Polypyrimidine tract-binding protein (PTB) is an abundant widespread RNA-binding protein with roles in regulation of pre-mRNA alternative splicing and 3′-end processing, internal ribosomal entry site-driven translation, and mRNA localization. Tissue-restricted paralogs of PTB have previously been reported in neuronal and hematopoietic cells. These proteins are thought to replace many general functions of PTB, but to have some distinct activities, e.g. in the tissue-specific regulation of some alternative splicing events. We report the identification and characterization of a fourth rodent PTB paralog (smPTB) that is expressed at high levels in a number of smooth muscle tissues. Recombinant smPTB localized to the nucleus, bound to RNA, and was able to regulate alternative splicing. We suggest that replacement of PTB by smPTB might be important in controlling some pre-mRNA alternative splicing events.

The importance of post-transcriptional mechanisms of gene regulation has been emphasized by the relatively modest number of genes in the human genome (1, 2). Alternative splicing, RNA editing, and alternative translational initiation all allow for more than one protein isoform to be produced by individual genes. Alternative splicing is the most prevalent of the post-transcriptional mechanisms for producing protein isoforms. Conservative estimates predict that one- to two-thirds of human genes are alternatively spliced, and some of these genes have the potential to produce thousands of isoforms (reviewed in Refs. 3–7).

Regulation of alternative splicing involves the interaction of cellular trans-acting factors with specific cis-acting regulatory elements within a target pre-mRNA (8, 9). These regulatory interactions influence the recognition of splice sites by the splicing machinery. Such regulation can be positive, involving activator factors and enhancer sequences. Conversely, repressor proteins can mediate their influence via silencer elements. Although some model systems of regulated splicing involve the presence or absence of a single regulatory protein, the majority of examples appear to be more complex, with regulatory decisions being achieved by particular combinations of regulatory factors, each of which is expressed more widely than the splicing event that is being regulated (7–9).

The heterogeneous nuclear ribonucleoproteins (hnRNPs) are a group of abundant and widespread nuclear proteins with diverse roles in pre-mRNA and mRNA function, including the regulation of alternative splicing (10, 11). Polypyrimidine tract-binding protein (PTB) (12, 13) reviewed in Refs. 12 and 13, also known as hnRNPI, is a prominent member of this family. PTB was originally identified as a potential splicing factor due to its ability to bind to polypyrimidine tracts at 3′-splice sites (14–16). However, it was subsequently recognized to act as a splicing repressor at particular splice sites (17–31). PTB also plays roles in nuclear pre-mRNA 3′-end processing (32, 33), cytoplasmic internal ribosomal entry site-driven translation (34), mRNA localization (35), and regulation of mRNA stability (36). Consistent with these varied roles, PTB can shuttle between the nucleus and cytoplasm, but is predominantly localized in the nucleus (37). The optimal RNA binding sequence for PTB (UCUU in a pyrimidine-rich context) is found within silencer elements that act by binding PTB (26). These elements are often found within the 3′-splice site polypyrimidine tract; and in some cases, PTB acts by directly competing for binding to the polypyrimidine tract with the splicing factor U2AF65 (23, 27). However, PTB-binding sites are also found in other locations in the region of PTB-regulated exons, so it may also be able to inhibit splicing in other ways (reviewed in Ref. 13).

Consistent with its expression pattern, most PTB-mediated repression of specific exons is widespread. Regulated selection of the exons occurs in a small subset of tissues where the repressive action of PTB is either absent or in some way modulated. PTB exists in two major alternatively spliced isoforms, termed PTB1 and PTB4, which arise from skipping or inclusion, respectively, of exon 9, which encodes a 26-amino acid insert. A minor isoform, PTB2, is produced by inclusion of exon 9 using an internal 3′-splice site, giving a 19-amino acid insert. In at least one case, these isoforms have differential activity, with PTB4 being more repressive than PTB2 or PTB1 (29). In addition to the PTB isoforms, at least two paralog genes, nPTB/brPTB and ROD1, with ~70% amino acid identity to PTB have been identified. nPTB/brPTB (38, 39) is expressed predominantly in neuronal cells, whereas ROD1 is expressed mainly in hematopoietic cells (40). Cells expressing nPTB or ROD1 tend to express less PTB; and in the case of the alternative N1 exon of c-src, nPTB is less repressive.
than PTB, contributing to the neuronal selection of the N1 exon (39). The effect of nPTB on other splicing events is similar to that of PTB (29). Although no functional data have been reported on ROD1, a reasonable proposal is that, in neuronal or hematopoietic cells expressing nPTB or ROD1, many PTB-repressed splicing events will be unaffected, whereas a specific subset will be altered. Alterations in the expression of the alternatively spliced isoforms of PTB or in the expression of paralog genes therefore provide one way in which PTB activity can be modulated.

We have been investigating two alternative splicing events that are regulated in smooth muscle (SM) cells. In α-actinin, a SM-specific exon is repressed by PTB in non-SM cells, leading to inclusion of the mutually exclusive alternative non-muscle (NM) exon (see Fig. 1A) (28, 41). In α-tropomyosin (α-TM), exon 2 is included only as a result of repression of the mutually exclusive exon 3 in SM cells (see Fig. 1C) (42). This repression is mediated in part by high affinity PTB-binding sites on either side of exon 3 (21, 26). In vitro splicing experiments have shown that PTB mediates a low level of exon 3 repression in non-muscle extracts (29). However, in vivo, full repression is observed only in SM cells. In this respect, PTB-mediated repression of an α-TM exon 3 differs from all other characterized splicing events regulated by PTB.

In an attempt to understand how the tropomyosin and actinin splicing events are regulated, we investigated the expression of PTB isoforms in differentiating rat aorta smooth muscle (RASM) cells. We found no change in the ratio of PTB1 to PTB2 is included only as a result of repression of the mutually exclusive exon 3 in SM cells (see Fig. 1C) (42). This repression is mediated in part by high affinity PTB-binding sites on either side of exon 3 (21, 26). In vitro splicing experiments have shown that PTB mediates a low level of exon 3 repression in non-muscle extracts (29). However, in vivo, full repression is observed only in SM cells. In this respect, PTB-mediated repression of an α-TM exon 3 differs from all other characterized splicing events regulated by PTB.

In an attempt to understand how the tropomyosin and actinin splicing events are regulated, we investigated the expression of PTB isoforms in differentiating rat aorta smooth muscle (RASM) cells. We found no change in the ratio of PTB1 to
which has not been reported before, would result in a nonfunctional EF-hand domain. By day 4, there were roughly equal amounts of the SM and NM isoforms, with a decrease in the amount of the skipped product. The cultured PAC-1 cells were similar to the day 4 cells, but with more NM than SM inclusion and no double-skipped product. RT-PCR analysis of vinculin (Fig. 1B) showed that the meta-vinculin isoform was expressed as a minor isoform only in day 0 RASM cells and was undetectable in day 4 and PAC-1 cells. Mutually exclusive splicing of α-TM exons 2 and 3 produced products of identical size. To differentiate them, the radiolabeled PCR products were digested with XhoI, which cuts within exon 2 to produce a 145-nucleotide product, or with PvuII, which cuts exon 3 products to produce a 150-nucleotide band (Fig. 1C). Double digests showed that the PCR product could be fully digested by both enzymes. The almost complete XhoI digestion and PvuII resistance of the day 0 PCR product showed that fully differentiated RASM cells predominantly expressed the exon 2-containing α-TM isoform. By day 4, PvuII digested a greater proportion of the PCR product compared with XhoI, indicating a substantial switch toward inclusion of exon 3 instead of exon 2. In comparison, in cultured PAC-1 cells, the majority of α-TM RNA contained exon 3. PAC-1 cells commonly show a greater degree of regulated splicing than observed here (21, 42, 48), but they served as a useful undifferentiated control sample. Taken together, the data indicate that the three alternative splicing events analyzed in RASM cells showed a substantial switch toward the non-SM pattern after 4 days in culture.

Identification and Expression of smPTB—Having observed the switch in alternative splicing of α-TM and α-actinin, both of which are regulated by PTB, we next analyzed expression of the PTB isoforms. RT-PCR was carried out using primers P37 and P38, which correspond to exons 8 and 11, respectively. This analysis allows the detection of alternative splicing of exon 9, which gives rise to the PTB1 and PTB4 isoforms. Unlike α-TM, α-actinin, and vinculin, alternative splicing of the PTB1 and PTB4 isoforms showed no significant changes between the day 0 and 4 RASM and PAC-1 samples (Fig. 2B). Therefore, despite the fact that PTB4 has been shown to be a more active repressor of α-TM exon 3 compared with PTB1 (29), changes in the ratio of the PTB isoforms do not cause the switch in α-TM and α-actinin splicing in dedifferentiating RASM cells. RT-PCR was not carried out under conditions that would allow quantitative analysis of absolute levels of expression. Nevertheless, we consistently observed that the levels of PTB products appeared to be lower in day 0 cells than in day 4 cells.

Further analysis of PTB expression using primers P37 and P33/2, which prime within exons 8 and 12, respectively, produced a strikingly different result. A novel band (labeled smPTB in Fig. 2C and D) larger than PTB4 was the major PCR product in day 0 samples, but not in day 4 or PAC-1 samples. Cloning and sequencing of this PCR product showed that it was derived from a PTB-related gene that was distinct from PTB and the known paralogs Rod1 (40) and nPTB (38, 39). We refer to this new PTB paralog as smPTB due to its high expression in a number of SM tissues (see below). RT-PCR using smPTB-specific primers P32 and P33 confirmed that it was expressed in day 0 RASM cells, but not in day 4 or cultured PAC-1 cells (Fig. 2D). smPTB was also expressed in other SM tissues such as the uterus and vas deferens.

SmPTB Is Expressed from an Intronless Gene—At the time that we identified smPTB, no corresponding sequences could be identified by BLAST searches of available expressed sequence tag or genomic data bases. However, using 5′- and 3′-RACE, full-length smPTB cDNA was isolated from day 0 RASM cell RNA. The open reading frame encodes a 588-amino acid protein with a predicted molecular mass of 63.7 kDa and with 53–74% amino acid identity to PTB, nPTB, and Rod1 (Fig. 3). Pairwise BLAST analyses showed that smPTB is more closely related to PTB than to either of the other genes. Subsequent to cloning the full-length smPTB cDNA, the corresponding rat gene sequence was identified using the Ensembl Trace Database, whereas the mouse gene was identified by BLAST analysis of annotated mouse genomic data bases and was located in contig 132920, corresponding to chromosome X A1.1. Both the rat and mouse genes are intronless. The mouse gene contains three possible polyadenylation addition signals giving a message size of 4.16, 5.09, or 6.53 kb. As determined by Northern blot analysis, the size of the rat smPTB mRNA is close to 6 kb (data not shown).

SmPTB has the same overall structural organization as PTB (Fig. 3), with four RRM domains and the same unusual fifth
The amino acid sequences of the four rat paralogs were aligned using Clustal, followed by some manual adjustment. Positions identical between the four genes are shown by asterisks, whereas conservative and semiconservative alterations in the RNP2 and RNP1 boxes are marked by colons and periods, respectively. The bipartite N-terminal nuclear localization signal of PTB is underlined. RRM domains are shown in blue, with the RNP2 and RNP1 boxes in red. RRs are as defined by PROSITE, with the exception that RRM3 is extended to include the fifth β-strand (underlined LTKD(Y/F) determined by NMR (49). The degree of identity to PTB is 78, 89, 71, and 75%, respectively. Within the characteristic RNP1 and RNP2 motifs, most amino acid changes are conservative (Fig. 3). PTB and nPTB have bipartite nuclear localization signals (37), the N-terminal half of which appears to be lacking in both smPTB and ROD1. smPTB contains sequences equivalent to PTB exon 9, which defines the PTB4 isoform. The larger size of smPTB is accounted for by additional inserts of 36 amino acids between RRM1 and RRM2 and 22 amino acids between RRM2 and RRM3. These two linkers are the most divergent regions both between smPTB and PTB, and also between rat and mouse smPTBs. Strikingly, the linker region between RRM3 and RRM4 is identical in smPTB and the other paralogs. This region was not observed in the NMR structure of PTB RRM3 and RRM4, suggesting that it is extremely flexible (49). The absolute conservation suggests that the linker serves an important role and perhaps takes up a defined structure upon RNA binding. PTB has three sites that can be cleaved by caspase-3 (50). The most efficiently cleaved site (LKTD138S) is conserved in nPTB and ROD1, but not in smPTB. However, the next major site (AAVD170A) in PTB is reasonably conserved in smPTB (SAVD178T), suggesting that it may also be a target of caspase-3 during apoptosis.

**Expression of smPTB**—Expression of smPTB relative to PTB was monitored across a range of rat tissues using PCR primer pair P37/Pd3. Expression of smPTB was most prominent in smooth muscle and skeletal muscle. The expression of PTB in these tissues was confirmed using primers specific for PTB exons 8 and 12. The identities of the bands are shown to the right.

mRNA, giving rise to the PTB1tr (where “tr” is truncated) and PTB4tr isoforms (Fig. 4).^2^ smPTB was expressed most promi-

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^2^ M. C. Wollerton and C. W. J. Smith, unpublished data.
nently in two SM tissues, the aorta (day 0) and uterus, where it was more abundant than PTB. It was also readily detected in the testis, thymus, skin, and lung (Fig. 4), although in these tissues, PTB was more abundant. It was not highly expressed in the stomach or small or large intestine, all of which contain SM cells. Longer exposures showed that smPTB expression could be detected in most tissues.

We next investigated smPTB expression by in situ hybridization, which allows direct analysis of expression without PCR amplification. Expression of smPTB was analyzed by in situ hybridization to day 10, 14, and 15 mouse embryo sections. This allowed us to conduct an unbiased survey of smPTB expression across multiple tissues at a stage of embryonic development when many SM cells express late markers of differentiation (e.g. SM myosin heavy chain). Whereas the sense smPTB control probe produced no signal, the antisense probes hybridized in a number of places, indicated by the light areas in the dark-field images (Fig. 5, lower panels). The most prominent signal was seen in the terminal bronchioles of the lung. The signal was readily detected at embryonic day 14.5 and was higher by embryonic day 15.5. Hybridization was also seen in the skin, intercostal muscles, and the venous plexus of the liver. In day 10 embryo sections, hybridization to the maternal uterus could be observed (Fig. 5, left panels). The lung, skin, and uterus are all tissues that showed relatively high levels of smPTB expression by RT-PCR (Fig. 4). The embryo sections did not include the aorta, which was one of the tissues with the highest smPTB signal upon RT-PCR (Fig. 4). Nevertheless, the in situ hybridization data were generally in agreement with the RT-PCR data and indicated that smPTB is differentially expressed in mouse embryos.

**smPTB Is a Nuclear Protein That Binds RNA**—To start to address the possible functions of smPTB, PAC-1 cells were transiently transfected with expression vectors for smPTB fused to GFP at either the C or N terminus (Fig. 6). Like PTB-GFP (12), GFP-smPTB localized almost completely to the nucleus, despite lacking sequences equivalent to the N-terminal half of the PTB bipartite nuclear localization signal (see above). Identical results were obtained with both N- and C-terminal GFP fusions and in HeLa cells. In a small number of PAC-1 cells, fluorescence was observed in the cytoplasm as well as the nucleus. These results demonstrate that smPTB is predominantly localized to the nucleus, consistent with a role in regulation of splicing, but that, like PTB, it may also be able to play additional cytoplasmic roles.

To examine the activities of smPTB in vitro, we overexpressed C-terminally His-tagged smPTB in E. coli. Recombinant smPTB migrated upon SDS-PAGE with an anomalously high mobility of 83 kDa compared with the expected size of 65 kDa. A similarly sized product was obtained by in vitro translation of smPTB in reticulocyte lysate (data not shown). Recombinant smPTB was analyzed for RNA binding by both UV cross-linking and electrophoretic mobility shift assays. RNA probes containing various regulatory elements from α-tropomyosin were used in UV cross-linking assays with recombinant
smPTB and PTB4 (Fig. 7, A and B). Both PTB and smPTB cross-linked to RNA probes that contained the PTB-binding elements P3 and DY (probes 1–4), but not to probe 5, which contains a UGC motif regulatory element. Whereas PTB cross-linked with roughly equal efficiency to probes 1–4, smPTB cross-linked more efficiently to probes 1 and 4, which contain the DY regulatory sequence. These data confirm that smPTB is an RNA-binding protein. To compare the affinity with which smPTB and PTB bind to RNA, we carried out a competitive UV cross-linking assay. PTB4 and smPTB were premixed before incubation together with α-TM RNA probe 1. While one protein was held at a constant concentration of 0.5 μM, the concentration of the other protein was varied. smPTB was readily able to displace PTB (Fig. 7C, lanes 1–6). In contrast, titration of PTB4 led to a more gradual increase in its own cross-linking signal, and there was little evidence of smPTB displacement (lanes 7–13). Similar results were seen with RNA probes 3 and 4 (data not shown). These data suggest that smPTB binds to the RNA probes tested with higher affinity compared with PTB.

**smPTB Is a Splicing Repressor**—We tested the activity of recombinant smPTB in a number of in vitro splicing assays, but were able to observe only nonspecific inhibitory activity (data not shown). At this stage, we do not know whether this is due to the recombinant protein lacking full activity or to the lack of an essential cofactor. We also tested the activity of smPTB as a splicing regulator by cotransfection with tropomyosin and actin splicing reporter constructs. It had relatively modest effects on splicing of α-TM constructs in PAC-1 SM and other cell types (data not shown). This could be because there is already abundant PTB in these cells, and smPTB does not have a significantly different activity on this substrate. We also tested the effects of smPTB cotransfection with the α-actin splicing reporter into HeLa cells. This reporter predominantly spliced to include the NM exon (Fig. 8). In control experiments, the α-actin reporter has been shown to be unresponsive to other overexpressed proteins, including β-galactosidase, hnRNP-C, hnRNP-L, and PTBtr1.2 Consistent with previous results (29), overexpression of PTB led to skipping of both mutually exclusive exons (Fig. 8, lanes 5 and 6). Overexpression of smPTB had a similar effect, leading to enhanced skipping of both the NM and SM exons (lanes 3 and 4), although it was not as potent compared with PTB. This result establishes that smPTB has the ability to act as a splicing repressor, with activity similar (but not identical) to that of PTB.

**DISCUSSION**

The data reported here confirm the existence and tissue-specific expression of mRNA for a fourth PTB paralog, smPTB, in rat and mouse. We have also observed additional cross-reactive bands in Western blots using anti-PTB antiserum and protein samples from day 0 RASM cells (data not shown). In the future, we aim to further analyze and verify smPTB expression using specific antisera raised against the recombinant protein. We have demonstrated that recombinant smPTB has various properties in common with PTB, including predominant nuclear localization (Fig. 6), RNA binding (Fig. 7), and splicing repression (Fig. 8). At present, it is not clear which alternative splicing events might be specific targets of endogenous smPTB. The correlation between smPTB mRNA expression and regulation of α-TM and α-actin alternative splicing in primary RASM cells and its high expression in some SM tissues (Figs. 1, 2, and 4) initially suggested that it may play a key role in regulating these two splicing events. However, some SM tissues such as the intestine did not express smPTB, and cotransfection of smPTB with either α-TM or α-actin reporters did not cause a significant switch toward the characteristic SM-specific splicing pattern of each gene. One possibility is that smPTB plays no role in these regulated splicing decisions. Other possibilities are that smPTB may simply replace the activity of PTB in these systems, that it may require a specific cofactor for activity in these systems, or that it may not be active in the presence of PTB. Currently, we cannot distinguish between these possibilities, although the fact that it binds RNA in vitro with higher affinity than PTB argues against the third possibility. In the future, it will be of interest to test the activity of smPTB using in vitro splicing and translation assays and also in cells after knockdown of endogenous PTB by RNA interference (31).
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