

# Polypyrimidine Tract Binding Protein Blocks the 5' Splice Site-Dependent Assembly of U2AF and the Prespliceosomal E Complex

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## Summary

Polypyrimidine tract binding protein (PTB) represses some alternatively spliced exons by direct occlusion of splice sites. In repressing the splicing of the *c-src* N1 exon, we find that PTB acts by a different mechanism. PTB does not interfere with U1 snRNP binding to the N1 5' splice site. Instead, PTB prevents formation of the prespliceosomal early (E) complex across the intervening intron by preventing the assembly of the splicing factor U2AF on the 3' splice site of exon 4. When the unregulated 5' splice site of the upstream exon 3 is present, U2AF binding is restored and splicing between exons 3 and 4 proceeds in spite of the N1 exon bound PTB. Thus, rather than directly blocking the N1 splice sites, PTB prevents the 5' splice site-dependent assembly of U2AF into the E complex. This mechanism likely occurs in many other alternative exons.

## Introduction

Alternative pre-mRNA splicing is a central mechanism for regulating gene expression in metazoans. Changes in splicing patterns can alter protein sequence and function or can turn off protein expression from a gene. These alterations in splicing are regulated during development, in different mature cell types, and in particular cells by extracellular signals (Black, 2003; Maniatis and Tasic, 2002; Shin and Manley, 2004).

Intron removal is carried out by the spliceosome, a dynamic multisubunit particle containing five small nuclear ribonucleoproteins (snRNPs) and many auxiliary proteins (Hartmuth et al., 2002; Jurica et al., 2002; Jurica and Moore, 2003; Makarov et al., 2002; Nilsen, 2003; Zhou et al., 2002a). In mammalian *in vitro* splicing systems, the snRNPs assemble onto each intron in an ordered manner (Brow, 2002; Burge et al., 1999). The initial splicing-specific complexes are the early com-

plexes, E' and E (Das et al., 2000; Kent et al., 2005; Reed, 2000). The E' complex assembles in the absence of ATP and contains the U1 snRNP bound at the 5' splice site and the SF1 protein at the branchpoint (Kent et al., 2005). The U2AF65/35 heterodimer binds the E' complex at the polypyrimidine tract to form the E complex, which also contains loosely associated U2 snRNP (Das et al., 2000; Kent et al., 2005). In yeast and mammals, this assembly of U2AF requires an interaction with the U1 snRNP (Abovich and Rosbash, 1997; Cote et al., 1995; Kent et al., 2005; Li and Blencowe, 1999; Will et al., 1996; Zhang and Rosbash, 1999). In the presence of ATP, the E complex converts into a prespliceosomal A complex in which the U2 snRNP is stably base paired to the branchpoint. The U4/5/6 tri-snRNP then joins the A complex to form the full-sized spliceosome called the B complex. This B complex undergoes an ATP-dependent rearrangement whereby the U1 and U4 snRNPs are lost and the U6 snRNA forms contacts with the 5' splice site and the U2 snRNA (Brow, 2002; Burge et al., 1999; Staley and Guthrie, 1998). This is the catalytic C complex spliceosome in which the two *trans*-esterification reactions of splicing occur, resulting in exon ligation and lariat intron release.

Alternative pre-mRNA splicing involves changes in the choice of splice sites and, hence, changes in the positions of spliceosome assembly (Black, 2003). Splice site choice is affected by a number of different determinants, including transcription rate, RNA secondary structure, and, most clearly, nonspliceosomal pre-mRNA binding proteins (Bentley, 2002; Black, 2003; Caceres and Kornblihtt, 2002; Maniatis and Tasic, 2002; Matlin et al., 2005; Proudfoot et al., 2002; Reed, 2003). The nonspliceosomal regulatory proteins, including members of the hnRNP and SR protein families, can act either positively or negatively to affect a particular splicing choice (Black, 2003; Graveley, 2000). These proteins are most often studied for their effects on overall splicing activity, as well as their sites of binding near particular exons. However, their action on spliceosome assembly has been difficult to assess. Most splicing regulators are thought to direct changes in the initial binding of the spliceosome components U1 snRNP and U2AF and in the formation of the E complex (Black, 2003; Graveley, 2000). *In vitro*, they bind to a pre-mRNA prior to the formation of the E complex and form the heterogeneous or H complex (Matlin et al., 2005). Some splicing repressors may prevent the assembly of spliceosomal components by blocking their RNA binding sites (Merendino et al., 1999; Valcarcel et al., 1993). However, the spliceosomal targets of most splicing activators and repressors are not known.

Polypyrimidine tract binding protein (PTB) is a splicing repressor that affects many alternative exons (Black, 2003; Wagner and Garcia-Blanco, 2001). These target exons are frequently surrounded by multiple PTB binding sites that are essential for repression (Carstens et al., 2000; Chan and Black, 1997; Charlet et al., 2002; Chou et al., 2000; Gromak et al., 2003; Southby et al., 1999). However, the mechanism of this PTB-mediated

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repression is not clear. One example of a highly tissue-specific splicing choice regulated by PTB is the N1 exon of the c-src pre-mRNA. The N1 exon is spliced into the c-src mRNA in most neurons but skipped in other cell types (Levy et al., 1987; Martinez et al., 1987). This exon is controlled by both intronic splicing enhancer and silencer elements (Chan and Black, 1995; Chou et al., 2000; Modafferi and Black, 1997, 1999). PTB-dependent repression of the N1 exon has been reconstructed in an in vitro splicing system that mirrors the regulation of the exon in cells (Black, 1992). Model c-src pre-mRNAs containing the N1 exon and its PTB binding sites are spliced in an extract from WERI-1 retinoblastoma cells, as is seen in the endogenous WERI-1 c-src mRNA. In contrast, splicing of N1 is repressed in an extract from nonneural HeLa cells. Depletion of PTB from the HeLa extract eliminates splicing repression (Chou et al., 2000). This repression requires PTB binding to sites in both the upstream and downstream introns (Amir-Ahmady et al., 2005; Chan and Black, 1995, 1997; Chou et al., 2000).

In examining the mechanism of splicing repression by PTB, we studied spliceosome assembly on the N1 exon containing RNAs in HeLa and WERI-1 extracts. We show here that the transition from the H complex to the E complex is a key regulatory step in splicing repression by PTB. We find that in contrast to some of the models for its action, PTB does not affect the binding of the U1 snRNP to the N1 exon 5' splice. Instead, PTB prevents the 5' splice site-dependent assembly of the essential splicing factor U2AF onto the intron downstream of N1.

## Results

### PTB Blocks Formation of the Prespliceosomal E Complex

As a first step in understanding the mechanism of repression, we wanted to identify the step in spliceosomal assembly blocked by PTB. The BS713 pre-mRNA shows regulation in the in vitro splicing system and contains the exons N1 and 4 and, importantly, the PTB binding sites in the upstream N1 exon 3' splice site and in the downstream intron (Figure 1A). The 5' splice site of the upstream exon 3 is deleted to ensure that splicing can occur only on the downstream intron and that the transcript assembles a single spliceosome. WERI-1 extract exhibits 8- to 10-fold lower PTB-dependent repression of splicing than HeLa extract (Amir-Ahmady et al., 2005; Chan and Black, 1997; Chou et al., 2000). Thus, the BS713 transcript is spliced efficiently in WERI-1 extract but is significantly repressed in HeLa extract (Figure 1B). For spliceosome assembly analysis, the BS713 transcript was incubated in HeLa or WERI-1 extracts in the presence of ATP, treated with heparin, and the spliceosomal complexes were separated on native agarose gels (Das and Reed, 1999). In HeLa extract, assembly did not proceed past the H complex (Figure 1C lanes 1–3). In WERI-1 extract, the expected time-dependent formation of A, B, and C complexes was observed on the downstream intron (Figure 1C lanes 4–6). Thus, PTB blocked spliceosome assembly prior to formation of the A complex.

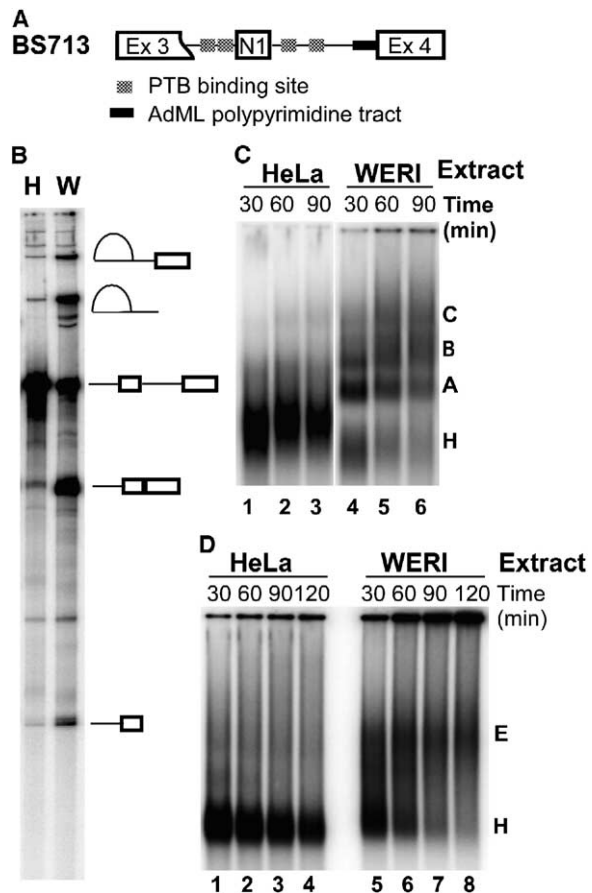


Figure 1. A Prespliceosomal E Complex Forms on the BS713 Pre-mRNA in WERI-1 Extract, but Not in HeLa Extract

(A) Map of the BS713 construct, which has the 5' splice site of exon 3 deleted and the AdML polypyrimidine tract introduced into the exon-4 3' splice site.

(B) In vitro splicing of the BS713 transcript in HeLa (H) and WERI-1 (W) extract. The RNA splicing products and intermediates are diagrammed to the right.

(C) Spliceosomal complex formation on the BS713 transcript in presence of ATP. BS713 pre-mRNA was incubated in HeLa (lanes 1–3) and WERI-1 (lanes 4–6) nuclear extract under splicing conditions for the indicated times and separated by native agarose gel. Positions of the H, A, B and C complexes are indicated.

(D) ATP independent prespliceosomal complex formation. BS713 pre-mRNA was incubated in HeLa (lanes 1–4) and WERI-1 (lanes 5–8) extract in the absence of ATP for the indicated times and separated on a 1.5% native agarose gel. The H and E complexes are marked.

The reaction and gel conditions that resolve the A, B, and C complexes do not distinguish the H complex from the prespliceosomal E complex. The formation of E complex is ATP independent and occurs at 30°C, but not at 4°C (Das and Reed, 1999). The U1 and U2 snRNPs are less tightly bound in the E complex than in later complexes, requiring separation conditions lacking heparin (Das and Reed, 1999). Under these reaction and gel conditions, the BS713 transcript assembled an E complex in WERI-1 extract (Figure 1D lanes 5–8). As seen by others, the E complex formed upon incubation at 30°C and was eliminated with heparin (data not

shown). This E complex required both splice sites in the pre-mRNA to form and contained the known components of the E complex (see below). Significantly, in HeLa extract, no E complex assembly could be detected on the BS713 RNA (Figure 1D lanes 1–4). This indicates that PTB prevents the transition from H to E complex.

Repression of N1 splicing requires the presence of both the upstream and downstream PTB binding elements. The upstream CU elements are within the larger polypyrimidine tract of the N1 exon 3' splice site and bind PTB with high affinity (Amir-Ahmady et al., 2005). The CU elements in the downstream intron are interspersed with binding sites for other proteins (Markovtsov et al., 2000). Previous mutagenesis experiments have shown that the ability of the upstream PTB binding region to function as the N1 3' splice site has no effect on the splicing of the downstream intron. Instead, it is the PTB sites in this region that are the key feature in N1 regulation (Amir-Ahmady et al., 2005; Chan and Black, 1995, 1997). To confirm that the block in the spliceosome pathway in the HeLa extract was dependent on PTB binding, we made the BS713D construct (Figure 2A). In BS713D, the PTB binding element in the 3' splice site of N1 is changed to a sequence that shows complete loss of PTB binding in an electrophoretic mobility shift assay (Amir-Ahmady et al., 2005). In HeLa extract, the BS713D RNA splices about 10-fold better than BS713 (Figure 2B lanes 3 and 4), and in WERI-1 extract both transcripts splice with equal efficiency (lanes 1 and 2). We also carried out native gel analysis for the formation of splicing complexes. Unlike BS713, the BS713D RNA forms the E complex, as well as the A, B, and C spliceosomal complexes in HeLa extract (Figure 2C). Thus, mutation of one set of the PTB binding sites leads to loss of splicing repression and allows assembly of the spliceosomal complexes in HeLa extract.

#### PTB Does Not Interfere with U1 snRNP Binding to the N1 Exon 5' Splice Site

To examine whether PTB was affecting the binding of splicing factors essential for the formation of the E complex, we purified the H and E complexes that assemble on the BS713 pre-mRNA in the two extracts. For this, we adapted the MS2 affinity tag procedure (Jurica et al., 2002; Zhou et al., 2002a, 2002b). Three RNA hairpins that bind the MS2 coat protein were introduced at the 3' end of exon 4 to create BS713-MS2. The addition of these MS2 hairpins does not alter the in vitro splicing pattern of the pre-mRNAs (data not shown). MS2-MBP fusion protein was prebound to the pre-mRNA followed by addition of nuclear extract lacking ATP, to prevent progression into later spliceosomes in the WERI-1 extract. The assembly process was monitored by native gel analysis until greater than 90% of the RNA was present in the H complex at 4°C or in the E complex at 30°C (Figure 1C and data not shown). After incubation, the reaction mixtures were separated by glycerol density gradient centrifugation and assessed for the sedimentation profile of the labeled pre-mRNA (Hartmuth et al., 2002; Jamison et al., 1992). The complex of the RNA with the MS2-MBP fusion protein, as-

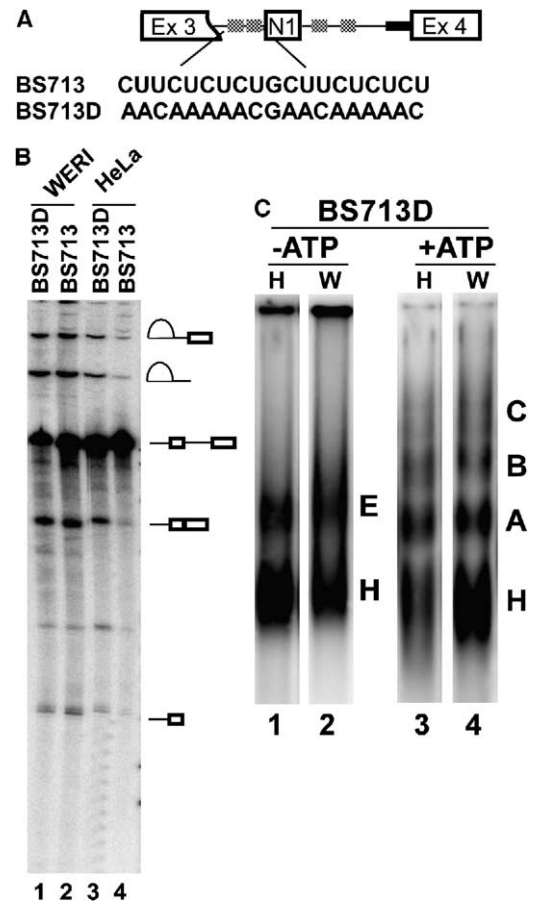


Figure 2. Mutation of One Set of PTB Binding Sites Allows E Complex Formation in HeLa Extract

(A) BS713D construct carries PTB binding site mutations in the N1 exon 3' splice site as shown. (B) In vitro splicing of the BS713D transcript (lanes 1 and 3) in WERI-1 and HeLa extract is compared to BS713 (lane 2 and 4). (C) Formation of spliceosomal complexes on the BS713D RNA in absence (lanes 1 and 2) and presence (lanes 3 and 4) of ATP in HeLa and WERI-1 extracts. Gel conditions are as described in Figure 1. Positions of the H, E, A, B, and C complexes are indicated.

sembled in a reaction lacking nuclear extract, had a sedimentation coefficient of less than 18S (Figure 3A). The H complexes that assembled on BS713-MS2 in the two extracts were indistinguishable: both sediment at 30S (Figure 3A). The E complex assembled on this RNA in WERI-1 extract was distinctly larger than the H complex, with a sedimentation coefficient of 40S. These gradient profiles agree with the observations by native gel and with previous observations on unregulated introns (Hartmuth et al., 2002; Jamison et al., 1992). Peak fractions were pooled for each complex, which was then purified on amylose resin (see the Experimental Procedures). Once isolated, the complexes were stable and showed the expected mobility on glycerol gradients or native gels (data not shown).

The purified complexes were then analyzed for their RNA content. RNA was extracted, 3' end labeled, and analyzed on denaturing gels (Figure 3B). As expected, no splicing intermediates or products were detected in

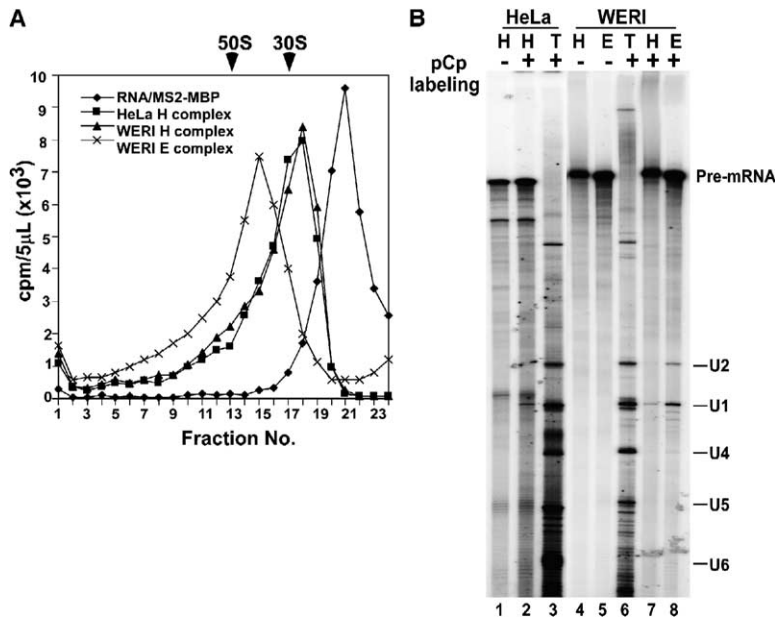


Figure 3. Purification and RNA Content of the H and E Complexes

(A) Prespliceosomal complexes assembled on the BS713-MS2 pre-mRNA in HeLa or WERI-1 extract were fractionated on 15%–30% glycerol density gradients. The position of the labeled pre-mRNA was determined by scintillation counting. The positions of the *E. coli* 50S and 30S ribosomes used as markers are indicated. The HeLa H and WERI-1 H complexes (squares and triangles) were assembled at 4°C and have equivalent mobility that is distinct from the MS2-MBP complex (diamonds) and the E complex (crosses) assembled at 30°C. The H and E complex fractions were pooled and purified on amylose resin.

(B) RNA was extracted from the purified H and E complexes and 3' end labeled with <sup>32</sup>P-pCp. Total RNA from nuclear extract (T) was used as markers for the U snRNAs. The positions of the U snRNAs and pre-mRNA are indicated.

either the H or the E complexes (Figure 3B, lanes 1, 4, and 5). The BS713 H complexes, from either the HeLa or the WERI-1 extract, contained U1 snRNA (Figure 3B, lanes 2 and 7). The WERI-1 E complex contained higher levels of U1 and also contained U2 snRNA (Figure 3B, lane 8).

To determine the site of binding of the U1 snRNP on the pre-mRNA, we performed psoralen crosslinking experiments. The BS713 RNA was incubated in HeLa or WERI-1 extracts at 30°C in the absence of ATP (Figure 4A). 4'-Aminomethyl-4,5',8-trimethylpsoralen (AMT psoralen) was then added and the reactions subjected to crosslinking (Tarn and Steitz, 1994). When resolved on a denaturing gel, intramolecular crosslinked products were seen in the absence of nuclear extract (lane 1). No crosslinked products were seen in the absence of psoralen or UV irradiation in either extract (lanes 2, 3, 7, and 8). In both HeLa and WERI-1 extracts, we observed one intermolecular crosslinked band (lanes 4 and 9). This product was eliminated in extract pretreated with RNase H and an oligonucleotide complementary to the 5' end of U1 snRNA (U1<sub>1–15</sub>; lanes 5 and 10) but was not affected by an oligonucleotide complementary to U2 snRNA (U2<sub>28–42</sub>; lanes 6 and 11).

To map the site of crosslinking precisely, we performed primer extension analysis with an antisense primer in the c-src intron (Figure 4B). This identified a crosslinking-dependent reverse transcriptase stop in both the HeLa and WERI-1 samples (Figure 4B, lanes 5, 6, and 8). No product was seen in extracts treated with the U1 oligonucleotide (lanes 7 and 9). Alignment with the sequencing ladder mapped the crosslink to nucleotide A<sub>7</sub> (the seventh nucleotide of the intron downstream of N1). This primer extension stop is precisely that predicted for the crosslinking of U1 snRNA to the N1 exon 5' splice site. Thus, the N1 exon 5' splice site is occupied by the U1 snRNP with equal efficiency in HeLa and WERI-1 extracts.

#### The U1 snRNP within Both the HeLa and WERI-1 Complexes Is Functional

H complexes have not always been described as containing U1 or other essential splicing components (Jurica and Moore, 2003). To examine whether the U1 snRNP in the H complex was functional, we tested the splicing activity of H complexes in extract in which the U1 snRNA was inactivated. The splicing activity of these complexes was compared with the activity of naked BS713-MS2 RNA and with purified E complex. Degrading the U1 snRNA with RNase H eliminated splicing of BS713-MS2 RNA in both HeLa and WERI-1 extracts (Figure 4C, lanes 1–4). When added back to WERI-1 extract, the purified H complex from HeLa and the H and E complexes from WERI-1 extract were active (Figure 4C, lanes 6, 8, and 10). When added back to HeLa extract, splicing of all the complexes was repressed (Figure 4C lanes 5, 7, and 9). This indicates that in WERI-1 extract, but not in HeLa extract, the U1 snRNP in the H complex allows the complex to progress through the splicing pathway.

The activity of each reaction above was determined by the extract the complex was added to and not the extract the complex was derived from. This was not surprising, as earlier work on constitutive spliceosome assembly found that H complexes are not committed precursors to the spliceosome (Jamison et al., 1992; Michaud and Reed, 1991). When competed with an excess of cold RNA containing a 5' splice site, the labeled RNA in these complexes can be prevented from assembling further, indicating that spliceosomal components can exchange out of these complexes. This prevented the assessment of these complexes for functional properties in crude extracts (Jamison et al., 1992; Michaud and Reed, 1991). We repeated these competition experiments on the BS713 RNA in our two extracts and obtained equivalent results (data not shown). We also carried out similar experiments with the purified

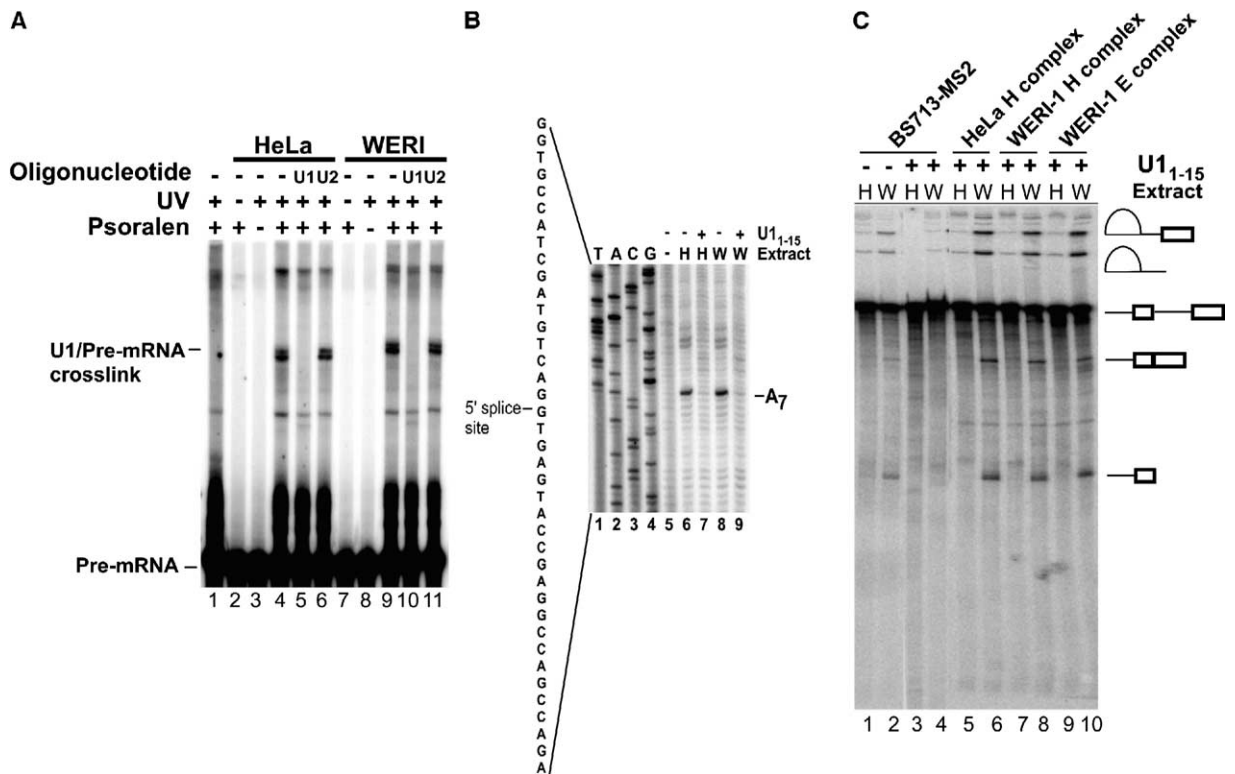


Figure 4. The U1 snRNP Is Base Paired to the N1 Exon 5' Splice Site in Both HeLa and WERI-1 Extract and Is Functional

(A) The BS713 RNA was incubated alone (lane 1) or in HeLa (lanes 2–6) or WERI-1 extract (lanes 7–11), in the absence of ATP, at 30°C for 90 min. Extracts were mock treated (lanes 4 and 9) or pretreated with oligonucleotide U1<sub>1-15</sub> (lanes 5 and 10) or oligonucleotide U2<sub>28-42</sub> (lanes 6 and 11). AMT-psoralen was added as indicated at the top. Lane 1 shows the position of intramolecular crosslinks. The U1/pre-mRNA crosslink is indicated to the right.

(B) The crosslinked site was mapped by primer extension using an oligonucleotide complementary to nucleotides 65–86 of the intron downstream of exon N1. Dideoxy sequencing reactions using the same oligonucleotide are shown: ddA (lane 1), ddT (lane 2), ddG (lane 3), and ddC (lane 4). Primer extension was carried out on RNA extracted from reactions in which the BS713/BamHI RNA was incubated in mock-treated (lanes 6 and 8) or oligonucleotide- (U1<sub>1-15</sub>) treated extract (lanes 7 and 9).

(C) The U1 snRNP in the purified complexes is active for splicing. HeLa and WERI-1 extract was preincubated in the presence (lanes 3–10) or absence (1 and 2) of DNA oligonucleotide complementary to U1 snRNA (U1<sub>1-15</sub>). Equivalent amounts of BS713 pre-mRNA (lanes 1–4), purified HeLa H complex (lanes 5 and 6), WERI-1 H complex (lanes 7 and 8), or WERI-1 E complex (lanes 9 and 10) were added to the treated extracts and incubation was continued for 2 hr. The splicing intermediates and products are shown at the right.

complexes in which the complexes were added back to the HeLa and WERI-1 extracts in presence of BS713 as a cold competitor RNA (Figure S1 available online with the Supplemental Data). Splicing activity in all the complexes could be competed away by the cold competitor, in agreement with the previous observations on the AdML RNA (Jamison et al., 1992; Michaud and Reed, 1991). However, the purified H complexes showed enhanced splicing kinetics compared to the naked BS713-MS2 RNA, indicating that these complexes do indeed contain components needed to progress to the E complex.

**PTB Blocks Assembly of the Essential Splicing Factor U2AF onto the Intron Downstream of Exon N1**  
To examine if PTB affected the binding of factors essential for E complex assembly other than U1 snRNP, we determined the protein composition of each of the purified H complexes. Proteins were precipitated from the purified H complexes, separated by SDS-PAGE,

and stained with colloidal blue (Figure 5A). Proteins from each band were identified by MALDI mass fingerprinting. Gel regions between stained bands were also analyzed to identify poorly stained proteins. Proteins specific to each complex as well as proteins common to both complexes are listed in Table S1. Each complex contained about 40 proteins, most of which were common to both complexes, including the U1 snRNP proteins and many hnRNP proteins. Each complex also contained several specific proteins. Most notably, both subunits of U2AF were found only in the WERI-1 H complex.

To confirm the results of the mass spec analysis, we carried out immunoblots for many proteins in the different BS713-MS2 complexes (Figure 5B). Probing for essential splicing factors and spliceosomal proteins showed that the U1 snRNP-specific protein U170K was present in all of the complexes, as was Splicing Factor 1 (SF1; Figure 5B). SF1 was missed in the MS analysis of the WERI-1 complex, presumably because it was ob-

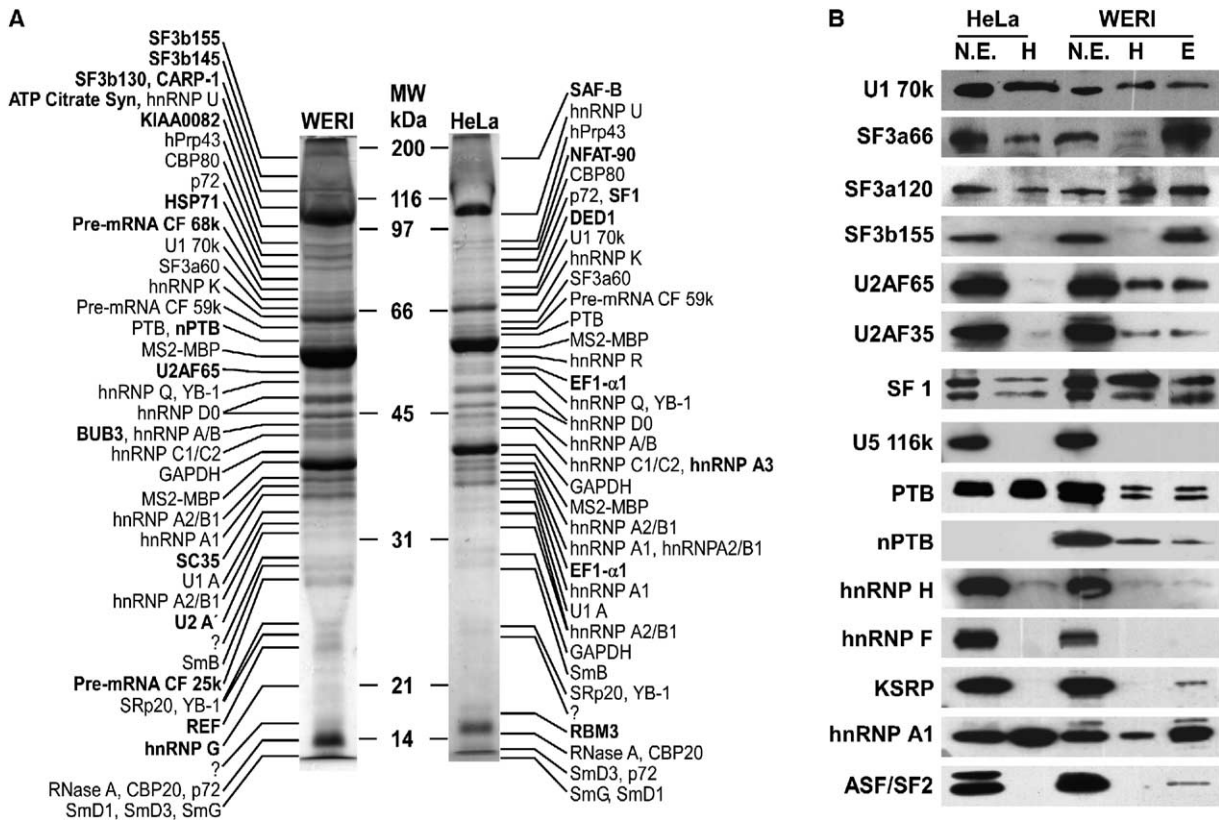


Figure 5. HeLa H Complex Lacks the Essential Splicing Factor U2AF

(A) The purified WERI-1 and HeLa H complexes were digested with RNase A, separated by 10% SDS-PAGE, and stained with colloidal Coomassie blue. Each band was excised and its protein components identified by mass spectrometry. The identity of each band is shown to the side. Proteins specific to each of the complexes are shown in bold. The question marks indicate proteins that could not be identified. Note that some proteins comigrating with other abundant species could be missed in the mass spectrometry identification. Some of these, such as U2AF35 and SF1, were confirmed by immunoblot.

(B) Proteins from the purified HeLa H complex and the WERI-1 H and E complexes were separated by SDS-PAGE, blotted to nitrocellulose, and probed with the antibodies against the indicated proteins. Total protein from HeLa and WERI-1 extracts (N.E.) was run in parallel lanes as controls.

scured by other proteins. Both U170K and SF1 are components of the E complex. The mature U2 snRNP is a 17S particle that contains the 12S core snRNP plus the trimeric SF3a complex and the eight proteins of the SF3b complex (Nesic and Kramer, 2001; Reed, 2000; Will et al., 2002). The presence of SF3b protein, SF3b155, in the WERI-1 E complex correlated well with the observed U2 snRNA (Figure 3). This protein was abundant in the E complex, very weakly present in the WERI-1 H complex, and not detected in the HeLa H complex. In contrast, proteins of the SF3a complex, SF3a66 and SF3a120, were present in the HeLa H complex in the apparent absence of the U2 snRNP. Most significantly, both subunits of the essential splicing factor U2AF (65 and 35) were present only in the H and E complexes assembled in WERI-1 extract, confirming the MS results that these proteins were absent from the HeLa H complex (Figures 5A and 5B).

We previously identified several proteins that interact with the c-src pre-mRNA and implicated some of these in the regulation of its splicing. These proteins include KSRP; FBP; PTB; nPTB; hnRNPs H, F, and A1; and the

SR protein ASF/SF2 (Chan and Black, 1997; Chou et al., 1999, 2000; Markovtsov et al., 2000; Min et al., 1995, 1997; Rooke et al., 2003). We also examined these proteins by immunoblotting (Figure 5B). The BS713 H complex from HeLa extract contained abundant PTB and hnRNP A1 and lower amounts of hnRNP H. These same proteins were present in the H complex from WERI-1 extract, which also contained the neuronal homolog of PTB, nPTB. All these proteins were also found in the E complex from WERI-1 extract, which interestingly also contained KSRP and a small amount of ASF/SF2. The hnRNP F protein, which binds to a short RNA sequence from the enhancer region in WERI-1 extract, was not seen in any of these complexes (Caputi and Zahler, 2002; Markovtsov et al., 2000; Min et al., 1995).

Heparin is often used as a competitor of nonspecific RNA-protein interactions in the purification of the mature spliceosomes (Hartmuth et al., 2002; Jurica et al., 2002; Makarov et al., 2002). To examine whether heparin treatment would compete away less tightly bound proteins in the H complex and reduce it to a stable core complex, we also isolated the BS713 H complexes after

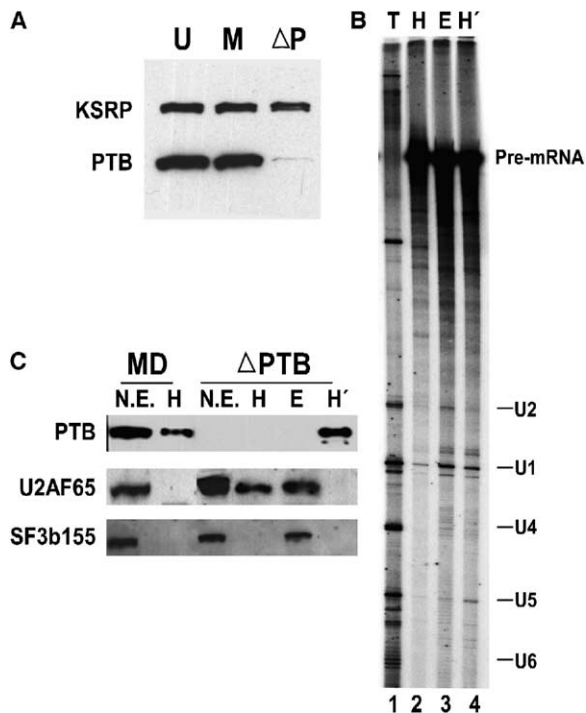


Figure 6. Depletion of PTB from HeLa Extract Allows Binding of U2AF to BS713-MS2 Pre-mRNA

(A) Immunodepletion of PTB from HeLa extract was carried out with the monoclonal anti-PTB antibody BB7. Immunoblot analysis of the untreated (U), the mock-depleted (M) and the PTB-depleted ( $\Delta$ P) extract used monoclonal antibodies AB5 (anti-KSRP) and BB7 (anti-PTB).

(B) The H and E complexes were assembled in PTB-depleted HeLa extract. Recombinant PTB was added back to the depleted extract to 500 nM and the H' complex was assembled in this extract by incubating the RNA at 30°C for 60 min. RNA from the purified complexes labeled with  $^{32}$ P-pCp is shown. Total RNA from nuclear extract (T) was used as markers for the U snRNAs. The positions of the U snRNAs and pre-mRNA are indicated.

(C) Total protein from the purified complexes assembled in the mock-depleted (MD) and PTB-depleted ( $\Delta$ PTB) nuclear extracts probed using antibodies against the proteins indicated. Total protein from nuclear extract (N.E.) was run in a parallel lane as control.

heparin treatment. In agreement with earlier results, we found that the associations of the U1 and U2 snRNPs in the H and E complexes were sensitive to heparin (data not shown). Importantly, heparin treatment did not just leave a core complex but, instead, led to the binding of new proteins to the pre-mRNA and a composition that was very different from the native complex (Table S2).

#### Depletion of PTB Allows U2AF65 Binding to BS713 RNA in HeLa Extract

If PTB was actively preventing U2AF assembly in HeLa extract, then U2AF binding should be restored by the removal of PTB. HeLa extract was immunodepleted for PTB using the monoclonal antibody BB7 (Figure 6A). This restored E complex formation and splicing as seen previously (data not shown and Chou et al., 2000). H and E complexes were assembled in the PTB-depleted

extracts and purified. As seen before, the H complex contains only the U1 snRNA, whereas the E complex contains U2 snRNA and additional U1 (Figure 6B, lanes 2 and 3). To confirm that the E complex formation resulted from the removal of PTB and not any other protein, recombinant PTB was added back to the immunodepleted extract. The BS713-MS2 RNA was incubated in this extract at 30°C and the assembled complex was purified. This was designated the H' complex to distinguish it from the H complex that is assembled at 4°C. The addback of PTB to the immunodepleted extract led to the loss of U2 snRNA binding but did not affect U1 binding (Figure 6B, compare lanes 3 and 4).

To confirm that PTB depletion allowed U2AF binding, we immunoblotted the H, E, and H' complexes for PTB, U2AF65, and SF3b155 proteins (Figure 6C). The H complex prepared from the mock-depleted extract contained PTB, but not U2AF65 and SF3b155 proteins. As expected, PTB was not present in the H and the E complexes prepared from the immunodepleted HeLa extract but was present in the H' complex assembled after PTB addback. The SF3B155 protein was present only in the E complex and was not present in the H and the H' complexes. On the other hand, the U2AF65 protein was present in both the H and the E complexes assembled in the depleted extract. Binding of U2AF65 was lost upon addback of PTB to form the H' complex. Thus, the assembly of the U2AF complex at the 3' splice site downstream of N1 is specifically prevented by PTB. There is no observable difference between the two extracts in the U2AF binding activity for this RNA.

#### PTB-Mediated Inhibition of U2AF65 Is Dependent on the Location of the 5' Splice Site

There were several possible mechanisms for how PTB might prevent U2AF assembly. In addition to binding at the N1 exon sites, it was possible that PTB was associating with the downstream polypyrimidine tract. Such an interaction has been observed, but is very weak relative to the binding of U2AF to this site (Singh et al., 1995). The more interesting possibility was that PTB was blocking an interaction of the U1 snRNP at the 5' splice site that is needed for U2AF assembly (Abovich and Rosbash, 1997; Cote et al., 1995; Kent et al., 2005; Li and Blencowe, 1999). In the normal src pre-mRNA, when the splicing of exon N1 is repressed, src exon 3 is joined to exon 4. This splicing event should also require U2AF binding to the 3' splice site of exon 4. Thus, if PTB was acting locally at the N1 exon, then the PTB bound to sites that inhibit N1 exon splicing should not inhibit U2AF binding during spliceosome assembly between exons 3 and 4. To examine this, we prepared the BS714 construct. BS714 has the 5' splice site of exon 3 restored and the 5' splice site of the N1 exon deleted (Figure 7A). This transcript has the PTB binding elements intact but offers an alternative functional 5' splice site. It should splice in either extract and still assemble a single spliceosome for analyzing on native gels and gradients. In vitro splicing assays, using the BS714 RNA, show that the major splicing event in both HeLa and WERI-1 extracts is the joining of exon 3 to exon 4 (Figure 7B). As seen previously on full three-exon RNAs, splicing of exon 3 to the N1 exon does not

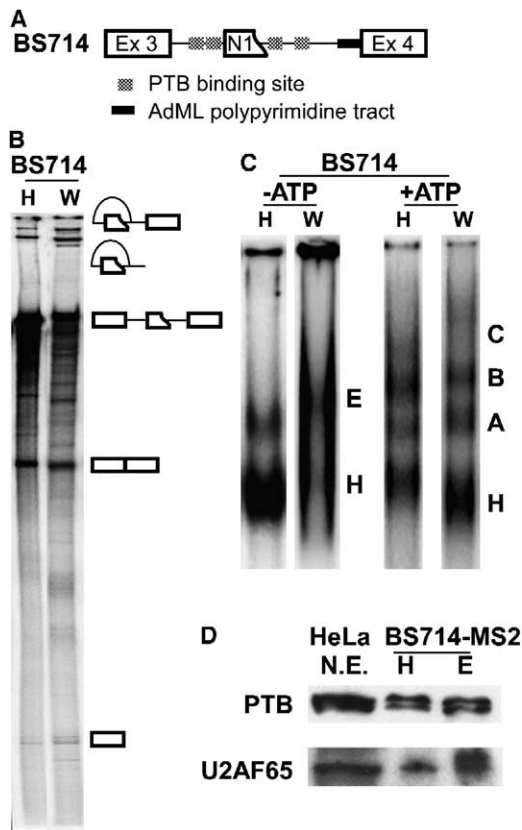


Figure 7. PTB-Mediated Inhibition of U2AF Binding Is Dependent on the Location of the 5' Splice Site

(A) Map of the BS714 pre-mRNA construct. The 5' splice site of Exon N1 is deleted and the 5' splice site of exon 3 is restored.

(B) In vitro splicing of the BS714 transcript was carried out in HeLa (H) and WERI-1 (W) extracts. The RNA splicing products and intermediates are shown to the right.

(C) Formation of the splicing complexes on the BS714 RNA in HeLa (H) and WERI-1 (W) extracts in the absence (-ATP) and presence (+ATP) of ATP.

(D) The H and E complexes assembled on BS714-MS2 RNA in HeLa extract and purified on amylose resin. Total protein from the purified complexes was probed with antibodies to PTB and U2AF65. Total protein from nuclear extract (N.E.) was run in a parallel lane as control.

occur (Chan and Black, 1997; Markovtsov et al., 2000). In the absence of ATP, BS714 forms an E complex in both HeLa and WERI-1 extracts. In the presence of ATP, it forms A, B, and C complexes as expected (Figure 7C).

The H and the E complexes assembled on the BS714-MS2 transcript in HeLa extract were purified and probed for PTB and U2AF65. Both proteins were present in both the H and E complexes. Thus, the repression of U2AF binding by PTB depends on the location of the 5' splice site. If only the 5' splice site for the repressed exon is present, U2AF does not assemble even though the U1 snRNP is bound to the transcript. If the unregulated 5' splice site from the upstream exon is present, PTB does not affect U2AF binding and spliceosomal complex formation. Thus, PTB is likely blocking a feature of the 5' splice site complex at the repressed exon that is required for U2AF assembly.

## Discussion

We examined the repression of a tissue-specific exon in the context of the spliceosome assembly pathway. The N1 exon is repressed by PTB in nonneural cells, but this repression is relieved in neuronal cells to allow splicing (Black, 1992; Chou et al., 2000; Modafferi and Black, 1999). Reconstituting this repression in vitro, we find that in neuronal WERI-1 cell extracts in which the N1 exon is spliced, spliceosome assembly proceeds through the typical pathway of E, A, B, and C complexes. In contrast, in nonneural HeLa cell extracts, the assembly of the spliceosome is blocked prior to the formation of the prespliceosomal E complex. Removal of PTB, either by depletion or by binding site mutations, allows E complex formation. Thus, the key event in splicing repression by PTB lies prior to the formation of the E complex.

The U1 and U2 snRNPs and the spliceosomal proteins SF1 and U2AF are all components of the E complex (Das et al., 2000; Kent et al., 2005). The U1 snRNP, SF1, and U2AF are all in direct contact with the RNA, whereas the U2 snRNP is loosely associated with the E complex via an interaction between SF3b155 and U2AF65 (Gozani et al., 1998). The 5' splice site and the branchpoint become juxtaposed during E complex formation, but the point in spliceosome assembly where splice site pairing becomes irreversible is an unresolved issue (Kent and MacMillan, 2002). The splicing of an intron becomes resistant to RNA competitors upon formation of the E complex (Jamison et al., 1992; Michaud and Reed, 1991). However, the choice of actual splice sites can still be altered by SR proteins up until the formation of the A complex (Lim and Hertel, 2004). Whether the choice becomes fixed at the E or A complex, by acting early in assembly to prevent formation of a particular E complex, PTB still allows other choices of splice site pairing.

It has been proposed that PTB represses splicing by directly blocking assembly of factors at splice sites (Black, 2003; Wagner and Garcia-Blanco, 2001). This is likely to occur in some cases (Liu et al., 2002). However, we find that in repressing the splicing of exon N1 to exon 4, PTB does not affect the binding of the U1 snRNP to the 5' splice site of the N1 exon. In contrast, U2AF, which needs to bind downstream from the PTB complex to the polypyrimidine tract of exon 4, does not assemble into the prespliceosome. Relative to U2AF, PTB can only weakly interact with the Adenovirus polypyrimidine tract present at exon 4 in these constructs (Chan and Black, 1995; Singh et al., 1995). It was possible that in the presence of the PTB complex upstream, such an interaction could inhibit splicing. However, when exon N1 is repressed, this exon 4 splice site needs to pair with the 5' splice site of exon 3. Indeed, we find that the presence of the upstream 5' splice site of exon 3 allows U2AF assembly and splicing to exon 4 even in the presence of the PTB complex around the N1 exon. Thus, PTB repression acts only on the U2AF needed to form an N1 exon E complex and does not affect U2AF binding to the same site for the exon 3 E complex.

Several studies have demonstrated cooperation between the 5' and 3' splice sites during E complex as-

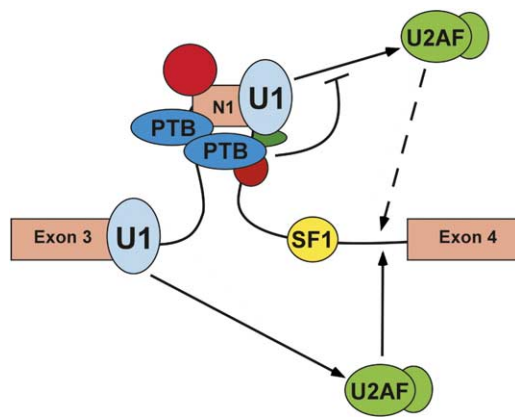


Figure 8. Model for PTB-Mediated Exon Silencing

PTB binds to the CU elements across N1 exon and forms a silencing complex. This complex does not affect U1 snRNP binding at the N1 exon 5' splice site but blocks the interaction of the U1 snRNP with U2AF and, thus, prevents assembly of U2AF at the downstream 3' splice site and formation of the E complex on this intron. The PTB complex does not interfere with the interaction of U2AF with the U1 snRNP at the 5' splice site of exon 3, allowing splicing of exon 3 to exon 4.

sembly (Kent and MacMillan, 2002; Kent et al., 2005; Lamond et al., 1987; Michaud and Reed, 1993; Reed, 2000). In both yeast and mammalian extracts, the binding of U2AF to the 3' splice site can require the U1 snRNP (Abovich and Rosbash, 1997; Cote et al., 1995; Kent et al., 2005; Li and Blencowe, 1999). The MacMillan laboratory recently showed that U2AF assembly requires the prior formation of the E' complex containing U1 and SF1 (Kent et al., 2005). Thus, in preventing U2AF binding, PTB could block either E' complex or E complex formation. Interestingly, SF1 in the HeLa H complex seems to be reduced relative to the WERI-1 H complex (Figure 5B; although we do not know the site of SF1 binding). U2AF is known to interact with SF1 bound at the branchpoint (Berglund et al., 1998; Kielkopf et al., 2004; Selenko et al., 2003). However, the nature of the bridging interaction between the 5' and 3' splice sites within the E' and E complexes is not known. We show here that this interaction can be a key point in the control of alternative splice site choice. This will be an interesting focus for further studies.

A model for the action of PTB is presented in Figure 8. Under PTB repression, the PTB silencing complex across the N1 exon prevents the U1 snRNP bound at the N1 5' splice site from interacting with U2AF and forming an E complex. In this situation, the U1 snRNP bound to exon 3 is still able to interact with U2AF. This promotes formation of an E complex bridging exons 3 and 4 and skipping N1. In the absence of PTB, the U1 at the N1 5' splice site functions normally to form an E complex between exons N1 and 4, promoting N1 exon splicing. This model of repression is applicable to many other examples of PTB-mediated splicing repression (Carstens et al., 2000; Charlet et al., 2002; Gromak et al., 2003; Southby et al., 1999; Wagner and Garcia-Blanco, 2001).

An important next step in these studies will be to

identify the interactions between the U1 snRNP and U2AF that are blocked by PTB. Components of the U1 snRNP are targets of several splicing regulatory proteins. TIA-1 protein interacts with the U1-C protein to promote recruitment of the U1 snRNP to some 5' splice sites (Forch et al., 2002). On the other hand, the P element somatic inhibitor protein (PSI) represses splicing via an interaction with the U1 70K protein (Labourier et al., 2001). Interestingly, both PTB and the U1 snRNP are components of a polyadenylation repressor complex, although their interaction is not known (Lou et al., 1999, 1998). Several studies have indicated that the repressor function of PTB is separable from its RNA binding activity (Liu et al., 2002; Wollerton et al., 2001). In particular, the ability to repress splicing with a PTB-MS2 fusion protein should allow the mapping of the PTB domains that interact with spliceosomal components (Gromak et al., 2003; Wagner and Garcia-Blanco, 2002).

Another intriguing question is why PTB activity is low in WERI-1 extracts. Both PTB and its neuronal homolog, nPTB, are present in the WERI-1 extract, although each is less abundant than PTB in HeLa extract. The addition of PTB to WERI-1 extract strongly inhibits splicing of PTB-dependent exons (Amir-Ahmady et al., 2005). Interestingly, the only cell type-specific factor identified in the WERI-1 complexes was nPTB. The nPTB protein appears to be neutral in its affect on splicing. It does not increase N1 splicing when added to HeLa or WERI-1 extract. Conversely, and unlike PTB, it does not inhibit splicing when added to WERI-1 extract or to PTB-depleted HeLa extract (Markovtsov et al., 2000 and data not shown). Thus, by substituting for PTB in WERI-1 extract, nPTB may prevent PTB-mediated exon silencing. It will be interesting to examine the crosslinking of these two PTBs to the different repressor sites within the BS713 complexes and to look at when certain proteins assemble at particular sites.

Several other proteins bind to the downstream regulatory region of N1 and have potential positive effects on N1 exon splicing, including KSRP, hnRNPs H and F, and the Fox proteins (Chou et al., 1999; Min et al., 1995, 1997, and J. Underwood and D.L.B., unpublished data). The Fox proteins bind to the important enhancer element UGCAUG but are mostly absent from WERI-1 cells. hnRNP H binds to a GGGGG element adjacent to the PTB binding site within the conserved core of the downstream regulatory region. Like PTB, hnRNP H was also observed in the H complexes. In contrast, KSRP was observed in the E complex, but not the H complex. Interestingly, the *Drosophila* homolog of KSRP (PSI) can engage in interactions with the U1 snRNP (Labourier et al., 2001). Given their interactions with nPTB and the location of their binding sites, these proteins also have potential as modulators of PTB function (Markovtsov et al., 2000).

Recent work by the Valcarcel group indicates that an exon bound PTB can inhibit exon definition (Izquierdo et al., 2005). In direct analogy to what we observe here for an intron, the inhibition of exon definition appears to involve prevention of a U2AF-U1 snRNP interaction. The constructs used in this study were designed to be short enough to allow assessment of the spliceosome assembly pathway as an intron definition process (Berget, 1995; Sterner et al., 1996). If exon 4 were able to

carry out exon definition prior to splicing, it is likely that the 3' splice site would assemble U2AF even in the presence of PTB upstream. When splice sites are recognized via an exon definition process, little is known about how the splicing components subsequently form E or A complexes across introns (Black, 2005). This pairing will presumably also require a U1-U2AF interaction that can be blocked by PTB. The characterization of the transition in spliceosome assembly from exon-defined splice sites to an intronic E complex is an important challenge for the future.

## Experimental Procedures

### In Vitro Splicing and Spliceosome Assembly

Pre-mRNAs were transcribed in vitro from plasmids BS713, BS713D, and BS714 (Figures 1A, 2A, and 7A; Supplemental Data). The N1 5' splice site was improved with changes at positions 180 (U to A), 193 (G to C), and 194 (U to C). The 3' splice site of exon 4 was replaced with a stronger Adenovirus major late exon 2 (AdML) polypyrimidine tract. These mutations improve the kinetics of splicing while maintaining the differential regulation of splicing between the two extracts (Chou et al., 2000).

Nuclear extracts from HeLa and WERI-1 cells were prepared as described previously (Black et al., 1998; Chan and Black, 1995; Dignam, 1990). In vitro splicing was carried out as described (Black, 1992). The spliceosomal complexes were separated and visualized using native agarose gels (Das and Reed, 1999; see Supplemental Experimental Procedures).

### Psoralen Crosslinking

After assembly of splicing complexes at 30°C for 90 min, the reactions were placed on ice. AMT-psoralen was added to a final concentration of 20 µg/mL and the reactions irradiated with 366 nm light for 10 min on ice (Tarn and Steitz, 1994). RNA from the reactions was extracted, ethanol precipitated, separated on 4% urea-PAGE (acrylamide: bisacrylamide ratio of 29:1), and visualized by Phosphorimager.

### Isolation and Analysis of Prespliceosomal Complexes

Purification of the pre-mRNP complexes was carried out as described by Reed and coworkers (Zhou et al., 2002a, 2002b), with some modifications as described in the Supplemental Data. RNA from the purified complexes was extracted with PCA and ethanol precipitated. The RNA was labeled with <sup>32</sup>P-pCp in a 10 µl reaction containing the RNA, RNAGuard, 10 units RNA ligase (NEB), 1 µl of 10x ligase buffer, and 2 µl of <sup>32</sup>P-pCp (3000 Ci/mmol) incubated at 4°C overnight. The RNA was extracted with PCA, ethanol precipitated, and separated on 8% urea-PAGE gel.

To obtain proteins, the complexes were treated with RNase A (0.05 mg/ml) at 37°C for 30 min, and TCA precipitated. The proteins were separated on 10% SDS-PAGE gels and stained with colloidal blue dye (Invitrogen). For Western blots, proteins from 0.3 pmol of complex were separated by SDS-PAGE, blotted to a nitrocellulose membrane, and probed with antibodies. The antibodies included affinity-purified peptide polyclonal antibodies against nPTB (N60IS2; KFKGEDKMDGAPS), hnRNP H (Chou et al., 1999), and hnRNP F (Min et al., 1995); rabbit polyclonal antiserum against full-length U2AF65 (D1770); and mouse monoclonal antibodies against U1 70k (anti-U170), PTB (BB7), KSRP (AB5), hnRNP A1. The following antibodies were kind gifts from other laboratories: AK-96 against ASF/SF2 (A. Krainer), anti-SF3a66 and anti-SF3a120 (A. Kraemer), anti-SF3b155 (R. Reed), anti-U5116k (R. Luhrmann), and anti-U2AF35 (K. Lynch).

Proteins present in the H complexes were identified by mass spectrometry. Proteins from 5 pmol of the HeLa and WERI-1 H complexes were separated on 10% SDS-polyacrylamide gel and stained with the colloidal blue stain (Invitrogen). Individual bands from the stained gels were excised and subjected to in-gel trypsin (Promega) digestion and peptide extraction (Jiménez et al., 1998).

MALDI mass fingerprinting is described in the Supplemental Experimental Procedures.

## Supplemental Data

Supplemental Data including Supplemental Experimental Procedures, one figure, and two tables are available with this article online at <http://www.molecule.org/cgi/content/full/19/4/485/DC1/>.

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