

Polypyrimidine tract binding protein controls the transition from exon definition to an intron defined spliceosome

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The polypyrimidine tract binding protein (PTB) binds pre-mRNAs to alter splice-site choice. We characterized a series of spliceosomal complexes that assemble on a pre-mRNA under conditions of either PTB-mediated splicing repression or its absence. In the absence of repression, exon definition complexes that were assembled downstream of the regulated exon could progress to pre-spliceosomal A complexes and functional spliceosomes. Under PTB-mediated repression, assembly was arrested at an A-like complex that was unable to transition to spliceosomal complexes. Trans-splicing experiments indicated that, even when the U1 and U2 small nuclear ribonucleoprotein particles (snRNPs) are properly bound to the upstream and downstream exons, the presence of PTB prevents the interaction of the two exon complexes. Proteomic analyses of these complexes provide a new description of exon definition complexes, and indicate that splicing regulators can act on the transition between the exon definition complex and an intron-defined spliceosome.

Alternative splicing is a common means of regulation for eukaryotic gene expression^{1–3}. Splicing choices are directed by proteins that bind to specific regulatory sequences in the pre-mRNA to alter spliceosome assembly, but the interactions of these proteins with the splicing apparatus are mostly unknown^{4–6}. Each intron to be excised from a pre-mRNA must be assembled into a spliceosome containing the five small ribonucleoprotein particles (snRNPs) U1, U2, U4, U5 and U6, and multiple auxiliary proteins^{7–9}. *In vitro*, the snRNPs sequentially bind the target intron to form discrete intermediate complexes termed H, E, A, B and C. The H complex contains the U1 snRNP bound to the 5' splice site of the pre-mRNA and many sequence-specific RNA binding proteins, including members of the heterogeneous nuclear ribonucleoprotein (hnRNP) group of proteins¹⁰. The subsequent E complex contains the U2 auxiliary factor (U2AF) and splicing factor 1 (SF1) bound to the 3' splice site and branchpoint, respectively. The U2 snRNP is also present in this complex, but is relatively loosely associated¹¹. In the E complex, the 5' and 3' splice sites are brought together via an interaction between the U1-containing complex and the U2AF complex^{12–14}. The first ATP-dependent step in spliceosome assembly results in the stable association of the U2 snRNP to the branchpoint through RNA base-pairing, and the formation of the A complex. Binding of the U4/U6-U5 tri-snRNP then forms the B complex. Several structural rearrangements in the B complex lead to loss of the U1 and U4 snRNPs, resulting in the C complex^{9,15,16}. Here

the U6 small nuclear RNA (snRNA) is base-paired to the 5' splice site, and the base-pairing between the U4/U6 snRNAs is replaced with a U2-U6 snRNA interaction. This creates the active conformation of the spliceosome; the two-transesterification reactions of splicing occur in the spliceosomal C complex.

Our knowledge of spliceosome assembly is mostly derived from *in vitro* studies of short single-intron splicing substrates, where assembly is primarily driven by cross-intron interactions. In this intron definition model, splice-site recognition and pairing are promoted by interactions between the components at the 5' and 3' splice sites, to form the E complex. However, most vertebrate pre-mRNAs contain several short exons separated by relatively long introns^{17,18}. Initial splice-site recognition in these transcripts is mediated by exon definition interactions, where the binding of splicing components to a 5' splice site promotes U2AF recognition of the 3' splice site lying upstream across the exon^{19,20}. This leads to the formation of an exon definition complex before assembly of a spliceosome across the intron. The conversion from these cross-exon interactions to an intron-defined complex, in which the splice sites are paired, must occur during the formation of an active spliceosome. However, this transition is poorly understood. The components of exon definition complexes have not been determined. For example, it is unclear how they differ from an intronic E complex and whether special factors might be required for their transition to intron defined

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spliceosomes. Understanding these steps will be essential to understanding how regulatory proteins can alter the choice of splice sites.

The polypyrimidine tract binding protein (PTB) has served as a model for a repressor of alternative splicing events in mammalian cells^{21,22}. PTB contains four RNA binding domains of the RNA recognition motif (RRM) type, each of which binds to a short CU-rich element²³. CU-rich sequences are clustered upstream and downstream of PTB-repressed exons, and are sometimes found within the exon itself. The binding of one or more PTB proteins to these elements can block spliceosome assembly by various potential mechanisms²². The binding of PTB to the polypyrimidine tract of a 3' splice site can directly occlude binding of U2AF, or PTB binding within the exon may block exon definition interactions required for U2AF assembly^{24–26}. However, PTB binding sites that flank the repressed exon and its splice sites also prevent its splicing^{27–30}. In this most common case, PTB must block splicing at steps later than the initial recognition of the splice sites¹⁰.

PTB regulates the splicing of many muscle- and neuron-specific exons, such as the neuron-specific N1 exon of the *c-src* pre-mRNA²⁸. The mechanism of N1 splicing has been studied in some detail in an *in vitro* system using extracts from neuronal WERI-1 retinoblastoma cells and non-neuronal HeLa cells^{30–32}. CU-rich elements flanking N1 bind to PTB and are essential for PTB-mediated repression of N1 splicing in HeLa extract. In contrast, in WERI extract PTB is mostly replaced with the inactive homolog nPTB, and N1 splices efficiently. We showed previously that in HeLa extract PTB does not interfere with U1 snRNP assembly on the N1 5' splice site¹⁰. Instead, PTB blocks formation of the E complex on the intron downstream of N1. To allow characterization of this E complex, these experiments used pre-mRNA substrates that do not carry out exon definition on the downstream exon 4. However, these exon definition reactions must occur on the endogenous transcript *in vivo*³³. To look at the role of exon definition in PTB-mediated repression, we have now characterized the exon definition complexes that assemble on exon 4. Under conditions of either splicing or PTB-mediated repression, we find that the U2 snRNP can assemble on the 3' splice site of exon 4 via exon definition interactions. However, this formation of a defined exon 4 complex downstream does not overcome the PTB-mediated repression of exon N1.

RESULTS

The exon 4 3' splice-site complex assembles via exon definition

Previously, we analyzed the PTB-mediated repression of N1 exon splicing in HeLa extract using the single-intron substrate, BS713, where splicing complex formation is driven by intron definition interactions (Fig. 1a). Comparing this to spliceosome assembly in WERI extract, where PTB-mediated repression is absent, we showed that PTB interfered with an intron definition interaction required for assembly of a spliceosome on the intron downstream of the N1 exon¹⁰. Although PTB does not prevent the binding of the U1 snRNP at the N1 exon 5' splice site, the binding of U2AF and the U2 snRNP downstream does not occur on this RNA, and formation of the E complex is blocked. Under normal conditions *in vivo*, the downstream exon 4 would carry a 5' splice site that is absent in our construct. We therefore wanted to test whether the 3' splice-site complex for exon 4 could assemble via exon definition interactions when the 5' splice site was included downstream of exon 4 (Fig. 1a). We could then examine the effect of this downstream exon definition complex on repression of N1 splicing by PTB.

To characterize the exon definition complex under the two regulatory conditions, we used the Ex4 RNA, which carries exon 4, its

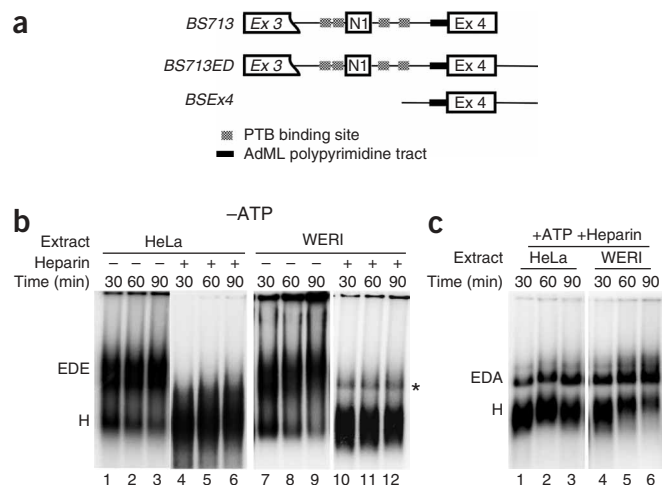


Figure 1 An exon definition complex forms in both HeLa and WERI extracts. (a) Maps of the BS713, BS713ED and BSEx4 constructs. BS713 has the 5' splice site of exon 3 deleted and the Adeno Major late (AdML) polypyrimidine tract introduced into the exon 4 3' splice site. In BS713ED, the 5' splice site is included downstream of exon 4. BSEx4 contains exon 4 and 50-nt sequences from the upstream and downstream introns. (b) Formation of the ATP-independent exon defined E (EDE) complex. Native agarose gel analysis of complex formation on the Ex4 RNA in HeLa (lanes 1–3) and WERI (lanes 7–9) extracts in the absence of ATP. Heparin treatment dissociates the EDE complex in both HeLa (lanes 4–6) and WERI (lanes 10–12) extracts. Positions of the H and EDE complexes are indicated. A minor heparin-resistant complex, indicated by an asterisk, is sometimes seen in the WERI extract, but this band is not consistently observed. (c) Formation of the ATP-dependent exon defined A (EDA) complex. Native agarose gel analysis of complex formation on the Ex4 RNA in the presence of ATP in HeLa (lanes 1–3) and WERI (lanes 4–6) extract after heparin treatment. Positions of the H and EDA complexes are indicated.

upstream 3' splice site and polypyrimidine tract as modified for the splicing substrate BS713, and the downstream 5' splice site (Fig. 1a). Splicing complex formation on this RNA was analyzed in the presence and absence of ATP in both HeLa and WERI extracts using native agarose gels³⁴. For the reactions lacking ATP, extracts were depleted of endogenous ATP by preincubation at room temperature for 30 min. The ATP-containing reactions were treated with heparin to improve the resolution of complexes. Gel analysis showed that complexes formed on the Ex4 RNA in both the presence and absence of ATP, and these complexes assembled with near equal efficiency in either HeLa or WERI extract (Fig. 1b,c). Heparin treatment disrupted complexes formed in the absence of ATP in both HeLa and WERI extract (Fig. 1b). However, in the presence of ATP, heparin-resistant complexes formed in both extracts (Fig. 1c). Analogous to the intron defined E and A complexes, we termed these ATP-independent and ATP-dependent complexes as the exon defined E (EDE) complex and the exon defined A (EDA) complex, respectively.

We then purified the EDE and EDA complexes using a phage MS2 RNA hairpin-tagged version of the Ex4 RNA (Supplementary Fig. 1a). Before assembly of the EDE and EDA complexes, the Ex4-MS2 RNA was prebound to a fusion protein consisting of MS2 phage coat protein and maltose binding protein (MS2-MBP). The complexes were assembled on the MS2-MBP-bound RNA, fractionated on glycerol density gradients and affinity purified on amylose resin, as described previously¹⁰. The EDE and EDA complexes from both HeLa and WERI extracts contained the U1 and U2 snRNAs, but none of the

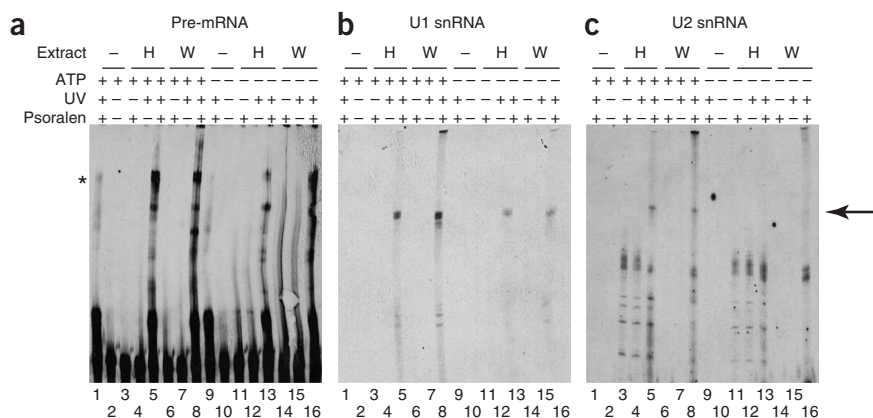


Figure 2 The U2 snRNA is base-paired to the pre-mRNA in the EDA complex. Northern analysis of the small nuclear RNAs (snRNAs) cross-linked to the Ex4 RNA in buffer DG (lanes 1, 2, 9 and 10), or in HeLa (H; lanes 3–5 and lanes 11–13) or WERI (W; lanes 6–8 and lanes 14–16) extract in the absence (lanes 1–8) or presence (lanes 9–16) of ATP, using probes recognizing the pre-mRNA RNA (a), U1 (b), or U2 snRNAs (c). The arrow at right indicates the cross-linked species. Note that the cross-linked U1 and U2 snRNA bands have similar migration but do not exactly overlap. The identity of the band indicated by the asterisk is not clear.

other spliceosomal snRNAs (**Supplementary Fig. 1b,c**). Psoralen cross-linking analysis was carried out to determine the base-pairing of the U1 and U2 snRNA to the Ex4 RNA in the EDE and EDA complexes. The Ex4 RNA was incubated in HeLa and WERI extracts in the presence of ATP. 4'-aminomethyl-4, 5',8-trimethylpsoralen (AMT-psoralen) was added and reactions were subjected to long-wave UV cross-linking. The cross-linked products were analyzed by northern blotting using probes against the pre-mRNA or the U1 or U2 snRNA (**Fig. 2**). No cross-linked bands were detected with any of the probes in the absence of psoralen or UV irradiation (**Fig. 1a–c**, lanes 2, 3, 6, 7, 10, 11, 14 and 15). In the presence of psoralen and UV irradiation, the naked pre-mRNA showed weak intramolecular cross-linking (**Fig. 1a**, lanes 1 and 9). Several cross-linked bands were detected with the pre-mRNA-specific probe in both HeLa and WERI extracts (**Fig. 2a**, lanes 5, 8, 12 and 16). One of these bands was also detected with the U1 snRNA probe in both the presence and absence of ATP (**Fig. 2b**, lanes 5, 8, 12 and 16). The U2 snRNA probe detected a cross-linked product in the presence of ATP, but not in its absence (**Fig. 2c**, lanes 5 and 8). Thus in the EDE complex only the U1 snRNA is base-paired to the pre-mRNA, whereas in the EDA complex both the U1 and U2 snRNAs are base-paired to the pre-mRNA.

Exon 4 complexes from the two extracts are similar

To compare the exon definition complexes under the two splicing regulatory conditions, we determined their protein composition using MS. As exon 4 is spliced under both regulatory conditions, this would also allow us to identify factors that bind to this region of the RNA and are not likely to determine the regulation. The purified complexes were treated with RNase A, precipitated with trichloroacetic acid (TCA) and digested with trypsin in solution. Peptides from each of the samples were identified by MS using the MudPIT procedure³⁵. The EDE and EDA complexes from both HeLa and WERI extract were purified and analyzed. Proteins present in the four complexes are listed in **Supplementary Table 1**. The compositions of the four complexes were similar. All contained the U1 and U2 snRNP-specific proteins and the Sm proteins. Not all components of a complex were detected in every MS experiment, but comparisons between these complexes, and additional complexes (see below), indicate that some undetected proteins were probably present. For example, the HeLa EDA and WERI EDE complexes showed no peptides for SmD2 but contained other components of the Sm core. Given that the HeLa EDE and WERI EDA complexes clearly contained SmD2, we infer its presence in the other two exon definition complexes. The many proteins that are common to both the EDE and EDA complexes from both extracts are listed in **Table 1**. The Ex4 RNA lacks the PTB binding

sequences involved in the regulation of N1 exon splicing, and the PTB protein was not detected in any of the complexes. Western blot analysis of the purified EDE complexes from HeLa and WERI extracts confirmed the presence of U2AF and the absence of the PTB proteins (**Supplementary Fig. 2**).

Notably, the proteins in the two WERI complexes were nearly identical to those in the HeLa complexes; the only protein detected in the HeLa complex but not in the equivalent WERI complex was the DEAD-box protein DDX1. The similarity of the two complexes indicates that the components of the exon 4 complexes are not likely to affect splicing regulation. This was not unexpected, as exon 4 must splice to an upstream exon in either of the two regulatory conditions. The addition of ATP to convert the complexes from EDE to EDA resulted in the apparent gain of some proteins, such as CGI-99 and RBM39 in the HeLa complex and hnRNP H and hnRNP A-B in both the HeLa and WERI complexes. However, their presence needs to be verified by immunoblotting.

In summary, exon definition interactions across exon 4 can induce 3' splice-site complex assembly for the intron downstream of the N1 exon. These complexes assemble with similar efficiency in HeLa and WERI extracts and are similar in their protein composition.

Table 1 Proteins present in both the exon defined E (EDE) and exon defined A (EDA) complexes purified from HeLa and WERI extracts

U1 snRNP specific proteins	hnRNP proteins	Others
U2 snRNP specific proteins	hnRNP U	PUF60
Sm proteins	hnRNP R	CAPER
Essential factors	hnRNP Q	CROP
CBP80	hnRNP L	LUC7L
CBP20	hnRNP K	CARP-1
SF1	FUS	ARS2b
U2AF65	YB-1	SR140
U2AF35	hnRNP G	BUB3
DEAD box proteins	hnRNP F	NFAT-90
DDX 5, p68	hnRNP D	NFAT-45
DDX 15, Prp43	hnRNP C	ZNF 207
DDX 17, p72	hnRNP A/B	RBM25
DDX 36	hnRNP A3	SPF31
DDX 46, Prp5	hnRNP A2/B1	TAT SF1
SR proteins	hnRNP A1	fSAP18
SRrp86, SFRS12	hnRNP A0	NELF E
SRp54, SFRS11	hnRNP RALY	CCAP1
SRp20, SFRS3	HuR	

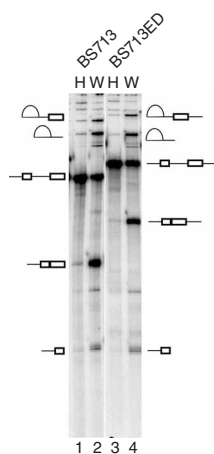


Figure 3 U2 small ribonucleoprotein particle assembly via exon definition does not overcome splicing repression. *In vitro* splicing of BS713ED RNA (lanes 3 and 4) in HeLa (H) and WERI (W) extract is compared to the BS713 (lanes 1 and 2). Splicing intermediates and products are indicated.

PTB represses splicing of N1 to the defined exon 4 complex

To examine splicing repression in the presence of the exon definition complex on exon 4, we made the BS713ED construct, which includes the 5' splice site downstream of exon 4 (Fig. 1a). Both the BS713 and BS713ED transcripts have one removable intron and can assemble a single functional spliceosome. Both transcripts splice efficiently in WERI extract (Fig. 3, lanes 2 and 4). However, both were strongly repressed for splicing in HeLa extract, despite the potential for exon definition in BS713ED (Fig. 3, lanes 1 and 3).

To confirm that splicing repression in HeLa extract was mediated by PTB, the PTB binding elements upstream of N1 were mutated to make BS713EDM (Fig. 4a). In WERI extract both the mutant and the wild-type RNA spliced with equal efficiency (Fig. 4b, lanes 1 and 2), but in HeLa extract splicing of the mutant RNA was about eight-fold more efficient than that of the wild-type RNA. PTB was also depleted from HeLa nuclear extract using the PTB-specific monoclonal antibody BB7. Western blot analysis confirmed efficient depletion of PTB in comparison to the mock-depleted extract, whereas the levels of two other proteins, Raver 1 and U1A, were unaffected (Fig. 4c). Splicing of the BS713ED RNA was stimulated about five-fold in the PTB-depleted extract compared to the mock-depleted extract (Fig. 4d, lanes 1 and 2). Adding back the recombinant PTB restored splicing repression (Fig. 4d, lane 3). Splicing of the mutant RNA was not affected significantly by PTB depletion or add-back (Fig. 4, lanes 4 and 5). Thus, similarly to the BS713 RNA, splicing of the BS713ED is repressed by PTB.

In contrast to the previous observations for BS713 RNA, the BS713ED RNA should form an exon definition complex on exon 4

in either extract¹⁰. Indeed, native gel analysis showed complex formation on BS713ED RNA in the absence of ATP in both HeLa (Fig. 5a, lanes 1–3) and WERI (Fig. 5a, lanes 4–6) extract. Because these complexes can potentially assemble via either intron or exon definition pathways, we refer to them as E-like (E') to distinguish them from an intron-defined E complex. These ATP-independent complexes were then purified by the MS2-affinity chromatography using the BS713ED-MS2 RNA. RNA 3' end labeling showed that the U1 and U2 snRNAs were present in both the HeLa (Fig. 5b, lane 2) and WERI (Fig. 5b, lane 5) complexes.

The addition of ATP to WERI extract caused the expected conversion of the E' complex into splicing complexes A, B and C (Fig. 6a, lanes 4–6). However, in the HeLa extract, ATP induced the conversion into a prespliceosomal A-like complex (A'), but this complex did not transition into the spliceosomal B and C complexes (Fig. 6a, lanes 1–3). Fractionation of these complexes on glycerol density gradients after heparin treatment showed clear separation of the A, B and C complexes in WERI extract (Fig. 6b). In HeLa extract, only the H and A' complexes were seen. The A' complex from HeLa extract and mixed spliceosomes from WERI extract were isolated without heparin treatment, as described previously¹⁰. Analysis of their RNA content showed that the WERI spliceosomes contained the expected splicing intermediates and products, as well as the U1, U2, U4 and U5 snRNAs (note that U6 often does not label well with pCp). In contrast, the HeLa A' complex lacked any spliced products and contained only the U1 and U2 snRNAs (Fig. 6c). Thus, in HeLa nuclear extract, the assembly of U2 snRNP on exon 4 via exon definition interactions is not sufficient to overcome splicing repression by PTB. In WERI extract, which lacks PTB repression, the U2 snRNPs bound at exon 4 can be converted into active spliceosomal complexes.

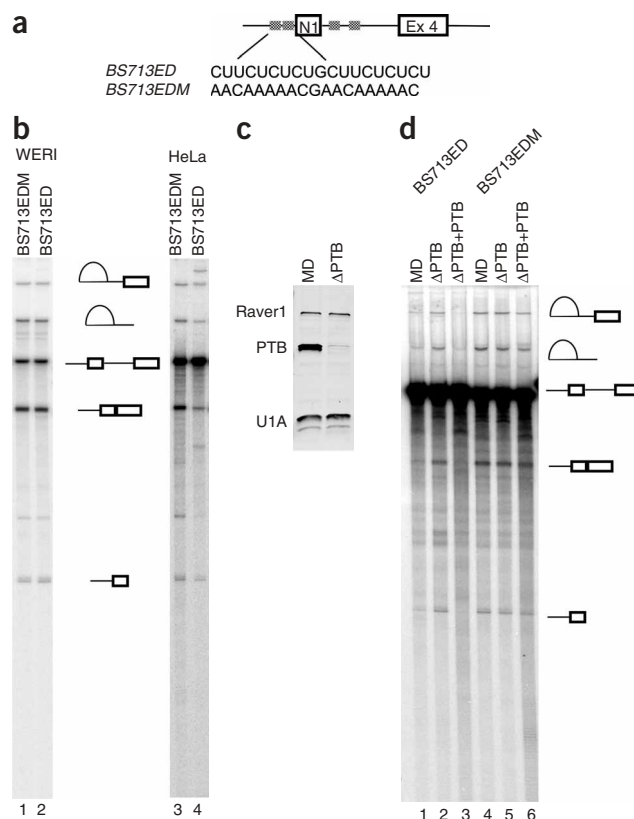


Figure 4 Splicing repression after exon definition in HeLa extract is dependent on polypyrimidine tract binding protein (PTB). (a) The BS713EDM construct carries a mutation in the N1 exon 3' splice site, as shown, that eliminates PTB binding. (b) *In vitro* splicing of the BS713EDM transcript (lanes 1 and 3) is compared to that of BS713ED (lanes 2 and 4) in HeLa and WERI extracts. (c) Western analysis of the mock-depleted (MD) and the PTB-depleted (Δ PTB) extract using antibodies against proteins PTB, U1A and Raver 1. (d) *In vitro* splicing of the BS713ED (lanes 1–3) and BS713EDM (lanes 4–6) transcripts in MD, Δ PTB and PTB-reconstituted (Δ PTB+PTB) HeLa extract is compared.

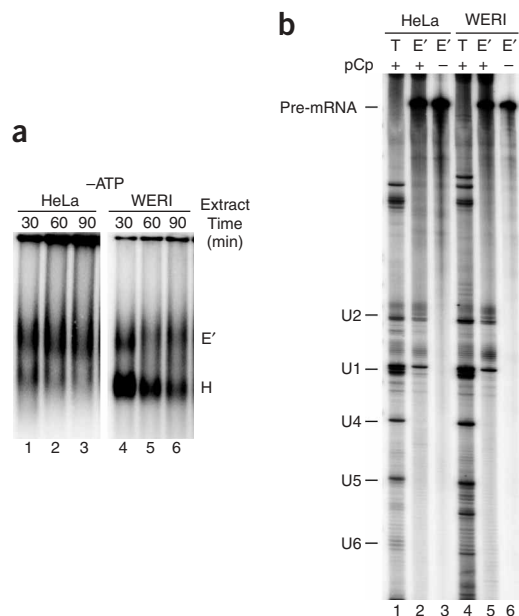


Figure 5 ATP-independent E-like (E') complex assembles in both HeLa and WERI extracts. **(a)** Native agarose gel analysis of complex formation on the BS713ED RNA in the absence of ATP in HeLa (lanes 1–3) and WERI (lanes 4–6) extract. Positions of the H and E' complexes are indicated. **(b)** ^{32}P -pCp labeling of RNA from the purified E' complexes. Total RNA from nuclear extract (T) was used as markers for the U snRNAs. The positions of the pre-mRNA and U snRNAs are indicated.

several other non-snRNP splicing proteins all bind to the complex (Table 2). The absence of some second-step factors such as PRP17, PRP18 and SLU7 in the MS analysis could be due to the underrepresentation of late spliceosomes in the mixture of complexes.

The A complex that assembles on an Adeno Major late (AdML) intron has been studied in some detail³⁷. Several proteins are present in both this functional HeLa A complex and the WERI spliceosomes, but are absent from the blocked HeLa A' complex. These different components, including the Srm160 and 300 proteins, and the proteins of the PRP19 complex give clues to how A' is blocked from further assembly. Notably, PRP3 and PRP4 proteins were present in the HeLa A' complex but no other U5 snRNP-, U4-U6 snRNP- or tri-snRNP-specific proteins were detected.

Trans-splicing occurs in WERI but not HeLa extract

The exon definition complex assembles properly in the HeLa extract but is not converted to a spliceosome. One possibility is that PTB blocks the interaction of the upstream and downstream complexes, preventing their conversion to an intron defined spliceosome. To look directly at the interaction between the 5' and 3' exon complexes, we used a trans-splicing assay that has been described by others^{38–40}. The BS713ED construct was split in the intron to produce 5' and 3' exon transcripts (Fig. 7a). Labeled 5' exon RNA and unlabeled 3' exon RNA were incubated in HeLa and WERI extracts. Denaturing gel analysis was used to assay formation of the Y-RNA and the detached 5' exon that result from the first step of the trans-splicing reaction. Incubation

Proteomic analysis identifies potential PTB corepressors

To compare the splicing complexes that assemble under the two regulatory conditions, we determined their protein composition. The purified HeLa E' and A', and the WERI E' and total spliceosomal complexes, were treated with trypsin and subjected to MS analysis. All proteins identified in these complexes are listed in Supplementary Table 1. The Ex4 exon definition complex proteins listed in Table 1 were identified in all the BS713ED complexes. These BS713ED complexes also contained proteins that presumably bind to the upstream sequence that is absent in the Ex4 RNA, including PTB, hnRNP E2 and Matrin 3. Unlike the Ex4 complexes, the E' complexes from both extracts changed significantly with addition of ATP.

In HeLa extract, conversion to the A' complex resulted in the loss and gain of multiple proteins. Proteins present in the HeLa complexes but not in the WERI complexes are potential PTB cofactors in splicing repression. Interesting proteins in this group were CUG triplet repeat, RNA binding proteins 1 and 2 (CUGBP1,2), muscleblind-like 1, 2 and 3 (MBNL1,2,3) and Raver 1 and 2 (Table 2). Raver 1, in particular, has been shown to cooperate with PTB in the repression of α -tropomyosin exon 3 (ref. 36).

Proteins detected in the WERI E' complex but not in the HeLa E' complex included SF2/ASF protein (also known as SRFS1), Ewing's sarcoma RNA binding protein (EWS), nPTB and FLJ38348. With ATP addition, this WERI E' complex efficiently transitions to functional spliceosomal A, B and C complexes. During this transition, PRP19 complex proteins, U5, U4-U6 and tri-snRNP-specific proteins, and

Figure 6 ATP-dependent splicing complex assembly in HeLa extract is stalled at the A-like (A') complex. **(a)** Native agarose gel analysis of complex formation on the BS713ED RNA in presence of ATP in HeLa (lanes 1–3) and WERI (lanes 4–6) extract. Positions of the H, A, A', B and C complexes are indicated. Note that the nature of the minor high molecular weight complex in HeLa extract is not clear. **(b)** Native agarose gel analysis of splicing complexes assembled on BD713ED RNA in HeLa and WERI extracts and fractionated on 15–30% glycerol density gradients. **(c)** ^{32}P -pCp labeling of RNA from the purified A' complex and from total spliceosomes (S). Total RNA from nuclear extract (T) was used as a marker for the U snRNAs. The positions of the pre-mRNA and U snRNAs are indicated.

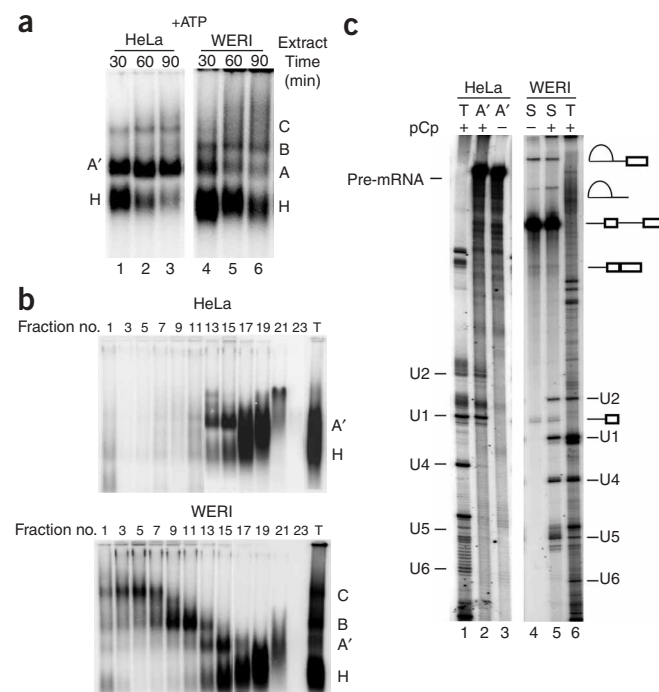


Table 2 Proteins present in the HeLa and WERI BS713ED complexes

Protein name	Acc. no.	Sequence motifs
Present only in the WERI spliceosomes		
U5 snRNP proteins		
U4/U6 snRNP proteins		
LSm proteins		
PRP19 complex		
Tri-snRNP protein		
110k, SART1, hSnu66	NP_005137	RS
DEAD box protein		
DDX39, UAP56	NP_004631	DEXD
SR proteins		
ASF/SF2, SFRS1	NP_008855	2 RRM, RS
9G8, SFRS7	NP_001026854	RRM, RS
hTra-2alpha	NP_037425	RRM, RS
hTra-2b, SFRS10	NP_004584	RRM, RS
Non-snRNP splicing proteins		
SRm160	AAC09321	PWI domain
SRm300	NP_057417	2 RS, S-rich
EWS	NP_005234	RRM, ZnF
DEK	NP_003463	SAP, DEK
Smu-1 homolog	NP_060695	WD40
RED	NP_006074	RED repeats
RBM22, FLJ0290	NP_060517	C3H1ZnF, RRM
RBM42, MGC10433	NP_077297	RRM
Aquarius, IBP160	NP_055506	Helicase SF
hPrp4k	NP_003904	S/T kinase
RBM26, Se70-2	NP_071401	RRM, C3H1 ZnF
WD33	NP_060853	WD40
PEP19	NP_006189	
BAT2	NP_542417	Pro-rich
PP1 γ	NP_002701	PP2Ac
NIPP1	NP_054829	FHA
hnRNP proteins		
nPTB, PTB-2	NP_067013	4 RRM
PSF	NP_005057	2 RRM, NOPS
p54nrb	NP_031389	2 RRM, NOPS
Present only in the HeLa A' complex		
DEAD box proteins		
DDX1, DED1	NP_004930	DEAD
DDX 36	NP_065916	DEAD
KIAA0052, SKIV2L2	NP_056175	DEXH
hnRNP proteins		
FBP	NP_003893	3 KH
SAF-B	NP_002958	RRM, SAP
CUGBP1	NP_941989	3RRM
MBNL3	NP_597846	C3H1 ZnF
MBNL2	NP_659002	C3H1 ZnF
MBNL1	NP_066368	C3H1 ZnF
Raver1	NP_597709	3 RRM
Raver2	NP_060681	3 RRM

of the 5' exon RNA in the absence of the 3' exon RNA did result in formation of a trans-spliced product in either extract (Fig. 7a, lanes 1 and 7). Similarly, we have not observed trans-splicing between exon 4 complexes, perhaps owing to the limited amount of sequence downstream of the 5' splice site in this RNA (data not shown; see also Fig. 1). When both 5' and 3' exon RNAs were mixed in the reaction,

trans-splicing was observed in the WERI extract but not in the HeLa extract (Fig. 7b). Thus, PTB assembling on the 5' exon transcript prevents its trans-splicing to the downstream exon.

One possibility is that, under PTB repression, the upstream exon could still pair with the downstream exon but could not proceed further in assembly. To examine this, we carried out affinity pull-down experiments using MS2-tagged exon 4 RNA, prebound to the MS2-MBP protein. Uniformly labeled 5' and 3' exon RNAs at 5-nM concentrations were incubated in HeLa or WERI extracts in the absence or presence of ATP. The reactions were incubated with amylose beads and the bound RNAs were isolated. In reactions lacking ATP, the 5' exon RNA could not be pulled down in association with the MS2-tagged 3' RNA (Fig. 7c, lanes 1–6). However, the addition of ATP induced the association of the 5' exon RNA with the tagged 3' exon RNA. This interaction was observed in WERI extract but not HeLa extract (Fig. 7c, lanes 7–12). Thus, the splicing complexes that assemble on the 5' and 3' exons stably interact only in WERI extract and in the presence of ATP.

DISCUSSION

Here our goal is to understand how splicing regulators interact with the spliceosome during its assembly to alter splicing choices. We find that, in repressing splicing of the *c-src* N1 exon to the constitutive exon 4 downstream, PTB prevents the interaction of the exon N1 5' splice-site complex with the 3' splice-site complex that is assembled on exon 4. This results in the accumulation of an A-like complex, A', which contains the U2 snRNP assembled through exon definition on exon 4 and the U1 snRNP assembled at exon N1, but is blocked from further assembly (Fig. 8). These results agree with earlier studies showing that under conditions of intron definition PTB blocks the assembly of U2AF, which requires an interaction with the U1 snRNP complex upstream to be incorporated into an intron defined spliceosome¹⁰. Under the exon definition conditions used here, the U2AF is stabilized by the 5' splice site of exon 4 downstream, but its interaction with the upstream N1 5' splice site still does not occur. Thus, PTB acts on the 5' splice-site side of this interaction (Fig. 8b).

The exon 4 complex has the same components whether PTB is present or not, allowing it to splice to the upstream exons 3 or N1, depending on the presence or absence of PTB. The transition from an exon definition complex to an intron defined spliceosome is poorly understood. The ability of PTB to prevent this step gives clues to general aspects of spliceosome assembly, as well as to PTB's specific regulatory targets.

Although they are major regulatory points in the assembly of the spliceosome, the compositions of the E and exon definition complexes (EDCs) have not been previously described. We find that both complexes contain the expected components of the U1 and U2 snRNPs and U2AF, as well as SR proteins that presumably bind within the exon. Although PTB is not present, the EDC does contain multiple hnRNP proteins whose binding sites are unknown and which do not seem to inhibit its splicing. The EDC also contains several ATP-dependent RNA helicases of the DEAD box family, including p68, PRP5 and others. Interestingly, when ATP is added to convert the EDC complex to an EDA complex with U2 base-paired to the branchpoint, there is little change in the components of the complex. Thus, one of the component DEAD-box proteins of the EDC could drive the ATP-dependent transition to a base-paired U2 snRNA. However, it is possible that another ATPase is involved that associates only transiently with the complex and is lost upon isolation.

Although the EDCs were similar in the two extracts, isolation of the complexes on the longer spliceable pre-mRNA identified several

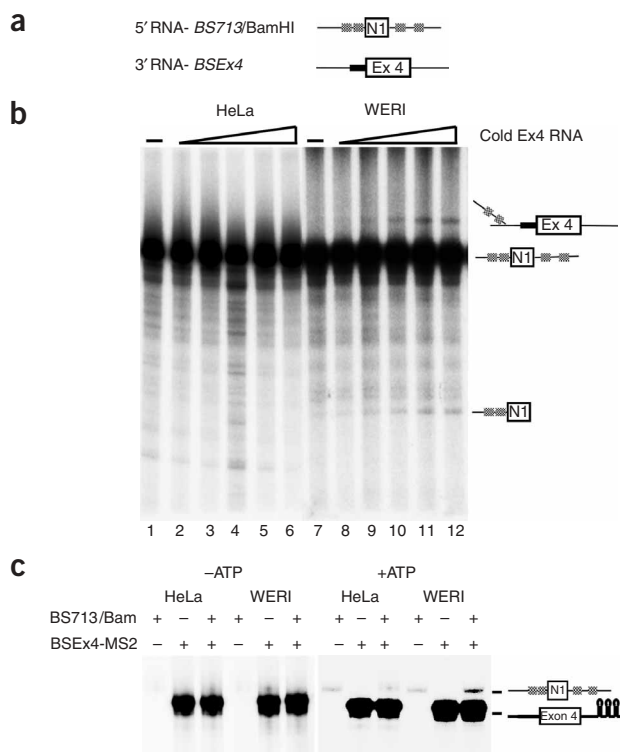


Figure 7 Trans-splicing occurs in WERI but not HeLa extract. **(a)** RNA substrates used in the trans-splicing assay. The 5' RNA was transcribed from BamHI cleaved BS713 and the 3' RNA was transcribed from BSEx4. **(b)** Trans-splicing analysis of uniformly ^{32}P -labeled 5' RNA alone (lanes 1 and 7) or in the presence of unlabeled 3' RNA in HeLa (lanes 1–6) and WERI (lanes 7–12) extract. **(c)** MS2-affinity tag-mediated pull-down of uniformly ^{32}P -labeled 3' RNA in the presence or absence of the 5' RNA in HeLa and WERI extract, in either the presence or absence of ATP.

proteins whose assembly depends on the regulatory conditions. As seen previously, it is clear that U2 can assemble properly at the branchpoint before the transition to intron bridging interactions. As this bridging interaction has apparently not formed in the PTB-repressed A' complex, it is interesting to compare the A' components to the functional A complexes studied by others³⁷. Nearly all proteins found in the A complex assembled on the AdML intron in HeLa extract are also seen in the mixed spliceosomes assembled here in WERI extract. Proteins present in these complexes but missing from the repressed HeLa A' complex are candidates for factors needed for this bridging interaction. The PRP19 complex is required for the transition to an active B complex, but it is present in the Adeno A complex and may have additional earlier functions^{37,41}. SRm160 and SRm300 are SR domain proteins shown to interact with components at both the 5' and 3' splice sites, and were proposed to act as the intronic bridge^{42,43}. Interestingly, both these proteins are present in the Adeno A complex and the WERI spliceosomes, but not in the HeLa A' complex, consistent with a role as bridging factors³⁷. Another protein present in the WERI spliceosomes but not the HeLa A' complex is DEK, which was shown to be important for proofreading of the U2AF-3' splice-site interaction⁴⁴. This is a step that could occur in the transition from exon definition to the intronic spliceosome. Other proteins previously proposed to mediate 5' and 3' splice-site interactions are the PRP5 and PRP40 proteins^{45–49}. These are present in all of our complexes (repressed or not). They could still be bridging factors that have lost the ability to contact one side of the intron in the repressed complex. Defining the roles of all of these proteins will require further study using depletion experiments and other approaches.

The pull-down and trans-splicing experiments indicate that PTB prevents the interaction of the 5' splice-site complex with the exon definition complex downstream. PTB could block contacts

on the U1 snRNP needed for this interaction, or it could prevent a required change in U1 conformation. The A' complex formed when splicing is repressed by PTB contains several proteins that are not found in the ATP-dependent exon definition complex. Some of these proteins are ATP dependent and include candidates for proteins that act as corepressors with PTB. Of these, several were previously shown to functionally interact with PTB, including CUGBP, MBNL and Raver. CUGBP and PTB have antagonistic effects on the splicing of exons in α -actinin and α -tropomyosin^{27,50}. Both MBNL and PTB repress the splicing of an exon in cardiac troponin-T⁵¹. Raver 1 is a known cofactor required for proper PTB repression of α -tropomyosin exon 3 (ref. 36). It will be interesting to test the involvement of these proteins in PTB-mediated repression of exon N1, although initial experiments do not indicate a requirement for Raver 1 (data not shown).

The action of PTB in blocking exon-defined complexes from forming functional spliceosomes is a potential mechanism for many splicing regulators. There are many intronic silencing elements that presumably recruit specific repressor proteins. Splicing repressors such as PTB, hnRNPs A1 and L can also sometimes bind in exons rather than introns^{52,53}. For hnRNP A1 and PTB, this binding can disrupt exon definition, or SR protein-dependent splicing enhancement. This may lead to the failure to recognize the exon at all. However, for CD45 exon 4 repression by hnRNP L, the exon definition complex does form, but cannot be converted to an intronic spliceosome, similar to what is observed here⁵⁴. It is interesting that these two mechanisms will allow normal exon definition on adjacent exons and their splicing, but will prevent the target exon from entering a productive assembly pathway.

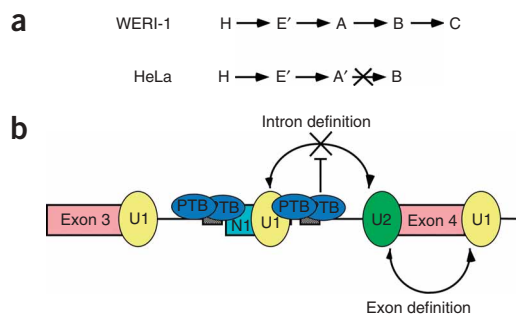


Figure 8 Model for polypyrimidine tract binding protein (PTB)-mediated splicing repression. **(a)** In WERI extract spliceosomal complexes H, E, A, B and C form efficiently, whereas in HeLa extract assembly is stalled at an A-like (A') complex. **(b)** Intron-bound PTB does not interfere with the binding of the U1 small ribonucleoprotein particle (snRNP) at the N1 exon 5' splice site, or with the assembly of an exon definition complex on the downstream exon. PTB prevents the intron-bound U1 and U2 snRNPs from interacting, and thus prevents intron definition and spliceosome assembly.

METHODS

Pre-mRNAs and affinity tags. Pre-mRNAs were transcribed *in vitro* from plasmids BS713, BS713ED, BS713EDM and BSEx4 (Fig. 1a). The BS713 construct has been described before³¹. The BS713ED construct includes the 5' splice site of *c-src* exon 4 and a 50-nucleotide (nt) region of the downstream intron. In the BS713EDM construct, the PTB binding element in the N1 exon 3' splice site is changed from 5'-CUUCUCUCUGCUUCUCUCU-3' to 5'-AACAAAAACGAACAAAAAC-3'. The BS713Bam RNA was transcribed from BS713 plasmid cleaved at the BamHI site in the intron downstream of N1 exon. Constructs BS713ED-MS2 and BSEx4-MS2 containing three MS2 affinity tags were prepared as described before.

In vitro splicing and spliceosome assembly. Nuclear extracts from HeLa and WERI-1 cells were prepared as described previously²⁸. *In vitro* splicing was carried out as described³³. Splicing complexes were assembled as described previously¹⁰. For the ATP-dependent complexes, at the indicated time, standard splicing reactions were stopped by addition of heparin to a final concentration of 0.2 mg ml⁻¹, and the incubation was continued for another 5 min. The splicing complexes were separated and visualized using native agarose gels. Of the 25- μ l splicing reaction, 4 μ l was loaded onto a 2% Seakem GTG agarose gel. The native gels (10 cm \times 14 cm) were cast and run in 25-mM Tris-glycine buffer at 100 V for 4 h at room temperature. The gels were fixed in a 10% (v/v) acetic acid, 10% (v/v) methanol solution for 30 min, dried under vacuum and visualized by Phosphorimager (Molecular Dynamics). For assembly of the ATP-independent complexes, the nuclear extracts were depleted of ATP by preincubating them at room temperature for 30 min. The assembly reactions lacked ATP and creatine phosphate. The complexes were resolved on a 1.5% native agarose gels without addition of heparin.

Trans-splicing was carried out as described³⁸. Briefly, uniformly ³²P-labeled 5' RNA at 5 nM and varying amounts of the unlabeled 3' RNA (0 nM, 0.1 nM, 0.5 nM, 1 nM, 2 nM and 5 nM) were added to standard splicing reactions and incubated at 30 °C for 90 min. RNA was extracted, ethanol precipitated, separated on 8% urea-PAGE and visualized by Phosphorimager. Reactions mixes for the pull-down assay contained 5 nM MS2-tagged 3' RNA, 50 nM of MS2-MBP protein, and 0 nM or 5 nM of 5' RNA in HeLa and WERI extract in the presence or absence of ATP. The reactions were incubated for 90 min at 30 °C. The MS2 hairpin-tagged RNA was pulled down using amylose beads, extracted and visualized as described above.

Psoralen cross-linking. The EDE and EDA complexes were assembled at 30 °C for 0 min in HeLa and WERI extracts. AMT-psoralen was added to a final concentration of 20 μ g ml⁻¹ and reactions were UV irradiated as described previously¹⁰. The pre-mRNA and U1 and U2 snRNA cross-linked RNAs were detected by northern blot analysis using digoxigenin-labeled probes⁵⁵. The pre-mRNA probe was directed against residues 1–153 of the Ex4 RNA, and the U1 and U2 snRNA probes were made as described⁵⁵.

Isolation of splicing complexes. Purification of the pre-mRNP complexes was carried out according to a published method⁵⁶, with some modifications as described before¹⁰. MS2 binding site-containing pre-mRNAs (10 nM) were first preincubated with a ten-fold excess of recombinant MS2-MBP fusion protein (a gift from J. Vilardell). The splicing complexes were then assembled on this pre-mRNA-MS2-MBP complex. For assembly of ATP-dependent complexes, a typical reaction (500 μ l) contained 10 nM pre-mRNA, 100 nM MS2-MBP, 20 mM ATP, 0.4 mM creatine phosphate, 2.2 mM MgCl₂, 10 U RNAGuard and 300 μ l of nuclear extract. The reaction mixture was incubated for 90 min at 30 °C. The ATP-independent complexes were assembled in extracts that had been depleted of ATP, and the assembly reactions lacked ATP and creatine phosphate. The reaction mix was then layered on a 15–30% (v/v) glycerol gradient prepared in buffer DG³³. The gradients were centrifuged at 24,000 rpm for 16 h, at 4 °C in a SW41 rotor in a Beckman L70 ultracentrifuge. The gradients were fractionated into 24 aliquots and the radioactivity was determined by scintillation counting. The peak fractions were pooled and passed twice through a column of amylose beads (500 μ l; New England Biolabs) pre-equilibrated in buffer DGM (buffer DG containing 2.2 mM MgCl₂). The column was washed with 10 column volumes of buffer

DGM. Bound complexes were eluted with 20 mM maltose in buffer DGM. Concentrations of the purified complexes were determined from the specific activity of the labeled transcript. The yield of the purified complexes using this method varied between 20–30% of the input transcript. *Escherichia coli* ribosomal subunits 50S and 30S were used as markers in parallel glycerol gradients.

RNA from the purified complexes was extracted with PCA (phenol:chloroform: isoamylalcohol) and ethanol precipitated. The RNA was labeled with ³²P-pCp in a 10- μ l reaction containing the RNA, RNAGuard, 10 units RNA ligase (NEB), 1 μ l of 10 \times ligase buffer and 2 μ l of ³²P-pCp (3,000 Ci mmol⁻¹) incubated at 4 °C overnight. The RNA was extracted with PCA, ethanol precipitated and separated on 8% urea-PAGE gel.

PTB purification and immunodepletion. Recombinant His-PTB was expressed in *E. coli* and purified using Ni-NTA agarose (Invitrogen). HeLa nuclear extract was immunodepleted for PTB using the mouse monoclonal anti-PTB antibody BB7 as described previously¹⁰. Western analysis was carried out using antibodies against PTB (BB7), Raver 1 and U1A.

Mass spectrometry. To obtain proteins, the complexes were treated with RNase A (0.05 mg ml⁻¹) at 37 °C for 1 h and TCA precipitated. Precipitated proteins were resuspended in 8 M urea, subjected to carboxyamidomethylation of cysteines, and digested with trypsin. A nano LC column was packed in a 100- μ m inner diameter glass capillary with an emitter tip. The column consisted of 10 cm of Polaris c18 5- μ m packing material (Varian), followed by 4 cm of Partisphere 5 SCX (Whatman), then another 2 cm of Polaris C18 to form a column for three-phase MudPIT analysis⁵⁷. The column was loaded by use of a pressure bomb and washed extensively with buffer A (see below). The column was then directly coupled to an electrospray ionization source mounted on a Thermo-Finnigan Decca XP Plus mass spectrometer. An Agilent 1100 HPLC equipped with a split line so as to deliver a flow rate of 30 nl per min was used for chromatography. Peptides were eluted using a 14-step MudPIT procedure⁵⁵. Buffer A was 5% (v/v) acetonitrile, 0.02% (v/v) heptafluorobutyric acid (HBFA); buffer B was 80% (v/v) acetonitrile, 0.02% (v/v) HBFA. Buffer C was 250 mM ammonium acetate, 5% (v/v) acetonitrile, 0.02% (v/v) HBFA; buffer D was the same as buffer C, but with 500 mM ammonium acetate. The programs SEQUEST and DTASELECT were used to identify peptides and proteins from the complete human database^{58,59}. Xcorr cutoffs of 1.8, 2.2 and 3.5 were used for inclusion of peptides with +1, +2 and +3 charge states, respectively. These cutoffs have been shown to yield peptide identifications with considerably less than a 1% false positive rate⁶⁰. Two or more peptides meeting these statistical criteria were required to consider a protein as positively identified. The resulting false positive rate for protein identification is calculated to be less than 0.01%.

Note: Supplementary information is available on the Nature Structural & Molecular Biology website.

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AUTHOR CONTRIBUTIONS

S.S. and D.L.B. designed the experiments and analyzed the data. S.S. performed the experiments. L.A.K. and D.C.R. performed and analyzed the MS experiments. A.D. contributed to Figure 2. S.S. and D.L.B. wrote the paper.

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