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The use of antibodies to the polypyrimidine tract binding protein (PTB) to analyze the protein components that assemble on alternatively spliced pre-mRNAs that use distant branch points

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ABSTRACT

We are using the rat β -tropomyosin (β -TM) gene as a model system to study the mechanism of alternative splicing. Previous studies demonstrated that the use of the muscle-specific exon is associated with the use of distant branch points located 147–153 nt upstream of the 3' splice site. In addition, at least one protein, the polypyrimidine tract binding protein (PTB), specifically interacts with critical *cis*-acting sequences upstream of exon 7 that are involved in blocking the use of this alternative exon in nonmuscle cells. In order to further study the role of PTB, monoclonal antibodies to PTB were prepared. Anti-PTB antibodies did not inhibit the binding of PTB to RNA because they were able to supershift RNA–PTB complexes. To determine if additional proteins interact with sequences within the pre-mRNA, ³⁵S-met-labeled nuclear extracts from HeLa cells were mixed with RNAs and the RNA–protein complexes were recovered by immunoprecipitation using antibodies to PTB. When RNAs containing intron 6 were added to an ³⁵S-met-labeled nuclear extract, precipitation with PTB antibodies showed a novel set of proteins. By contrast, addition of RNAs containing introns 5 or 7 gave the same results as no RNA, indicating that these RNAs are unable to form stable complexes with PTB. These results are in agreement with our previous studies demonstrating that PTB interacts with sequences within intron 6, but not with sequences within introns 5 and 7. When ³⁵S-met-labeled HeLa nuclear extracts were mixed with biotinylated RNA containing intron 6 and the RNA–protein complexes were recovered using streptavidin-agarose beads, an identical pattern of proteins was observed when compared with the immunoprecipitation assay. Analysis of the proteins that assembled on introns 5, 6, or 7 using biotinylated RNA revealed a unique set of proteins that interact with each of these sequences. The composition of proteins interacting with sequences associated with the use of the 3' splice site of intron 6 included proteins of 30, 40, 55, 60, 65, 70, 80, and 100 kDa. Microsequencing identified two of the proteins to be Sam68 and the Far Upstream Element Binding Protein (FBP) from the *c-myc* gene. In addition, a comparison of the proteins that assemble on introns from the α - and β -TM genes that utilize distant branch points revealed common as well as unique proteins that assemble on these introns. These studies identify a set of proteins, in addition to PTB, that are likely involved in the use of distant branch sites associated with the use of alternatively spliced introns.

Keywords: alternative RNA splicing; polypyrimidine tract binding protein

INTRODUCTION

Alternative splicing of primary RNA transcripts is a widespread mechanism that has been identified in the genes of a variety of organisms including *Drosophila*, *Caeno-*

rhabditis elegans, and vertebrates. Alternative splicing functions predominantly in two ways. The first is in the on/off control of gene expression, that is, in regulating the production of a functional or nonfunctional protein product, usually by the inclusion of a stop codon. Examples in *Drosophila* include genes that encode the proteins of sex determination, *suppressor-of-white-apricot*, and *P-element transposase*. The second function of alternative splicing is in the formation of multiple protein isoforms. A growing number of cellular and viral genes have been characterized that encode multiple

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protein isoforms via the use of alternatively spliced exons (for reviews see Adams et al., 1996; Wang et al., 1997). In many cases, alternative RNA splicing contributes to developmentally regulated and tissue-specific patterns of gene expression. In the case of viruses, the use of alternative splicing may facilitate the expression of a larger number of proteins from a relatively small genome. The organization of genes that are subject to alternative processing is diverse and can be due to the use of alternative promoters and alternative exons located in internal regions as well as at the 3' end of genes (reviewed in Smith et al., 1989).

Despite great advances in our understanding of the general splicing reaction, the mechanisms responsible for alternative splice selection are largely unknown. Significant progress has been made in *Drosophila* systems, such as in the genes of the sex determination pathway, *suppressor of white apricot*, and *P-transposase*. In these systems, alternative splicing is subject to regulation by factors that either activate or inhibit splice site usage (reviewed in Adams et al., 1996; Wang et al., 1997). In contrast, much less is known in vertebrate systems about the mechanisms and cellular factors involved in splice site selection. A number of features in the pre-mRNA can contribute to alternative splice selection, including intron size, the relative strength of 5' splice sites, the pyrimidine content of the 3' splice sites, the location of branch points, the presence of multiple alternative branch points, and specific exon sequences. In addition, the secondary structure of a pre-mRNA may play a role in alternative splicing by regulating the accessibility of different exons to the splicing machinery (reviewed in Adams et al., 1996; Wang et al., 1997). A number of cellular factors, including SR proteins and hnRNP proteins, have been implicated in regulated alternative splicing. SR proteins bind to purine-rich exonic enhancer sequences and can promote the choice of proximal versus distal 5' splice sites (Ge & Manley, 1990; Krainer et al., 1990). This latter effect is antagonized by hnRNPA1 as well as hnRNP A/B proteins (Mayeda & Krainer, 1992; Caceras et al., 1994; Mayeda et al., 1994; Yang et al., 1994). hnRNP I, also known as polypyrimidine tract binding protein (PTB), is implicated in the regulation of a number of alternatively spliced pre-mRNAs, including α - and β -tropomyosin (β -TM) genes (Mulligan et al., 1992; Lin & Patton, 1995; Singh et al., 1995; Perez et al., 1997), fibronectin (Norton, 1994), neuron-specific src (Chan & Black, 1997), and the γ 2 receptor of the GABA receptor (Ashiya & Grabowski, 1997). hnRNP F and a protein related to hnRNP K, referred to as KSRP, are part of a multiprotein complex involved in the regulation of the c-src gene (Min et al., 1995, 1997).

We have been using the rat 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. 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 (non-muscle) systems revealed an ordered pathway of splicing in which the internal alternatively spliced exon 6 must first be joined to the downstream common exon 8 before they could be spliced to the upstream common exon 5 (Helfman et al., 1988; Tsukahara et al., 1994). Characterization of the branch points used during in vitro splicing of exon 5 to exon 7 (skeletal muscle type splice) revealed the use of multiple branch points that are located an unusually long distance (>140 nt) from the 3' splice site of exon 7 (Helfman & 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 nt upstream of the 3' splice site that specifies the location of the distant branch points used upstream of exon 7. The second element, referred to as the intron regulatory element (IRE), is located between the polypyrimidine tract and the 3' splice site of exon 7. The IRE 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 (Helfman et al., 1990; Guo et al., 1991; Guo & Helfman, 1993). Extensive mutational analysis 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; Guo & Helfman, 1993). 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 in vivo in HeLa cells disrupt the binding of these proteins to the pre-mRNA 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 characterize the precise nature of the factors that interact with sequences in the β -TM pre-mRNA, we purified to apparent homogeneity a protein that interacted specifically with sequences within the IRE upstream of muscle-specific exon 7 (Mulligan et al., 1992). This protein was found to be identical to the PTB that other studies have indicated to be involved in splicing (Garcia-Blanco et al., 1989; Patton et al., 1991; Lin & Patton, 1995; Singh et al., 1995).

The present study was performed in order to further understand the role of PTB in regulated splicing. Mono-

clonal antibodies (mAbs) to PTB were prepared and used to analyze the interaction of PTB with sequences within the pre-mRNA, and to immunoprecipitate RNA–protein complexes in HeLa nuclear extracts. A group of proteins, in addition to PTB, were found to assemble on sequences within the regulated intron, but not on sequences in introns 5 and 7 of β -TM pre-mRNAs. Comparison of the proteins that assemble on introns that utilize distant branch points from the α - and β -TM genes revealed some common, as well as unique, proteins that assemble on these introns. These studies identify a group of proteins, in addition to PTB, that are likely to be involved in use of distant branch sites that are associated with alternatively spliced exons.

RESULTS

Monoclonal antibodies to PTB

In order to address the possible functions of PTB, we have generated a panel of mAbs using purified recombinant human PTB as an antigen. Hybridoma supernatants were initially screened for binding to purified PTB in a dot blot assay, and 18 different clones were positive. This panel of mAbs was further analyzed by western blot assay using HeLa nuclear extracts. To further localize the epitope in PTB recognized by the mAbs, we performed additional western blot assays using recombinant fragments of PTB that were partially purified from bacterial lysates containing full-length or the C-terminal portion of PTB (amino acids 292–532). Fourteen mAbs specifically recognized the C-terminal region of PTB and two mAbs recognized the N-terminal portion of PTB (amino acids 1–291). Further characterization of the mAbs to PTB using immunoprecipitation with ^{35}S -methionine-labeled nuclear extracts demonstrated that these antibodies could specifically precipitate the native form of PTB (data not shown). In addition, under relatively mild conditions (absence of RIPA buffer), each of these antibodies co-precipitated a 100-kDa protein that presumably corresponds to the PTB-associated splicing factor, termed PSF (Patton et al., 1993). No other proteins were detected using western blot and immunoprecipitation assays, demonstrating the specificity of these antibodies for PTB in nuclear extracts.

We reported previously that PTB interacts with intron sequences upstream of exon 7, and that this interaction is likely involved in the negative regulation of this muscle-specific exon in nonmuscle cells (Helfman et al., 1990; Guo et al., 1991; Mulligan et al., 1992). It was of interest to determine whether anti-PTB mAbs to PTB affect the interactions of PTB with RNA. A mobility-shift assay was performed to determine the effect of anti-PTB mAbs on the binding of PTB to RNA using the 180 wt RNA described previously (Mulligan et al., 1992). The RNA–protein complex formed upon the addition of purified PTB is not inhibited by pre-incubation with mAb

and therefore does not reduce the ability of PTB to bind RNA (data not shown). Thus, soluble PTB is recognized by all the mAbs, and this binding does not prevent the interaction of PTB with RNA.

Immunoprecipitation of RNA–protein complexes with anti-PTB antibodies

As described above, at least one protein, PTB, has been shown to interact with *cis*-acting elements involved in alternative splicing of the β -TM pre-mRNA (Mulligan et al., 1992). In order to determine if additional proteins interact with the pre-mRNA, we used two different methods to detect RNA–protein interactions. First, we have developed an assay based on the ability of PTB antibodies to immunoprecipitate RNA–protein complexes. Antibodies to PTB were bound to Protein G Sepharose beads and then added to a reaction mixture of [^{35}S]-met-labeled HeLa cell nuclear extracts and unlabeled RNA to recover the RNA–protein complexes. The RNA–protein complexes that bound to the beads were washed to eliminate nonspecific binding of proteins and those that bound were analyzed by SDS-PAGE followed by autoradiography.

We performed the immunoprecipitation assay using mAbs to PTB or tropomyosin in the absence or presence of added RNAs. The antibody to tropomyosin served as a control for nonspecific binding to immunoglobulin molecules. The various RNAs used in these studies are diagrammed in Figure 1. When the immunoprecipitation was performed using mAbs to PTB, PTB and a 100 kDa protein, which likely corresponds to PSF (Patton et al., 1993), were recovered (Fig. 2, lane 3). No specific proteins were recovered using the antibody to the cytoplasmic cytoskeleton protein tropomyosin (Fig. 2, lane 4). It is worth noting that one protein slightly larger than the PTB doublet was observed in all lanes. This protein of approximately 60 kDa likely represents nonspecific binding to the beads because it was detected using beads alone (Fig. 2, lane 2). When the nuclear extract was first mixed with an RNA that was able to interact with PTB, it was possible to recover the resulting RNA–protein complex. Lanes 5–7 in Figure 2 show the results obtained in the presence of exogenous 260 wt RNA, which contains sequences from the branch point of intron 6, exon 7, and 25 nt of intron 7 (see Fig. 1). Immunoprecipitation using PTB antibodies resulted in the recovery of an RNA–protein complex containing PTB, a 100-kDa protein that is likely PSF, and some prominent proteins of molecular mass ranging from 35 to 80 kDa (Fig. 2, lane 6). By contrast, these proteins were not detected in immunoprecipitations containing the 260 wt RNA, beads, and no PTB antibody (Fig. 2, lane 5) or with anti-TM mAb (Fig. 2, lane 7). To control for nonspecific interaction simply due to the addition of RNA, a different RNA was used containing sequences from exon 5 through exon 6 of

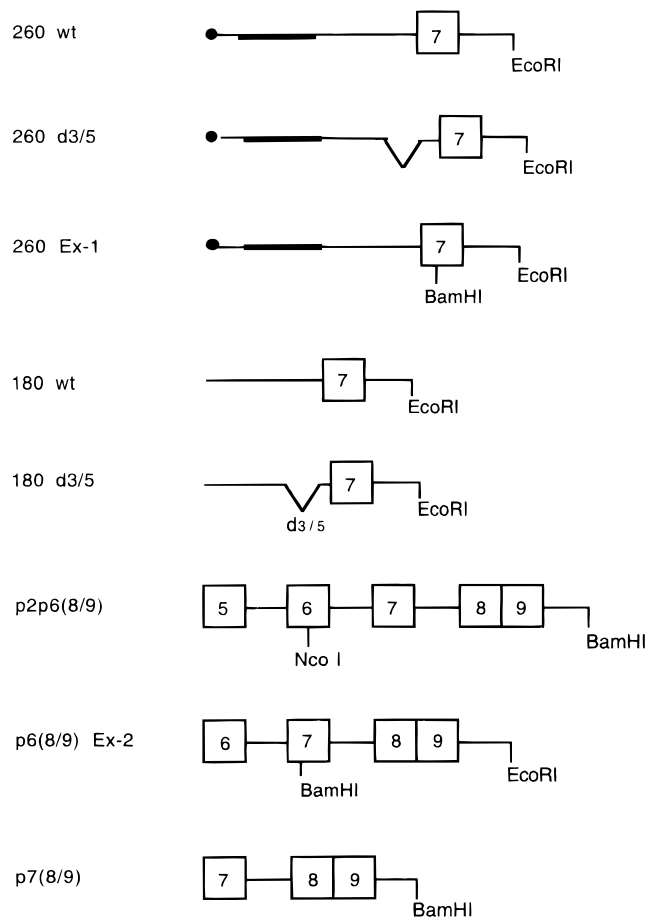


FIGURE 1. Schematic representation of RNAs used. RNA constructs are derived from the rat β -TM gene (Helfman et al., 1986). Boxes represent exons and lines represent introns. Filled circles represent the position of the branch points. Black bar represents the polypyrimidine tract.

the β -TM gene, called p2p6(8/9) (Fig. 2, lanes 8–10). Control RNA did not result in co-precipitation of additional proteins (compare Fig. 2 lanes 2–4 versus lanes 8–10). These results are in agreement with our previous studies demonstrating that PTB does not bind to sequences within intron 5 of the β -TM pre-mRNA (Mulligan et al., 1992). Thus, these data demonstrate that the co-precipitation assay is specific to different regions of the β -TM pre-mRNA and that many proteins assemble on sequences involved in alternative splicing of β -TM RNA.

Recovery of RNA–protein complexes using biotin and streptavidin

In order to confirm the results obtained using antibodies to recover RNA–protein complexes, we also used biotinylated RNA and recovered the RNA–protein complexes with streptavidin beads. This assay has been used extensively to study the assembly of various components on pre-mRNAs (Bennett et al., 1992). Four

different RNA constructs derived from the β -TM gene were used in these experiments (see Figs. 1, 3). A protein of approximately 47 kDa was recovered using streptavidin beads and nuclear extract without the addition of exogenous RNA (Fig. 3, lane 2). We then compared the proteins that assembled on four different biotinylated RNAs (Fig. 3, lanes 3, 5, 7, 9). For controls, we used nonbiotinylated RNAs (Fig. 3, lanes 4, 6, 8, 10), which are similar to the results obtained with no RNA (Fig. 3, lane 2) where no additional proteins were recovered. However, when the assay was performed in the presence of biotinylated RNA, a number of additional proteins were recovered that were specific for different regions of the RNA, demonstrating the specificity of these RNA–protein interactions. The proteins that bound to 260 wt or p6(8/9)Ex-2 RNAs were similar to the proteins recovered in the assay using PTB antibodies (compare Fig. 2, lane 6 and Fig. 3, lanes 3 and 5). It is also worth noting that PTB was found to interact with the two RNAs containing sequences in intron 6 (Fig. 3, lanes 3 and 5), but not with those containing introns 5 or 7 (Fig. 3, lane 7 and 9). This result was also confirmed by western blot analysis of the recovered proteins (data not shown). These data are in agreement with previous studies using gel mobility shift assays showing that PTB interacts with sequences within intron 6, but not introns 5 or 7 (Mulligan et al., 1992).

Protein assembly on intron 6

The regions in the β -TM pre-mRNA involved in regulating the use of exon 7 in nonmuscle cells have been localized to a 260-nt region including sequences in intron 6, exon 7, and 25 nt of intron 7 (Guo & Helfman, 1993). It was important to further dissect the regions in intron 6 that interacted with proteins in HeLa cell nuclear extracts. We next compared the binding of proteins to different regions in the intron using the immunoprecipitation assay (Fig. 4) and the biotinylated RNA precipitation assay (Fig. 5). The various RNAs used in these assays are shown schematically in Figure 1. Both assays detected similar proteins. Previous studies demonstrated that mutations and deletions in the region immediately upstream of exon 7 (the IRE) or within exon 7 resulted in the activation of exon 7 (skeletal muscle-type splice) in nonmuscle cells (Helfman et al., 1990; Guo et al., 1991; Guo & Helfman, 1993). Therefore, we compared the proteins that assembled on 260 wt versus mutant 260 d3/5, which contains a deletion of 25 nt in the IRE. The 260 wt RNA contains all the sequences necessary and sufficient for regulation in a nonmuscle (HeLa cell) environment (Guo & Helfman, 1993). Surprisingly, a comparison of the proteins that assembled on these two RNAs did not show any qualitative difference in the proteins that bound (Figs. 4, 5, lanes 5 and 6). Whether this is because all of the factors that interact with these sequences cannot be iden-

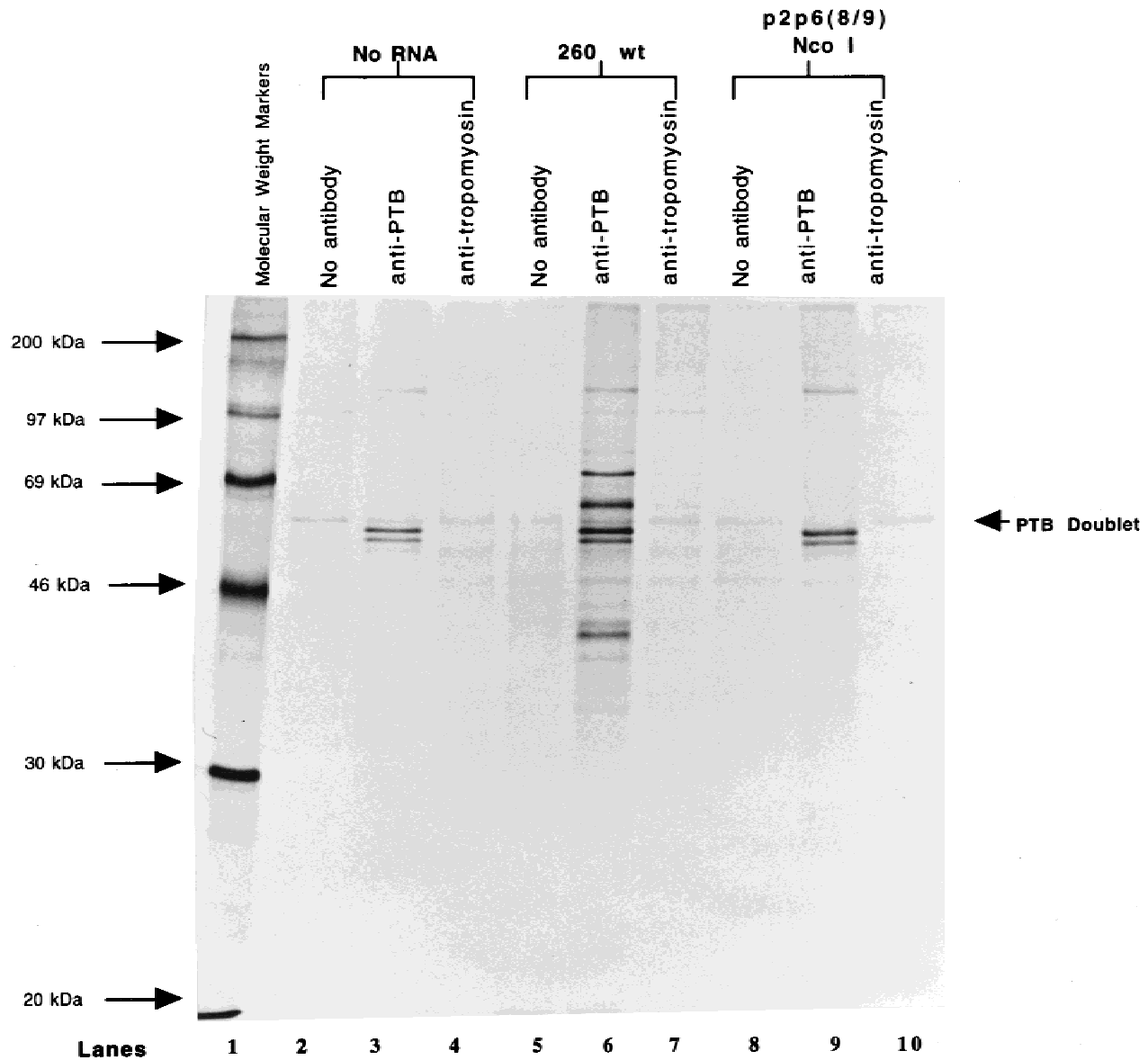


FIGURE 2. Immunoprecipitation of RNA-protein complexes. Ten microliters of [³⁵S]-methionine-labeled nuclear extract were added to 2 μ g of in vitro-transcribed RNA and precipitated with either a PTB antibody bound to Protein G Sepharose beads or an antibody against tropomyosin. Different RNA constructs were used to compare what proteins bound to different regions of the pre-RNA. Lane 1, molecular weight standard; lanes 2–4, no RNA added in the assembly reactions; lanes 2, 5, 8, no antibody bound to the beads; lanes 5–7, assembly in the presence of 260 wt RNA; lanes 8–10, assembly in the presence of the p2p6(8/9) *Nco* I RNA; lanes 3, 6, 9, PTB antibody bound to Protein G Sepharose beads; lanes 4, 7, 10, tropomyosin antibody bound to the beads.

tified with these assays remains to be determined. Interestingly, the majority of the proteins interacted with sequences between the branch point region and the 3' splice site (Figs. 4, 5, lane 7). Similar results were obtained when the RNA containing mainly the polypyrimidine tract, which is associated with the use of the distant branch point sequence (10 nt upstream of the branch point through 90 nt downstream of it, data not shown), was used. These results demonstrate that a multiprotein complex assembles on the sequences associated with distant branch point usage.

Identification of proteins assembling on β -TM RNA

To determine the identity of the polypeptides that assembled on the 260 wt RNA, we recovered the proteins from the assembly assay and analyzed them by western blot analysis. Some of the proteins were gel-purified and subjected to microsequencing. In addition to PTB, three additional polypeptides were identified. Western blot analysis using antibodies to the 65 kDa subunit of U2AF demonstrated that this protein was also present

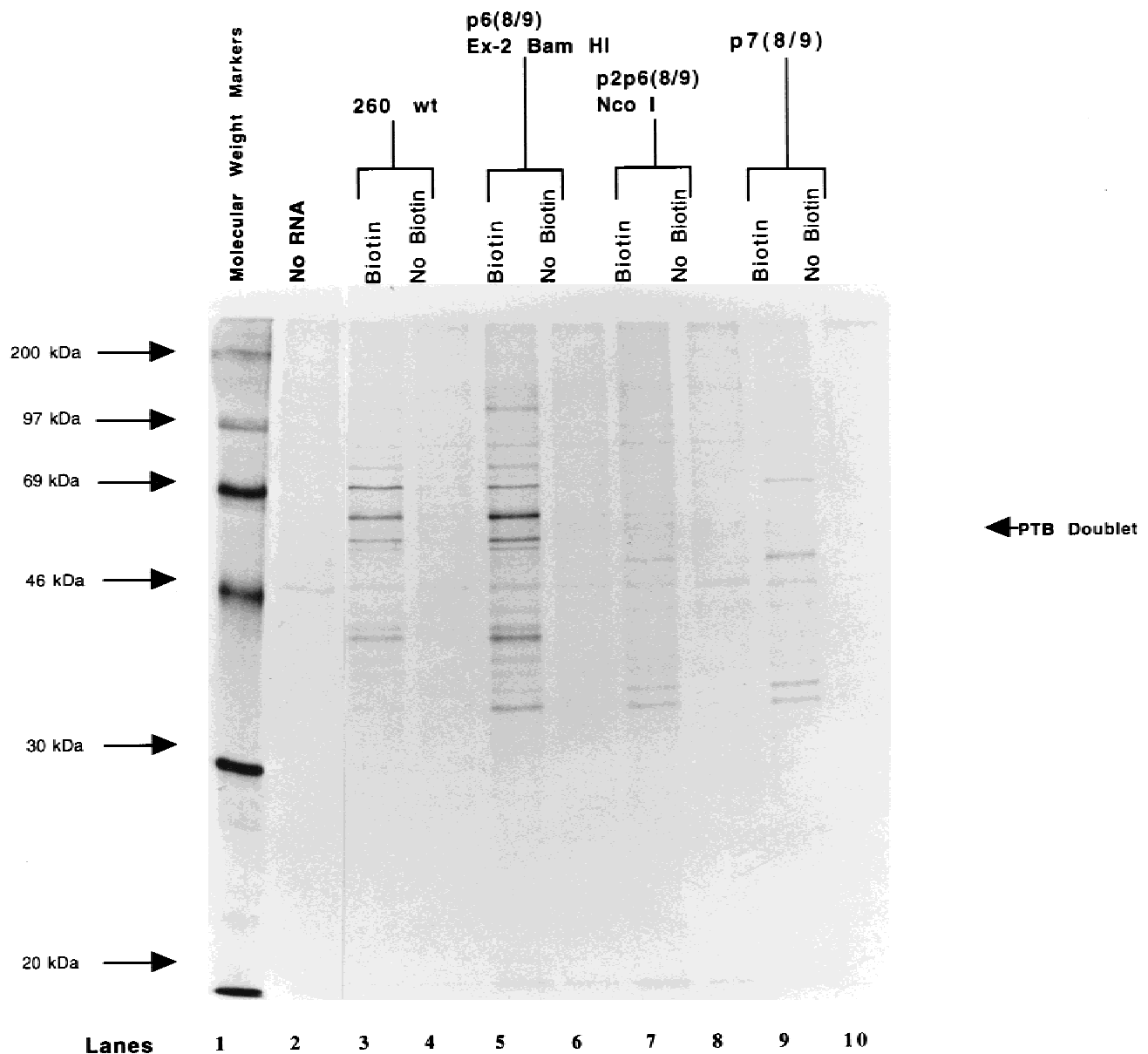


FIGURE 3. Recovery of RNA–protein complexes using biotinylated RNA. Ten microliters of [³⁵S]-methionine-labeled nuclear extract were incubated with 2 μ g of in vitro-transcribed biotinylated RNA and the complexes were recovered using streptavidin beads. RNAs used contained different introns of the β -TM pre-mRNA (see Fig. 1). p2p8(8/9) *Nco* I contained intron 5, p6(8/9) *Bam*H 1 contained intron 6, and p7(8/9) contained intron 7. A comparison is shown using RNAs transcribed in the presence and absence of biotin–UTP. Lane 1, molecular weight markers. Assembly reactions were performed without RNA (lane 2), with 260 wt RNA (lanes 3, 4), p6(8/9) *Ex-2 Bam*H 1 RNA (lanes 5, 6), p2p6(8/9) *Nco* I RNA (lanes 7, 8), or p7(8/9) RNA (lanes 9, 10).

in the complex (data not shown), and was responsible, at least in part, for the prominent band just above PTB (Figs. 4, 5). The biotin–streptavidin binding assay was scaled up and the proteins were recovered, separated by SDS-PAGE, and visualized using Coomassie-blue stain. A minor band of 65 kDa and a major band of 70 kDa were recovered and subjected to microsequencing (Table 1). Searching the database showed that two peptides from the 65-kDa band were highly homologous (7 of 7 and 8 of 10 amino acid residues matched,

respectively) to the human Sam 68 tyrosine phosphoprotein, whose cDNA sequence was originally cloned as the Ras-GAP associated tyrosine phosphoprotein p62 (Wong et al., 1992; Courtneidge & Fumagalli, 1994). All four peptides analyzed from the 70-kDa band showed complete sequence homology to a 67-kDa protein that was first reported to bind to the far upstream element (FUSE) of the *c-myc* gene and is therefore called the FUSE binding protein (FBP) (Duncan et al., 1994, 1996). FBP has been reported to bind to single-stranded DNA

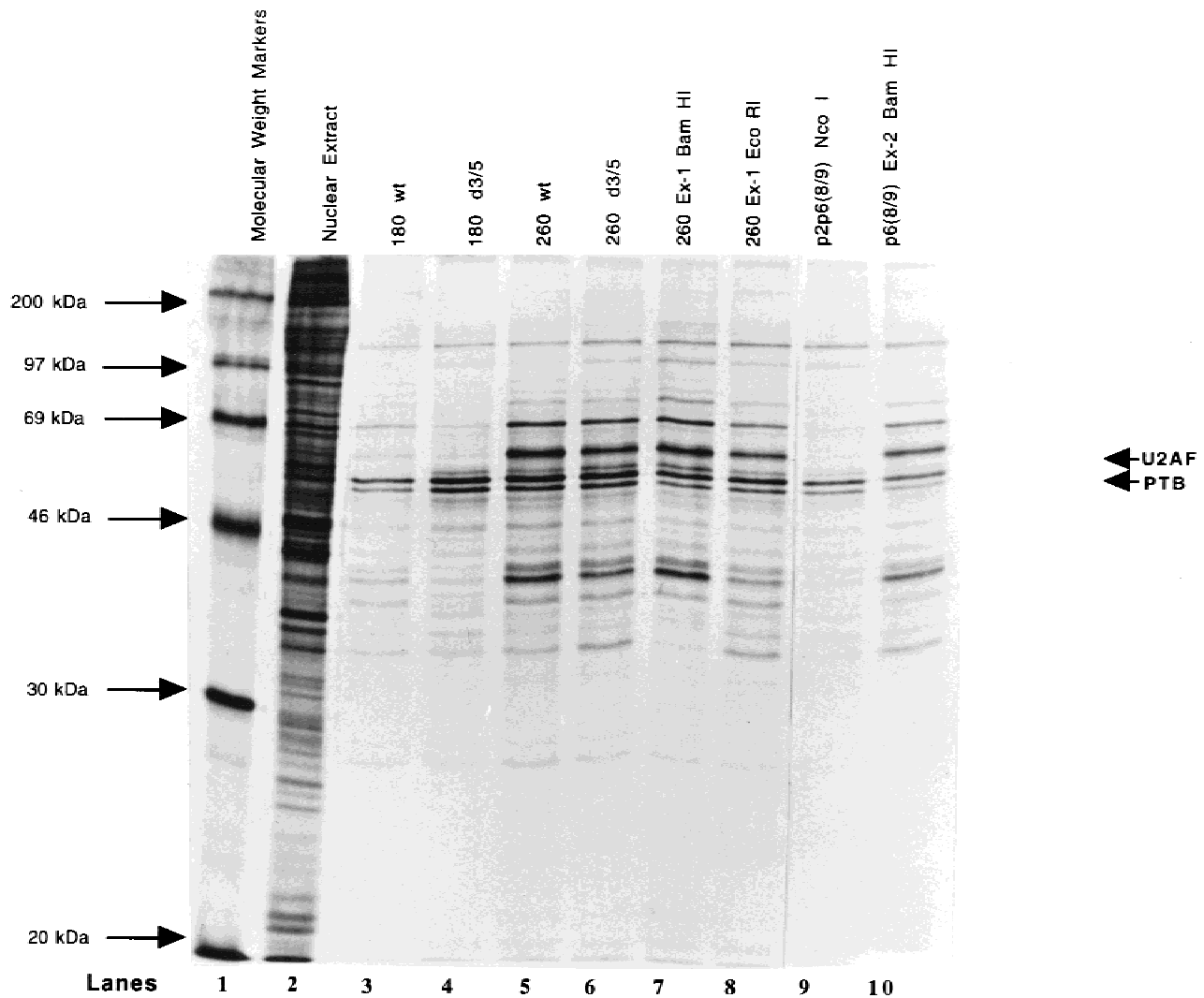


FIGURE 4. Distinct proteins interact with different RNAs. Ten microliters of [35 S]-methionine-labeled nuclear extract were incubated with 2 μ g of in vitro-transcribed RNA before the precipitation of proteins with the PTB antibody bound to Protein G Sepharose beads. Lane 1, high molecular weight markers; lane 2, 0.5 μ L of the [35 S]-methionine-labeled nuclear extract; lanes 3–10, proteins assembled on RNAs containing different regions of the β -TM pre-mRNA. All the RNAs were represented schematically in Figure 1.

and RNA and was originally characterized as a transcriptional enhancer for c-myc.

A group of proteins assemble on α - and β -TM pre-mRNAs that utilize distant branch points

Distant branch sites are common to alternative introns in the rat α - and β -TM genes (Helfman & Ricci, 1989; Smith & Nadal-Ginard, 1989). Both introns contain a long polypyrimidine tract associated with the use of a distant branch point located 150–175 nt upstream of the 3' splice site. In addition, intron 2 in the α gene is efficiently spliced in nonmuscle cells, whereas intron 6 in the β gene is not. It was therefore of interest to determine if there were some common factors that assemble on introns associated with distant branch points,

as well as differences that might provide insight into the differential use of these introns. We therefore performed assembly assays using two different RNAs, one containing the sequence of β -TM gene intron 6 and the other containing the sequence of the α -TM gene intron 2. Figure 6 shows the results obtained using either the biotinylated RNA or the immunoprecipitation assay and nuclear and cytoplasmic fractions derived from HeLa cells. Although there were similar patterns of proteins that assembled on both RNAs, two proteins of approximately 70 and 80 kDa assemble preferentially on the β -TM RNA. The 70-kDa protein corresponds to FBP. The identity of the 80-kDa protein remains to be established. Further work will be required to determine if these proteins play a role in regulated alternative splicing of β -TM pre-mRNA. In addition, these results suggest

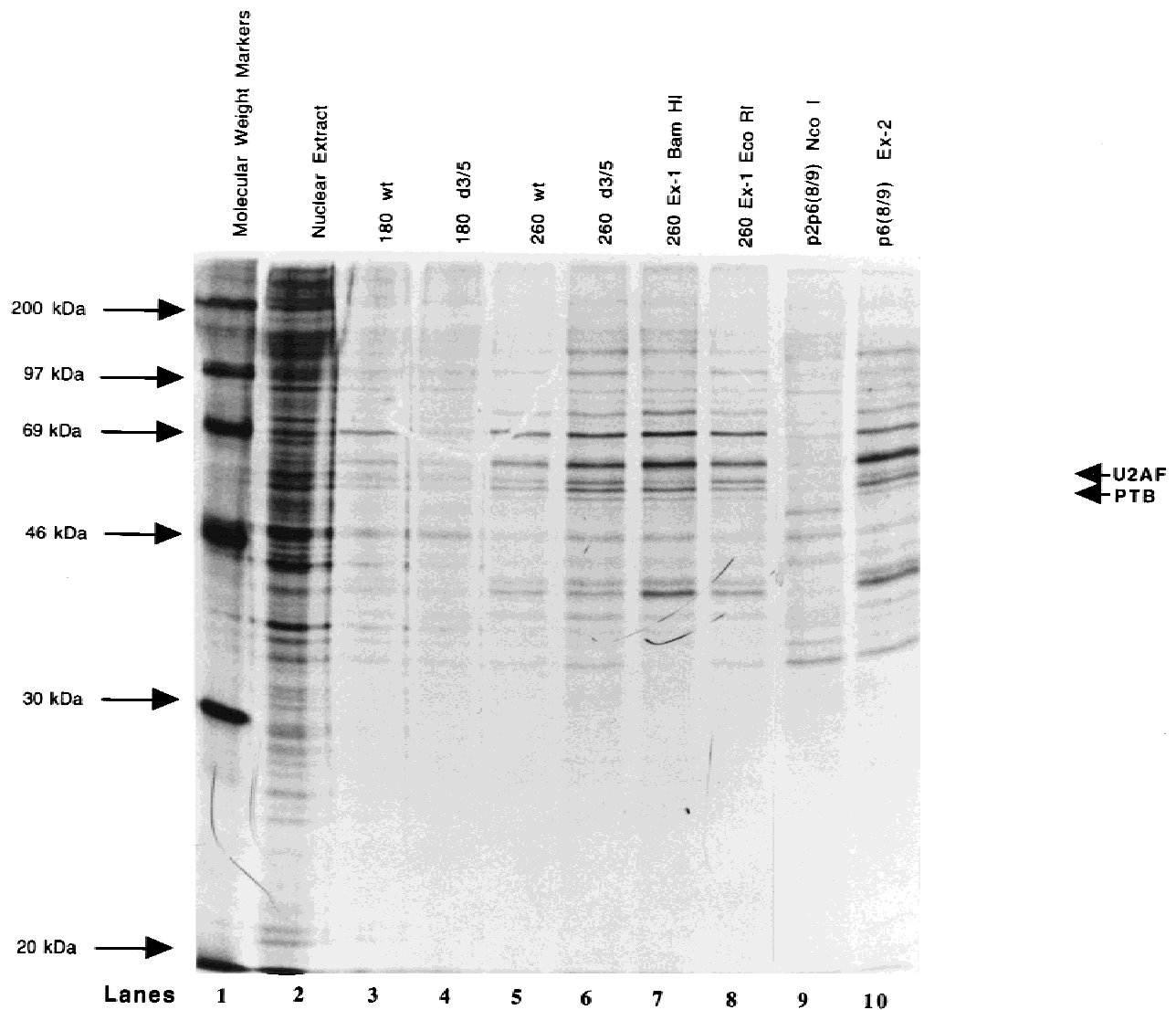


FIGURE 5. Recovery of RNA–protein complexes utilizing biotinylated RNA. Ten microliters of [³⁵S]-methionine-labeled nuclear extract were incubated with 2 μ g of in vitro-transcribed biotinylated RNA before the precipitation of proteins by streptavidin beads. RNA constructs used are identical to the construct seen in Figure 4.

that binding of a common set of proteins might be a common feature of introns that use distant branch sites.

DISCUSSION

Immunoprecipitation of RNA–protein complexes with anti-PTB antibodies

We previously reported that two distinct *cis*-acting elements are located between the 3' splice site of exon 7 and the distant branch points (Helfman et al., 1990; Guo et al., 1991). The first element comprised a polypyrimidine tract located 89–143 nt upstream of the 3'

TABLE 1. Peptide sequences.^a

65 KDa Polypeptide	70 KDa Polypeptide
GAYREHPYxRY	LFTIRGTPQQIDY
ILGPQGNTIK	AWEEYYK
	IGGDAGTSLNSNDY
	EMVLELIRDQGGFREVNRNEYGxxIGxN
Sam 68 protein	FUSE-binding protein

^aThe peptide sequences obtained from the 65-kDa polypeptide and of the 70-kDa polypeptide, both of which assemble on the 260 wt RNA, which contains intron 6 downstream of the branch point sequence, exon 7, and 25 nt of intron 7, in a biotin–streptavidin binding assay are shown. They were found to correspond to the Sam 68 protein and the FUSE-binding protein (FBP), respectively, by searching the protein database. “x” indicates ambiguities in the sequence.

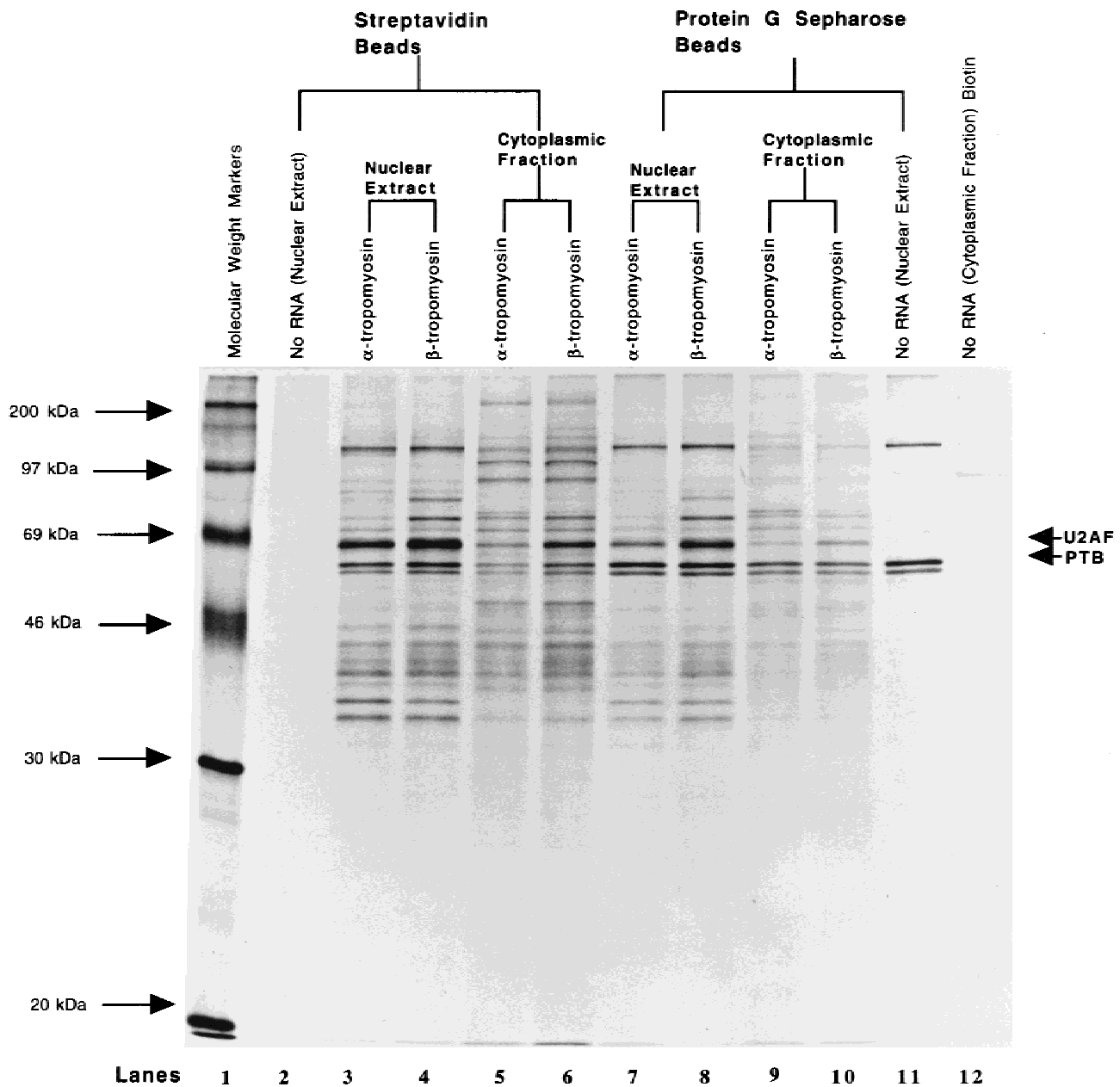


FIGURE 6. Comparison of RNA-protein complexes that form on RNA from the α and β genes. Ten microliters of [35 S]-methionine-labeled nuclear extract or S100 fraction were incubated with 2 μ g of in vitro-transcribed RNA, with or without biotin-UTP and either precipitated by streptavidin beads or PTB antibody bound to Protein G Sepharose beads. Lane 1 shows molecular weight markers. Lanes 2–6 are obtained from the biotinylated RNA assay, whereas lanes 7–11 are from the Protein G Sepharose immunoprecipitation assay. Lanes 2 and 11 show assembly results without added RNA. Lane 12 is assembly using nonbiotinylated RNA. Lanes 3, 4, 7, and 8 are the results obtained using the nuclear extract, whereas lanes 5, 6, 9, and 10 are the results obtained using S100 cytoplasmic fraction.

splice site and specified the location of the distant branch points. The second element is located between the polypyrimidine tract and the 3' splice site of exon 7 and is herein referred to as the IRE. The IRE contains an important determinant in alternative splice site selection because deletions or clustered point mutations in this regulatory region result in the use of the muscle-specific exon in nonmuscle cells (Helfman et al., 1990; Guo et al., 1991; Guo & Helfman, 1993). Previous stud-

ies have demonstrated that PTB bound independently to both of these *cis*-acting elements (Mulligan et al., 1992). It was therefore of interest to determine if other proteins, in addition to PTB, interacted with these *cis*-acting regulatory elements. We utilized two assays to identify proteins that interact with the critical regulatory sequences at the 3' end of intron 6. The first assay took advantage of antibodies against PTB to immunoprecipitate RNA-protein complexes. The second method

utilized biotinylated RNA and streptavidin beads to recover the proteins associated with RNA. These two assays yielded the same results. In agreement with our previous studies, PTB bound stably to sequences within intron 6, but not introns 5 or 7 in β -TM pre-mRNA. These studies further suggest a role for PTB in the use of this intron. Furthermore, a number of proteins, in addition to PTB, was found to interact with sequences at the 3' end of intron 6.

The use of antibodies to a particular protein to co-precipitate RNA-protein complexes will be of general use to investigators studying the interaction of proteins on a given sequence. In particular, it is important to use two independent methods to confirm that a group of proteins is interacting on the same RNA. In this regard, it is possible that, although multiple proteins might interact with an RNA target, it is difficult to determine if they are all bound to the same RNA or if there are different populations containing subsets of RNA-protein complexes. By carrying out the co-precipitation assay with antibodies to various proteins known to interact with a particular RNA, it will be possible to substantiate that all the interacting proteins are assembling on the same RNA molecule. In addition to studies in β -TM pre-mRNA, PTB has been reported to play a role in a number of alternatively spliced RNAs, including α -TM, calcitonin, fibronectin, n-src, and the GABA receptor (Mulligan et al., 1992; Norton, 1994; Lin & Patton, 1995; Ashiya & Grabowski, 1997; Chan & Black, 1997; Perez et al., 1997). It will be of interest to determine what proteins, in addition to PTB, assemble on these RNAs.

Regulation of alternative splicing of β -TM 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; Guo & Helfman, 1993). This blockage requires the sequences in the IRE and within exon 7 (Helfman et al., 1990; Guo et al., 1991; Guo & Helfman, 1993). In our previous studies, we demonstrate that the IRE can interact with PTB in a sequence-specific manner (Mulligan et al., 1992). It is possible that other factors also bind to these sequences. Because several fractions obtained during purification exhibited mobility shift activity specific for the wild-type RNA probe, it remained to be determined if any of these fractions contain sequence-specific RNA-binding proteins. In the present studies, we found that PTB is part of a multiprotein complex that assembles on the RNA and includes the far-upstream element-binding protein (FBP), SAM 68, and U2AF 65.

The cDNA sequence of the human Sam 68 tyrosine phosphoprotein was originally cloned for p62, a protein associated with the *ras* GTPase-activating protein (GAP) p120. Courtneidge and co-workers demonstrated that this sequence corresponds to Sam 68, which is a sub-

strate of c-src-kinase during mitosis (Fumagalli et al., 1994). Still, both proteins are considered to be related because they also show similar properties. Interestingly, the amino acid sequence in the N terminus of Sam 68 has a strong homology to a putative hnRNP protein called GRP33 and has been shown to bind RNA (Wong et al., 1992; Courtneidge & Fumagalli, 1994). Although tyrosine phosphorylation at its C-terminus does not appear to be directly related to the RNA binding properties of Sam 68, altered protein-protein interactions by tyrosine phosphorylation may affect mRNA processing (Wong et al., 1992). Because Sam 68 is present at all stages of the cell cycle, but it is only phosphorylated and associated with c-src during mitosis, it is likely to be located in the nucleus, like hnRNP proteins and other RNA binding proteins. Its interaction with the membrane-associated c-src is therefore only possible upon the breakdown of the nuclear envelope in mitosis. Thus, it is plausible that Sam 68 is a component of a protein complex that assembles on intron 6 and exon 7, potentially regulating alternative splicing of the β -TM exon 7. At this point, nonspecific binding still cannot be ruled out completely. Furthermore, because only two peptides were matching sequences of Sam 68, it is still possible that the isolated 65-kDa polypeptide is only a related protein to Sam 68 and p62. The other two peptides from the 65-kDa protein band did not correspond to any known polypeptides except one that showed some homology to ribosomal proteins and could resemble nonspecific binding to RNA.

As shown by two-dimensional gel analysis (data not shown), the prominent 70-kDa band on the one-dimensional SDS-acrylamide gel consists of only one protein and all peptides sequenced from this band were identical to FBP. FBP was reported initially as a sequence-specific single-stranded DNA binding protein (Duncan et al., 1994) that binds to the noncoding strand of the FUSE region and serves as a transcriptional activator for c-myc. Its homology to a repeated sequence, the KH-domain in the hnRNP K protein, also suggested a role in RNA binding, which was subsequently confirmed. It has been found recently that FBP exists in three different isoforms, all of which are involved in ssDNA and RNA binding (Davis-Smyth et al., 1996). Considering their apparent specificity for RNA, at least one of the three different isoforms may be involved in the regulation of alternative splicing and this property is currently being investigated by a number of laboratories (D. Levens, pers. comm.).

The biological significance of these proteins binding to sequences upstream of exon 7 remains to be established. We previously demonstrated that mutations in the IRE lead to activation of the skeletal muscle-specific exon *in vivo*, and this correlated with the ability of PTB to bind to these sequences (Guo et al., 1991; Mulligan et al., 1992). Here we show that mutations in the IRE did not result in a detectable loss of the proteins bound

to the 260 wt RNA (Fig. 4, lanes 5–8; Fig. 5, lanes 5–8). However, when RNA containing the mutant 180 d3/5 was used, which contains a 25-nt deletion in the IRE in the 180 RNA (Fig. 5, lanes 3 versus 4), there was a loss of proteins bound to this RNA. It is worth noting that the d3/5 mutation in the 180 wt RNA disrupts the binding of PTB (Mulligan et al., 1992), and therefore may not be amenable to the immunoprecipitation assay using anti-PTB antibodies. The use of biotin–RNA overcomes this limitation. A comparison of the proteins recovered using the wt and mutant 180 RNAs (Fig. 5, lanes 3 and 4) revealed a loss of the proteins when the IRE was mutated. Thus, in addition to PTB, other proteins are associated with sequences within the IRE. Interestingly, the same patterns of proteins were detected using 260 wt and 260 mutants. The similarities in composition between wild-type and mutant RNAs might reflect multiple binding sites for proteins in the RNA. It is also worth noting that our previous analysis of *cis*-acting regulatory elements in β -TM pre-mRNA indicates 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 formation of the multiprotein complex identified in the present studies because the same pattern of proteins was detected using both wild-type and mutant RNAs in these sequences. It remains to be determined if these exon sequences interact with other factors.

How the interaction of proteins within the regulated intron contributes to alternative splice site selection is unclear at this time. Because mutations in the IRE result in activation of the muscle-specific exon, it is possible that these mutations function in part to alter the binding of specific proteins that in turn could change the spatial organization of the pre-mRNA. The distant branch point might play a critical role by maintaining the distance between the branch point and the exon. Thus, these sequences could function, in part, by binding to specific hnRNP proteins that are required for the spatial organization of the pre-mRNA. Any perturbation in their binding could alter the regulation. For example, a mechanism for a regulated intron being based on distance has been reported in the case of the alternative splicing of *Drosophila* double sex (*dsx*) pre-mRNA (Tian & Maniatis, 1994). Although the use of the 3' female-specific *dsx* splice is normally dependent on the binding of Tra and Tra2 proteins, the use of this 3' splice becomes Tra/Tra2 independent when the *dsx* enhancer is located closer to the 3' splice site of the exon. It is therefore possible that mutations or deletions of the β -TM pre-mRNA function, in part, to change the spatial organization of the pre-mRNA, thereby activating the muscle-specific exon. Whether skeletal muscle cells will repress some or all of the components expressed in nonmuscle cells, or whether these muscle cells express a factor that promotes the inclusion of the muscle-specific exon remains to be determined.

Cellular factors involved in distant branch point location in α - and β -TM genes

We also compared the proteins that assembled on intron 2 of the α -TM gene with those that interact with intron 6 of the β -TM gene. Both introns contain similar *cis*-acting sequences, including a long polypyrimidine tract associated with the use of distant branch points located 150–172 nt upstream of a 3' splice site (Helfman et al., 1989; Smith & Nadal-Ginard, 1989). Comparing the proteins that assembled on the two introns revealed a common group of proteins. These results bring up the possibility that remote branch points will involve the use of a conserved group of protein factors. In addition to α - and β -TM genes, a number of recent studies have demonstrated that the use of branch points located a relatively long distance from a 3' splice site of alternatively spliced viral and cellular genes. Splicing of adenovirus E1A RNA was found to use multiple branch points located 51–59 nt from the 3' splice site (Gattoni et al., 1988), and alternative splicing of the fibronectin gene exon EIIIB uses branch sites 62–76 nt upstream of the 3' splice site (Norton, 1994). These studies raise the possibility that the use of branch points located a relatively long distance from a 3' splice site may be an essential feature of some alternatively spliced exons.

In summary, the present studies identify proteins, in addition to PTB, that assemble around the 3' splice site of exon 7 in the β -TM pre-mRNA. Work is currently underway to further characterize these proteins and determine their role in alternative pre-mRNA splicing.

MATERIALS AND METHODS

Construction of plasmids

DNA templates for use in *in vitro* transcription are derived from the rat β -TM gene (Helfman et al., 1986, 1988, 1990; Helfman & Ricci, 1989; Guo et al., 1991).

Synthesis of pre-mRNA

[³²P]-labeled SP6/tropomyosin transcripts were synthesized *in vitro* and primed with CAP analog as described (Konarska et al., 1984). [³²P]-labeled RNAs were further purified on polyacrylamide/urea gels.

Nuclear extracts

For standard splicing reactions, nuclear extracts were prepared as described previously, starting with 8 L of HeLa cells that were grown to a density of 1×10^6 /liter (Krainer et al., 1984). All steps were performed at 4 °C. For experiments using [³⁵S]-met, 4 L of HeLa cells were grown to a density of 5×10^5 cells per mL. The cells were recovered by centrifugation, washed with methionine-free media (DMEM, Gibco BRL), and labeled overnight at 37 °C in 200 mL of methionine-free DMEM with 2% bovine fetal serum, 8% dialyzed calf

serum, 1% L-glutamine, 1% sodium pyruvate, and 50 mCi/mL [³⁵S]-methionine. All protein concentrations were determined by the method of Bradford, using albumin as a standard. Quantitative amino acid analysis indicated that absorption assays underestimated the concentration of PTB by approximately fivefold.

RNA mobility-shift assay

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

Immunoprecipitation of RNA–protein complexes

A mixture of 1 mL each of hybridoma supernatants containing mAbs against either the amino- or carboxy-terminal regions of the PTB were incubated with 100 μ L of Protein G Sepharose beads (Pharmacia) and mixed overnight at 4 °C. The anti-PTB beads were washed three times in RIPA buffer [150 mM NaCl, 1.0% (w/v) NP-40, 0.5% deoxycholate, 0.1% SDS, 50 mM Tris, pH 8.0] to remove unbound antibody, and equilibrated in binding buffer (buffer D that contains 20 mM Hepes, pH 8.0, 20% glycerol, 100 mM KCl, and 0.2 mM EDTA) with 0.05% Triton X-100. Ten microliters of ³⁵-methionine-labeled nuclear extract was incubated on ice with 1 mg/mL of heparin, 0.05% Triton X-100, and 2 μ g of unlabeled RNA derived from the α - or β -TM genes in a total of 15 μ L and incubated on ice or at 30 °C for 30 min. Ten microliters of packed anti-PTB beads were added to the 15- μ L reaction and incubated on ice for 30 min. The Protein G Sepharose beads were then washed three times in buffer D containing 0.05% Triton X-100. Bound proteins were recovered with the addition of 50 μ L of 2 \times Laemmli sample buffer [100 mM Tris-HCl, pH 6.8, 200 mM DTT, 0.2% (w/v) bromophenol blue, and 20% (v/v) glycerol] and boiled for 3–5 min followed by centrifugation for 30 s and the supernatant recovered. Twenty microliters were loaded on a 12% SDS-polyacrylamide gel and electrophoresed in running buffer (192 mM glycine, 25 mM Tris, 0.02% SDS). Gels were fixed in a solution containing 8% (v/v) methanol and 8% (v/v) acetic acid for 30 min, dried, and the proteins were visualized by autoradiography.

Recovery of RNA–protein complexes using biotin

Proteins were recovered from labeled extracts under similar conditions for the immunoprecipitation assay except RNA was synthesized using biotinylated UTP and the RNA–protein complexes were recovered using streptavidin beads (Gibco, BRL).

Large-scale biotin–streptavidin binding assay and peptide sequencing

For purification of polypeptides from Coomassie blue-stained gels and subsequent peptide sequencing, the amounts of the biotin–streptavidin assay were scaled-up. Six-hundred milligrams of biotinylated RNA were incubated with 2 mL of unlabeled HeLa cell nuclear extract, 1 mg/mL heparin, and 0.05% Triton X-100 for 30 min at 4 °C. The mixture was added to 1.5 mL of packed streptavidin beads and incubated while rotating for 30 min at 4 °C. Washes were performed with 15 mL buffer D containing 0.05% Triton X-100 as described above. To recover the bound proteins, the beads were loaded on a column and eluted with three column volumes of buffer D containing 1 M NaCl and 0.05% Triton X-100, and one column volume of 6 M urea in 50 mM Tris, pH 7.4. The elution fractions were analyzed by SDS-PAGE and visualized by silver staining according to Sambrook et al. (1989) with the exception that 0.03 mM DTT was added instead of ethanol. To concentrate the proteins, Centricon-10 spin columns (Amicon) were used. The salt concentration was reduced by adding buffer D without mM KCl and repeating the centrifugation step twice. The concentrated samples were separated by SDS-PAGE and stained with Coomassie brilliant blue G (Sigma).

For protein sequencing, performed at the protein sequencing facility at Cold Spring Harbor Laboratory, two bands were excised and subjected to in-gel digestion with *Achromobacter* protease (Lysylendopeptidase; 50 ng/ μ L in 50 mM Tris-HCl, pH 9.0) as described previously (Sueyoshi et al., 1995). The resulting polypeptides were separated by HPLC using a Vydac C18 column (1.0 \times 250 mm, 10 μ m, 300 Å) and sequenced by automated protein sequencers (Applied Biosystems 470 and 477).

Miscellaneous

PTB was purified from HeLa cells using the method of Mulligan et al. (1992). Immunoblot analysis used standard procedures (Harlow & Lane, 1988). UV crosslinking was performed essentially as described (Garcia-Blanco et al., 1989).

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