interactions with the CUG-BP binding region of the CLCB EN 3′ splice site. The minimal binding site for CUG-BP is reiterated 3× and used as a substrate for binding in the HeLa (H) and neural (B) extracts as detected by UV crosslinking. Lanes −: Binding reactions without RNA competitor. Self RNA competitor was added at 4, 8, and 16 pmol (wedge). RNA substrate is present at 1 pmol in each reaction. Arrowhead indicates CUG-BP.

CLCB, and NMDA E5, interact functionally with PTB at the 3′ splice site, in contrast to NMDA E21, which is negatively regulated during the same time frame. Preferential binding of PTB to neural compared to adjacent, nonneural 3′ splice sites is also demonstrated in HeLa splicing extracts as predicted for interactions that control exon selection. These discriminatory features of PTB may help to explain how splicing regulation is coordinated in vivo during development. Although PTB is expected to mediate the coordinate repression of the γ2, CLCB, and NMDA E5 splicing events in nonneural cells, it is feasible for a variety of mechanisms to counteract PTB function and lead to positive regulation in neural cells. In this regard, it is intriguing that obvious changes in PTB expression occur during postnatal development as documented by Western blot analysis of stage-specific cerebellar nuclear extracts (Fig. 10). It will be of interest in future studies to test, more directly, the effects of these changes on splicing regulation and to determine how developmental changes in PTB expression are achieved in vivo.

It has previously been shown in nonneural cells that increased levels of the neural spliced mRNA of CLCB accumulates upon expression of exogenous CLCB and ASF/SF2 genes (Cáceres et al., 1994). In this previous study, the level of regulation with ASF/SF2 overexpression was found to be inferior to that observed in the animal, suggesting that additional neural factors may be required for full splicing regulation. From the results of the present study, an alternative possibility is that PTB, which is normally abundant in nonneural cells, may exert a level of repression that is not completely overcome by the overexpression of ASF/SF2.

How PTB discriminates among its regulatory targets is an interesting puzzle. In the framework of the neural exons tested here, pyrimidine content appears to be a contributing factor because the 3′ splice site sequences of the PTB regulated neural exons γ2, CLCB, and NMDA E5 share a pyrimidine content equal to 66% (Fig. 1D, top), and this contrasts with a pyrimidine content of 49% for the 3′ splice site of NMDA E21 (Fig. 3C). In addition, the pyrimidine content of the PTB-regulated, neural 3′ splice site regions is generally higher than that of their downstream partners (neural/downstream: γ2, 66%/59%; CLCB, 78%/46%; and NMDA E5, 68%/64%). Nonetheless, this illustration shows that pyrimidine content alone is not a reliable predictor of 3′ splice sites that would be regulated by PTB.

Of the eight 3′ splice site regions tested here, the common feature of the PTB-regulated sites is the presence of pyrimidine-rich regions with multiple copies of the core PTB consensus motif UCUU(C) identified by in vitro selection (Pérez et al, 1997; see Figs. 1D and 3C). In addition, we note that the PTB-regulated, but not downstream, 3′ splice sites contain one or more copies of UUCUCU identified previously as a candidate PTB splicing repressor motif (Ashiya & Grabowski, 1997). We find that, like UCUU, this additional motif induces the switch to the neural pathway when reiterated and tested in HeLa splicing reactions (not shown). Thus, there is good agreement in the extent of splicing

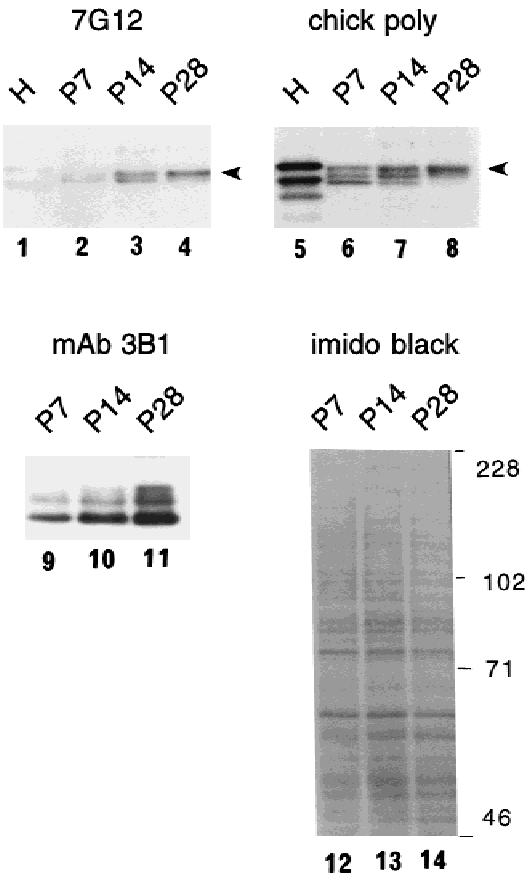


FIGURE 10. Western blot analysis documents changes in PTB and CUG-BP expression during development in nuclear extracts of rat cerebellum tissue. Protein samples, 24 μg, contain nuclear extract prepared from rat cerebellum tissue at P7, P14, and P28. Reference samples contain HeLa nuclear extract (H). Parallel blots are developed with PTB-specific chicken polyclonal or 7G12 monoclonal antibodies (lanes 1–8). Arrowhead indicates position of p59/PTB. mAb 3B1 is specific for CUG-BP (lanes 9–10). Imido black stain for total protein on parallel blot in the 228- (top) to 46- (bottom) kDa range (lanes 12–14).

repression by PTB and the density of these PTB binding motifs in the 3′ splice site regions.

A diverse group of hnRNP proteins associate with pre-mRNA in the nucleus, and it has been proposed that these hnRNP complexes favor functional RNA annealing interactions, with snRNPs for example, and disfavor nonproductive RNA folding (for a review, see Burd & Dreyfuss, 1994). The idea that pre-mRNA processing is facilitated by hnRNP complex formation stems from NMR experiments that show RNA in an extended, and hence more accessible, conformation upon protein binding (Görlach et al., 1992). In agreement with this idea, a variety of hnRNP proteins, including PTB, have been shown to associate with model pre-mRNAs in H complex, but dissociate upon assembly of complex A (Bennett et al., 1992). Thus, PTB may associate widely with pre-mRNAs and contribute to these general features of hnRNP function, as distinct from its regulation of a more restricted population of pre-mRNAs.

Does PTB impose splicing repression by itself or in collaboration with additional protein partners? The branchpoints in the neural 3′ splice sites of γ2, CLCB, and NMDA E5 pre-mRNAs are uniformly located in close proximity to the candidate PTB-binding, splicing repressor motifs (Fig. 1D). This architecture suggests a general mechanism of interference with spliceosome assembly at or prior to branch site recognition by U2 snRNP. Nonetheless, the results shown here do not provide insight about the precise structure of PTB that induces splicing repression. For example, it is not known if corepressors are needed to stabilize a network of PTB interactions surrounding the branch site. Previous studies have shown that human PTB is expressed as three isoforms, PTB1, PTB2, and PTB4/hnRNPI, each containing four RNA binding domains (RBD), but differing in the hinge region between the second and third RBD (Gil et al., 1991; Patton et al., 1991; Ghetti et al., 1992). PTB has been shown to dimerize in vivo by yeast two hybrid analysis, but whether or not these isoforms assemble into higher order complexes on RNA repressor sequences is not understood (Oh et al., 1998). One interaction of PTB with the related protein hnRNP L has been reported, without the demonstration of functional consequence of such an interaction (Hahm et al., 1998). The copurification of PTB with the PTB-associated splicing factor PSF has also been reported, but the interaction of these proteins in vivo has not been confirmed (Patton et al., 1991).

A neural splicing extract reproduces in vivo splicing regulation in the context of an altered PTB status and differential CUG-BP binding

To identify regulatory factors that specify the neural pathway of splicing, we have developed splicing competent extracts of rat brain nuclei. These extracts stimulate the neural pathway of splicing for the trio of PTB-regulated exons without the requirement for exogenous RNA fragments to titrate PTB. The status of PTB in these extracts is predominantly the neural p59/PTB form as shown by Western blot analysis with a chicken polyclonal antibody that recognizes the full set of rat PTB isoforms. Because of the difficulty in achieving a quantitative depletion of PTB from HeLa splicing extracts, these neural extracts provide a unique test of the inhibitory effect of the human PTB1 isoform. These results strengthen the evidence that PTB specifically represses neural exon selection. Although the neural extracts require a concentration step to become splicing active, HeLa extracts differ in that they retain exon-skipping activity whether the extracts are concentrated or diluted several fold. One caveat is that, compared to the natural protein, the GST portion of the human PTB1 recombinant tested here may alter its biochemical properties. In this respect, mechanisms that result in repression of splicing in vitro may differ from in vivo

mechanisms. Future experiments are required to address this issue.

Since the control of the neural splicing events studied here is likely to be mediated by a balance of opposing positive and negative RNA elements, future studies aimed at identifying positive elements should be aided by the neural splicing extracts developed here. In general, the identification of neural activities involved in positive splicing regulation has been difficult. Previous reports have shown that neural splicing extracts prepared from a WERI-1 cell line promote the neural splicing pathway for *c-src* pre-mRNA (Min et al., 1995). As a result, the WERI-1 extracts have been valuable in the identification of hnRNP F and the splicing regulator KSRP, which interact specifically with an intronic enhancer sequence to promote neuron-specific exon selection (Min et al., 1995, 1997). In comparison, the neural extracts described here are derived from whole rat brain tissue. Nonetheless, the extracts are highly enriched in neural protein markers and must contain activities from a variety of differentiated neuronal cell types. The procedure we describe should also be a useful step toward preparation of stage-specific splicing extracts from more defined tissues, such as cerebellum.

The neural extracts in this study have been used to begin to identify RNA binding proteins that interact with the neural 3′ splice site regions of γ2, CLCB, and NMDA E5 pre-mRNAs. Both UV crosslinking/competition and affinity-selection approaches demonstrate the selective interaction of CUG-BP with the CLCB and NMDA E5 RNAs, which contain multiple CUG triplets. The interesting features of these results include the location of binding to two of the neural, but not nonneural, splice site regions, and the apparent extract dependence of binding. Although these features are what would be expected for a regulatory factor involved in promoting a subset of neural splicing events, no tests are provided here to demonstrate the functional relevance of the CUG-BP interactions. Also of interest is the observation that PTB interacts with the minimal CUG-BP binding site in the context of the HeLa extract under the same conditions that CUG-BP is observed to bind this sequence in the neural extract. The natural binding site for CUG-BP in the CLCB substrate suggests that the differential binding interactions may be due to adjoining binding sites for PTB (UCUU) and CUG-BP (CUG). Such an architecture suggests that CUG-BP may contribute to mechanisms that counteract PTB function. The observed increase in CUG-BP expression during rat cerebellar development as shown by Western blot analysis suggests that this notion is worthy of further investigation.

CUG-BP is identical to the ubiquitous hnRNP protein hNAB50, which has been demonstrated to bind to CUG triplet sequences (Timchenko et al., 1996). Recent work has shown that CUG-BP is a regulator of an alternative splicing event in embryonic skeletal muscle involving the cardiac troponin T (cTNT) pre-mRNA (Philips et al., 1998). In the case of cTNT, CUG-BP appears to promote exon selection, because mutation of the CUG triplets disrupts exon selection and CUG-BP binding. In addition, regulation of cTNT splicing is disrupted in tissues of patients with myotonic dystrophy, which contain CUG triplet repeat expansions believed to titrate CUG-BP in muscle tissue. Based on the neuromuscular pathology of myotonic dystrophy, it has been predicted that genes regulated at the level of splicing by CUG repeats would be found in neural tissue (Singer, 1998). The results shown here suggest that the CLCB and NMDA E5 neural splicing events are candidate targets of regulation by CUG-BP or related activities.

MATERIALS AND METHODS

Construction of RNA substrates and competitors

Splicing substrates rCL25 and rNM25 were derived from rGγ25 (Zhang et al., 1996). *Not*I-*Bam*HI fragments containing either CLCB regulated exon EN and flanking introns or NMDAR1 exon 5 and flanking introns were generated by PCR using CLCB and NMDAR1 genomic DNA clones as templates. These fragments were inserted into rGγ25, replacing the 24-nt exon and flanking intron segment. EN3, E51, NM21, Gγd1, CLd1, NMd1, and NM22 were generated by PCR using γ2, CLCB, and NMDA genomic DNA clones as templates. PCR products were gel purified, cleaved with *Hin*dIII and *Eco*RI, and subcloned into a pBS vector (Stratagene). EN8 and EN9 were generated in the same manner as EN3 except using *Not*I and *Eco*R1 as the restriction enzymes. The sequence of all clones generated by PCR was verified by dideoxy sequence analysis. Pre-mRNA substrates (capped at the 5′ end with GpppG) and RNA competitors (uncapped) were transcribed in 25-μL reactions and quantified as described (Ashiya & Grabowski, 1997).

Splicing reactions and extract preparation

Splicing reactions contained 44% (v/v) HeLa or rat brain nuclear extract, 2.0 mM $MgCl_2$, 1.5 mM ATP, 5 mM creatine phosphate, and 150,000 cpm of ^{32}P [UTP]-labeled RNA substrate in 25-μL volumes. Reactions were incubated at 30 °C for 60 min, followed by proteinase K digestion (100 units/mL, 10 min, 30 °C), phenol/chloroform extraction, and ethanol precipitation. RNA competitors were preincubated with nuclear extract on ice for 5 min prior to addition of the labeled RNA substrate in reactions containing RNA competitor.

HeLa nuclear extract was prepared as described (Dignam et al., 1983). Neural extracts were prepared from whole rat brain tissue, (six Sprague-Dawley rats, age 28 days) by homogenization and sucrose-gradient purification of the nuclei exactly as described (Ashiya & Grabowski, 1997). Sprague-Dawley rats at P28 are used for extract preparation because splicing regulation is maximized at this age in cerebellum tissue for neural splicing events of γ2, CLCB, and NMDA E5 (Wang & Grabowski, 1996). After dialysis into Dignam D buffer,

the neural extracts were concentrated to 4 mg/mL in Centricon 3 concentrators (Amicon) by centrifugation at 4 °C to achieve splicing activity. To facilitate concentration, Dignam D buffer was adjusted to 5% glycerol. Nuclear extracts were flash frozen in liquid nitrogen and stored at −80 °C. Cerebellar nuclear extracts from P7, P14, and P28 rats were prepared from cerebellum tissue as described for whole rat brain nuclear extracts, except that the volume of buffer C to the volume of tissue was adjusted to maintain the same conditions for the nuclear extraction procedure.

UV crosslinking/competition

Reactions (25 μL) contained 300,000 cpm of freshly prepared 2P-labeled RNA substrate. RNA competitors were preincubated with nuclear extract on ice for 5 min prior to addition of labeled RNA substrate. Binding reactions were carried out at 30 °C for 5 min prior to UV crosslinking. Samples were then irradiated on ice in open Eppendorf tubes with 1.2 J using a Stratalinker (Stratagene) at a distance of 6.5 cm from the UV source. RNase A (Pharmacia) was added to a final concentration of 1 mg/mL and incubated at 30 °C for 20 min. SDS sample buffer was added, the samples were heated at 95 °C for 5 min and separated by SDS-PAGE on 12.5% polyacrylamide gels. The gel was fixed in 45% methanol and 9% acetic acid for 2 h and dried. Crosslinked proteins were detected by autoradiography.

Affinity selection and Western blot analysis

RNA substrates were transcribed in the presence of biotin-16-UTP (Gibco-BRL) and purified on Sephadex G50 spin columns. Binding reactions (150 μL) contained 44% (v/v) rat brain nuclear extract, in 1× NET buffer (150 mM NaCl, 50 mM Tris-HCl, pH 7.5, 5 mM EDTA). Streptavidin-agarose (Sigma) was prewashed in 1× NET buffer and Blocking buffer (0.01% NP40, 0.5 mg/mL bovine serum albumin, 0.05 mg/mL glycogen, 0.05 mg/mL *Escherichia coli* tRNA) prior to loading RNA binding reactions. Biotin-streptavidin selection was carried out for 2 h at 4 °C, followed by 5 washes in 0.5 mL of 1× NET buffer + 0.01% NP40. After the final wash, 40 μL of 1.5× SDS sample buffer were added and the samples were boiled for 5 min prior to loading onto 12.5% SDS-PAGE gels. Western blot analysis was carried out by electroblotting gel-separated proteins onto Immobilon P membrane (Millipore), followed by blocking the membrane in a solution of 5% (w/v) nonfat dry milk. Primary and secondary antibody binding were performed sequentially, 1 h each, at room temperature. See figure legends for primary antibody dilutions. Secondary antibodies were anti-IgG-HRP conjugates (Promega). Antibody binding was detected with Western blot Chemiluminescence reagents (NEN Life Sciences).

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REFERENCES

Ashiya M, Grabowski PJ. 1997. 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. *RNA 3*:996–1015.

Bennett M, Michaud S, Kingston J, Reed R. 1992. Protein components specifically associated with prespliceosome and spliceosome complexes. *Genes & Dev 6*:1986–2000.

Black DL. 1998. Splicing in the inner ear: A familiar tune, but what are the instruments? *Neuron 20*:165–168.

Buckanovich RJ, Yang YYL, Darnell RB. 1996. The onconeural antigen Nova-1 is a neuron-specific RNA-binding protein, the activity of which is inhibited by paraneoplastic antibodies. *J Neurosci 16*:1114–1122.

Burd CG, Dreyfuss G. 1994. Conserved structures and diversity of functions of RNA-binding proteins. *Science 265*:615–621.

Cáceres 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. 1997. The polypyrimidine tract binding protein binds upstream of neural cell-specific *c-src* exon N1 to repress the splicing of the intron downstream. *Mol Cell Biol 17*:4667–4676.

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.

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

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.

Ghetti A, Piñol-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, Smith CWJ. 1998. Role of an inhibitory pyrimidine element and polypyrimidine tract binding protein in repression of a regulated α-tropomyosin exon. *RNA 4*:85–100.

Görlach M, Wittekind M, Beckman RA, Mueller L, Dreyfuss G. 1992. Interactions of the RNA binding domain of the hnRNP C proteins with RNA. *EMBO J 11*:3289–3295.

Grabowski PJ. 1998. Splicing regulation in neurons: Tinkering with cell-specific control. *Cell 92*:709–712.

Hahm B, Cho OH, Kim JE, Kim YK, Kim JH, Oh YL, Jang SK. 1998. Polypyrimidine tract-binding protein interacts with HnRNP L. *FEBS Lett 425*:401–406.

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.

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

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

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-tropo-

myosin 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.

Oh YL, Hahm B, Kim YK, Lee HK, Lee JW, Song OK, Tsukiyama-kohara K, Kohara M, Nomoto A, Jang SK. 1998. Determination of functional domains in polypyrimidine-tract-binding protein. *Biochem J 331*:169–175.

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.

Pérez I, Lin CH, McAfee JG, Patton JG. 1997. Mutation of PTB binding sites causes misregulation of alternative 3′ splice site selection in vivo. *RNA 3*:764–778.

Philips AV, Timchenko LT, Cooper TA. 1998. Disruption of splicing regulated by a CUG-binding protein in myotonic dystrophy. *Science 280*:737–741.

Singer RH. 1998. Triplet repeat transcripts: A role for RNA in disease. *Science 280*:696–697.

Stamm S, Casper D, Dinsmore J, Kaufman CA, Brosius J, Helfman DM. 1992. Clathrin light chain B: Gene structure and neuron-specific splicing. *Nucleic Acids Res 20*:5097–5103.

Timchenko LT, Miller JW, Timchenko NA, DeVore DR, Datar KV, Lin L, Roberts R, Caskey CT, Swanson MS. 1996. Identification of a (CUG)n triplet repeat RNA-binding protein and its expression in myotonic dystrophy. *Nucleic Acids Res 24*:4407–4414.

Valcárcel J, Gebauer F. 1997. Post-transcriptional regulation: The dawn of PTB. *Curr Biol 7*:R705–R708.

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

Wei N, Lin CQ, Modafferi EF, Gomes WA, Black DL. 1997. A unique intronic splicing enhancer controls the inclusion of the agrin Y exon. *RNA 3*:1–14.

Whiting P, McKernan RM, Iversen LL. 1990. Another mechanism for creating diversity in γ-aminobutyrate type A receptors: RNA splicing directs expression of two forms of γ2 subunit, one of which contains a protein kinase C phosphorylation site. *Proc Natl Acad Sci USA 87*:9966–9970.

Wong DH, Ignatius MJ, Parosky G, Parham P, Trojanowski JQ, Brodsky FM. 1990. Neuron-specific expression of high-molecular weight clathrin light chain. *J Neurosci 10*:3025–3031.

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

Zukin RS, Bennett MVL. 1995. Alternatively spliced isoforms of the NMDAR1 receptor subunit. *Trends Neurosci 18*:306–313.