Polypyrimidine Tract Binding Protein Interacts with Sequences Involved in Alternative Splicing of β-Tropomyosin Pre-mRNA*

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Previous studies of alternative splicing of the rat β-tropomyosin gene have shown that nonmuscle cells contain factors that block the use of the skeletal muscle exon 7.(Guo W., Mulligan G. J., Wormsley S., and Helfman, D. M. (1991) Genes & Dev. 5, 2095–2106) Using an RNA mobility-shift assay we have identified factors in HeLa cell nuclear extracts that specifically interact with sequences responsible for exon blockage. Here we present the purification to apparent homogeneity of a protein that exhibits these sequence specific RNA binding properties. This protein is identical to the polypyrimidine tract binding protein (PTB) which other studies have suggested is involved in the recognition and efficient use of 3′-splice sites. PTB binds to two distinct functional elements within intron 6 of the β-tropomyosin pre-mRNA: 1) the polypyrimidine tract sequences required for the use of branch points associated with the splicing of exon 7, and 2) the intron regulatory element that is involved in the repression of exon 7. Our results demonstrate that the sequence requirements for PTB binding are different than previously reported and shows that PTB binding cannot be predicted solely on the basis of pyrimidine content. In addition, PTB fails to bind stably to sequences within introns 5 and intron 7 of β-TM pre-mRNA, forms a stable complex with sequences in intron 6, which is not normally spliced in HeLa cells in vitro and in vivo. The nature of the interactions of PTB within this regulated intron reveals several new details about the binding specificity of PTB and suggests that PTB does not function exclusively in a positive manner in the recognition and use of 3′-splice sites.

The splicing of pre-mRNA requires small nuclear ribonucleoproteins (snRNPs; U1, U2, U4, U5 and U6), as well as a large number of non-snRNP factors, and occurs in a complex termed the spliceosome (Kainer and Maniatis, 1988; Green, 1991; Guthrie, 1991; Ruby and Abelson, 1991). In mammalian cells, only a few non-snRNP factors have been identified that are required for splicing (Ast et al., 1991; Fu and Maniatis, 1990; Kainer and Maniatis, 1985; Kainer et al., 1990b; Patton et al., 1991; Perkins et al., 1986; Kramer and Utans, 1991; Zamore and Green, 1989, 1991). Proteins that interact with the 3′-splice site include U2AF (U2 auxiliary factor), which is necessary for the binding of U2 snRNP to the branch point and for splicing complex assembly (Zamore and Green, 1989, 1991). In addition, a number of RNA-binding proteins have been identified, some of which exhibit sequence-specific binding to pre-mRNA, and are associated with spliceosomes (Choi et al., 1986; Garcia-Blanco, 1989; Gerke and Steitz, 1986; Patton et al., 1991; Tazi et al., 1986). Two proteins that UV cross-link to 5′-splice sites may play a role in splice site selection (Stolon and Berget, 1991). The critical pyrimidine stretch also has specific interactions with hnRNP proteins A1, C, and D (Bandyulious, 1989; Choi et al., 1986; Dreyfus, 1986; Pinol-Roma et al., 1988), a 70–100-kDa intron binding protein (Gerke and Steitz, 1986; Tazi et al., 1986), and PTB (Garcia-Blanco et al., 1989; Mullen et al., 1991; Patton et al., 1991). In vitro experiments have indicated that U2AF (Zamore and Green, 1989, 1991), PTB (Patton et al., 1991), and some hnRNP proteins (Choi et al., 1986; Mayeda and Kainer, 1992; Sierakowski et al., 1986) can play a functional role in splicing. Despite the partial characterization of these activities it is still mechanistically unclear how they contribute to the high degree of specificity and fidelity observed in general splice site selection.

Considerable progress has been made on alternative splicing in Drosophila, where genetic approaches have allowed the identification and subsequent characterization of developmentally regulated proteins that specifically alter splicing patterns. In genes of the sex determination pathway, suppressor of white apricot, suppressor of white apricot, alternative splicing is subject to regulation by factors that either inhibit or activate the use of alternative 5′- or 3′-splice sites (Kainer and Maniatis, 1991; McKeown, 1990; Maniatis, 1991). For example, the sex lethal (Sxl) protein is a female-specific activity that binds to sequences in the pre-mRNA and blocks the use of a particular 3′-splice site (Kainer and Maniatis, 1991). This allows the use of a competing downstream 3′-splice site and the production of a protein required for female-specific differentiation. In the suppressor of white apricot transcript a 3′-splice site is blocked by the suppressor of white apricot protein, thereby autoregulating its own production (Bingham et al., 1988).

In contrast, much less is known about the cellular factors that mediate alternative splicing in vertebrate systems. Tissue-specific regulators of splicing, similar to those identified in Drosophila systems, have not yet been identified in mammalian cells. The lack of functional systems that reproduce tissue-specific alternative splicing patterns in vitro has hind-
ized the biochemical characterization of cell-type specific factors (Maniatis, 1991). At present, the only factors reported to alter alternative splice selection include studies of the essential splicing factor ASF/SF2 and hnRNP protein A1 (Ge and Manley, 1990; Krainer et al., 1990a; Mayeda and Krainer, 1992). In vitro studies have demonstrated that at high concentrations ASF/SF2 promotes the use of proximal 5'-splice sites (Ge and Manley, 1990; Krainer et al., 1990a), and the hnRNP protein A1 can antagonize this effect (Mayeda and Krainer, 1992). These studies show that modulation of alternative splice site selection can, in principle, be achieved by changes in the levels of general splicing factors.

We have been using the rat β-tropomyosin (β-TM) gene as a model system to study the molecular basis for developmental and tissue-specific alternative RNA splicing (Helfman et al., 1986). This gene spans 10 kb of DNA with 11 exons and encodes two distinct isoforms (Fig. 1A). Exons 1–5, 8, and 9 are common to all mRNAs expressed from this gene. Exons 6 and 11 are used in fibroblasts as well as smooth muscle, whereas exons 7 and 10 are used exclusively in skeletal muscle. Our previous studies of tropomyosin pre-mRNA splicing with HeLa cell (nonmuscle) systems revealed an ordered pathway of splicing in which either of the internal alternatively spliced exons (exons 6 or 7) must first be joined to the downstream common exon before they can be spliced to the upstream common exon (Helfman et al., 1988). Characterization of the branch points used during \textit{in vitro} splicing of exons 5 to 7 (skeletal muscle type splice) revealed the use of multiple branch points that are located at an unusually long distance (>140 nucleotides) from the 3'-splice site of exon 7 (Helfman and Ricci, 1989). Subsequent investigations of the sequences between the 3'-splice site of exon 7 and the distant branch points demonstrated that two distinct functional elements are present in this region (Helfman et al., 1990). The first element comprises a polypyrimidine tract located 89–143 nucleotides upstream of the 3'-splice site, which specifies the location of the distant branch points used upstream of exon 7. The second element is located between the polypyrimidine tract and the 3'-splice site of exon 7. This region contains an important determinant in alternative splice site selection, because deletions or clustered point mutations in this regulatory element result in the use of the skeletal muscle specific exon in nonmuscle cells (Guo et al., 1991; Helfman et al., 1990).

Extensive mutational analyses indicated that the critical cis-acting elements that block the use of exon 7 in nonmuscle cells are confined to sequences within exon 7 and the upstream intron (Guo et al., 1991). Using partially purified protein fractions we found that the regulatory sequences in the intron upstream of exon 7 interact with RNA-binding proteins in HeLa nuclear extracts (Guo et al., 1991). We also demonstrated that mutations in the pre-mRNA that result in the use of the skeletal muscle exon \textit{in vitro} in HeLa cell disrupt the binding of these proteins to the pre-mRNA \textit{in vitro} (Guo et al., 1991). This led us to propose that nonmuscle cells contain factors that interact with specific regulatory sequences in the pre-mRNA to block the use of the skeletal muscle exon.

To further study the precise nature of the factors that interact with sequences in the β-TM pre-mRNA, we have purified to apparent homogeneity a protein that interacts specifically with the intron regulatory element upstream of muscle specific exon 7. This protein is identical to the PTB which other studies have indicated to be involved in the recognition and efficient splicing of 3'-splice sites (Garcia-Blanco et al., 1989; Patton et al., 1991). Our analysis of several different introns in the tropomyosin pre-mRNA indicates that PTB binds only to the intron upstream of the skeletal muscle-specific exon 7. The nature of the interactions in this intron reveals several new details about the binding specificity of PTB and suggests that PTB does not function exclusively in a positive manner in the recognition and use of 3'-splice sites.

![FIG. 1. A, schematic diagram of the rat β-tropomyosin gene. Open boxes represent constitutive exons; hatched and solid boxes represent tissue-specific exons, as indicated. Horizontal lines represent introns (not drawn to scale). The amino acids encoded by each exon are indicated. The cap site and polyadenylation sites are also indicated. B, nucleotide sequence of the β-tropomyosin pre-mRNA. The nucleotide sequence of exon 5 through exon 8 are shown. The long polypyrimidine tract that functions to position the branch points upstream of exon 7 is underlined. The position of the lariat branch points used upstream of exon 7 are indicated by an asterisk above the “Ex 7” probe is indicated by > and <, at the 5' - and 3'-ends, respectively. The nucleotides deleted from probe d3-5 are bracketed from nucleotides 543–569. The number of nucleotides from the beginning of exon 5 is indicated in the right margin.](image-url)
RNA-binding Proteins in Alternative Splicing

MATERIALS AND METHODS

Construction of Plasmids—The DNA templates for use in vitro transcription are derived from the rat β-tropomyosin gene (Helfman et al., 1986, 1988, 1990; Helfman and Ricci, 1989; Guo et al., 1991). Synthesis of Pre-mRNA—The 32P-labeled SPG/tropomyosin transcripts were synthesized in vitro primed with CAP analog as described (Konarska et al., 1984). The 32P-labeled RNAs were further purified on polyacrylamide/urea gels.

Nuclear Extract and Fractionation—Nuclear extracts were prepared as previously described starting with 8 liters of HeLa cells that were grown to a density of 1×10^6/liter (Krainer et al., 1990b). All steps were carried out at 4°C. The crude nuclear extract material was dialyzed against Buffer D (20 mM Hepes, pH 8.0, 100 mM KCl, 0.2 mM Na2EDTA, and 0.5 mM diithiothreitol), and then brought to 20% final concentration by the addition of solid ammonium sulfate (10.6 g/100 ml). The solution was gently stirred for 30 min then centrifuged for 30 min at 2300 × g to remove the precipitated proteins. The supernatant was brought to 50% final concentration by the addition of solid ammonium sulfate (17.5 g/100 ml). The solution was gently stirred for 30 min, and the precipitate was recovered by centrifugation for 30 min at 2300 × g. This 20-50% ammonium sulfate pellet was resuspended in Buffer D containing 0.02% Nonidet P-40, and 0.5 mM phenylmethylsulfonyl fluoride. The protein concentration was adjusted in the same buffer to 4 mg/ml and loaded on a Whatman P11 phosphocellulose column (15 × 1.5 cm). The column was washed with 30 ml of Buffer D, and then eluted step-wise in Buffer D containing 150 mM KCl and 1000 mM ammonium sulfate. The column was washed with 15 ml of Buffer D and then eluted step-wise in Buffer D containing 150 mM KCl at 4°C. Each step was collected in 3 × 5-ml fractions and assayed for RNA-binding proteins. Most of the binding activity was found in the 350 mM KCl fraction. The material from this fraction was diluted to 100 mM KCl and loaded on a 10-ml Pharmacia LKB Biotechnology Inc. S-Sepharose column. The column was washed with 15 ml of Buffer D and then eluted step-wise in Buffer D containing 150 mM KCl and 1000 mM KCl. Each step was collected in 3 × 5-ml fractions and assayed for RNA-binding proteins. The majority of the RNA binding activity was found in the 150 mM eluate which was diluted to 100 mM KCl and fractionated by affinity chromatography on a 2-ml poly(U)-Sephadex (GIBCO-Bethesda Research Laboratories) column. This column was washed with 3 ml of Buffer D, and eluted with Buffer D containing 0.4 mM KCl, 0.8 mM KCl, 1 mM KCl, and 2 mM guanidine hydrochloride. Each step was collected in 3 × 1-ml fractions. Essentially all of the specific binding activity eluted at 0.8 mM KCl. Before assaying for binding, fractions were adjusted to 100 mM KCl by dialysis or gel filtration using Sephadex G-25. All protein concentrations were determined by the method of Bradford (1976), using albumin as a standard. Quantitative amino acid analysis indicated that absorption assays underestimated the concentration of PTB by approximately 5-fold.

RNA Mobility-shift Assay—The RNA mobility-shift assays were performed essentially as previously described (Guo et al., 1991; Konarska and Sharan, 1986). The protein-RNA complexes were separated by native gel electrophoresis using 4% polyacrylamide gels (acylamide:bis, 29:1) using TBE (89 mM Tris-borate, 2 mM Na2EDTA) as the running buffer. The gels (25 × 15 cm) were pre-electrophoresed at 10 V/cm for 1 h prior to loading samples. Binding reactions were performed in a 25-μl reaction containing 20 mM Hepes, pH 8.0, 5% glycerol, 70 mM KCl, 3 mM MgCl2, 3 units of RNasin, 0.5 mM ATP, 20 mM creatine phosphate, 0.02-1.0 μg of protein, 10 μg of tRNA, 150 μg of heparin, and 32P-labeled RNA probe. In its most purified form PTB binding activity is labile, but can be stabilized by the addition of bovine serum albumin. In all assays of purified protein bovine serum albumin was added to a final concentration of 1 mg/ml. After incubation for 10-20 min at 30°C, 1 μl of loading buffer containing 97% glycerol, 0.01% bromophenol blue, and 0.01% xylene cyanol is added just before loading the sample on the gel. The gel was electrophoresed at 120 V for 2-4 h at room temperature. The gels were dried and visualized by autoradiography.

Miscellaneous—PTB was purified using the method of Patton et al. (1984) with the addition of rabbit polyclonal antibodies to the purified doublet (see Fig. 3), and immuno blot analyses used standard procedures (Harlow and Lane, 1988). UV cross-linking was carried out essentially as described (Garcia-Blanco et al., 1989).

RESULTS

Purification of a Factor That Binds to the Intron Regulatory Sequences in β-Tropomyosin Pre-mRNA—We reported previously that sequences upstream of and within exon 7 are involved in negative regulation of this muscle specific exon in nonmuscle cells (Guo et al., 1991; Helfman et al., 1990). We also demonstrated the nuclear extracts derived from HeLa cells contain protein(s) that bind specifically to these regulatory sequences (Guo et al., 1991). This led us to hypothesize that the interaction of these factors with the pre-mRNA is involved in regulated alternative splicing and that this interaction is required for blocking the use of the skeletal muscle exon in nonmuscle cells (Guo et al., 1991).

To purify the binding factor(s) that interact specifically with the intron regulatory element, we fractionated HeLa cell nuclear extracts and assayed each fraction using an RNA mobility shift on nondenaturing polyacrylamide gels to detect the binding of 32P-labeled wild-type and mutant RNAs (Guo et al., 1991). The wild-type probe, termed Ex 7, contains 88 nucleotides of intron sequences upstream of exon 7, exon 7 (76 nucleotides), and 26 nucleotides of intron sequences downstream of exon 7. The mutant RNA probe, termed d3-5, contains a deletion of 26 nucleotides in the intron regulatory element in the Ex 7 RNA, which was previously shown to disrupt binding using partially purified fractions (Guo et al., 1991). The crude nuclear extract was fractionated with ammonium sulfate, and chromatography on phosphocellulose, S-Sepharose, and poly(U)-Sephadex (purification details under “Materials and Methods”). The binding assays of the various fractions are shown in Fig. 2. Most of the specific binding was found in the 20-50% ammonium sulfate fraction (Fig. 2B). This activity was loaded on a phosphocellulose column, and the 0.35 M KCl eluate contained most of the binding activity (Fig. 2C). The 0.35 M KCl eluate from the phosphocellulose column was loaded onto an S-Sepharose column and an active fraction that eluted at 0.15 M KCl contained the binding

FIG. 2. Mobility-shift assay of binding activity through the purification steps. 32P-Labeled RNAs were incubated with the indicated fractions and separated on nondenaturing 4% polyacrylamide gels. Positions of free RNA probe and RNA-protein complexes are indicated. Incubation and electrophoresis conditions are detailed under “Materials and Methods.” A, diagram of RNA probes. The number of nucleotides in the intron and exon regions are indicated. Each fraction was incubated with both 32P-labeled wild-type (odd-numbered lanes) and mutant (even-numbered lanes) RNA probes. B, ammonium sulfate fractions were assayed using 0.02-0.33 μg of protein. C, phosphocellulose column fractions were assayed using 0.7-0.9 μg of protein. D, S-Sepharose column fractions were assayed using 0.6-0.8 μg of protein. E, poly(U)-Sephadex column fractions were assayed using 0.03-0.05 μg of protein.
activity characterized in Guo et al. (1991). This activity was further fractionated by affinity chromatography on poly(U)-Sephadex. This column was eluted with Buffer D containing 6.4 M, 0.8 M, and 1 M KCl, and then 2 M guanidine hydrochloride (Fig. 2E). The vast majority of binding activity emerges in the 0.8 M KCl eluate. The protein profile of the Poly U column as analyzed by SDS-PAGE and visualized with silver stain is shown in Fig. 3A. The 0.8 M KCl fraction contains a protein doublet of approximately 60 kDa. Fig. 3B shows the Coomassie Blue-stained SDS-PAGE of the protein components in the crude nuclear extract and the subsequent fractions containing peak binding activity: ammonium sulfate 20–50%, phosphocellulose 0.35 M KCl wash, S-Sepharose 0.15 M KCl wash, and poly(U)-Sephadex 0.8 M KCl wash (lane 5). The final active fractions obtained by affinity chromatography on poly(U)-Sephadex contained a protein doublet of approximately 60 kDa (Fig. 3B).

The Protein That Binds to Regulatory Sequences in the \(\beta\)-Tropomyosin Pre-mRNA Is Identical to PTB—The relative mobility and Poly U binding properties of the purified protein suggested a possible relationship to the PTB previously reported (Garci-Blanco et al., 1989; Mullen et al., 1991; Patton et al., 1991; Wang and Pederson, 1990). However, the purification of PTB by this assay was unexpected for two reasons. First, the RNAs used in this study were intended to preclude the binding of PTB, which we initially thought would only interact with sequences contained in the polypyrimidine tract. Therefore, the Ex 7 RNA used in the mobility-shift assay lacks all polypyrimidine tract sequences underlined in Fig. 1B. Second, although the intron regulatory element is pyrimidine-rich, the sequence is unlike polypyrimidine tracts previously shown to interact with PTB (see “Discussion”). To determine whether the protein purified in Fig. 3 was related to authentic PTB, we purified authentic PTB using the protocol of Patton et al. (1991). In agreement with the published procedure, we obtained a partially purified fraction containing PTB and at least two other proteins; a 100-kDa protein with associated breakdown products, and a 35-kDa protein (Fig. 4A, lane 2). A comparison of the polypeptide composition of the authentic PTB and our purified protein show that both preparations contain protein doublets of approximately 60 kDa, which co-migrate on SDS-PAGE (Fig. 4A). Although the molecular basis for this size heterogeneity is not fully understood, it is likely the result of multiple forms of PTB expressed via alternative RNA splicing (Patton et al., 1991; Gil et al., 1991). Immunoblot analysis was used to confirm the identity of our purified protein using antibodies to authentic PTB (provided by Mariano Garcia-Blanco), and antibodies that we prepared against the protein purified in the present studies. As shown, our antibody specifically reacts with the same proteins in both fractions (Fig. 4B, panel A, lanes 1 and 2) and a 60-kDa doublet in crude nuclear extract (lane 3). Similarly, the polyclonal antibody against murine PTB cross-reacts with the identical 60-kDa proteins (Fig. 4B, panel B). The Amido Black stain (Fig. 4B, panel C) demonstrates that equal amounts of protein were transferred to nitrocellulose, and therefore the antibodies cross-react in a quantitative as well as qualitative fashion. We also determined the ability of authentic PTB to bind to sequences in the \(\beta\)-tropomyosin pre-mRNA. Fig. 4C demonstrates that authentic PTB exhibits a binding activity specific for wild-type Ex 7 RNA (lanes 1 and 2), similar in mobility to that obtained with our purified protein (lanes 3 and 4) and crude nuclear extract (lanes 5 and 6). Finally, we carried out a competition experiment in order to determine if the same form of PTB is able to bind to both the polypyrimidine tract and the intron regulatory element (Fig. 4D). Binding to the \(^{32}\)P-labeled Ex 7 probe was competed with unlabeled polypyrimidine tract RNA or the intron regulatory element (Ex 7 RNA). As shown in Fig. 4D, either RNA was able to compete for the binding of the Ex 7 RNA. Collectively these experiments demonstrate that the protein purified in our present studies is identical to the polypyrimidine tract binding protein.

Binding Specificity of PTB for Sequences in \(\beta\)-Tropomyosin Pre-mRNA—The specific nucleotides that are required for the interaction of PTB with RNA are not known. Although previous studies would predict that the length polypyrimidine tract in intron 6 (see sequences underlined Fig. 1B) would interact with PTB, it was surprising that PTB binds to the intron regulatory element in the Ex 7 RNA. This led us to examine what other regions in the \(\beta\)-TM pre-mRNA can interact with PTB. Five different \(^{32}\)P-labeled RNAs of approximately equal size (190–250 nucleotides) corresponding to sequences between exons 5 through exon 8 were analyzed (Fig. 5A). RNAs containing sequences between exon 5 and exon 6 (lane 2) and exon 7 and exon 8 (lanes 8 and 10) did not form stable complexes with PTB in the mobility-shift assay. In contrast, RNAs containing sequences derived from the 5'-half of intron 6 (lane 4) as well as sequences in the 3'-
half of intron 6 (lane 6) formed a stable complex with PTB. Therefore, between exons 5 through 8 of the β-TM pre-mRNA, PTB specifically binds to sequences within intron 6.

In order to examine more fully the specificity of PTB for sequences in the β-TM pre-mRNA, a number of additional RNAs were analyzed (Fig. 5B). Our previous studies have shown that the 3′ half of intron 6 contains two distinct functional elements, of which only the intron regulatory element is contained in the Ex 7 RNA probe. As described above, an RNA probe containing both of these elements forms a stable complex with PTB (Fig. 5A, lane 6). When analyzed individually, both the polypyrimidine tract (Fig. 5B, lane 10) and the intron regulatory element (Fig. 5B, lane 12) could stably bind to PTB. Sequences around exon 8 and exon 6, containing both the 3′- and 5′-splice sites (lanes 1–4), failed to form stable complexes with PTB. These results are in agreement with our previous studies using a partially purified fraction (Guo et al., 1991). Previous studies of PTB used UV cross-linking to detect the interaction of PTB to 32P-labeled RNAs. Accordingly, the RNAs shown in Fig. 5B were also analyzed by UV cross-linking (Fig. 5B, lower panel). In agreement with the mobility-shift assay, both functional elements upstream of exon 7 can be efficiently cross-linked to PTB (lanes 6, 10, and 12). By contrast, RNAs containing sequences surrounding exons 6 or 8, were cross-linked poorly to PTB. Collectively, these studies demonstrate that between exons 5 through 8 of the rat β-TM pre-mRNA, PTB preferentially interacts with sequences contained within intron 6.

In Fig. 5C we compare the ability of PTB to interact with three distinct RNAs from the β-TM pre-mRNA each of which contains a high content of pyrimidines; the intron regulatory element (lanes 1–4), the lengthy polypyrimidine tract associated with branch point use (lanes 5–8), and the intron sequences upstream of exon 6 (lanes 9–12). Each RNA is incubated with increasing concentrations of PTB. Both the intron regulatory element and the polypyrimidine tract exhibit a mobility shift at all concentrations of PTB analyzed. In contrast, the intron sequences upstream of exon 6 fail to form a stable complex with PTB under any conditions. The results obtained with the latter RNA are somewhat surprising since this RNA contains a number of pyrimidine tracts that are longer than those present in the intron regulatory element (see Fig. 1B). These results indicate that the interaction of

(1991) were analyzed by 10% SDS-PAGE and Coomassie Blue staining. Lane 1, the 60-kDa doublet; lane 2, authentic PTB; and lane 3, molecular mass markers (in kilodaltons). B, antibodies to the 60-kDa doublet and PTB cross-react with both proteins. The 60-kDa doublet (lane 1), authentic PTB (lane 2), and nuclear extract (lane 3) were separated using 10% SDS-PAGE, and the proteins were electrophoretically transferred to nitrocellulose and analyzed using antibodies raised against the polypyrimidine tract and nuclear extract (lanes 1–5). Lane 7, authentic PTB fraction (0.15 pg); lanes 8 and 9, Buffer D control. lane M, molecular mass markers (in kilodaltons). C, PTB specifically binds β-TM RNA sequences in the mobility-shift assay. 32P-Labeled wild-type (odd-numbered lanes) and mutant (even-numbered lanes) RNA probes were incubated with the three different fractions and separated on nonnondenaturing 4% polyacrylamide gel. Lanes 1 and 2, authentic PTB fraction (0.15 μg); lanes 3 and 4, 60-kDa doublet from the Poly U column (0.04 μg); lanes 5 and 6, nuclear extract (0.5 μg); lanes 7 and 8, Buffer D control. Lane M, molecular mass markers (in kilodaltons). D, PTB specifically binds to sequences within intron 6 formed a stable complex with PTB. Therefore, between exons 5 through 8 of the β-TM pre-mRNA, PTB specifically binds to sequences within intron 6.

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FIG. 4. The 60-kDa doublet is identical to the polypyrimidine tract binding protein (PTB). A, the 60-kDa doublet from the Poly U column and authentic PTB purified according to Patton et al.
PTB with an RNA involves more than the length of consecutive pyrimidines.

Our previous studies demonstrated that deletions (such as d3-5), as well as clustered point mutations which substituted three to five nucleotides in the intron regulatory element, allow the use of the skeletal muscle specific exon 7 in nonmuscle cells in vivo (Guo et al., 1991). In addition, an RNA probe containing a four-nucleotide clustered point mutation did not compete as well as a wild-type competitor for the binding of the Ex 7 RNA by a partially purified fraction (Guo et al., 1991). Therefore, it was of interest to determine if this clustered point mutation (int3) would similarly affect the binding by purified PTB. The ability of wild-type and mutant RNAs to compete with the binding of Ex 7 RNA by PTB was analyzed (Fig. 6). Wild-type Ex 7 sequences can compete for PTB binding at the lowest concentration analyzed, while a similar level of competition by the mutated sequence requires a four times greater concentration. These results correlate with our previous studies, and strongly suggest that PTB plays a role in blocking the use of exon 7 in nonmuscle cells.

**DISCUSSION**

In this paper we describe the purification to apparent homogeneity of a protein from HeLa cell nuclear extracts that binds to regulatory sequences in the rat β-TM pre-mRNA.

**FIG. 5. Specificity of PTB binding to sequences in the β-TM pre-mRNA.** A, the 32P-labeled RNA probes shown at the top of the figure were incubated in the absence (−) or presence (+) of 0.02 µg of purified PTB from the Poly U column and analyzed on a nondenaturing 4% polyacrylamide gel. RNAs A–E correspond to nucleotides 1–258, 258–423, 421–595, 591–768, and 769–905 plus 63 nucleotides of exon 9, respectively, shown in Fig. 1B. B, the 32P-labeled probes shown at the right of the figure were incubated in the absence (−) or presence (+) of 0.02 µg of purified PTB and analyzed using either the mobility-shift assay or UV cross-linking assay. C, stable complexes of different apparent affinities are formed with two distinct functional elements in intron 6. Probes I, II, and III as diagrammed are incubated with increasing concentrations of PTB: 0, 0.02, 0.04, and 0.08 µg and analyzed using the mobility-shift assay.

These sequences were previously shown to be involved in blocking the use of a skeletal muscle specific exon in nonmuscle cells (Guo et al., 1991; Helfman et al., 1990). Unexpectedly, this protein was found to be identical to the polypyrimidine tract binding protein (PTB). The ability of PTB to bind to these particular RNA sequences raises a number of questions concerning the sequence requirements for PTB-RNA interactions and the potential roles of PTB in RNA processing.

**PTB Binding to RNA—**PTB was first identified in HeLa cell nuclear extracts based on its ability to UV cross-link the polypyrimidine tract associated with the 3′-splice site of pre-mRNAs (Garcia-Blanco et al., 1989). Although the precise RNA sequence required for PTB binding is not known, this interaction was shown to be specific for RNAs containing introns. The protein has a higher affinity for uridines than cytidines and does not bind to RNAs with polypyrimidine tracts shorter than 10 nucleotides (Garcia-Blanco et al., 1989; Patton et al., 1991). The binding does not require branch point sequences or the AG dinucleotide of a 3′-splice site (Garcia-Blanco et al., 1989; Patton et al., 1991). In the present study we used a mobility-shift assay to follow the purification of an RNA-binding protein that interacts with an intron regulatory element in the β-TM pre-mRNA. The purification of PTB by this assay was unexpected because the RNA probes used are different from sequences previously shown to interact with PTB. The intron regulatory element is C-rich and contains no more than a single stretch of 7 consecutive pyrimidines at the 5′-end of the RNA. These 7 pyrimidines are not sufficient for binding to PTB as mutations located further downstream of this pyrimidine stretch disrupt binding. Our results demonstrate that the sequence requirements for PTB binding are different than previously reported and show that PTB binding cannot be predicted solely on the basis of pyrimidine content.

The amino acids in PTB that contact RNA remain to be determined. Recently, cDNAs encoding PTB proteins have been cloned and sequenced (Bothwell et al., 1991; Gil et al., 1991; Patton et al., 1991). PTB is ubiquitously expressed in all cell types and tissues examined, and multiple forms of PTB are expressed via alternative RNA splicing (Bothwell et al., 1991; Gil et al., 1991; Patton et al., 1991). To date there is no evidence that these mRNAs are produced in a tissue-specific manner or that different PTB isoforms will bind to different RNA sequences, yet this needs to be further investigated. At this point it is clear that the same PTB fraction that binds polypyrimidine tracts associated with branch point use, can also specifically bind the intron regulatory element which plays a part in splice site blockage (Figs. 4 and 5). In
light of these functionally distinct binding sites for PTB, it is of interest to consider the predicted protein sequence of PTB which exhibits significant homology to human hnRNP L protein (Pino-Roma et al., 1989) and Drosophila elav (Robinson et al., 1988). Like hnRNP L protein, PTB contains nonconsensus RNA binding domains, with two distinct sequences that weakly match the RNP-1 and RNP-2 boxes of the RNP domain, a protein motif that is common to RNA-binding proteins (Bandzhuulis et al., 1989; Query et al., 1989). The RNP boxes are usually separated by 30–35 amino acids, and the crystal structure of the RNP domain in the U1 A protein supports the importance of these two RNP boxes in the recognition of a binding site (Nagai et al., 1990). In PTB, however, the RNP-1 and RNP-2 like boxes are separated by 137 amino acids. Further work is required to determine if these relatively distant motifs act cooperatively or if instead each might act as separate binding domains with distinct targets and affinities. This latter possibility could explain the differing affinities observed in Fig. 5C, but we cannot distinguish between this and the possibility that the two RNAs used in these studies differ in their number of binding sites or a match to an unknown consensus binding site.

Role of PTB in RNA Splicing—The function of PTB in splicing remains to be established. UV cross-linking studies in nuclear extracts under splicing conditions have been used to study the binding of PTB to the β-globin intron 1 (Wang and Pederson, 1990), intron 2, adeno major late transcript, human immunodeficiency virus tat precursor, a fibroblastic intron (Garcia-Blanco et al., 1989), and α-TM introns 1 and 2 (Mullen et al., 1991; Patton et al., 1991). The binding of PTB to RNA was found to correlate with efficient splicing of mRNA precursors and led to the hypothesis that PTB is involved in splicing (Garcia-Blanco et al., 1989; Patton et al., 1991). Subsequent biochemical complementation studies indicated that nuclear extract depleted by Poly(U) affinity chromatography are splicing incompetent, but upon addition of a fraction containing PTB, as well as an associated 100-kDa protein, splicing can be restored (Patton et al., 1991). It has been hypothesized that PTB functions in a complex to recognize the pyrimidine stretch in the 3′-splice site region, and through a U1 snRNP interaction forms the earliest commitment complex (Bothwell et al., 1991). Additional properties ascribed to this molecule include the ability to bind to single-stranded DNA (Brunel et al., 1991).

While further studies are needed to define the role that PTB might play in the RNA processing pathway, the present work provides new information concerning the possible role of PTB in splicing. Although the 3′-splice site of exon 7 is not used in HeLa cells, sequences upstream of this 3′-splice site bind efficiently to PTB (Fig. 5). These results are in contrast to previous studies that show a direct correlation of PTB binding with splicing efficiency (Garcia-Blanco et al., 1989; Mullen et al., 1991; Patton et al., 1991). Furthermore, although exons 6 and 8 are efficiently spliced, sequences contained within their 3′-splice sites are unable to form stable complexes with PTB. These RNAs, however, can be weakly cross-linked to PTB (Fig. 5). Prior studies have relied only on cross-linking assays to analyze the interaction of PTB with various RNAs (Garcia-Blanco et al., 1988; Mullen et al., 1991; Patton et al., 1991; Wang and Pederson, 1990). It is possible that cross-linking methods may detect transient as well as stable interactions, while the mobility-shift assay requires stable interactions. Therefore, it is unclear at present if use of the 3′-splice sites of exons 6 and 8 will involve PTB. These studies suggest that the interaction of PTB will be required for splicing of some but not all introns, and that PTB might also function in a negative manner (see below).

Regulation of Alternative Splicing β-Tropomyosin Pre-mRNA—The mutually exclusive splicing of exons 6 and 7 in nonmuscle cells is regulated, in part, by factors that block the use of exon 7 (Guo et al., 1991). This blockage requires sequences in the intron regulatory element and within exon 7 (Guo et al., 1991; Helfman et al., 1990). In the present studies we demonstrate that the intron regulatory element can interact with PTB in a sequence-specific manner. However, we cannot rule out the possibility that other factors will also bind to these sequences. For example, a number of other proteins have been identified that interact specifically with the poly-pyrimidine tract/3′-splice site of introns. These include hnRNP A1, C, and D (Dreyfus, 1986), U2AF (Zamore and Green, 1989, 1991), and IBP (Gerke and Steitz, 1986; Tazi et al., 1986). Since several fractions obtained during this purification exhibited mobility-shift activity specific for the wild-type RNA probe, it remains to be determined if any of these fractions contain these or other RNA-binding proteins. In addition, interactions involving other RNA-binding proteins may not have been detected by the mobility shift or UV cross-linking assays used in the present studies. It is also worth noting that our previous analyses of cis-acting regulatory elements in β-TM pre-mRNA indicate that sequences in exon 7 also participate in blocking the use of this exon in nonmuscle cells (Guo et al., 1991). However, these exon sequences do not appear to be required for the interaction of PTB (Fig. 5A), and mutations in this exon do not disrupt the binding of PTB (data not shown). It remains to be determined if these exon sequences interact with other factors.

Although the biological significance of PTB binding to sequences upstream of exon 7 remains to be established, there is a strong correlation between the ability of PTB to bind to these sequences and our previous results demonstrating that mutations in this region lead to activation of the skeletal muscle-specific exon in vivo (Guo et al., 1991). The same mutations that result in the use of exon 7 in nonmuscle cells in vivo, also disrupt the binding of PTB in vitro (Fig. 6). Thus, the interaction of PTB with the intron regulatory element in the β-tropomyosin pre-mRNA may account for the regulation of splice site selection. We previously demonstrated that the poly-pyrimidine tract upstream of the 3′-splice site (sequences underlined, Fig. 1B) specifies the location of the branch points used upstream of exon 7 (Helfman et al., 1990). PTB binds stably to this element (Fig. 4R), and this interaction is likely to be required for branch point formation (Mullen et al., 1991). On the other hand, the regulatory sequences downstream of this poly-pyrimidine tract can also bind to PTB (Fig. 5B), but this interaction correlates with blockage of this exon in nonmuscle cells. The results suggest both a positive and negative role of PTB in the regulation of alternative splicing of exon 7 in skeletal muscle and nonmuscle cells.

How these dual functions of PTB might be achieved is not clear, but we can offer a few speculations. Since PTB can exist as multiple isoforms via alternative RNA splicing, skeletal muscle and nonmuscle cells could express a different set of PTB isoforms with different binding specificities, which thereby lead to tissue-specific splice site selection. At present we cannot distinguish if all of the alternatively spliced forms of PTB interact with the same RNA sequences. PTB could also be subject to post-translational modifications in different cell types that alter its binding properties. However, we have thus far been unable to detect differences in isoform expression in different cell types,2 or in the binding of Ex7 RNA in nuclear extracts obtained from undifferentiated and differentiated myogenic cells (data not shown). At least one form of PTB corresponds to the hnRNP I protein and therefore may function in the packaging of pre-mRNA into an hnRNP

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2 S. Wormsley and D. M. Helfman, unpublished observations.
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In conclusion, having identified that PTB can bind to specific regulatory sequences in the β-TM pre-mRNA, it is possible that the binding of PTB to both the particle (Ghetti et al., 1992). The proper assembly of the β-tropomyosin pre-mRNA with hnRNP proteins might be essential for regulation of tissue-specific splicing. A number of hnRNP proteins exhibit sequence-specific interactions with the pre-mRNA (Chung and Wooley, 1986; Dreyfus, 1986). It is also possible that the binding of PTB to both the polypropylene glycol and regulatory element is constant in all cell types. PTB bound to the RNA could then serve as a platform for the interaction of other factors. The distribution or number of PTB binding sites along a region of RNA could also provide different recognition sites for the interactions of additional binding factors, which might be expressed in a tissue-specific manner.

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