

Multisite RNA Binding and Release of Polypyrimidine Tract Binding Protein during the Regulation of *c-src* Neural-Specific Splicing

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Summary

We studied the role of polypyrimidine tract binding protein in repressing splicing of the *c-src* neuron-specific N1 exon. Immunodepletion/add-back experiments demonstrate that PTB is essential for splicing repression in HeLa extract. When splicing is repressed, PTB cross-links to intronic CUCUCU elements flanking the N1 exon. Mutation of the downstream CU elements causes dissociation of PTB from the intact upstream CU elements and allows splicing. Thus, PTB molecules bound to multiple elements cooperate to repress splicing. Interestingly, in neuronal WERI-1 cell extract where N1 is spliced, PTB also binds to the upstream CU elements but is dissociated in the presence of ATP. We conclude that splicing repression by PTB is modulated in different cells by a combination of cooperative binding and ATP-dependent dissociation.

Introduction

Alternative splicing allows the production of multiple mRNAs from a single gene through the selection of different combinations of splice sites within a precursor mRNA (pre-mRNA). In many cases, alternative splicing contributes to developmentally regulated and cell type-specific patterns of gene expression. Although the control of alternative splicing is poorly understood, specific regulatory proteins have been identified in several systems. These splicing regulatory proteins are thought to bind to sequence elements in a pre-mRNA and positively or negatively affect spliceosome assembly at nearby splice sites (Caceres et al., 1994; Adams et al., 1997; Wang and Manley, 1997; Wang et al., 1997; Lopez, 1998).

Polypyrimidine tract binding protein (PTB or hnRNP I) is a 57 kDa hnRNP protein that binds to pyrimidine-rich elements, often in the 3' splice site region of introns (Garcia-Blanco et al., 1989; Patton et al., 1991; Ghetti et al., 1992; Valcarcel and Gebauer, 1997). PTB is ubiquitously expressed in mammalian tissues and contains four RNA recognition motifs (RRMs). PTB has been implicated in the regulation of tissue-specific alternative splicing of several genes, including α - and β -tropomyosin, fibronectin, *c-src*, GABA_A receptor γ 2, and α -actinin (Mulligan et al., 1992; Norton, 1994; Lin and Patton, 1995; Singh et al., 1995; Ashiya and Grabowski, 1997; Chan

and Black, 1997; Perez et al., 1997a; Gooding et al., 1998; Grossman et al., 1998; Southby et al., 1999). A common feature of these systems is that PTB binding sites are clustered near the branch site of the alternatively spliced exon. Studies of alternative 3' splice site selection in the α - and β -tropomyosin genes indicate that PTB can block binding of the required splicing factor U2AF to a polypyrimidine tract, thus inhibiting splicing (Lin and Patton, 1995; Singh et al., 1995). However, in some cases, additional PTB binding sites located elsewhere in the transcript are also essential for the regulation (Chan and Black, 1995; Ashiya and Grabowski, 1997; Southby et al., 1999).

PTB is similar in some respects to another polypyrimidine element binding protein, Sex-lethal (SXL), which regulates the splicing of transformer (*tra*) pre-mRNA in *Drosophila* (Valcarcel et al., 1993). In female flies, SXL binding to an alternative 3' splice site in the *tra* pre-mRNA apparently blocks access by U2AF and induces the selection of a downstream 3' splice site. SXL binding also induces skipping of an exon in its own transcript, but this requires multiple SXL binding sites both upstream and downstream of the regulated exon (Sakamoto et al., 1992; Horabin and Schedl, 1993). Unlike SXL, which is expressed only in female flies where repression occurs, PTB is ubiquitously expressed. It is unclear how the repression effect of PTB is negated for particular exons in particular cell types.

We are studying the mouse *c-src* transcript as a model for understanding neuron-specific splicing regulation. The *src* primary transcript contains an 18-nucleotide exon (N1) that is inserted between the constitutive exons 3 and 4 in neurons but is skipped in other cells (Levy et al., 1987; Martinez et al., 1987). N1 splicing is both positively regulated in neurons and negatively regulated in nonneuronal cells (Modafferi and Black, 1999). In neurons, inclusion of the N1 exon requires an intronic splicing enhancer sequence lying downstream of the exon (Black, 1992; Modafferi and Black, 1997). This enhancer, although stronger in neuronal cells, can activate splicing in either cell type. The central, most conserved portion of this enhancer sequence (nucleotides 38–70 downstream of the exon) is called the downstream control sequence (DCS). The DCS binds to a complex of regulatory proteins that is thought to be important in allowing N1 splicing in vitro (Min et al., 1995). The proteins hnRNP F, H, and the KH-type splicing regulatory protein (KSRP) were identified as components of the DCS complex (Min et al., 1995, 1997; Chou et al., 1999). These proteins are present in both cell types and appear to be required for exon N1 splicing. However, their precise roles in the regulation are not clear.

In nonneuronal cells, the skipping of exon N1 is regulated by four conserved CUCUCU sequences (CU elements) that flank the exon and are required for splicing repression in nonneuronal extracts (Chan and Black, 1995; Modafferi and Black, 1999). Two CU elements are located within the polypyrimidine tract of the N1 3' splice

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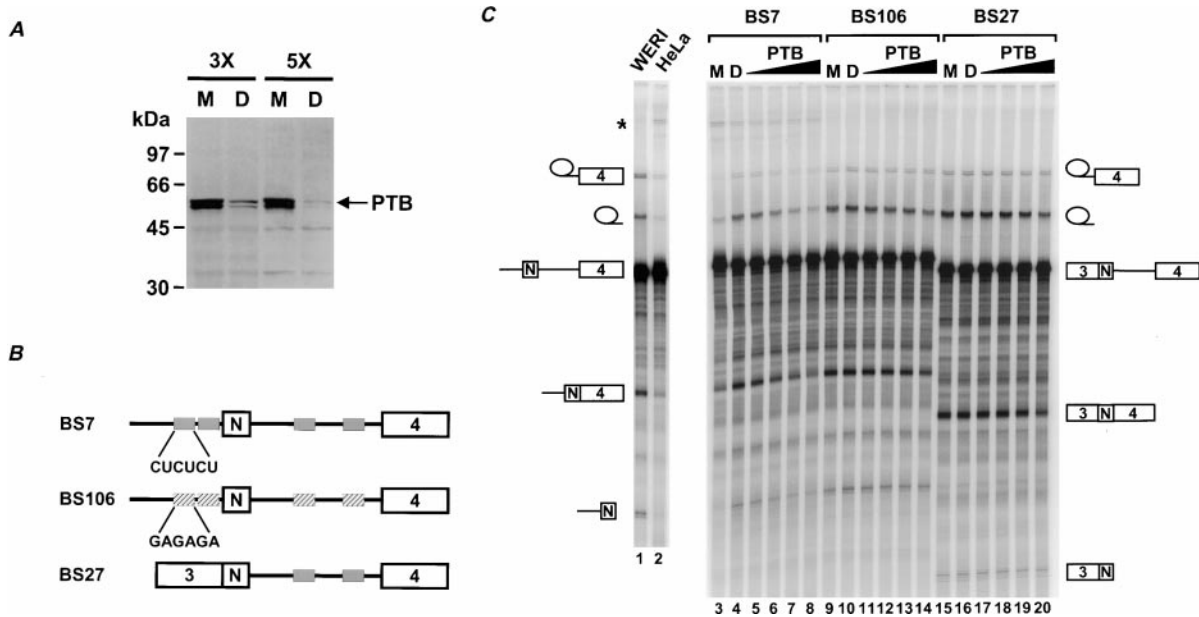


Figure 1. Immunodepletion of PTB Derepresses N1 Exon Splicing In Vitro
 (A) Western blot analysis of the mock depleted (M) or PTB-depleted (D) HeLa nuclear extract. Extracts were passed either three (3×) or five (5×) times over the protein A Sepharose beads coupled either with (D) or without (M) BB7 anti-PTB antibody.
 (B) Maps of the *src* pre-mRNA constructs. The BS7 transcript has wild-type sequence with the CU elements shown as shaded boxes. The BS106 transcript carries mutations in the CU elements (shown as striped boxes with the nucleotide sequence indicated). The BS27 transcript has exon 3 spliced to exon N1 removing the upstream CU elements.
 (C) In vitro splicing in PTB-depleted and mock depleted extract. Lanes 1 and 2, the BS7 transcript incubated in untreated WERI-1 and HeLa extracts. Lanes 3–20 contain splicing reactions in the mock depleted HeLa extract (M), PTB-depleted extract (D), or PTB-depleted extract supplemented with increasing amounts of native PTB (5, 10, 50, and 200 ng). The indicated *src* transcripts were used: BS7, lanes 3–8; BS106, lanes 9–14; and BS27, lanes 15–20. The precursor and product bands for each RNA are denoted at the side. The asterisk marks the product of a cryptic splicing reaction seen in the BS7 RNA when normal splicing is repressed.

site, and two are located downstream of exon N1, including one within the DCS motif that is crucial for the activation of N1 splicing in neurons. PTB binds specifically to the CU elements within the upstream 3' splice site (Chan and Black, 1997). Moreover, these upstream CU elements are required for the repression of the intron downstream of N1, indicating that PTB does not simply block U2AF binding to the N1 polypyrimidine tract, but also disrupts spliceosome assembly on the downstream intron.

In this paper, we demonstrate through immunodepletion/reconstitution experiments that PTB is essential for the repression of N1 splicing in nonneuronal cells. We follow the PTB binding to individual sites during splicing in extracts from two different cell types. We find that multiple CU elements are necessary for stable PTB binding, and that when splicing is derepressed, there is an active, ATP-dependent process that removes PTB from the RNA.

Results

PTB Is Essential for the Repression of N1 Splicing in HeLa Cell Extract

Previously, we showed that PTB repressed N1 splicing when added to a HeLa nuclear extract that was derepressed by an excess of a CU element RNA competitor (Chan and Black, 1997). While suggestive of a role for PTB in splicing repression, it was possible that the added PTB was not acting directly on the *src* pre-mRNA

but instead binding the RNA competitor and sequestering it from the actual repressor. To show a direct role for PTB, we developed monoclonal anti-PTB antibodies that allow specific immunodepletion of PTB from the extract. Of several anti-PTB hybridomas, one (BB7) was chosen for immunodepletion experiments. An affinity column was prepared by attaching BB7 antibodies to protein A Sepharose. After several passages of the extract through the affinity column, the extent of the PTB depletion was determined by Western blotting. After three and five passages through the BB7 column, the PTB was reduced to 17% and 3%, respectively, of that in the mock depleted extract (Figure 1A). Although the PTB removal is almost complete after five passages through the column, both the mock and the PTB-depleted extracts lost significant splicing activity under these conditions (data not shown). We therefore tested the three-passage extract where the mock depleted extract had good splicing activity on unregulated substrates.

Several pre-mRNA substrates were tested for the effect of PTB removal (Figure 1B). The BS7 transcript containing the exon N1 and both the upstream and downstream CU elements is normally spliced in the neuronal WERI-1 cell extract, but repressed in nonneuronal HeLa cell extract (Figure 1C, lanes 1 and 2) (Chan and Black, 1995). The BS7 transcript showed equally low splicing activity in the mock depleted and the untreated HeLa extract (lanes 2 and 3). In contrast, splicing of BS7 increased 5-fold in the PTB-depleted nuclear extract

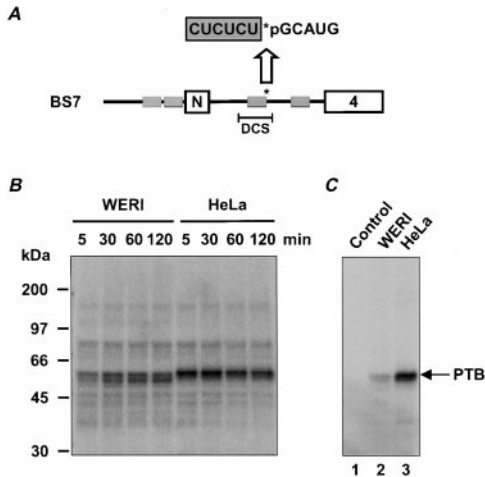


Figure 2. PTB Binds to the CU Element Downstream of Exon N1
(A) Map of the labeled *src* BS7 construct. The CU elements are shown as shaded boxes, and the DCS is indicated with a bar. The single ^{32}P -labeled guanosine is indicated with an asterisk.
(B) The labeled BS7 transcript was incubated with WERI-1 and HeLa extracts under splicing conditions over a 2 hr time course as indicated. After UV cross-linking, reaction mixtures were digested with RNase A, and radiolabeled proteins were separated by SDS-PAGE.
(C) Immunoprecipitation of the UV cross-linked proteins with the BB7 anti-PTB antibody. Reactions from the WERI-1 (lane 2) and the HeLa (lane 3) extracts at the 30 min incubation time were UV cross-linked, RNase A digested, and immunoprecipitated with the BB7. Proteins were separated by SDS-PAGE and autoradiographed. The 60 kDa PTB band is indicated. Lane 1 contains the UV cross-linking reaction from the HeLa extract incubated with protein A beads alone.

(Figure 1C, lane 4). To confirm that the increased splicing was due to the loss of PTB rather than other proteins, we added back purified PTB to the depleted extract. The addition of PTB to the immunodepleted extract strongly repressed splicing of *src* BS7 pre-mRNA (Figure 1C, lanes 5–8). These experiments provide a clear demonstration that PTB is essential in the repression of N1 splicing *in vitro*.

Previous results showed that mutation or deletion of either the upstream or downstream CU elements results in exon N1 inclusion in HeLa extract (Chan and Black, 1995). The BS106 transcript is equivalent to BS7 except that all four CU elements are mutated. The BS27 transcript has *src* exon 3 already joined to exon N1, thus removing the upstream intron and its two CU elements (Figure 1B). To confirm that the PTB repression is mediated by the CU elements, we tested these mutant transcripts in the mock and PTB-depleted extracts. Both BS106 and BS27 were spliced well in the mock depleted extracts, and this did not change in the PTB-depleted extract. Adding back PTB to the immunodepleted extract had virtually no effect on BS27 and induced a weak repression of splicing for the BS106 transcript (Figure 1C). This may be due to PTB binding to additional sites in the RNA. These results confirm that PTB-mediated repression requires the CU elements in the pre-mRNA.

Bound PTB Molecules Flanking the N1 Exon Work Cooperatively to Repress N1 Splicing

PTB is known to bind to the CU elements within the 3' splice site upstream of N1 (Chan and Black, 1995). Since

splicing is derepressed when the downstream CU elements are mutated, it was thought that PTB also bound to the downstream CU elements, including that in the conserved downstream control sequence (DCS). Recently, we showed that PTB and a WERI cell protein related to PTB both bound to the CU element of a short DCS RNA (V. Markovtsov and D. L. B., unpublished data). However, this binding was weak compared to an RNA with the two upstream CU elements. Given these results with short RNA probes, it seemed likely that in a full length pre-mRNA, normal PTB would bind to both the downstream and the upstream CU elements. To examine this, we placed a single-labeled nucleotide at the CU element within the DCS of the full-length BS7 RNA (Figure 2A). This site-specific labeled transcript was incubated in either WERI-1 or HeLa extract under splicing conditions and irradiated with short-wave UV light at different time points. After UV cross-linking and RNase treatment, labeled proteins were separated by SDS-PAGE. As shown in Figure 2B, a 60 kDa protein was cross-linked to this site at all time points in the HeLa extract. Immunoprecipitation with anti-PTB antibody confirmed that this protein is PTB (Figure 2C, lane 3). In the WERI-1 extract, a slightly different 60 kDa band is seen as well as a lower band at about 58 kDa. The upper protein band was weakly immunoprecipitated with the BB7 antibody (Figure 2C, lane 2). This band presumably contains the previously identified WERI cell PTB protein, which does not bind well to the BB7 antibody, and possibly normal PTB, which is also present in this extract. The lower band may be hnRNP H, which is known to bind near this site (Chou et al., 1999; and V. Markovtsov and D. L. B., unpublished data). Thus, PTB binds to this downstream CU element when splicing of the BS7 pre-mRNA is repressed in HeLa extract.

The requirement for both upstream and downstream CU elements for splicing repression implies a cooperation between the PTB molecules binding to these elements. To investigate this hypothesis, we placed a single-labeled nucleotide between the upstream CU elements of the *src* transcript to follow PTB binding at this site. We asked whether the downstream CU elements affected PTB binding to the upstream CU elements. Two transcripts were constructed: the wild-type *src* BS7 and the *src* BS103 transcript carrying mutations in the downstream CU elements. We showed previously that BS103 is derepressed for splicing in HeLa extract (Chan and Black, 1995). As expected, PTB cross-linked strongly to the upstream CU elements of BS7 in HeLa extract (Figure 3A, lanes 1 and 2). In the absence of ATP, PTB also bound well to BS103 (lane 3). However, when ATP was added to the extract, the amount of PTB cross-linked to the upstream CU elements was greatly reduced on the *src* BS103 transcript (lane 4). A small reduction in PTB binding also occurred on BS7, but this was not as dramatic as seen with BS103. Thus, mutation of the downstream CU elements in BS103 led to the loss of PTB binding to the upstream CU elements. Interestingly, this loss of PTB from the upstream CU elements is ATP dependent; PTB cross-links well to either transcript when incubated in the absence of ATP (Figure 3A, lanes 1 and 3).

We next tested whether the upstream CU elements affected PTB binding to the downstream CU elements.

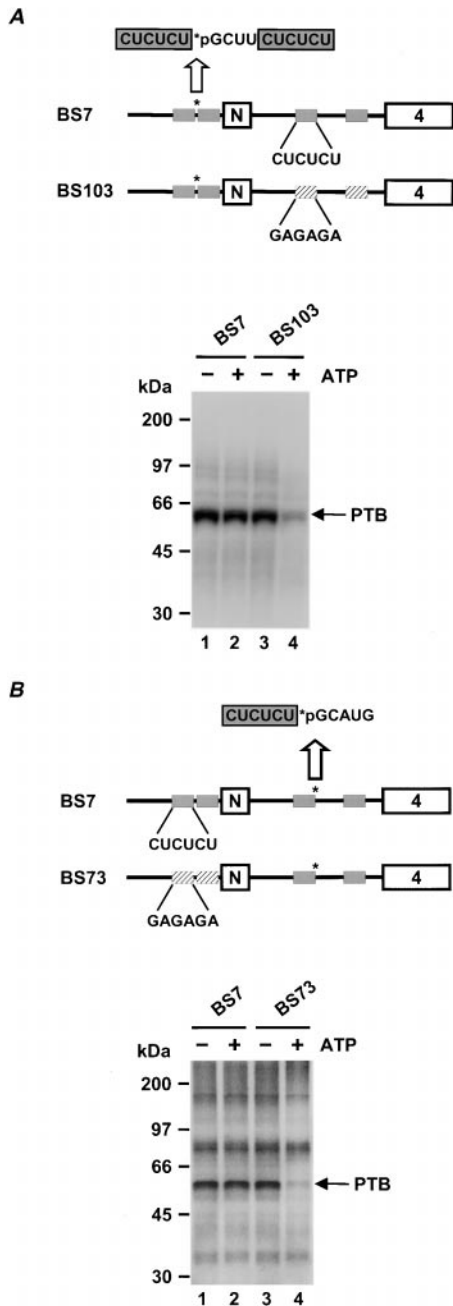


Figure 3. Both Upstream and Downstream CU Elements Are Required to Stabilize PTB Binding

(A) (Upper panel) Map of BS7 and BS103 transcripts carrying a labeled nucleotide within the upstream CU elements. The mutant downstream CU elements in the *src* BS 103 transcript are shown as striped boxes with the sequence indicated. (Lower panel) UV cross-linking assays of the above transcripts in HeLa extract. Reactions were incubated either under splicing conditions (lanes 2 and 4) or in the absence of ATP, MgCl₂, and creatine phosphate (lanes 1 and 3) for 30 min before UV cross-linking.

(B) (Upper panel) Map of BS7 and BS73 transcripts carrying a labeled nucleotide within the DCS CU elements. CU elements are shown as shaded boxes, and the ³²P-labeled guanosine is indicated with an asterisk. The mutant downstream CU elements in the *src* BS 73 transcript are shown as striped boxes with sequence as indicated. (Lower panel) UV cross-linking assays of the above transcripts in HeLa extract. Reactions were incubated either under splicing conditions (lanes 2 and 4) or in the absence of ATP, MgCl₂, and creatine phosphate (lanes 1 and 3) for 30 min before UV cross-linking.

The *src* BS73 transcript, carrying mutations in the upstream CU elements, was labeled in the downstream CU element within the DCS motif (Figure 3B). This BS73 transcript is derepressed for N1 splicing in HeLa extract (Chan and Black, 1995). Protein UV cross-linking to the BS73 in the HeLa extract again showed that PTB binding was lost from the downstream CU element in an ATP-dependent manner (Figure 3B, lanes 3 and 4), as compared with the wild-type BS7 transcript (lanes 1 and 2). This indicates that the upstream CU elements can stabilize PTB binding to the downstream CU elements. Thus, the PTB molecules flanking the N1 exon are likely to interact cooperatively to repress N1 splicing in non-neuronal extracts. Besides PTB, a 75 kDa protein doublet was also found to cross-link to this downstream site. This protein is thought to be the previously identified DCS binding protein KSRP (Min et al., 1997). The intensity of this band is variable, but it can also be seen in Figure 2.

In other assays, we have observed PTB binding to one or two CU elements. The N1 3' splice site containing two CU elements forms a stable PTB complex observable by gel shift assay (Chan and Black, 1997). PTB can also bind to an RNA affinity column containing a short DCS RNA (V. Markovtsov and D. B., unpublished data). It may be that these assays are more sensitive than UV cross-linking, thus allowing detection of weaker binding. However, it is also possible that the ATP-dependent loss of PTB observed here requires a full-length spliceable RNA.

In Neuronal Extracts, PTB Dissociates from the Upstream CU Elements in an ATP-Dependent Manner

In the above experiments, the activation of N1 splicing correlates with the loss of PTB binding to CU elements. To examine whether this occurs during normal splicing of N1 in neurons, the BS7 transcript labeled in the upstream CU elements was incubated in the neuronal WERI-1 extract and the cross-linked proteins were analyzed. Similar to the HeLa extract, PTB binds to the upstream CU elements in WERI-1 extract in the absence of ATP (Figure 4, lanes 2 and 4). The addition of ATP to the WERI-1 extract causes PTB to dissociate from the upstream CU elements even when the downstream CU elements are intact (Figure 4, lane 3). These results indicate that activation splicing of N1 in neuronal extract (Figure 1C, lane 1) is also connected with PTB dissociation from the upstream repressor elements and that this dissociation requires ATP. It is not clear in this experiment whether the observed protein is PTB or the WERI cell form of PTB (lane 4), which are both present in the extract. Due to the location of the label, these transcripts are digested with RNase T1 and not RNase A. This produces a larger cross-linked band and prevents the resolution of the PTB isoforms. It is also not clear whether the PTB/WERI PTB dissociates from the downstream sites in this case. As seen in Figure 2, there is still significant PTB binding to the downstream CU element in neuronal extract after ATP addition. The protein composition of this region is complex, and other proteins may stabilize PTB/WERI PTB binding at this downstream site. Nevertheless, it is clear that activation of N1 splicing

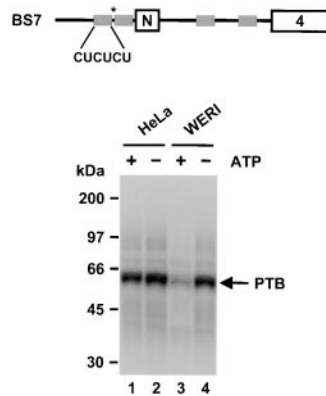


Figure 4. PTB Dissociates from the Upstream CU Elements in WERI-1 Extract during the Splicing Reaction

The *src* BS7 transcript carrying a single-labeled nucleotide within the upstream CU elements (shown in the upper panel) was incubated in either HeLa or WERI-1 extracts under splicing conditions (lanes 1 and 3) or in the absence of ATP, MgCl₂, and creatine phosphate (lanes 2 and 4) for 30 min before UV cross-linking. The cross-linked proteins were then separated by SDS-PAGE and autoradiographed as shown.

in WERI-1 extract involves changes in the PTB interaction with the upstream 3' splice site.

In other experiments, the pre-mRNA was incubated in extract without ATP. The nucleotide was then added and the mixture incubated further. In this situation, PTB cross-links to the 3' splice site prior to nucleotide addition, but cross-linking is lost when the ATP is added (data not shown). This indicates that PTB already bound to the RNA can be affected by the ATP.

U2 snRNP Binding Does Not Drive the ATP-Dependent Loss of PTB

The upstream CU elements in *src* pre-mRNA are within the 3' splice site of the N1 exon. Since assembly of a U2 snRNP complex on the branchpoint upstream of these elements should require ATP, we asked whether the dissociation of PTB required binding of the U2 snRNP. In previous experiments (Chan and Black, 1995), we showed that the N1 3' splice site weakly assembled a spliceosomal A complex in WERI but not HeLa extract. Removing the downstream CU elements allowed A complex formation in either extract. We also used streptavidin pulldown assays to examine whether U2 binding to the N1 exon region was different in the WERI-1 and HeLa extracts (M.-Y. C., unpublished results). A biotinylated partial BS7 transcript containing the upstream branchpoint/3' splice site region, the N1 exon, and the downstream CU elements, but lacking the downstream 3' splice site of exon 4, bound to significantly more U2 snRNA in the WERI-1 extract than in HeLa extract (data not shown). The lack of U2 snRNP binding to the N1 transcript in HeLa extracts supports the idea that PTB is blocking splicing of the upstream intron as well as the downstream.

Under conditions where splicing is allowed, the binding of U2 at the 3' splice site could displace PTB from the CU elements. Since U2 assembly is ATP dependent, this would explain the ATP dependence of PTB dissociation. We next examined whether U2 snRNA was required

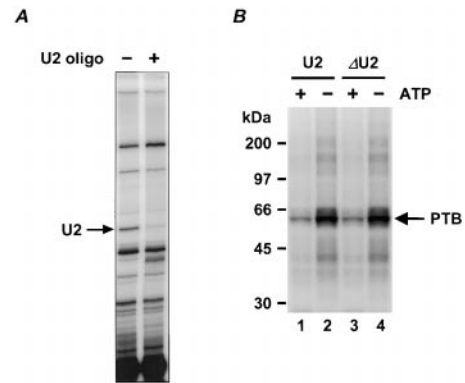


Figure 5. The Loss of U2 snRNP Does Not Prevent the ATP-Dependent Loss of PTB Binding to the Upstream CU Elements

(A) Oligo-directed RNase H degradation of U2 snRNA in neuronal extracts. WERI-1 nuclear extract was incubated in the absence or presence of a DNA oligo complementary to U2 snRNA. After incubation, samples were treated with RNase H followed by RNA extraction. The RNAs were then 3' end labeled and separated on a 12% denaturing polyacrylamide gel.

(B) Cross-linking of proteins to the upstream CU elements in untreated and RNase H-treated extracts. *Src* BS7 RNA labeled within the upstream CU elements was incubated in normal WERI-1 extract in the absence or presence of ATP as indicated, or the U2 snRNA-degraded extract (Δ U2). ATP was removed after the RNase H treatment by addition of hexokinase and glucose.

for the dissociation of PTB in neuronal extract. The WERI-1 nuclear extract was treated with an oligonucleotide complementary to U2 snRNA to degrade the endogenous U2 with RNase H (Figure 5A, lane 2). This U2-degraded extract does not splice the *src* BS7 transcript, indicating the functional loss of U2 snRNP (data not shown). Similarly, it does not splice the adenovirus major late first intron and does not form a prespliceosomal A complex on this intron. A BS7 transcript carrying a label within the upstream CU elements was then incubated in the U2 snRNA degraded extract. As shown in Figure 5B, the loss of U2 snRNA did not prevent the ATP-dependent loss of PTB binding (lanes 3 and 4). This indicates that the PTB dissociation is not driven by the U2 snRNP, as it occurs in the absence of functional U2.

PTB Can Block Downstream Intron Splicing after Assembly of a U2 Complex on the Downstream 3' Splice Site

During repression of splicing by PTB, U2 snRNP is blocked from assembling on the upstream 3' splice site, whereas U1 snRNP is seen binding to the N1 transcript whether PTB is bound or not (data not shown). Since the repression of splicing is primarily directed at the downstream intron (Black, 1992; Chan and Black, 1995), we wanted to examine the assembly of U2 on the downstream 3' splice site adjacent to exon 4. In the wild-type *src* substrate, this is a relatively weak site that does not strongly assemble a spliceosome, as measured by native gel electrophoresis (Chan and Black, 1995; and data not shown). We introduced the strong polypyrimidine tract from the adenovirus major late (AdML) pre-mRNA into this downstream 3' splice site to make the

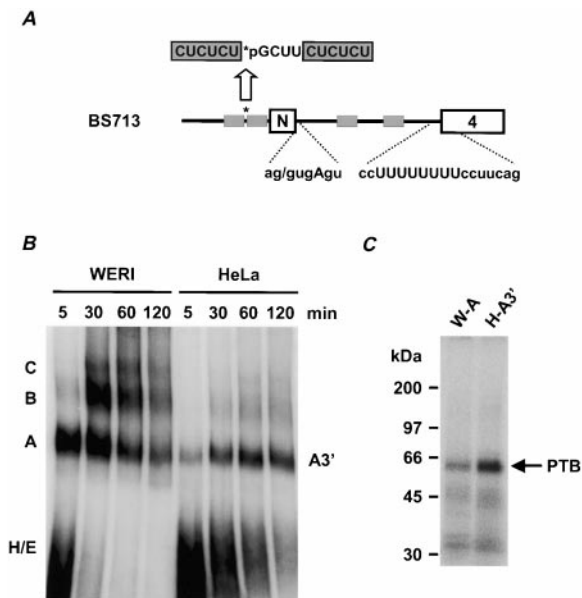


Figure 6. UV Cross-Linking of Splicing Complexes Assembled on the Site-Specific Labeled *src* Pre-mRNA

(A) Map of the *src* BS713 construct. The CU elements are shown as shaded boxes, and the ³²P-labeled guanosine residue is indicated above. The altered nucleotide sequences of the 5' splice site of exon N1 and the 3' splice site of exon 4 are also indicated.

(B) Kinetics of splicing complex formation on the BS713 transcript. BS713 RNA was incubated in either WERI-1 or HeLa extract under splicing conditions for the times indicated. Heparin (0.25 mg/ml) was added before UV cross-linking, and the reaction mixtures were then loaded on a 3% composite agarose-polyacrylamide native gel. Bands corresponding to the H/E, A, B, C and A3' complexes are indicated.

(C) Gel slices containing the WERI-1 A complex and the HeLa A3' complex at the 30 min incubation time were excised and electroeluted from the gel. Equivalent radioactivity from each complex was treated with RNase T1 for 30 min at 37°C. UV-cross-linked proteins were then resolved by 10% SDS-PAGE.

src BS713 transcript (Figure 6A). This 3' splice site forms a prespliceosome complex (or A3' complex) containing the U2 snRNP even in the absence of an upstream 5' splice site (Konarska and Sharp, 1986; Lamond et al., 1987; Bennett et al., 1992; Michaud and Reed, 1993). To further improve this downstream intron, we introduced a point mutation in the 5' splice site to match it to the consensus. These mutations increased the overall level of splicing but did not affect the regulation of N1 exon inclusion in the two extracts (data not shown). We could thus examine whether the upstream PTB complex affected the downstream spliceosomal A complex by native gel electrophoresis. These experiments were performed using site-specific labeled *src* transcript, allowing us to analyze PTB binding to the upstream CU elements in particular gel-isolated complexes.

The labeled BS713 transcript was incubated in either WERI-1 or HeLa extract for different times, and the assembled splicing complexes were then subjected to UV cross-linking and separated by native gel electrophoresis. In WERI-1 extract, splicing complexes A, B, and C are assembled sequentially on the downstream intron during the time course (Figure 6B). In HeLa extract, the

A complex is assembled with slower kinetics than in WERI extract but ultimately is formed fairly efficiently. This partial A complex does not progress to functional spliceosomes, indicating that further spliceosome assembly on the downstream intron is blocked in HeLa extract. This HeLa complex is presumably the A3' complex assembled on the 3' splice site of the downstream intron. In a transcript with an exon upstream of N1, this HeLa A3' complex would be expected to assemble into full spliceosomes using the unregulated upstream 5' splice site.

To examine the binding of PTB in these complexes, UV-cross-linked proteins derived from the WERI-1 A complex and the HeLa A3' complex were isolated from the gel and analyzed by SDS-PAGE. As shown in Figure 6C, the 60 kDa PTB/WERI PTB protein band was only weakly present in the WERI-1 A complex, indicating that PTB is mostly dissociated from the upstream CU elements at this stage of spliceosome assembly. In contrast, in the HeLa A3' complex, PTB was strongly cross-linked to the upstream CU elements. Thus, PTB bound to the repressor elements allows U2 snRNP assembly on the downstream 3' splice but apparently prevents formation of a more mature spliceosome.

Discussion

PTB Is a Modulated Splicing Repressor

The splicing of the *c-src* N1 exon is negatively controlled in nonneuronal cells by CU elements that flank the N1 exon and bind to PTB. We demonstrate here that PTB is an essential factor in the repression of N1 splicing *in vitro* and that PTB binding to individual CU elements is tightly controlled during splicing repression and derepression. PTB cross-linking to individual CU elements requires repressor elements on both sides of the N1 exon. Presumably these PTB molecules bind cooperatively to the downstream and upstream CU elements, interacting with each other across the N1 exon. The stoichiometry of PTB to its binding sites is not known. PTB forms homodimers in solution under RNA-free conditions (Perez et al., 1997b; Oh et al., 1998). Thus, one PTB dimer could bind to each pair of CU elements. These dimers may then assemble into a stable higher order structure, resulting in an N1 exon RNA loop bridged by PTB.

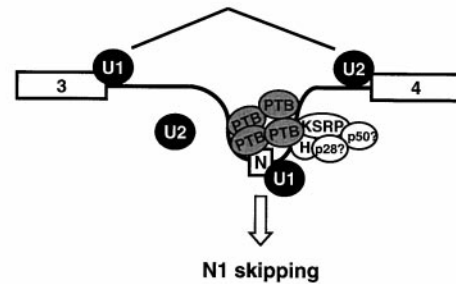
The mechanism by which the PTB complex blocks splicing is not yet clear. In a streptavidin pulldown experiment of biotinylated RNA, U2 snRNP was not bound to the N1 exon region in nonneuronal extracts. Thus, the formation of the PTB-RNA complex may block access of the U2 snRNP to the N1 3' splice sites. Although the U1 snRNP is associated with the transcript under these conditions, it is not clear that U1 is bound to the correct 5' splice site. Since PTB prevents downstream intron splicing, this U1 is not functional. A possible U1/PTB interaction was also seen in the calcitonin/calcitonin gene-regulated peptide (CT/CGRP) transcript where a PTB-containing polyadenylation enhancer complex was shown to recruit U1 snRNP to an adjacent binding site (Lou et al., 1999). Thus, the interactions between PTB and various spliceosomal components will be an interesting point of inquiry in a number of systems.

We show here that the dissociation of PTB from the repressor elements in neurons requires ATP. Spliceosome assembly requires ATP hydrolysis for the phosphorylation of splicing factors and for conformational rearrangements catalyzed by ATP-dependent helicases (Staley and Guthrie, 1998; Misteli, 1999). Since U2 binding is evidently not needed for PTB removal, the dissociation of PTB apparently occurs before the complete assembly of the A complex, the first defined ATP-dependent step in spliceosome assembly. However, we do not know whether factors needed earlier in A complex formation are involved. The assembly of the U2-containing A complex requires a number of U2-associated factors as well as the U2AF protein, SF1 protein, and a DEAD box helicase, UAP56 (Kramer and Utans, 1991; Brosi et al., 1993; Champion-Arnaud and Reed, 1994; Gozani et al., 1996; Fleckner et al., 1997; Berglund et al., 1998; Gozani et al., 1998; Rutz and Seraphin, 1999). The ATP dependence of PTB dissociation may reflect a change in RNA or RNP structure similar to RNA rearrangements catalyzed by helicases. Alternatively, PTB removal may result from the regulation of splicing factors activated through protein kinases. This is an attractive model for controlling the tissue specificity of splicing through the neuron-specific phosphorylation of PTB or other factors, although we have not yet found evidence for the direct phosphorylation of PTB (data not shown).

Whatever the source of the ATP effect, it is clear that although PTB is present and binds to the transcript in WERI extract, it can subsequently be removed. The disruption of PTB binding is very interesting because activities that alter the hnRNP complex before assembly of the spliceosome have not been described. It would appear that very specific and controlled changes in the hnRNP complex that take place after its initial assembly are important in the regulation of splice site choice. This model is different from the regulated transcripts in the *Drosophila* sex determination cascade where the mere presence or absence of a regulatory protein determines the assembly of an enhancer or repressor complex on the target RNA. For example, SXL protein appears to repress splicing of its target RNAs wherever it is expressed. In contrast, PTB is present in all cells, but its ability to regulate splicing is modulated to affect particular exons in particular tissues.

Similar to the PTB binding to the wild-type substrate in neurons, in nonneuronal cells the loss of PTB binding to the upstream repressor elements in the absence of the downstream elements was ATP dependent. This suggests (1) that the cooperative binding of PTB is not present in neurons and/or (2) PTB removal activities are present in both cell types. There is a candidate WERI-specific factor that could interfere with the cooperativity of PTB binding. The first downstream CU element is located within the DCS motif of the intronic enhancer. The DCS is composed of at least three different functional elements, GGGGGCUG, CUCUCU, and UGCAUG (Min et al., 1995; Modafferi and Black, 1997, 1999). A short DCS RNA binds to a set of generally expressed proteins including hnRNP F, H, and KSRP. Most significantly, the DCS also binds a brain-enriched protein related to PTB. This protein binds to the CU element in the DCS in place of normal PTB, which is also present in the extract (V. Markovtsov and D. L. B., unpublished

In non-neuronal cells



In neurons

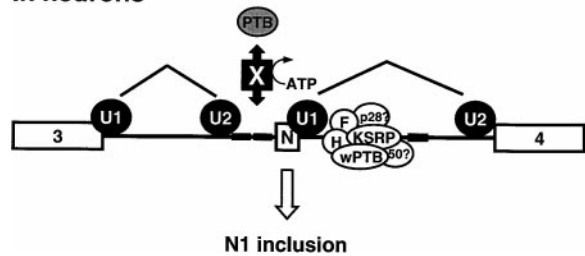


Figure 7. A Model for the Role of PTB in Controlling Spliceosome Assembly on the *src* pre-mRNA in Neuronal and Nonneuronal Cells. CU elements are shown as black boxes. Factor X indicates an unknown factor that drives PTB off the upstream CU elements in the presence of ATP. Other known molecules binding the transcript are indicated including the U1 and U2 snRNPs and the DCS binding proteins, hnRNP F (F), hnRNP H (H), KSRP, and WERI PTB, as well as unidentified factors.

data). It is tempting to speculate that this WERI PTB prevents the cooperative interaction of PTBs bound at the flanking repressor elements, allowing their removal by a non-tissue-specific factor. However, the functional differences between these two PTBs are not yet clear. Moreover, the WERI form of PTB does not activate splicing of the N1 exon when added to HeLa extract, and so it cannot be the sole factor that prevents PTB repression (V. Markovtsov and D. B., unpublished data).

A Model for PTB-Dependent Splicing Repression

Based on the results presented here, we propose a model for the role of PTB binding and release in regulating spliceosome assembly on the introns that flank the N1 exon (Figure 7). In nonneuronal cells, PTB molecules bind to the repressor elements flanking the N1 exon. The cooperative assembly of PTB onto these repressor elements creates an oligomeric PTB complex that bridges the N1 exon. This stable PTB complex blocks U2 binding to the N1 exon branchpoint and also blocks the U1 bound to the N1 5' splice site from assembling into a spliceosome. In the presence of this PTB complex, the U2 snRNP can still assemble on the 3' splice site of the downstream exon 4. This allows splicing of exon 4 to exon 3 and results in N1 exon skipping. In neuronal cells, PTB dissociates from the upstream CU elements in an ATP-dependent process prior to or early in A complex formation. The role of ATP in the dissociation of PTB is not clear, but it is apparently mediated by a factor other than the U2 snRNP. It could result from a phosphorylation event by an unknown kinase. Alternatively, there

could be an energy-dependent enzyme similar to an RNA helicase that actively removes PTB. The dissociation of PTB allows U2 snRNP binding to the N1 branchpoint as well as splicing of the N1 5' splice site to exon 4, leading to exon N1 inclusion. Under these conditions, there is also a change in the regulatory proteins binding to the downstream enhancer. This may enhance spliceosome assembly on the downstream intron, as well as affect the cooperativity of PTB binding. Future experiments will look at the interactions of PTB with both the spliceosome and the enhancer proteins. This should illuminate its central role in controlling N1 exon inclusion.

Experimental Procedures

Plasmids and Site-Specific Labeled *src* Pre-mRNAs

Plasmids BS7, BS27, BS103, and BS106 were described previously (Chan and Black, 1995). BS713 is the same as BS7 except that the 5' and the 3' splice site sequences are changed to the sequences shown in Figure 6A. Site-specific labeled *src* pre-mRNA containing a single ³²P-labeled guanosine was synthesized according to Moore and Sharp (1992). DNA templates encoding the 5' and 3' portions of the transcript were generated by PCR. After T7 transcription, the 5' end of the 3' transcript was dephosphorylated with alkaline phosphatase and rephosphorylated with [γ -³²P]ATP and T4 polynucleotide kinase. The two transcripts were ligated using a DNA-bridging oligo and T4 DNA ligase (Amersham). The DNA-bridging oligos were 5'-AGGGCCAGCGAGAGAGAAGCAGAGAGAAGGAGAGCTGAGGTGCGAAG-3' for the upstream label (Figure 3A); and 5'-GCCGTGGAGGAAGCACATGCAGAGAGAGCCCCAGCCTC-3' for the downstream label (Figures 2 and 3B).

In Vitro Splicing and Immunodepletion Assays

Nuclear extracts were prepared, and in vitro splicing reactions carried out as described previously (Chan and Black, 1995). Immunodepletion and reconstitution experiments were performed as described by Zuo and Maniatis (1996). In brief, 0.5 ml of protein A-Sepharose beads (Pharmacia) were mixed with an equal volume of BB7 mouse ascites for 1 hr at 4°C. The gel slurry was packed into a column, and the column was washed with 5 ml of DG buffer. One milliliter of HeLa nuclear extract was passed through the column either three or five times at room temperature. The eluate was then passed through a fresh protein A column (0.25 ml in bed volume) twice to remove residual IgG.

UV Cross-Linking and Splicing Complex Analysis

Conditions for the UV cross-linking and the native gel analysis of splicing complexes were the same as for splicing except that polyethylene glycol was omitted. Some reactions were run in the absence of ATP, creatine phosphate, and magnesium, and some in the absence of ATP and creatine phosphate. The results with these two conditions were the same. After incubation, heparin sulfate was added to each reaction at a final concentration of 0.25 mg/ml and incubated for an additional 5 min on ice before UV cross-linking. Samples were digested with either RNase A, for the transcript labeled in the downstream CU element, or RNase T1, for the transcript labeled within the upstream CU elements. Cross-linked proteins were separated by 10% SDS-PAGE. For the analysis of splicing complexes, UV cross-linked samples were separated by a native gel as described (Chan and Black, 1995). Splicing complexes were visualized by autoradiography, excised, electroeluted from the gel, and precipitated with acetone. Equivalent radioactivity from each complex was treated with RNase T1 at 37°C for 30 min before SDS-PAGE.

Monoclonal Anti-PTB Antibodies and Immunoblotting

Mouse monoclonal anti-PTB antibodies against human PTB1 were produced using bacterially expressed recombinant PTB as an antigen. Immunization, cell fusion, and hybridoma screening followed a standard protocol (Harlow and Lane, 1988). One of the positive clones (BB7; subclass IgG_{2b}) was selected for the immunodepletion

experiment. Immunoblotting after the immunodepletion was performed with a rabbit polyclonal anti-PTB antibody described previously (Chan and Black, 1997) and visualized by the ECL detection system (Amersham).

PTB Purification and U2 snRNA Degradation

The purification of PTB from HeLa nuclear extracts was performed as described (Patton et al., 1991). Oligo-directed degradation of U2 snRNA was performed as described previously (Black et al., 1985). DNA oligo 5'-CAGATACTACTTG-3' complementary to nucleotides 28–42 of U2 snRNA was used. Full degradation of U2 snRNA by RNase H required addition of ATP to the extract. To deplete ATP after RNase H treatment, nuclear extracts were incubated in the presence of 50 U/ml yeast hexokinase (Boehringer Mannheim), 1 mM MgCl₂, and 5 mM glucose for 30 min at 30°C.

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