Differential alternative splicing activity of isoforms of polypyrimidine tract binding protein (PTB)

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ABSTRACT

Polypyrimidine tract binding protein (PTB) is an RNA-binding protein that regulates splicing by repressing specific splicing events. It also has roles in 3'-end processing, internal initiation of translation, and RNA localization. PTB exists in three alternatively spliced isoforms, PTB1, PTB2, and PTB4, which differ by the insertion of 19 or 26 amino acids, respectively, between the second and third RNA recognition motif domains. Here we show that the PTB isoforms have distinct activities upon α -tropomyosin (TM) alternative splicing. PTB1 reduced the repression of TM exon 3 in transfected smooth muscle cells, whereas PTB4 enhanced TM exon 3 skipping in vivo and in vitro. PTB2 had an intermediate effect. The PTB4 > PTB2 > PTB1 repressive hierarchy was observed in all in vivo and in vitro assays with TM, but the isoforms were equally active in inducing skipping of α -actinin exons and showed the opposite hierarchy of activity when tested for activation of IRES-driven translation. These findings establish that the ratio of PTB isoforms could form part of a cellular code that in turn controls the splicing of various other pre-mRNAs.

Keywords: alternative splicing; gene regulation; nPTB; pre-mRNA splicing; PTB

INTRODUCTION

Alternative splicing is a common mechanism of gene control (Chabot, 1996; Cooper & Mattox, 1997; Lopez, 1998; Jiang & Wu, 1999; Smith & Valcárcel, 2000). Up to a third of human genes are estimated to be alternatively spliced (Grabowski, 1998; Hanke et al., 1999; Mironov et al., 1999), and in some spectacular examples, thousands of distinct protein isoforms can be produced from a single gene (Schmucker et al., 2000). Switches in alternative splicing patterns are often controlled with strict cell-type or developmental-stage specificity. Therefore understanding the mechanisms of alternative splicing is an essential component of attempts to understand programs of cell differentiation and development in molecular terms.

Switching of splicing patterns can involve activation or inhibition of particular splice sites. These regulatory events are mediated by *cis*-acting sequences, which can either be distinct enhancer or silencer elements, or

can be specific variants of the consensus splice site sequences. Binding of *trans*-acting factors to these elements either promotes or inhibits spliceosome assembly. Initial expectations were that cell-specific alternative splicing events would be determined by the activity of dedicated cell-specific regulators. Indeed, the well-characterized *Drosophila* female-specific sex-lethal and transformer proteins act as repressors and activators, respectively, of specific splice sites (Lopez, 1998). Investigation of mammalian model systems has tended to support a view in which alternative splicing is regulated by particular combinations of widespread factors (Smith & Valcárcel, 2000). Individual regulatory factors do not necessarily show the same cell-type restriction as the alternative splicing events that they regulate. Two families of such general factors are the RS-domain proteins and hnRNP proteins. RS domain proteins are characterized by domains that are enriched in the amino acids arginine and serine (Fu, 1995; Gravely, 2000). These proteins include a number of essential splicing factors as well as a number of activating regulators. Most of them contain RNA-binding domains of the RRM type. Members of the SR-family, a subset of RS-domain proteins, bind to splicing enhancer sequences and have

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well-established roles in regulating splice site selection (Manley & Tacke, 1996; Tacke & Manley, 1999).

HnRNP proteins are a diverse group of RNA-binding proteins with various roles in pre-mRNA and mRNA function (Krecic & Swanson, 1999; Smith & Valcárcel, 2000). A number of hnRNP proteins have roles in alternative splicing. HnRNP A1 and hnRNP H can cause exon skipping when recruited to particular splicing silencer sequences (Caputi et al., 1999; Chen et al., 1999; Del Gatto Konczak et al., 1999). HnRNP A1 can also antagonize the activity of SR proteins in alternative splice site selection (Chen et al., 1999). In contrast, hnRNP F and H assemble into a neural specific enhancer complex in the *c-src* pre-mRNA, promoting inclusion of the neural specific N1 exon (Min et al., 1995; Chou et al., 1999). Polypyrimidine tract binding protein, also known as hnRNP-I, was originally thought to be a splicing factor (Garcia-Blanco et al., 1989; Gil et al., 1991; Patton et al., 1991), but has subsequently been found to be a repressive splicing regulator (reviewed in Valcárcel & Gebauer, 1997). It acts as a splicing repressor of exons in a number of genes including *c-src*, γ -GABA_A receptor $\gamma 2$, α - and β -tropomyosins, fibronectin, α -actinin, and fibroblast growth factor receptors (FGFR)-1 and -2 (Mulligan et al., 1992; Norton, 1994; Lin & Patton, 1995; Singh et al., 1995; Ashiya & Grabowski, 1997; Chan & Black, 1997; Perez et al., 1997a; Gooding et al., 1998; Southby et al., 1999; Wagner et al., 1999; Zhang et al., 1999; Carstens et al., 2000; Jin et al., 2000). Exons regulated by PTB are usually associated with high-affinity binding sites for PTB that are identical or similar to the optimal binding site for PTB (UCUU in a pyrimidine-rich context; Perez et al., 1997a). In most of these examples, PTB is involved in widespread repression of an exon, except in a limited range of cell types. For instance, in actinin, the smooth-muscle-specific exon of a mutually exclusive pair is widely repressed by PTB and as a result is only selected in SM cells (Southby et al., 1999). In contrast, in the α -tropomyosin (TM) system, which is coregulated with actinin (Roberts et al., 1996), PTB mediates a low level of repression upon exon 3 in most cells, but in smooth muscle cells only this repression is enhanced (Perez et al., 1997a; Gooding et al., 1998; Fig. 1B). In addition to its roles as a splicing repressor, PTB activates IRES-driven translation (Kaminski et al., 1995; Hunt et al., 1999), activates some poly(A) addition sites (Moreira et al., 1998; Lou et al., 1999), and can also localize some mRNAs (Cote et al., 1999).

Structurally, PTB has four RRM-like RNA-binding domains (Fig. 1A) and is dimeric (Perez et al., 1997b). The solution structure of RRMs 3 and 4 shows that these maintain the typical RRM domain fold, but that RRM 3 has an additional fifth beta strand and that the two RRMs are joined by a highly flexible linker (Conte et al., 2000). Structure–function mapping has shown that the N-terminal half, containing RRMs 1 and 2, is

responsible for PTB dimerization, whereas the C-terminal RRMs 3 and 4 mediate high-affinity RNA binding (Perez et al., 1997b; Oh et al., 1998). PTB has three alternatively spliced isoforms, PTB1, PTB2, and PTB4 (Fig. 1; the original sequence reported as PTB3 appears to have contained a single base sequencing error distinguishing it from PTB4 (Gil et al., 1991; Ghetti et al., 1992)). Compared to PTB1, PTB2 and PTB4 contain an additional 19 or 26 amino acids, respectively, between RRMs 2 and 3, arising from the optional inclusion of PTB exon 9 using one of two alternative 3' splice sites (Fig. 1A). In addition to these alternatively spliced isoforms, two PTB homolog genes encode proteins with 70–80% amino acid identity to PTB. ROD1 contains a PTB4-like insert and is expressed predominantly in haematopoietic cells, although its function remains unclear (Yamamoto et al., 1999). nPTB (also referred to as brPTB) lacks a PTB4-like insert and is expressed at highest levels in neuronal cells, where it appears to play a role in antagonizing the activation of some exons by Nova and relieving the repressive activity of PTB on other neuronal specific exons (Ashiya & Grabowski, 1997; Chan & Black, 1997; Markovtsov et al., 2000; Polydorides et al., 2000). Most experimental work with PTB to date has used the shortest isoform, PTB1. When different isoforms were tested, no differences in activity were reported (Lou et al., 1999; Carstens et al., 2000).

In this study, we compared the activity of PTB isoforms. We find that the three isoforms have distinct activities in regulating TM, but not actinin, splicing. Whereas overexpression of PTB1 antagonized repression of TM exon 3, PTB4 enhanced exon 3 skipping in vivo and in vitro. The isoforms showed the opposite hierarchy of activity in an in vitro IRES-dependent translation assay. This is the first report of distinct functional activities of the PTB alternatively spliced isoforms and establishes their ratio as a potential cellular determinant of some alternative splicing patterns.

RESULTS

PTB depletion and addback alters TM alternative splicing

PTB has been implicated as a repressor of TM exon 3 by: (1) addition of excess recombinant PTB to in vitro splicing reactions (Lin & Patton, 1995; Singh et al., 1995), (2) correlations between loss of regulation and PTB binding upon mutation of PTB binding sites in the TM exon 3 polypyrimidine tract (P3) and DY element (Fig. 1B; Perez et al., 1997a; Gooding et al., 1998), (3) titration of PTB in vitro by the addition of competitor RNAs, and restoration of inhibition by addition of recombinant PTB to the competitor-challenged extracts (Gooding et al., 1998). A more rigorous approach is to selectively deplete extracts and then test whether re-

FIGURE 1. Alternative splicing of PTB and tropomyosin. **A:** PTB contains four RRM-type RNA-binding domains. Three alternatively spliced isoforms exist via alternative splicing of exon 9. Complete skipping of exon 9 produces PTB1. Inclusion of exon 9 using one of two competing 3' splice sites produces PTB2 and PTB4, which have an extra 19 or 26 amino acid insert, respectively, between RRMs 2 and 3. **B:** α -tropomyosin (TM) has a pair of mutually exclusive exons, one of which is selected with smooth muscle specificity. PTB binding sites in the polypyrimidine tract (P3) and in a downstream pyrimidine tract (DY) (indicated by arrowheads) are involved in repressing exon 3 in SM cells (Perez et al., 1997a; Gooding et al., 1998). The sequences of P3 and DY are shown below. The branch point of exon 3 is the bold A; optimal PTB binding sites involved in repression are bold and underlined. In addition there is an upstream regulatory element (URE) containing CUG repeats, and a second CUG-repeat region adjacent to DY (black ovals). The branch points and associated pyrimidine tracts of exons 2 and 3 are indicated by the semicircles and rectangles upstream of each exon.

combinant PTB is sufficient to restore the original splicing pattern to depleted extract. We have used this approach to establish the role of PTB as a repressor of the actinin SM exon (Southby et al., 1999). PTB was depleted from HeLa nuclear extracts using biotinylated RNA containing repeats of the optimal PTB binding site. Approximately 85% of the PTB was removed by this procedure (Fig. 2A); both characteristic PTB doublet bands were equally depleted. The residual PTB could not be removed without inactivation of the splicing activity of the extract (Southby et al., 1999); similar results were found with immunodepletion of PTB (Markovtsov et al., 2000).

Although TM exon 3 is only highly repressed in SM cells, we have previously found that in nonmuscle cells there is a low background level of repression mediated by the same *cis*-acting regulatory elements that confer SM-specific regulation (Gooding et al., 1994, 1998).

We therefore tested the effect of PTB depletion upon the splicing pattern of TM transcripts. We used a test RNA containing exons 1–3–4, with all the elements flanking exon 3 that are necessary for regulated exon skipping. In HeLa extracts, the predominant splicing intermediates and products correspond to splicing of exon 3 to exons 1 and 4. However, there is also a small amount of 1–4 splicing, as indicated by the diagnostic lariat product band (indicated by the asterisk in Fig. 2B). The corresponding 1–4 spliced product is much smaller and less intense and does not provide a reliable diagnostic band. In PTB-depleted extracts, the 1–4 lariat was reduced, whereas various products of exon 3 splicing were increased, as compared with mock-depleted extracts (Fig. 2B, lanes 2 and 3). In particular, bands corresponding to splicing of exon 1 to exon 3 were markedly increased upon PTB depletion, suggesting that the major target of PTB repression in the extracts

FIGURE 2. Recombinant PTB restores TM splicing to PTB-depleted extracts. **A:** Western blot for PTB of samples containing 25 or 50 ng of pQE-PTB1 (lanes 1 and 2), 0.25, 0.5, 1.0, 1.5, and 2.0 μ L of HeLa nuclear extract (lanes 3–7) and 2.3 μ L of mock-depleted and PTB-depleted nuclear extract (equivalent protein content to 2 μ L of undepleted extract) in lanes 8 and 9. Depletion removes 85–90% of PTB, with both the characteristic PTB doublet bands being equally depleted. **B:** In vitro splicing of TM1–3–4 RNA in normal (N), mock-depleted (M) and PTB-depleted nuclear extracts. The 1–4 splicing pathway (i.e., exon 3 skipping) is indicated by the diagnostic lariat product at the top of the gel (asterisk). Unspliced RNA is shown in lane 1. Various other products and intermediates of exon 3 splicing are indicated, including the fully spliced 134 product. In PTB-depleted extract, exon skipping is reduced whereas various products of exon 3 splicing are increased. Addition of recombinant pQE-PTB1 (0.1, 0.5, 1, 2, and 3 μ M, lanes 4–8) restored the splicing pattern of undepleted extract.

is the upstream polypyrimidine tract of exon 3. Addition of recombinant His-tagged PTB1 at concentrations similar to those in the original undepleted extract restored the original splicing pattern (0.5 μ M added PTB, Fig. 2B, lanes 5). Higher concentrations of PTB were usually without further effect (Fig. 2B, lanes 6–8), although they sometimes produced a small increase in exon 3 skipping. These data provide strong support for the mediation by PTB of the low background level of repression upon TM exon 3 in non-SM cells.

Opposite effects of PTB isoforms upon TM exon 3 in vivo

The only experiments to date that have addressed the potential role of PTB in SM cells involved mutation of binding sites for PTB in TM constructs (Perez et al., 1997a; Gooding et al., 1998). We therefore decided to test the effects of PTB overexpression upon the splicing of TM constructs in SM cells. High-level overexpression of PTB1 was obtained in transiently transfected cells as judged by western blot (see below; Fig. 4). The western blot detects total endogenous and over-

expressed PTB, but only 10–20% of cells are routinely transfected, as determined using LacZ or GFP reporters. Therefore we estimate that in transfected cells, PTB levels are significantly elevated, in the order of 5–10-fold above normal. We next looked at the effects of PTB overexpression upon alternative splicing of a minigene construct containing TM exons 1–3–4. In most cells, the default splicing pattern is predominant inclusion of exon 3. In the PAC-1 partially differentiated SM cell line, exon 3 is skipped in ~15–30% of transcripts. Contrary to expectation, PTB1 overexpression caused an approximately threefold decrease in skipping of TM exon 3 in SM cells (Fig. 3, lanes 1 and 2). When we used a test construct with a shortened polypyrimidine tract (pTΔPy), which also exhibits regulated splicing but with a higher background of exon skipping in non-SM cells, PTB1 overexpression was found to antagonize exon skipping in both SM and HeLa cells (Fig. 3, lanes 7, 8, 10, and 11).

These results suggested either that overexpression of PTB1 titrates out a corepressor of TM exon 3 in both cell types, or that a more potent repressor acts upon TM exon 3 in vivo and that overexpressed PTB1 can

FIGURE 3. PTB1 overexpression reduces exon 3 skipping. PAC1 smooth muscle (SM) and HeLa cells were transiently transfected with wild-type TM reporter construct (pTS3D) or a mutant construct with a truncated exon 3 pyrimidine tract, which is still regulated but with generally elevated levels of exon skipping (pTΔPy). Transfections were carried out alone (–, lanes 2, 5, 8, and 11) or with coexpression of PTB1 (+, lanes 1, 4, 7, and 10). Lanes 3, 6, 9, and 12 are mock transfections. Harvested RNA was analyzed by RT-PCR. The positions of products corresponding to 134 splicing and 14 splicing are indicated. The asterisks denote bands that correspond to 134 and 14 splicing using a cryptic 3' splice site 29 nt upstream of exon 4. These bands appear in similar ratios to the major bands, so splicing using the exon 4 cryptic splice site is appropriately regulated (Gooding et al., 1994, 1998). Values below each lane refer to the percentage of exon 3 skipping, and are the mean $\pm$ SD of three experiments except in lanes 1 and 2, which are the values of only the experiment shown. The data shown are all from the same experiment; the black lines indicate positions where unnecessary lanes have been removed from the image. PTB1 cotransfection in each case decreased exon skipping.

compete for binding to the same sites. The alternatively spliced PTB2 and PTB4 isoforms were candidate proteins that would bind to the same sequences, possibly with different regulatory consequences. We therefore compared the effects upon TM alternative splicing of overexpression of PTB1, PTB2, and PTB4 (Fig. 4). Western blots showed that the three isoforms were overexpressed and that PTB1 corresponds to the lower band of the doublet observed in most cell types, whereas PTB2 and PTB4 both comigrate with the upper doublet band (Fig. 4A). Whereas PTB1 consistently reduced skipping of TM exon 3, PTB4 caused an increase in exon skipping in SM cells (Fig. 4B). PTB2 had an effect intermediate between that of PTB1 and PTB4. In non-SM cells such as mouse L cells or HeLa cells, qualitatively similar results were obtained. Although the starting level of exon skipping was much lower, PTB4 increased exon skipping, whereas PTB1 either decreased exon skipping or had no effect. The PTB4 >

PTB2 > PTB1 hierarchy of repressive activity was always observed using a variety of cell lines and reporter constructs. The hierarchy was observed over a 1000-fold concentration range of transfected PTB expression plasmid; there was no concentration of PTB4 plasmid that gave a lower level of exon skipping than any concentration of PTB1 plasmid (data not shown). Transfection with mixtures of the different PTB isoforms produced levels of exon skipping intermediate between the amounts obtained with each isoform individually (data not shown). These data are most easily explained by competition between different PTB isoforms for binding to available regulatory sites in TM pre-mRNA, with PTB4 causing most and PTB1 least repression. By comparison with their effect on TM exon 3, none of the PTB isoforms had any effect upon the splicing of constructs containing TM exons 1–2–4 (Fig. 4C, pTX2); the level of exon 2 inclusion was insensitive to PTB cotransfection.

FIGURE 4. PTB isoforms have different effects upon TM splicing in vivo. **A:** Western blot for PTB of PAC1 SM cells, L, and HeLa cells that were transfected with expression vectors for PTB1, PTB2, PTB4, or control untransfected cells (-). Compared with the control samples, PTB1 expression led to an enhancement of the lower band of the PTB doublet, whereas PTB2 and PTB4 led to enhancement of the upper doublet band. **B:** The wild-type TM reporter construct TS3D was cotransfected with PTB1, PTB2, or PTB4 in SM, L, and HeLa cells and RNA analyzed by RT-PCR. Values below each lane represent mean $\pm$ SD with $n = 3$ for SM, 4 for L, and 3 for HeLa. Lanes 5, 10, and 15 are mock transfections in which no reporter construct was transfected. In cell type, there was a hierarchy of activity, with PTB4 producing the greatest and PTB1 the least amount of exon skipping. **C:** A control construct, pTX2, containing TM exons 1–2–4 was transfected into HeLa cells along with PTB1, PTB2, and PTB4. Values for exon skipping are mean $\pm$ SD for five experiments. PTB transfection had no effect upon the levels of exon skipping.

A large number of TM 1–3–4 constructs are available containing mutations of the regulatory elements (Gooding et al., 1994, 1998). We therefore tested whether the effects of overexpressed PTB were associated with particular *cis*-acting elements. We tested PTB cotransfection using reporter TM constructs carrying mutations in the URE (CUG repeats), the exon 3 polypyrimidine tract (PTB binding sites) or the PTB sites of DY (the DRE) (see Fig. 1). Although the basal levels of exon skipping were much reduced in most of these constructs, consistent with a reduced ability of endogenous PTB to induce exon skipping, overexpressed PTB isoforms still had an effect. The PTB4 > PTB2 > PTB1 hierarchy of repressive activity was in all cases maintained, with PTB4 cotransfection usually resulting in approximately threefold more exon skipping than PTB1 (Table 1). The inability to completely abolish the effect of PTB is partly because each mutant still retained some functional PTB sites in the polypyrimidine tract of exon 3. In contrast to the TM exon 3 constructs, an exon 2 reporter (pTX2) was unresponsive to cotransfection with any of the PTB isoforms.

Equal effect of PTB isoforms on skipping of actinin exons

In contrast to our data on the effects of PTB isoforms on TM splicing, previous reports have not detected any differences between the activities of PTB1, PTB2, and PTB4 (Lou et al., 1999; Carstens et al., 2000). We therefore tested the effects of PTB1, PTB2, and PTB4 on another alternatively spliced system. PTB has been shown by *in vitro* depletion and reconstitution to repress the actinin SM exon in non-SM cells (Southby et al., 1999). Actinin minigene constructs do not show high levels of regulation in transfected SM cells (J. Southby & C.W.J. Smith, unpubl. observations). In all cell types tested, the predominant splicing pathway is use of the NM exon, although in SM cells 5% SM exon inclusion was observed (Fig. 5, lane 4). Consistent with previous data showing that PTB represses the SM exon (Southby et al., 1999), overexpression of PTB1, PTB2, or PTB4 reduced selection of the SM exon to less than 1% (Fig. 5). The more dramatic effect of PTB overexpression was to induce skipping of both the NM and

TABLE 1. Summary of data from cotransfection of PTB isoforms with TM reporter constructs.

Construct	Mutation	Cell type	PTB1	PTB2	PTB4	Control
pTS3D	Wild type	SM	5.4 ± 0.1	11.2 ± 1.1	19.5 ± 0.7	14.7 ± 0.7
		L	2.6 ± 0.4	3.8 ± 0.2	6.6 ± 1.1	2.4 ± 0.3
		HeLa	0.8 ± 0.1	1.15 ± 0.02	1.7 ± 0.1	1.1 ± 0.1
pT2M2	URE point mutations	SM	1.4 ± 0.03	3.1 ± 0.1	4.7 ± 0.02	1.9 ± 0.1
		HeLa	<1	<1	<1	<1
pTS3F	DY deletion	SM	1.6 ± 0.3	2.6 ± 0.5	4.7 ± 0.4	1.9 ± 0.6
		HeLa	<1	<1	<1	<1
pP3-2TS3D	P3 point mutation	SM	3.4 ± 0.3	6.2 ± 0.1	9.3 ± 0.7	6.0 ± 0.2
		HeLa	<1	<1	<1	<1
pTΔPy	P3 partial deletion	SM	30 ± 1.7	45 ± 1.8	61 ± 1.4	65 ± 0.5
		HeLa	2.7 ± 0.2	4.9 ± 0.5	7.9 ± 0.5	8.1 ± 0.6
pTΔPy.3F	DY deletion	SM	2.3 ± 0.2	5.0 ± 0.05	6.6 ± 0.8	1.7 ± 0.3
	P3 partial deletion	HeLa	<1	<1	<1	<1
pTX2	Exon 2 reporter	HeLa	8.9 ± 0.7	8.7 ± 0.7	8.3 ± 0.8	10.3 ± 0.9

All constructs are derived from the regulated wild-type vector pTS3D, which contains TM exons 1, 3, and 4. The mutations are summarized in column 2. Columns 4–7 show the percentage exon skipping ($\pm$ SD) in various experiments ($n = 3$, except pTS3D in L cells, $n = 4$, and pTX2, $n = 5$). pT2M2, pTΔPy, and pTX2 were described in Gooding et al. (1994). pTS3F was described in Gooding et al. (1998). pP3-2TS3D was derived from pTS3D by including the P3-2 mutation of Perez et al. (1997a). pTΔPy.3F combines the P3 deletion of pTΔPy with the DY deletion of pTS3F.

FIGURE 5. PTB isoforms repress actinin alternative exons. The construct pA, contains actinin exons EF1a-NM-SM-EF2. Branch points and associated pyrimidine tracts of the NM and SM exons are indicated by semicircles and rectangles. The sequences between the branch points (bold As) and 3' splice sites are shown to the right, with potential PTB binding sites (UCUU) bold underlined. PTB sites between the SM branch point and exon are known to be involved in repression (Soutby et al., 1999). The role of potential PTB binding sites upstream of the NM exon has not been previously investigated. pA was transfected in PAC1 SM cells along with PTB1, PTB2, and PTB4 expression vectors. Values below the lanes give the percentage of SM exon splicing and of skipping of both exons and represent mean $\pm$ SD for five experiments. Samples were loaded to show approximately equal amounts of the EF1a-NM-EF2 product (the percentage of this product can be calculated from the amounts of the other products). The data are all from the same experiment; the black line between lanes 3 and 4 indicates a position at which unnecessary lanes were removed from the image. All PTB isoforms reduced SM exon splicing and induced skipping of both NM and SM exons.

FIGURE 6. Recombinant PTB isoforms. Samples of 0.5 and 1 μ g of purified His-tagged pQE-PTB1 (lanes 1 and 2), PTB2 (lanes 3 and 4), PTB4 (lanes 5 and 6), and nPTB (lanes 7 and 8) were separated by SDS PAGE alongside molecular weight markers (lane 9, "M") and visualized by Coomassie Blue staining. Sizes of markers in kilodaltons are shown to the right.

SM exons (i.e., EF1a-to-EF2 splicing). This splicing pathway is observed in a proportion of actinin transcripts in SM tissue samples (C. Gooding, unpubl. observations). This suggests that PTB might act as a repressor of the NM exon, in addition to its role in repressing the SM exon. In marked contrast with the distinct effects of the PTB isoforms upon TM splicing, they were equally

effective in inducing skipping of the NM and SM exons (Fig. 5) over a 1000-fold concentration range of transfected expression plasmid (data not shown). Thus, the differential activity of PTB isoforms is not general, but is specific to particular regulated splicing events.

PTB repressive hierarchy in vitro

The preceding data showed that overexpressed PTB isoforms exhibited differences in their effects upon TM alternative splicing in transfected cells, and between the TM and actinin systems. To study this effect in more detail, and, in particular, to ascertain whether the effects of overexpressed PTB isoforms were direct, we turned to in vitro splicing assays in HeLa cell nuclear extract.

His-tagged recombinant PTB1, PTB2, PTB4, and also the neuronal PTB homolog, nPTB, were expressed in *Escherichia coli* and purified (Fig. 6). The proteins were then added into PTB-depleted nuclear extracts (Fig. 7). All four proteins were able to restore the splicing pattern characteristic of mock-depleted extracts. However, when added in excess to the extracts (conditions analogous to transient overexpression in cultured cells) there were clear difference between the proteins. PTB4 was more potent than the other proteins at inducing skipping of exon 3. For instance, at 3 μ M added protein (approximately 10-fold excess), PTB4 gave a 10-fold

FIGURE 7. Differential activity of PTB isoforms upon in vitro TM splicing. TM 1–3–4 RNA was spliced in vitro in whole nuclear extract (N), mock-depleted (M) and PTB-depleted extracts. Recombinant pQE-PTB1, PTB2, PTB4, and nPTB were added to the depleted extract to final concentrations of 0.1, 0.25, 0.5, 1, 2, and 3 μ M (lanes 4–9 in each panel). Each of the proteins was able to restore the original splicing pattern to the extracts (lanes 5 and 6). Splicing precursor, intermediates, and products are shown schematically to the left. The lariat product diagnostic of exon 3 skipping is indicated by the asterisk. At higher levels, PTB4 was much more effective than the other proteins in inducing exon skipping and inhibiting exon 3 splicing. In contrast PTB1 generally had no further effects at higher concentrations.

higher ratio of the lariat product for exon 3 skipping compared with the 1–3–4 spliced product than PTB1 (Fig. 7, lanes 9). Similar results were obtained when the recombinant proteins were added directly to un-depleted nuclear extracts (data not shown). That the differences in activity could be due to differences in the fraction of active protein in the different preparations is ruled out by the observation of a distinct hierarchy of activity in translation assays (see below). Thus, the *in vitro* data support the cell-transfection data and show that PTB4 is more repressive upon TM exon 3 than PTB1. Gel shift and UV-crosslinking experiments indicated no differences in the RNA-binding properties of the four proteins (data not shown). Therefore the enhanced inhibitory activity of PTB4 does not appear to reflect enhanced RNA binding, but rather a property of the protein once it has bound to the RNA.

FIGURE 8. Differential activity of PTB isoforms in promoting translation from the HRV-2 IRES. **A:** The dicistronic RNA contains a cyclin cistron that can be translated by normal 5'-end-dependent scanning, and a 3' NS' cistron that is translated using the IRES. **B:** Lane 1 contains unsupplemented rabbit reticulocyte lysate (RRL), lane 2 contains RRL supplemented with 20% (by vol) HeLa extract (H), and lane 3 contains RRL supplemented with 2.5 ng/μL unr. Lanes 4–6 and 7–9, 10–12 and 13–15, and 16–18 and 19–21 contain increasing concentrations (2.5, 5, 10 ng/μL) of PTB1, PTB2, and PTB4, respectively. Lanes 7–9, 13–15, and 19–21 also contain 2.5 ng/μL unr. The positions of cyclin and NS' are shown to the right of the autoradiograph. **C:** Graphical representation of densitometric analysis of the intensity of the NS' band. Lane numbers correspond to those of the autoradiograph. PTB caused a dose-dependent increase in translation (most obvious in the presence of unr), with PTB1 being more active than PTB2 and PTB4.

PTB isoforms have differential activity in IRES-driven translation

As a control to test that the differential activity of the recombinant PTB isoforms upon TM splicing was not simply due to differences in the proportion of active protein in each PTB sample, we wished to test the PTB isoforms in another functional assay. PTB also plays a role in the cytoplasmic translation driven by the internal ribosome entry sites (IRESs) of several picornaviruses (Kaminski et al., 1995; Hunt & Jackson, 1999). We therefore tested the effects of PTB1, PTB2, and PTB4 upon translation driven by the human rhinovirus-2 (HRV-2) IRES in a dicistronic mRNA assay (Fig. 8A). In this assay, the HRV-2 IRES drove expression of the downstream NS' protein whereas the upstream 5' end-dependent cistron expressed cyclin. Although translation of cyclin occurs efficiently in reticulocyte lysate without the addition of any further proteins (Fig. 8B,C, lane 1), efficient translation of NS' by the HRV-2 IRES requires additional factors present in HeLa extracts (Fig. 8B,C, lane 2). The HeLa activating factors are PTB and a second RNA-binding protein, *unr* (Hunt & Jackson, 1999; Hunt et al., 1999; Fig. 8B,C). Addition of both PTB and *unr* to reticulocyte lysate produced a significant activation of IRES-driven NS' translation (Fig. 8B,C, lanes 7–9, 13–15, and 19–21) compared with either protein alone or no addition to the reticulocyte lysate (Fig. 8B,C, lanes 1, 3–6, 10–12, and 16–18). Significantly, the PTB isoforms showed differential activity, with PTB1 being more active than PTB2 and PTB4 (Fig. 8B,C, lanes 7–9, 13–15, and 19–21). Thus the hierarchy of activity in the translation assay was the opposite of that obtained in the TM alternative splicing assay. This result is of interest to the role of PTB in internal initiation of translation, but also provides a stringent control for the differential activity of the PTB isoforms upon TM splicing (Fig. 7).

DISCUSSION

In this article we have shown for the first time that different PTB isoforms can have distinct activities in both alternative splicing and IRES-dependent translation. Previous reports have shown that the ratios of PTB isoforms vary between different cell types (Wagner et al., 1999; Jin et al., 2000), but experimental investigations that have compared the activity of PTB isoforms have detected no differences (Lou et al., 1999; Carstens et al., 2000). The functional differences that we have now detected between PTB isoforms, coupled with differences in the ratio of isoforms in different cell types, suggests that their ratio may be a cellular determinant of some alternative splicing events.

Our data showed that PTB4 is more repressive than PTB1 upon TM exon 3 in both *in vivo* and *in vitro* assays (Figs. 4 and 6; Table 1). PTB2 had intermediate

activity, but it is likely that this isoform has limited physiological significance. It is undetectable by RT-CPR in a range of rat tissues, and is only a very minor isoform in HeLa cells (C. Gooding & C.W.J. Smith, unpubl. observations). Although the PTB4 > PTB2 > PTB1 repressive hierarchy upon TM exon 3 was maintained in all experiments, there were some apparent discrepancies that are noteworthy. Principal among these is the observation that PTB1 overexpression in SM cells led to decreased skipping of exon 3. Although it is formally possible that overexpressed PTB1 acts as an activator of exon 3, the simplest interpretation is that it is less repressive than PTB4 (or PTB4-containing heterodimers), and that by displacing PTB4 from regulatory elements, it leads to lower levels of exon skipping.

Although many alternative splicing events are influenced equally by PTB1 and PTB4 (Fig. 5; Wagner et al., 1999; Jin et al., 2000), our results suggest that a specific subset may be influenced by this ratio. Ironically, although we have demonstrated this potential using TM minigene constructs, we do not believe that the PTB1/4 ratio is a major deciding factor between inclusion or skipping of TM exon 3. First, the ratio of PTB1 and PTB4 shows no correlation with alterations in TM splicing between fully differentiated and dedifferentiated rat aorta cells (C. Gooding & C.W.J. Smith, unpubl. observations). Secondly, overexpression of PTB4 was not able to cause complete switching to the differentiated SM-specific splicing pattern (Fig. 4). Moreover, when added back to PTB-depleted extracts, the PTB isoforms were equally effective at restoring the original splicing pattern at physiological concentrations (Fig. 7). It was only at higher concentrations (5–10-fold excess) that PTB4 was more effective at inducing TM exon 3 skipping. In addition to PTB binding sites, clusters of CUG motifs on either side of TM exon 3 are required for regulation. The putative corepressor factors that interact at these sites have yet to be identified. It is possible that in the presence of an appropriate corepressor, PTB4 would prove to be a more potent repressor of TM exon 3 at lower concentrations. Despite our incomplete understanding of the mechanism of TM splicing regulation, the clear implications of our results are that the alternative splicing activity of PTB1 and PTB4 are distinct in a subset of PTB-regulated splicing events and that changes in this ratio could play a role in regulating these events.

Early models for repression by PTB suggested that it may compete with U2AF⁶⁵ for binding at certain polypyrimidine tracts (Mulligan et al., 1992; Lin & Patton, 1995; Singh et al., 1995), in a fashion analogous to the competition between U2AF⁶⁵ and sex-lethal protein at the transformer-regulated 3' splice site (Valcárcel et al., 1993). However, most exons that are regulated by PTB have multiple binding sites for PTB within and flanking the exons (Ashiya & Grabowski, 1997; Chan & Black, 1997; Perez et al., 1997a; Gooding et al., 1998;

Zhang et al., 1999; Carstens et al., 2000). In the case of the n-src exon, cooperative binding of PTB to sites on both sides of the exon is required for repression (Chou et al., 2000). This is reminiscent of the cooperative binding of SXL protein to its own pre-mRNA (Wang & Bell, 1994), and of the binding of hnRNP-A1 to high affinity sites flanking a regulated exon in its own pre-mRNA (Blanchette & Chabot, 1999). One model for splicing repression by PTB is that it establishes "zones of repression" bounded by high-affinity PTB binding sites (Carstens et al., 2000). However, given that high-affinity PTB sites are often found within the polypyrimidine tract, it remains possible that PTB interferes with U2AF function by a mechanism other than binding competition. The isoform-specific effects of PTB are not consistent with simple mutually exclusive binding of PTB or U2AF to the polypyrimidine tract of TM exon 3. Rather, they suggest that repression by PTB involves additional interactions once it is bound to RNA. Indeed, this has been suggested by previous work showing that PTB and U2AF⁶⁵ bind concurrently to repressed β -tropomyosin RNAs (Grossman et al., 1998). Moreover, we have recently found that although PTB cross-linking shows an inverse correlation with the efficiency of splicing in a number of mutant actinin RNAs, the binding of U2AF⁶⁵ is independent of splicing efficiency (J. Southby & C.W.J. Smith, unpubl. observations).

The difference between PTB1 and PTB4 is restricted to the 26 additional amino acids between RRM2 and 3. Secondary structure predictions suggest that other than one short beta-strand, most of it is nonhelical or beta-sheet. This region could allow PTB4 to interact with an essential corepressor. However, this corepressor would not be generally necessary because PTB1 is equally repressive to PTB4 in the actinin (Fig. 5) and FGF-receptor (Carstens et al., 2000) model systems. Alternatively, the insert could influence the conformational flexibility between the N- and C-terminal halves of PTB, or between individual PTB monomers within a dimer (Perez et al., 1997b; Oh et al., 1998). This could then be important in allowing PTB4 to interact with splicing factors or other repressors, or it may facilitate the cooperative binding of PTB at widely separated regulatory sites flanking exon 3. It is possible that at some alternative exons, PTB1 would be the more active isoform, as it is for IRES-driven translation. The role of PTB binding at IRESs is thought to be, in part, to promote the optimal tertiary configuration of the RNA, by binding to widely spaced loop sequences (Jackson, 1996; Belsham & Jackson, 2000). In this context, the greater activity of PTB1 could be simply explained by a constrained flexibility, either between the N and C-terminal halves of the protein or between the two monomers of a PTB dimer, being more favorable for appropriate RNA folding.

In addition to alternatively spliced isoforms, PTB homologs probably have a major role in alternative

splicing. Already two such homologs with a 70–80% amino acid identity to PTB have been identified, each with a distinct cell-type specificity. ROD 1 is expressed predominantly in hematopoietic cells, although its function remains unclear (Yamamoto et al., 1999). nPTB is most highly expressed in neurons and it has been implicated as a regulator of neuronal-specific splicing events (Ashiya & Grabowski, 1997; Chan & Black, 1997; Markovtsov et al., 2000; Polydorides et al., 2000). It has RNA-binding properties subtly distinct from PTB, and it appears, in part, to counter the repressive activity of PTB upon the N1 exon of *c-src*. Our in vitro data show nPTB to be equally effective as PTB1 in repressing TM exon 3, in contrast to the *c-src* N1 exon, where it is less repressive (Markovtsov et al., 2000). It is an attractive possibility that the PTB homologs could have overlapping but distinct RNA-binding specificities to PTB, and that when binding, they could act in either a more or less repressive way than PTB. Changes in the ratio of PTB and various cell-type restricted PTB homologs could thereby alter specific subsets of PTB-regulated alternative splicing events while leaving others unaffected. The complexity of PTB isoform and homolog expression will prove to be even more complicated than at present. We have identified another alternative splicing event within the PTB gene, involving skipping of exon 11 (M.C. Wollerton & C.W.J. Smith, unpubl. observations), and another PTB homolog expressed in smooth muscle cells from a novel gene (C. Gooding & C.W.J. Smith, unpubl. observations). This new homolog has a PTB4-like insert, as well as additional inserts upstream of RRM2s 2 and 3 that are not present in PTB, nPTB, or ROD1. We are currently investigating the activities of these new PTB variants.

MATERIALS AND METHODS

Plasmid cloning

Most of the splicing reporter constructs have been described previously (Gooding et al., 1994, 1998; Southby et al., 1999). P3-2pTS3D is a derivative of pTS3D with a UCUU-to-CCCC mutation within the second PTB binding site of the exon 3 polypyrimidine tract (Perez et al., 1997a). pTΔPy.3F is a vector combining the P3 pyrimidine tract partial deletion of pTΔPy with the complete DY deletion of pTS3F (Gooding et al., 1994, 1998). pPTB1 is a construct for expression of human PTB1. The PTB1 open reading frame was PCR amplified using the PTB5'1/PTB3'1 primer pair and PTB clone 121 as template (Patton et al., 1991). The fragment was subcloned into pCMV-SPORT-βgal (Gibco BRL) as an *Asp718-SalI* fragment. A fragment containing the PTB4 insert was subcloned from an EST (AA305300) into pPTB1 as a *SacII/PpuMI* fragment to give pPTB4. pPTB2 was cloned by introducing a synthetic oligonucleotide fragment into the unique *SacII* and *NarI* sites of pPTB1. pnPTB contained the nPTB open reading frame amplified by PCR using the nPTB5'1/nPTB3'1 primer pair and nPTB template N-60 Weri cDNA (supplied by D. Black).

PTB1, PTB2, PTB4, and nPTB open reading frames were also cloned into the pQE vector for expression of His-tagged proteins in *E. coli*. Oligonucleotides for amplifying PTB and nPTB were:

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PTB5'1: 5'-GCGCGGTACCATGGACGGCATTGTCCCA
        GATATA-3',
PTB3'1: 5'-GCGCGCGCGTCGACCTAGATGGTGGACT
        TGGAGAAGGA-3',
nPTB5'1: 5'-GCGCGGTACCATGGACGGAATCGTCACT
        GAAGTT-3',
nPTB3'1: 5'-GCGCGTCGACTTAAATTGTTGACTTGGA
        GAAAGA-3'.
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Cell culture and transfection

Transfections were either carried out by the calcium phosphate method (Mullen et al., 1991; Gooding et al., 1994, 1998), or with lipofectamine (Gibco BRL). For a typical lipofectamine transfection, 35-mm plates were seeded from a confluent 10-cm plate 24 h before transfection at 1:10 to 1:20 for PAC1 smooth muscle cells (Rothman et al., 1992) and 1:20 to 1:40 for L and HeLa cells. Three to four hours before transfection, cells were refed with fresh medium. One microgram supercoiled plasmid DNA (typically in a 1:4 ratio of reporter:effector plasmid) was added to 100 μL Optimem reduced serum medium (Gibco BRL). This mixture was added to 100 μL Optimem containing 4.5 μL lipofectamine for SM and 3 μL lipofectamine for L and HeLa and incubated at room temperature for 30 min. Optimem (0.8 mL) was added and the resulting 1 mL of precipitate added to a plate of cells (which had been washed with Optimem). After incubation at 37 °C for 5 h, the precipitate was removed and the cells fed with standard growth medium and incubated at 37 °C.

Preparation of cellular RNA

RNA was routinely isolated using the TRI reagent method (Sigma). TRI reagent (1 mL; Sigma, catalog number T9424) was added to each 35-mm plate, prewashed with PBS, and incubated at room temperature for 5 min. The cell lysate was passed several times through a pipette to form a homogenous lysate, which was then transferred to a microcentrifuge tube. Chloroform (0.2 mL) was added, the mixture vortexed for 15 s, and then incubated at room temperature for 10 min, and centrifuged for 15 min. We transferred 0.5 mL of the aqueous phase to a fresh microcentrifuge tube, 0.5 mL of propan-2-ol was added, and the mixture vortexed and incubated at room temperature for 10 min. The RNA precipitate was collected by centrifugation for 10 min, washed with 70% (v/v) ethanol, and resuspended in water. RNA yield was determined by absorbance spectrophotometry at 260 and 280 nm.

Analysis of cellular RNA and protein

Splicing patterns of construct RNAs were analyzed by RT-PCR as described previously (Gooding et al., 1994, 1998; Southby et al., 1999), except that Superscript II (Gibco BRL) was used for the reverse transcription step. The oligonucle-

otide SV3'RT (5'-GCAAACCTCAGCCACAGGT-3') was used for reverse transcription. PCRs were carried out using a [³²P]-end-labeled primer, and analyzed by phosphorimager after denaturing polyacrylamide gel electrophoresis. Oligonucleotides for PCR were:

SV5'1: 5'-GAGCTATTCCAGAAGTAGTGAGGAG-3',
SV3'1: 5'-ACTCACTGCGTTCCAGGCAATGCT-3',
SV5'2: 5'-GGAGGCCTAGGCTTTTGCAAAAAG-3',
3'Act: 5'-ACATGAAGTCAATGAAGGCYTG-3'.

SV5'1 and SV3'1 were used for all RT-PCRs. For analysis of actin splicing, a second round of nested PCR using SV5'2 and 3'Act was carried out (Southby et al., 1999).

For analysis of PTB overexpression, cells were washed two times with PBS, collected by scraping with a razor blade covered in tape, and transferred with a pipette to a microcentrifuge tube on ice. Cells were pelleted by centrifugation for 30 s, and resuspended in 10 volumes of sample buffer containing 2% (w/v) SDS, 80 mM Tris-HCl, pH 6.8, 10% (v/v) glycerol, 5% (v/v) β -mercaptoethanol. Samples were boiled for 5 min, DNA sheared by passage several times through a 0.5-mm needle (Terumo), insoluble material collected by centrifugation at $10,000 \times g$ for 10 min and the supernatant (containing protein) transferred to a fresh microcentrifuge tube before running on an SDS gel. Western blotting using PTB antibodies was carried out by a standard immunoblotting procedure with either alkaline phosphatase (Roberts et al., 1996) or ECL (Enhanced Chemiluminescence, Amersham) detection.

Expression and purification of recombinant PTB proteins

Recombinant His₆-tagged proteins were expressed in *E. coli* M15 cells. The cells were cracked by passage through a French pressure cell, and the soluble and insoluble fractions of the homogenate separated by centrifugation. The recombinant His₆-tagged proteins were purified from the soluble fraction under nondenaturing conditions using Ni-NTA agarose beads (Qiagen) as previously described (Lin & Patton, 1995; Perez et al., 1997b) with minor modifications. Recombinant His₆-tagged PTB1 and PTB4 were further purified using oligo(dT) cellulose (Roche). Peak fractions from the Ni-NTA purification were pooled and dialyzed overnight into Buffer A (20 mM HEPES, pH 7.9, 2 mM MgCl₂, 0.1 mM EDTA, 1 mM DTT, 10% (v/v) glycerol, 0.05% NP-40), and any precipitate removed by centrifugation. This fraction was applied to a 1-mL column of oligo(dT) cellulose previously washed with 10 mL Buffer A + 150 mM KCl. The sample on the column was further washed with 10 mL Buffer A + 150 mM KCl, and then the protein eluted with a gradient of KCl (0.2–1.0 M) in Buffer A. Peak fractions were pooled and dialyzed overnight into Buffer E (20 mM HEPES, pH 8, 100 mM KCl, 0.2 mM EDTA, 5% (v/v) glycerol, 0.5 mM DTT).

In vitro transcription and splicing reactions

[³²P]-labeled RNA transcripts for splicing were transcribed from pGEM vectors with SP6 or T7 polymerase (Smith & Nadal Ginard, 1989). HeLa cell nuclear extracts used for splic-

ing were prepared from suspension cells using the modifications of Abmayr et al. (1988). In vitro splicing reactions contained 15–20 fmol of ³²P RNA transcript in a 10- μ L reaction with 2 mM MgCl₂, 500 μ M ATP, 20 mM creatine phosphate, 10 U RNasin, 12 mM HEPES, pH 7.9, 12% (v/v) glycerol, 60 mM KCl, 0.12 mM EDTA, 0.3 mM DTT, 2.6% polyvinyl alcohol, and 20–30% nuclear extract, and were incubated at 30 °C for 3 h. The reactions were then proteinase K digested, phenol extracted, and ethanol precipitated. The reaction products were run on a 8 M urea–4% polyacrylamide gel.

Depletion of PTB from HeLa nuclear extracts

PTB was depleted from nuclear extracts as previously described (Southby et al., 1999), with some modifications. Biotinylated CUCUU₈ RNA was transcribed using 100 μ M biotin-14-CTP and trace labeled to allow for quantitation. The biotinylated RNA was then bound to streptavidin magnetic beads (ratio of 100 pmol RNA:50 μ L Dynabeads) in $2 \times$ BW buffer (10 mM Tris, pH 7.5, 1 mM EDTA, 2 M NaCl). The biotinylated RNA beads were titrated in an in vitro splicing reaction to determine the optimal bead concentration to give maximal competition in 20% extract. Depletion was carried out incubating the RNA beads with extract for 5 min at room temperature and then the beads separated from the extract using a magnetic particle concentrator. Two rounds of depletion were generally necessary; the RNA beads were titrated in an in vitro splicing reaction for a second time to determine the optimal quantity for depletion. Mock-depleted extracts were prepared with no RNA bound to the beads. The protein concentration of depleted, mock-depleted and complete extracts was determined by the Bradford method. Western blot analysis with anti-PTB antibodies was performed either using ECL (Amersham) or as described previously (Roberts et al., 1996).

Translation

In vitro translation was carried out as previously described (Hunt & Jackson, 1999) in rabbit reticulocyte lysate (Promega) that had been micrococcal nuclease treated and supplemented with 15 μ M haemin, 50 μ g/mL creatine phosphokinase, and 60 μ g/mL calf liver tRNA (Boehringer Mannheim). Translation was carried out in the presence of [³⁵S]methionine (1,000 Ci/mmol; Amersham Pharmacia) and was analyzed by SDS PAGE and autoradiography.

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