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A Testis Cytoplasmic RNA-Binding Protein That Has the Properties of a Translational Repressor

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Translation of the mouse protamine 1 (*Prm-1*) mRNA is repressed for several days during male germ cell differentiation. With the hope of cloning genes that regulate the translational repression of *Prm-1*, we screened male germ cell cDNA expression libraries with the 3′ untranslated region of the *Prm-1* RNA. From this screen we obtained two independent clones that encode Prbp, a *Prm-1* RNA-binding protein. Prbp contains two copies of a double-stranded-RNA-binding domain. In vitro, the protein binds to a portion of the *Prm-1* 3′ untranslated region previously shown to be sufficient for translational repression in transgenic mice, as well as to poly(I) · poly(C). Prbp protein is present in multiple forms in cytoplasmic extracts prepared from wild-type mouse testes and is absent from testes of germ cell-deficient mouse mutants, suggesting that Prbp is restricted to the germ cells of the testis. Immunocytochemical localization confirmed that Prbp is present in the cytoplasmic compartment of late-stage meiotic cells and haploid round spermatids. Recombinant Prbp protein inhibits the translation of multiple mRNAs in a wheat germ lysate, suggesting that Prbp acts to repress translation in round spermatids. While this protein lacks complete specificity for *Prm-1*-containing RNAs in vitro, the properties of Prbp are consistent with it acting as a general repressor of translation.

Translational regulation is a common mechanism of gene regulation in gametogenesis and in early embryonic development (9, 40). One reason for translational control is that it permits changes in protein levels to occur in the absence of new transcription; this is a necessity in murine spermatogenesis, since transcription ceases well before most of the dramatic morphological changes that accompany spermatid differentiation occur (21, 37). Genes known to be under translational control during murine spermatogenesis include those involved in mediating nuclear condensation, which occurs in the final stages of spermiogenesis. These include the genes for the two transition proteins, *TP1* and *TP2* (25, 52), and the protamines, *Prm-1* (1, 24) and *Prm-2* (18). Translational repression of *Prm-1* mRNA is essential for normal spermatid differentiation, since premature translation of *Prm-1* mRNA leads to dominant male sterility in transgenic mice (30).

Despite the widespread occurrence of translational regulation in many developmental processes, relatively little is known about the molecular mechanisms which regulate mRNA behavior. However, a major discovery in recent years has been the demonstration that 3′ untranslated regions (3′ UTRs) play a critical role in regulating mRNA transport, localization, stabilization, and translation (reviewed in references 8 and 19). Further understanding of how 3′ UTRs mediate such diverse effects on RNA behavior necessitates the identification of molecules within the cell that bind to the 3′ end of the message.

Sequences contained within the 3′ UTR of the *Prm-1* mRNA are required for its translational repression in round spermatids (4). As little as 62 nucleotides (nt) of the *Prm-1* 3′ UTR is able to confer a translational delay on a heterologous reporter human growth hormone mRNA (*hGH* mRNA) in round spermatids, whereas the 3′ UTR of *hGH*, or the 3′-most 23 nt of the *Prm-1* 3′ UTR, is not able to confer such a delay (3, 4). The mechanism by which the *Prm-1* 3′ UTR inhibits its translation is unknown. Presumably proteins or antisense RNAs interact with sequences in the 3′-most 62 nt of the 3′ UTR and prevent translation initiation from occurring at the 5′ end of the message. The involvement of antisense RNA in mediating translational control via the 3′ UTR has recently been demonstrated for the *lin-14* mRNA in *Caenorhabditis elegans* (30a, 51a). We have been unable to detect the presence of *Prm-1* 3′ UTR antisense RNAs in total testis RNA preparations by Northern (RNA) blotting (29a), suggesting that antisense RNAs are not involved in *Prm-1* translational repression.

Proteins that bind to the 3′ UTRs of *Prm-1* and *Prm-2* RNAs have been previously identified. Kwon and Hecht (27a, 27b) have described two regions in the 3′ UTRs of *Prm-1* and *Prm-2* mRNA, the Y and H boxes, that interact with proteins present in testis extracts and have shown that the Y box in *Prm-2* can be UV cross-linked to a protein of 18 kDa. We (12a) have characterized proteins of 48 and 50 kDa that are present in the cytoplasmic fraction of pachytene spermatocytes and round spermatids and have shown that they bind to a conserved sequence in the *Prm-1* and *Prm-2* 3′ UTRs. Surprisingly, neither the Y box nor the H box (27a), nor the binding site for the 48- and 50-kDa proteins (12a), is contained within the minimal 62-nt region of the *Prm-1* 3′ UTR RNA that is sufficient for translational repression in transgenic mice (3). These observations suggest that there may be redundancy in the *cis*-acting elements that control the translational repression of *Prm-1* mRNA or that the proteins and the binding sites thus far identified are involved in other aspects of *Prm-1* and *Prm-2* RNA behavior besides their translational repression. We have recently shown that mRNAs that contain the binding site for the 48- and 50-kDa proteins and that lack all but the 3′-most 23 nt of the *Prm-1* 3′ UTR are also under translational repression in transgenic mice (unpublished data). Thus, two different re-

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1    CCGGGCTTGGCCCCGGCCCTAGCTCCTCGGCGGTGTACTGGGGTGCGTGGAGGCTGTAGTCACGGTGGCGCCCGCGGGGACGGAGGAGGGA

92   ATG AGT GAA GAG GAT CAG GGC TCC GGC ACT ACT ACA GGC TGC GGG CTG CCC AGC ATA GAG CAA ATG CTG
     M   S   E   E   D   Q   G   S   G   T   T   T   G   C   G   L   P   S   I   E   Q   M   L    23

161  GCC GCC AAC CCG GGC AAG ACC CCG ATC AGC CTT CTT CAG GAG TAT GGG ACC AGA ATA GGA AAG ACG CCC
     A   A   N   P   G   K   T   P   I   S   L   L   Q   E   Y   G   T   R   I   G   K   T   P    46

230  GTG TAC GAC CTT CTC AAA GCC GAG GGC CAA GCC CAT CAA CCT AAT TTC ACC TTT CGG GTC ACC GTT GGC
     V   Y   D   L   L   K   A   E   G   Q   A   H   Q   P   N   F   T   F   R   V   T   V   G    69

299  GAC ACC AGC TGC ACT GGT CAG GGC CCC AGC AAG AAG GCA GCC AAG CAC AAG GCA GCT GAG GTG GCC CTC
     D   T   S   C   T   G   Q   G   P   S   K   K   A   A   K   H   K   A   A   E   V   A   L    92

368  AAA CAC CTC AAA GGG GGG AGC ATG CTG GAA CCA GCC CTG GAG GAC AGC AGT TCT CTT TCT CTC CTA GAC
     K   H   L   K   G   G   S   M   L   E   P   A   L   E   D   S   S   S   L   S   L   L   D   115

437  TCT TCA CCG CCT GAG GAC ACT CCT GTC GTT GCT GCA GAA GCT GCT GCC CCT GTT CCA TCT GCT GTA CTA
     S   S   P   P   E   D   T   P   V   V   A   A   E   A   A   A   P   V   P   S   A   V   L   138

506  ACC AGG AGC CCT CCC ATG GAG ATG CAG CCC CCT GTC TCT CCT CAG CAG TCT GAG TGC AAC CCC GTC GGT
     T   R   S   P   P   M   E   M   Q   P   P   V   S   P   Q   Q   S   E   C   N   P   V   G   161

575  GCT CTG CAG GAG CTG GTG GTG CAA AAA GGC TGG CGT TTG CCA GAG TAC ATG GTG ACC CAA GAG TCT GGG
     A   L   Q   E   L   V   V   Q   K   G   W   R   L   P   E   Y   M   V   T   Q   E   S   G   184

644  CCT GCT CAC CGC AAA GAG TTC ACC ATG ACT TGC CGG GTG GAG CGT TTC ATT GAG ATT GGC AGT GGC ACT
     P   A   H   R   K   E   F   T   M   T   C   R   V   E   R   F   I   E   I   G   S   G   T   207

713  TCC AAA AAG CTG GCA AAG CGT AAC GCA GCA GCT AAG ATG CTC CTT CGA GTG CAC ACT GTA CCT CTG GAT
     S   K   K   L   A   K   R   N   A   A   A   K   M   L   L   R   V   H   T   V   P   L   D   230

782  GCC CGG GAT GGC AAT GAG GCA GAG CCT GAT GAC GAT CAT TTT TCC ATT GGC GTG AGC TCC CGC CTG GAT
     A   R   D   G   N   E   A   E   P   D   D   D   H   F   S   I   G   V   S   S   R   L   D   253

851  GGA CTG AGG AAT CGT GGG CCA GGC TGC ACC TGG GAT TCC TTG CGG AAT TCT GTG GGA GAA AAG ATC CTA
     G   L   R   N   R   G   P   G   C   T   W   D   S   L   R   N   S   V   G   E   K   I   L   276

920  TCC CTT CGC AGT TGC TCC GTG GGC TCT CTA GGG GCT CTG GGC TCT GCC TGC TGC AGT GTC CTC AGT GAG
     S   L   R   S   C   S   V   G   S   L   G   A   L   G   S   A   C   C   S   V   L   S   E   299

989  CTC TCT GAG GAG CAG GCT TTC CAT GTC AGC TAT CTG GAT ATT GAG GAA CTG AGC CTG AGT GGG CTC TGC
     L   S   E   E   Q   A   F   H   V   S   Y   L   D   I   E   E   L   S   L   S   G   L   C   322

1058 CAG TGC CTA GTG GAA CTG TCC ACC CAG CCA GCC ACT GTG TGT TAT GGT TCT GCA ACC ACC AGG GAG GCA
     Q   C   L   V   E   L   S   T   Q   P   A   T   V   C   Y   G   S   A   T   T   R   E   A   345

1127 GCC CGA GGT GAT GCT GCT CAC CGC GCC CTA CAG TAC CTC AGG ATC ATG GCG GGT AGC AAG TAG CATCCCA
     A   R   G   D   A   A   H   R   A   L   Q   Y   L   R   I   M   A   G   S   K   .           365

1197 CTGCAGTGATGGATATGCATCTTTTACTTCTTGCTCCTTCTGCCCCTGGGTCCATGTATCCACCTAGCTCTGGTACCCTCCAGAGGTGCCA

1288 TCTCTACCTCTGACACAGCCTGTCTGCCTTGAGACTGAGGAAGGCACAGGCAAGCAAGGAGCCATGAACCACAGGGCCCCAGCCAGCACAG

1379 GATTTGTCCTCATTCCGTGGGTGATGGATGAATCTATTGGAGTCCTGAATAAATGCTGCTCTTTGGCTTCCAAAACCCTGCTCTCTCTGGC

1470 CTTGGGTCATGAGGGTATAGGAATAAACATGGTGAGCTTGTTCTCCTTTTG
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FIG. 1. (A) Nucleotide and deduced amino acid sequences of the *Prbp* cDNA. Nucleotides are numbered on the left, and amino acids are numbered on the right. An upstream in-frame stop codon (TAG) is indicated by a double underline. Two copies of the polyadenylation signal, AATAAA, are underlined. (B) Alignment of the amino acid sequences of Prbp and human TRBP (15). Numbers refer to amino acid position. Boxes indicate the positions of the conserved dsRNA-binding domains (14). A 24-amino-acid peptide that is sufficient for RNA binding is indicated by the underline (14).

gions of the *Prm-1* 3′ UTR are sufficient for translational repression in vivo, demonstrating that there is redundancy in the *cis*-acting elements that mediate its translational repression.

In an attempt to clone cDNAs that encode *Prm-1* 3′ UTR RNA-binding proteins, we screened germ cell cDNA libraries with the 3′ UTR of *Prm-1* RNA. We have recently described two of the genes isolated from this screen. One of these genes, *Spnr*, encodes an RNA-binding protein that is localized to a spermatid-specific microtubule array called the manchette,

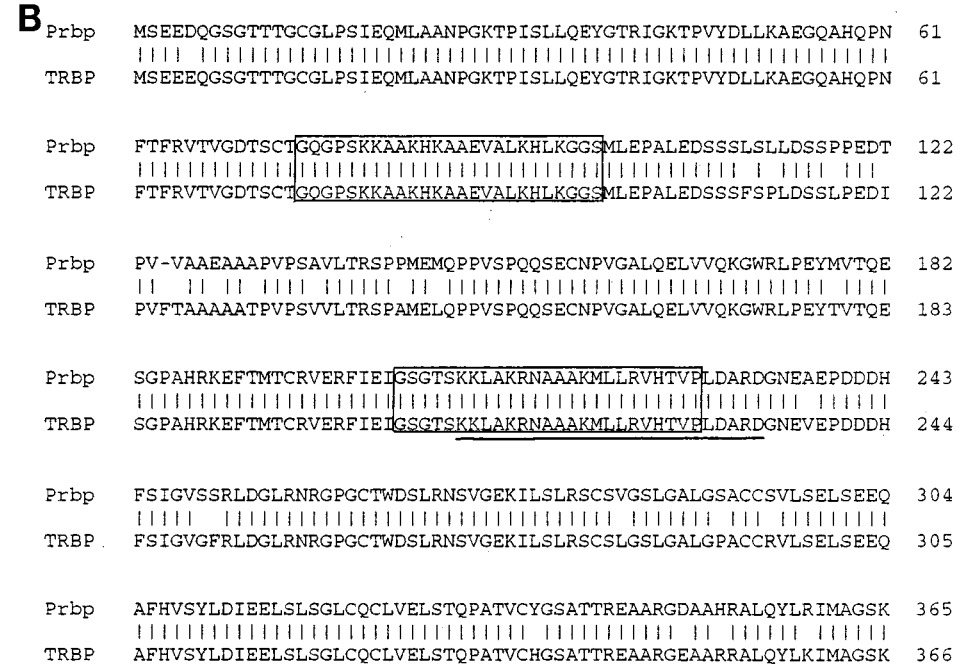

FIG. 1—*Continued.*

suggesting that it is involved in the transport and translational activation of spermatid mRNAs (46). The product of the second gene, *Tenr*, also encodes an RNA-binding protein, and it is localized in a lattice-like network within the spermatid nucleus (45). The Tenr protein contains a region with similarity to known double-stranded RNA (dsRNA) adenine deaminases, suggesting that it may function in the editing of spermatid-specific RNAs. We report here the cloning and characterization of a third gene from this screen, whose product has the properties expected of a protein that represses the translation of numerous mRNAs, including the *Prm-1* message, in the testis.

MATERIALS AND METHODS

Mice. B6SJLF_1/J and B6CBAF_1/J mice, used for the preparation of tissue protein extracts, RNA, or testis sections, were obtained from the Jackson Laboratory (Bar Harbor, Maine).

cDNA cloning. Mouse mixed germ cell, pachytene spermatocyte, and round spermatid cDNA expression libraries were screened as described previously for DNA-binding proteins (47, 51), except that filters were probed with RNAs labeled with either digoxigenin-UTP (Boehringer Mannheim, Indianapolis, Ind.) (100 ng/ml) (primary screen) or [α-^{32}P]UTP (1×10^6 to 3×10^6 cpm/ml) (secondary and tertiary screens) in 0.25% nonfat milk in binding buffer. The probes, either the 156-nt full-length *Prm-1* 3′ UTR RNA or the 145-nt full-length *hGH* 3′ UTR RNA, were prepared by in vitro transcription with SP6 or T7 RNA polymerase. After extensive washing in binding buffer, positive phage plaques were detected by autoradiography or by immunological reaction with an antidigoxigenin antibody conjugated to alkaline phosphatase according to the protocol supplied by the manufacturer (Genius nucleic acid detection kit [Boehringer Mannheim]).

Seven overlapping restriction fragments of the *Prbp* cDNA were gel isolated and subcloned into the pBluescript KS(−) vector (Stratagene, La Jolla, Calif.) for sequencing. Double-stranded DNA templates were sequenced by the dideoxy chain termination method (43) with a Sequenase kit (U.S. Biochemical, Cleveland, Ohio). Databases were searched by using the Intelligenetics suite (Intelligenetics, Mountain View, Calif.) and Blast (National Center for Biotechnology Information, Bethesda, Md.) programs.

RNA analysis. Total RNA was isolated from dissected tissues as described previously (5) with some modifications. Briefly, tissues were homogenized in solution D (4 M guanidinium thiocyanate, 25 mM sodium citrate [pH 7.0], 0.5% sarcosyl, 0.1 M 2-mercaptoethanol) and extracted with the addition of 0.1 volume of 2 M sodium acetate (pH 4.0)–1 volume of H_2O-saturated phenol–0.2 volume of chloroform-isoamyl alcohol (49:1). After being cooled on ice for 15 min, samples were microcentrifuged for 20 min at 4°C. RNA in the aqueous phase was precipitated with 1 volume of isopropanol. Following centrifugation, the RNA pellet was dissolved in 10 mM Tris-HCl (pH 7.5)–5 mM EDTA–1% sodium dodecyl sulfate (SDS), extracted with a 4:1 mixture of chloroform and 1-butanol, and precipitated with 2 volumes of absolute ethanol. RNA samples were separated on a 1.5% agarose gel containing 2.2 M formaldehyde, transferred to a nitrocellulose membrane, fixed by being baked at 80°C, and hybridized at 45°C in 50% formamide–5× SSC (1× SSC is 0.15 M NaCl plus 0.015 M sodium citrate)–50 mM $NaPO_4$ (pH 6.5)–250 μg of single-stranded DNA per ml–1× Denhardt's solution–0.5% SDS–6.25% dextran sulfate with α-^{32}P-labeled probes prepared from cDNA by random hexamer labeling. The blot was washed at a final stringency of 0.1× SSC and 0.5% SDS at 50°C.

Preparation of tissue extracts. Total cell extracts were prepared as described previously (42) with some modifications. Briefly, tissues were dissected and homogenized in 5 volumes of triple-detergent lysis buffer (50 mM Tris-HCl [pH 8.0], 150 mM NaCl, 0.1% SDS, 1% Nonidet P-40, 0.5% sodium deoxycholate, and protease inhibitors [1 μg of aprotinin per ml, 1 mM benzamidine, 0.5 μg of leupeptin per ml, 1 μg of pepstatin A per ml, 0.5 mM phenylmethylsulfonyl fluoride]) by 20 to 30 strokes of a Dounce homogenizer. After incubation for 30 min on ice, the homogenates were centrifuged at 12,000 × *g* for 2 min at 4°C. Each supernatant was aliquoted, quickly frozen in liquid N_2, and stored at −70°C.

For nuclear and cytoplasmic extracts, tissues were dissected and processed as described previously (10) except that for the cytoplasmic extracts, we omitted the high-speed centrifugation step prior to dialysis. Protein concentrations were determined by the Bradford assay (Bio-Rad, Hercules, Calif.), and aliquots were quickly frozen in liquid N_2 and stored at −70°C.

Fusion proteins. A 1.4-kb *Sma*I-*Eco*RI fragment from the *Prbp* cDNA was gel isolated and subcloned into the pMAL-c2 expression vector (New England BioLabs, Beverly, Mass.). Synthesis of a maltose-binding protein (MBP)-Prbp fusion protein in *Escherichia coli* DH5α was induced with IPTG (isopropyl-β-D-thiogalactopyranoside) at room temperature. The fusion protein was affinity purified with an amylose resin column, as described by the supplier (New England BioLabs). Fractions containing the fusion protein were aliquoted and stored at −70°C. An MBP–β-galactosidase (MBP-βgal) fusion protein was prepared from the pMAL-c2 vector with no insert in a similar manner.

RNA band shift analysis. Labeled RNAs were synthesized in vitro with [α-^{32}P]UTP (3,000 Ci/mmol; NEN-Dupont, Boston, Mass.) by in vitro transcription by a standard protocol (Promega, Madison, Wis.). Full-length products were isolated from nondenaturing polyacrylamide gels as previously described (12a).

RNA band shift assays were performed as described previously (31). RNA (2×10^5 to 5×10^5 cpm) was heat denatured at 70°C for 5 min in RNA-binding buffer (10 mM HEPES [*N*-2-hydroxyethylpiperazine-*N*′-2-ethanesulfonic acid] [pH 7.6], 120 mM KCl, 3 mM $MgCl_2$, 2 mM dithiothreitol, 5% glycerol) and cooled slowly to room temperature. After incubation with protein extract (300 to

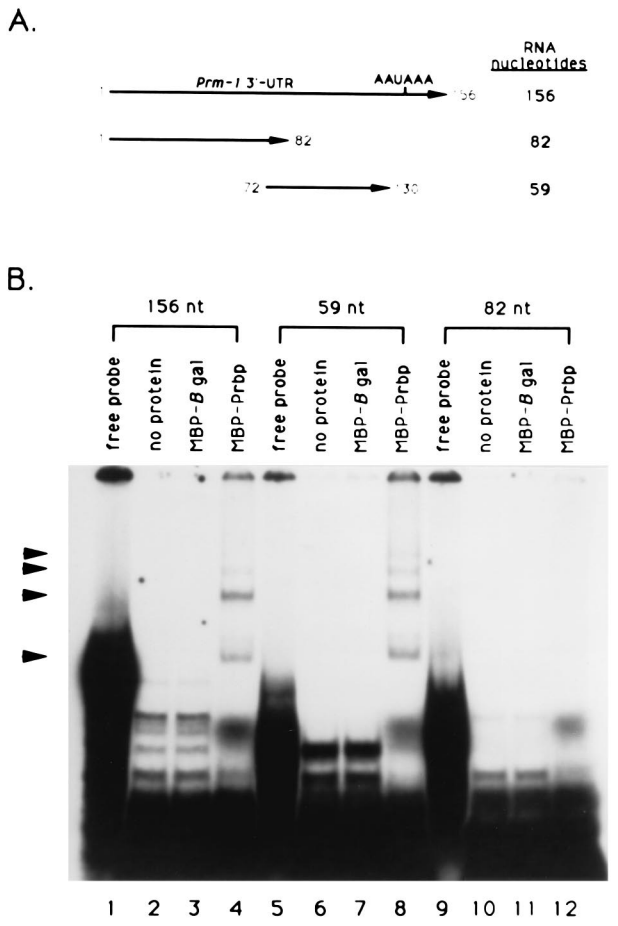

FIG. 2. (A) RNA probes used in band shift analysis. ^{32}P-labeled RNA probes corresponding to the full-length 156-nt *Prm-1* 3′ UTR, an 82-nt 5′ portion, and a 59-nt 3′ portion were prepared as described in Materials and Methods. Each of the RNAs contained additional sequence contributed by the polylinker sequence contained in the pGEM2 plasmid (Promega), as indicated in Fig. 3. (B) Band shift analysis of *Prm-1* 3′ UTR RNAs with an MBP-Prbp fusion protein. Lanes 1, 5, and 9 contain each of the untreated RNA probes. The remaining lanes contain RNA probes that were incubated in the absence of protein, or with approximately 300 ng of affinity-purified protein (MBP-βgal or MBP-Prbp) as indicated above each lane, prior to treatment with RNase T_1 and heparin as described in Materials and Methods. Arrowheads show the positions of MBP-Prbp–RNA complexes.

500 ng of fusion proteins or 30 to 50 μg of testis extract) for 25 min, the reaction mixture was subject to RNase T_1 digestion (0.5 U; Calbiochem, La Jolla, Calif.) for 10 min. Nonspecific protein binding to RNA probes was minimized by incubating the reaction mixture with heparin at 10 mg/ml. RNA-protein complexes were resolved on a 4% polyacrylamide gel in 0.5× Tris-borate-EDTA at 4°C and detected by autoradiography.

In the competition band shift assays, the protein extracts were preincubated with unlabeled competitor RNAs for 15 min prior to addition of radiolabeled *Prm-1* 59-nt RNA. The concentration of poly(I) · poly(C) (Boehringer Mannheim, Indianapolis, Ind.) RNA was determined by the optical density at 260 nm. Concentrations of cold competitor *Prm-1* 59-nt RNA were determined by including trace amounts of [^{3}H]UTP in the in vitro run-on transcription reaction and measuring trichloroacetic acid-precipitable incorporated counts.

Antibodies. Rabbit antiserum was raised by immunization with an MBP-Prbp fusion protein, using the services of the R & R Rabbitry (Stanwood, Wash.). An anti-MBP-Prbp antibody was affinity purified by using antigen immobilized on a nitrocellulose filter as described previously (42).

Western blot (immunoblot) analysis. Total protein (approximately 50 to 150 μg) was resolved on SDS–10% polyacrylamide gels that were then electroblotted onto nitrocellulose filters. Blots were blocked with 5% nonfat dry milk in phosphate-buffered saline (BPBS) and then incubated overnight at 4°C in the affinity-purified anti-MBP-Prbp antibodies diluted in BPBS. After being washed once in BPBS supplemented with 0.05% Tween 20 and twice in BPBS for 20 min, the blots were incubated with goat anti-rabbit immunoglobulin G antibody conjugated to horseradish peroxidase (Bio-Rad). After washing as above, the presence of enzyme was revealed by chemiluminescence with luminol and 4-iodophenol as a luminogen as described previously (44).

Cell-free translation. Capped *Prbp* mRNA synthesized in vitro from cDNA was translated in a rabbit reticulocyte lysate with [^{35}S]methionine as suggested by the supplier (Promega). Five microliters of the reaction mixture was loaded per lane.

Immunocytochemistry. Testes and epididymides were dissected from sexually mature males, fixed in Bouin's fixative (0.2% picric acid and 2% paraformaldehyde in 1× PBS) overnight, and embedded in paraffin by standard procedures. Tissue sections were treated with primary antibody as previously described (4). Biotinylated goat anti-rabbit immunoglobulin G and streptavidin conjugated to horseradish peroxidase were used as recommended by the manufacturer (Zymed Laboratories, Inc., South San Francisco, Calif.). Peroxidase activity was visualized with the chromogen aminoethyl carbazole. Tissue sections were counterstained with hematoxylin where indicated. The developmental stage of seminiferous tubules was determined by treating adjacent sections with periodic acid-Schiff stain and Harris' hematoxylin as previously described (38, 41).

In vitro translation inhibition. Synthesized RNA products contained the *hGH* mRNA fused to either the full-length *hGH* 3′ UTR or the full-length *Prm-1* 3′ UTR. In vitro transcription reactions were performed in 50-μl reaction volumes with 5 μg of linearized plasmid template, 0.5 mM ATP, CTP, and UTP, 50 μM GTP, 0.5 mM $m^7G(5')ppp(5')G$, and 10 μCi of [^{3}H]UTP (36 Ci/mmol; NEN-Dupont). Samples were phenol and chloroform extracted and then ethanol precipitated with 0.2 M NaCl and 2.5 volumes of pure ethanol. RNA pellets were collected by microcentrifugation, washed in 70% ethanol, and resuspended in 25 μl of H_2O. RNA templates were quantitated by trichloroacetic acid precipitation, and full-length integrity was assessed by gel electrophoresis. In a 10-μl reaction volume, various amounts of MBP-Prbp fusion protein were incubated with 0.25 μg of RNA template for 15 min at room temperature. Following this incubation, in vitro translation was performed for 2.0 h at 30°C in 25 μl of wheat germ reaction mixture (Promega) supplemented with 0.5 mCi of translation grade [^{35}S]methionine (NEN-Dupont) per ml. Control reactions were performed in the presence of 1.0 μg of MBP alone and an equivalent amount of protein storage buffer alone. Following addition of SDS-polyacrylamide gel electrophoresis (SDS-PAGE) loading buffer, samples were electrophoresed in SDS–15% polyacrylamide gels and autoradiographed.

Further controls were performed to evaluate translation product proteolysis and RNA template stability in the presence of the MBP-Prbp fusion protein. MBP-Prbp-induced proteolysis of the hGH translation product was examined by adding 1.0 μg of MBP-Prbp fusion protein to a standard translation reaction mixture (0 μg of MBP-Prbp fusion protein) following the 2.0 h of incubation at 30°C. This sample was incubated for an additional 20 min at 30°C prior to SDS-PAGE analysis. The stability of the RNA template in the presence of MBP-Prbp was examined as follows. In the same 10-μl reaction mixture described above, 1.0 μg of MBP-Prbp fusion protein was incubated with 0.25 μg of RNA template for 15 min at room temperature; this was followed by phenol and chloroform extraction and RNA recovery via ethanol precipitation. The RNA pellets were resuspended in 10 μl of H_2O, and in vitro translation was performed.

RESULTS

Expression screen. To clone genes that encode potential *Prm-1* translational control factors, we screened lambda gt11 cDNA expression libraries prepared with RNA from pachytene spermatocytes, round spermatids, and mixed germ cells with a 156-nt *Prm-1* 3′ UTR RNA probe labeled with digoxigenin as described in Materials and Methods. To enrich for clones that encode *Prm-1* RNA-binding proteins and to minimize the number of clones that encode general RNA-binding proteins, only those plaques that upon rescreening did not hybridize with a digoxigenin-labeled 145-nt *hGH* 3′ UTR RNA probe were retained for subsequent analysis. Of a total of $\sim 7.5 \times 10^5$ plaques screened, we identified 19 plaques that bound to the *Prm-1* 3′ UTR RNA and did not bind, or bound significantly less well, to the *hGH* 3′ UTR RNA. Characterization of each of the cDNA clones by DNA cross-hybridization revealed that they represented cDNAs for five different genes. The cDNA from one of these genes, which we refer to as *Prbp* (for *Prm-1* RNA-binding protein), was isolated once from the pachytene spermatocyte library and once from the round spermatid library.

Prbp is a member of a known family of RNA-binding proteins. The largest of the *Prbp* cDNAs was sequenced and shown to be 1,521 bp long and to contain an open reading frame that would encode a protein of 365 amino acids (Fig.

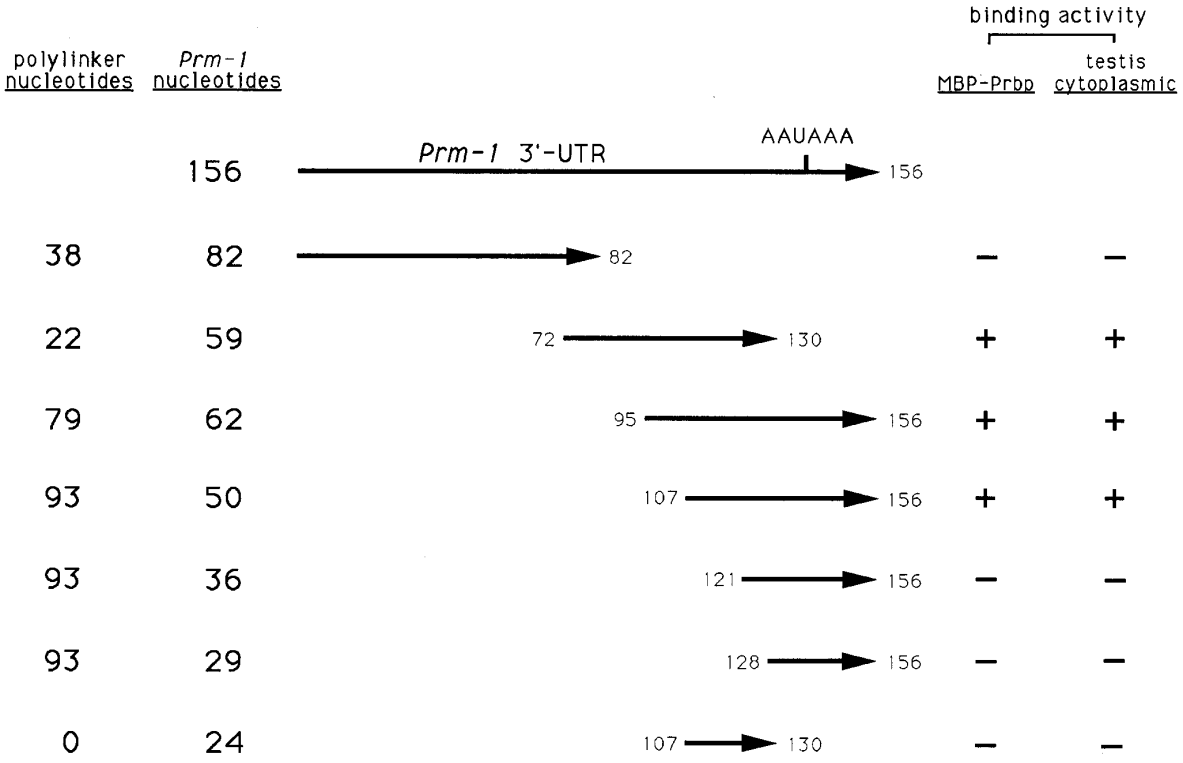

FIG. 3. Summary of RNA band shift analysis performed with deletion variants of the *Prm-1* 3′ UTR. Radiolabeled RNA probes containing the indicated regions of the *Prm-1* 3′ UTR and polylinker sequence contributed by the pGEM2Z plasmid (Promega) were prepared as described in Materials and Methods and used in RNA band shift assays. Band shift assays were performed with either 300 ng of affinity-purified MBP-Prbp fusion protein or approximately 30μg of testis cytoplasmic extract. Each RNA probe either showed maximum binding (+), as illustrated in Fig. 2 for the 156- and 59-nt RNAs, or completely failed to bind (−), as illustrated for the 82-nt RNA.

1A). The cDNA contains one in-frame stop codon 72 nt upstream of the putative translation initiation codon, two copies of the polyadenylation signal AATAAA, and a poly(A) tract, suggesting that this cDNA is likely to encode the full-length Prbp protein.

A search of the GenBank database revealed that the Prbp protein has 93% amino acid identity with the human TAR RNA-binding protein (TRBP) (Fig. 1B), strongly suggesting that Prbp is the mouse homolog of TRBP. TRBP is a cellular protein that was cloned first on the basis of its ability to bind to the human immunodeficiency virus type 1 (HIV-1) TAR RNA in vitro (15) and a second time on the basis of its ability to bind to the HIV-1 Rev-responsive element (39). Prbp and TRBP contain two copies of a lysine- and arginine-rich motif of 24 amino acids (boxed in Fig. 1B) that is also found in the human interferon-induced and dsRNA-activated protein kinase, PKR (16), and in numerous other demonstrated or putative dsRNA-binding proteins (14, 49).

RNA-binding properties of Prbp. To study the in vitro RNA-binding properties of Prbp, a fusion protein of MBP and Prbp (containing amino acids 28 through 365) was constructed, expressed in *E. coli*, and affinity purified as described in Materials and Methods. In an RNA band shift assay the MBP-Prbp fusion protein bound to the full-length 156-nt *Prm-1* 3′ UTR RNA, producing multiple complexes (Fig. 2B, lane 4) that all migrated more slowly than the free probe (Fig. 2B, lane 1). A negative control, an affinity-purified fusion protein containing MBP and β-galactosidase (MBP-βgal), failed to bind to the full-length *Prm-1* 3′ UTR RNA (Fig. 2B, lane 3). To determine if Prbp binds to a specific region of the *Prm-1* 3′ UTR RNA, band shift assays were performed with an RNA containing 59 nt of the 3′-most region, and an RNA containing 82 nt of the 5′-most region, of the *Prm-1* 3′ UTR (Fig. 2A). While the 59-nt RNA bound as well as the full-length 156-nt *Prm-1* 3′ UTR RNA (Fig. 2B, lane 8), the 82-nt RNA completely failed to bind to the MBP-Prbp fusion protein (Fig. 2B, lane 12).

To further map the Prbp-binding site, a series of deletion variants containing different portions of the 3′-most region of the 3′ UTR were constructed and tested in the band shift assay. As summarized in Fig. 3, the deletion analysis suggested that sequences between nt 107 and 130 (nt 1 being the first nucleotide in the 3′ UTR) are important for protein binding. To determine if the 24-nt region defined by the deletions is sufficient for MBP-Prbp binding, oligonucleotide-directed in vitro transcription was used to synthesize an RNA containing only the *Prm-1*-specific 24-nt region and no ribonucleotides contributed by the polylinker sequences in the plasmid vector. RNA band shift analysis revealed that the 24-nt target RNA failed to bind to MBP-Prbp (Fig. 3), suggesting either that the 24-nt RNA target is too small an RNA for protein binding or that additional sequences on either side of the 24-nt region are required for binding (see Discussion).

As mentioned above, the Prbp protein contains two copies of an amino acid motif found in numerous dsRNA-binding proteins, including the human TRBP protein. To determine if the MBP-Prbp fusion protein binds to dsRNA, competition assays were performed with poly(I) · poly(C) RNA. Poly(I) · poly(C) RNA competed for binding of radiolabeled *Prm-1* 59-nt RNA (Fig. 4B), as did unlabeled 59-nt *Prm-1* RNA (Fig. 4A), suggesting that, in vitro, Prbp binds dsRNA.

Prbp-binding activity is present in testis cytoplasmic extracts. Protein extracts prepared from mouse testis contain two

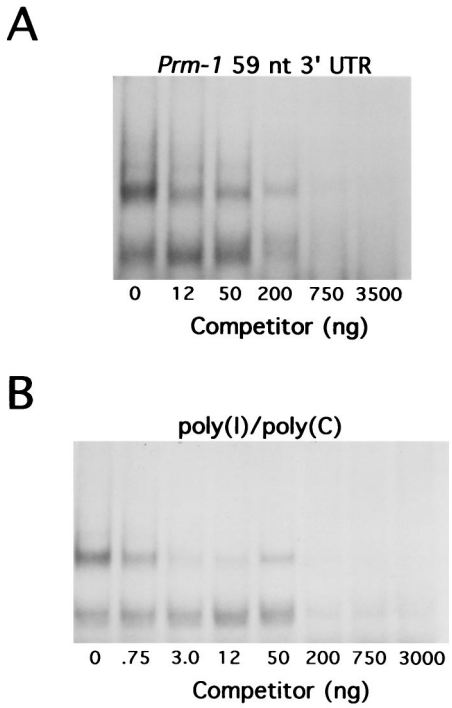

FIG. 4. Competition of MBP-Prbp–*Prm-1* 59-nt RNA band shift complexes. Band shift assays were performed with radiolabeled *Prm-1* 59-nt RNA as described in Materials and Methods except that prior to addition of the radiolabeled probe, the testis cytoplasmic extracts were preincubated for 15 min with unlabeled competitor RNAs. The competitors used were cold *Prm-1* 59-nt RNA (A) and poly(I) · poly(C) RNA (B).

separate activities that bind to two distinct regions of the *Prm-1* 3′ UTR RNA in a band shift assay (2). One of these activities binds in a sequence-specific manner to a 22-nt site in the 5′ half of the *Prm-1* 3′ UTR (Fig. 5, lane 2, asterisk) (12a), and the other binds to a 59-nt RNA derived from the 3′ half of the 3′ UTR (Fig. 5, lane 5, closed arrow). To map the binding site for the 3′-most binding activity, RNA band shift assays were performed with the same set of *Prm-1* 3′ UTR deletion variants previously used to map the MBP-Prbp-binding site. As shown in Fig. 3, the activity present in testis extracts binds the same set of deletion variants as the MBP-Prbp fusion protein, suggesting that the activity present in testis extracts is Prbp.

To determine if the 3′ binding activity detected in testis cytoplasmic extracts is Prbp, RNA band shift assays were performed in the presence of a rabbit polyclonal anti-MBP-Prbp antibody prepared as described in Materials and Methods. Addition of the MBP-Prbp antibody to the sample containing the 59-nt probe and the testis cytoplasmic extract (Fig. 5, lane 6) or the MBP-Prbp fusion protein (Fig. 5, lane 8) eliminated the bands previously detected with the testis extract (Fig. 5, lane 5) and MBP-Prbp alone (Fig. 5, lane 7, arrowheads) and instead resulted in the retention of a significant percentage of the RNA-protein complex in the well (open arrow). Addition of the anti-MBP antibody or preimmune serum to testis cytoplasmic extracts did not affect detection or migration of the complex (data not shown). To confirm that the complex detected with the 5′-most 82-nt RNA probe does not contain Prbp and that the anti-MBP-Prbp antibody is binding specifically to the Prbp complex formed with the 59-nt probe, the anti-MBP-Prbp antibody was added to the sample containing the 82-nt probe and the testis cytoplasmic extract. No difference in the mobility of the 5′-most complex could be detected in the presence of the anti-MBP-Prbp antibody (Fig. 5, compare lanes 2 and 3). These data strongly support the conclusion that the Prbp protein is present in testis cytoplasmic extracts and that sequences in the 3′-most region of the *Prm-1* 3′ UTR RNA are required for binding. In addition, optimal binding of both the testis activity and the MBP-Prbp fusion protein was achieved at approximately 100 mM KCl (data not shown), further supporting the conclusion that the 3′-most binding activity is Prbp.

Prbp protein exists in multiple forms in the testis and is expressed only in germ cells. Hybridization of a Western blot containing total and cytoplasmic testis protein extracts with the anti-MBP-Prbp antibody detected a cluster of four major bands around 40 kDa and two minor bands of approximately 35 and 33 kDa (Fig. 6, lanes 3 and 4). No hybridizing bands were detected in testis nuclear extracts (Fig. 6, lanes 5 and 6). To determine which of the proteins detected were translation products of *Prbp* mRNA, *Prbp* RNA synthesized from the cDNA was in vitro translated in the presence of [^{35}S]methionine with a rabbit reticulocyte lysate, and the products were compared to those identified in the Western blot. All four bands of approximately 40 kDa, as well as the 33-kDa product, were detected as products of the in vitro translation reaction (Fig. 6, lane 2). Only the minor band of 35 kDa was detected in testis extracts by Western blotting and not by in vitro trans-

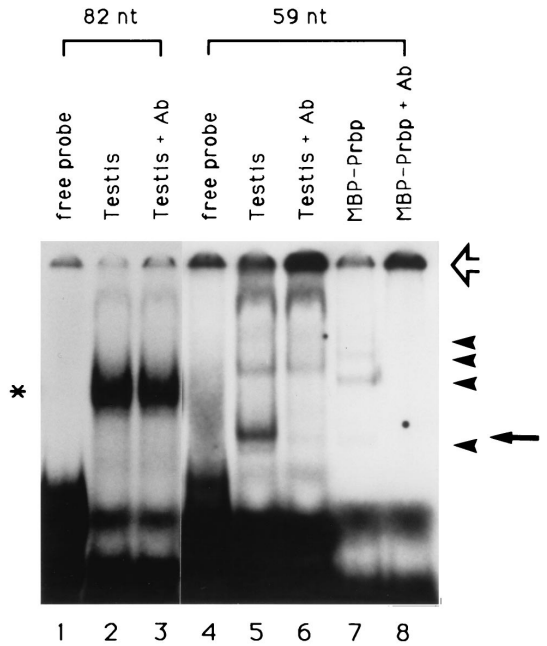

FIG. 5. Testis extracts contain Prbp RNA-binding activity. RNA band shift assays were performed with an 82-nt RNA derived from the 5′-most region of the *Prm-1* 3′ UTR (lanes 1 to 3) or a 59-nt RNA derived from near the 3′-end of the *Prm-1* 3′ UTR (lanes 4 to 8). The positions of both RNAs are shown schematically in Fig. 3. Band shift assays were performed with either testis cytoplasmic extracts or the affinity-purified MBP-Prbp fusion protein as indicated above each lane. Following heparin treatment, the samples that were eventually loaded in lanes 3, 6, and 8 were incubated for an additional 30 min with 1 μl of an affinity-purified anti-MBP-Prbp antibody (Ab). The asterisk indicates the position of a previously described *Prm-1* 3′ UTR-binding activity (12a). The closed arrow indicates the position of the binding activity mapped by using the RNAs shown in Fig. 2 and shown schematically in Fig. 3. The closed arrowheads indicate RNA-protein complexes detected with the MBP-Prbp fusion protein. The open arrow indicates the appearance of additional RNA-protein complexes retained in the wells in lanes 6 and 8 following treatment with the anti-MBP-Prbp antibody.

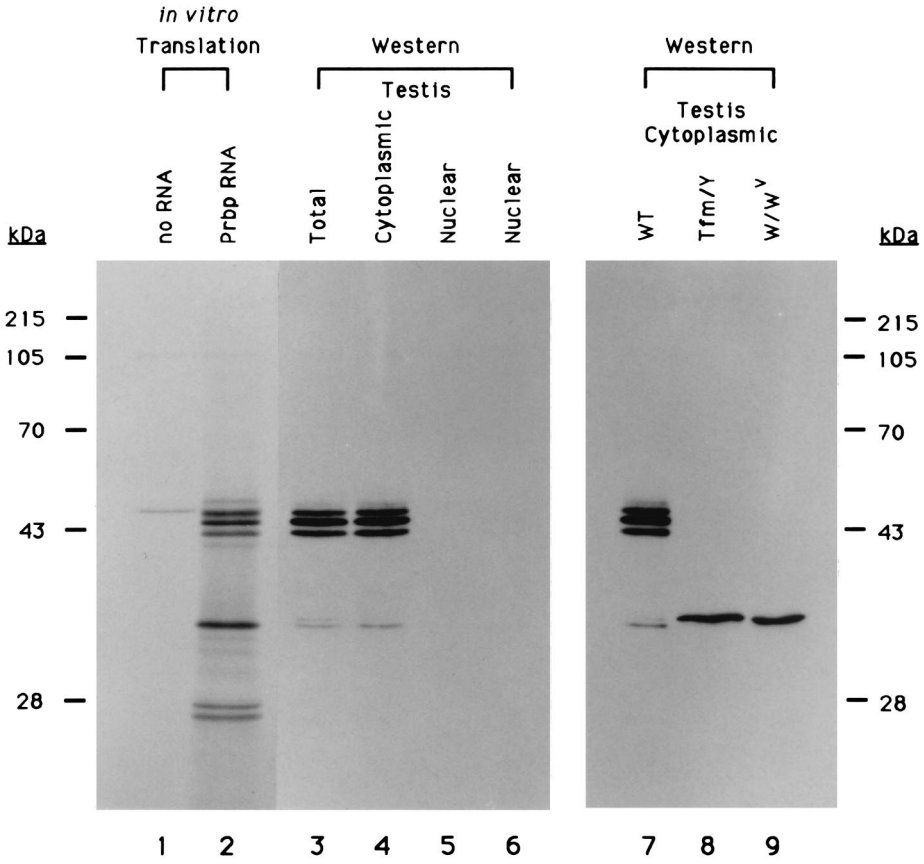

FIG. 6. Prbp protein exists in multiple forms in germ cells. Lane 1, [^{35}S]methionine-labeled in vitro translation products of a rabbit reticulocyte lysate (Promega) with no exogenous RNA added. Lane 2, approximately 50 ng of capped *Prbp* mRNA was synthesized in vitro with T7 polymerase and in vitro translated in the presence of [^{35}S]methionine. Lanes 3 to 9, Western blot analysis of Prbp protein. Approximately 100 μg of total and cytoplasmic testis extracts prepared from normal (lanes 3, 4, and 7) or mutant (lanes 8 and 9) mice and 10 μg (lane 5) and 50 μg (lane 6) of testis nuclear extracts were separated by SDS–10% PAGE, electroblotted onto nitrocellulose, and hybridized with affinity-purified anti-Prbp antibody as described in Materials and Methods. Prestained molecular mass standards (Gibco BRL, Gaithersburg, Md.) were loaded in an adjacent lane, and their electrophoretic positions are indicated on the right and left.

lation of *Prbp* mRNA. The detection of the same quartet of bands of 40 kDa by both Western blotting and in vitro translation of *Prbp* mRNA suggests that they are all translation products of *Prbp* mRNA. In addition, hybridization of a Northwestern (RNA-protein) blot containing testis protein extracts with a radiolabeled *Prm-1* 3′ UTR RNA probe revealed the same quartet of bands at 40 kDa (data not shown), demonstrating that they can all bind to *Prm-1* RNA in vitro. The 33-kDa protein also appears to be a product of *Prbp* mRNA, but it is likely to be a proteolytic fragment of one of the 40-kDa forms, as its relative abundance varies between testis cell extracts (data not shown).

To determine if Prbp is expressed within the somatic or the germ cell compartment of the testis, Western blot analysis was performed on protein extracts prepared from testes of mouse mutants that either lack germ cells entirely (*W/W^v* [36]) or contain diploid spermatogonial cells but lack meiotic and postmeiotic germ cells because of an arrest at the beginning of meiosis I (*Tfm/Y* [32]). None of the four major Prbp proteins of around 40 kDa, or the minor 33-kDa protein, were detected in extracts prepared from either of the mutants (Fig. 6, lanes 8 and 9), strongly suggesting that expression of Prbp is restricted to the meiotic and/or postmeiotic germ cells. The 35-kDa protein was detected in *W/W^v* and *Tfm/Y* testes, suggesting that it is not restricted to germ cells and providing further evidence that it is unlikely to be a product of the *Prbp* gene. In addition, unlike the 40-kDa protein forms, the 35-kDa protein does not bind the full-length *Prm-1* 3′ UTR RNA in Northwestern blot analysis (data not shown).

Developmental expression of Prbp during spermatogenesis. Spermatogenesis occurs within seminiferous tubules and has been described as consisting of 12 stages based on the particular cell associations present in a given tubule, on spermatid nuclear shape, and on the extent of acrosome morphogenesis (38, 41). Within these 12 stages of the cycle one can describe 16 steps of spermatid differentiation (38). To determine the stages and cell type in which Prbp is expressed, immunocytochemistry was performed on testis sections by using an anti-MBP-Prbp antibody, and serial sections were stained with hematoxylin (a nuclear stain) and periodic acid-Schiff reagent (an acrosomal stain) (38). Representative staining of Prbp in a stage II to IV seminiferous tubule is shown in Fig. 7. The Prbp protein was first detected in stage VII pachytene spermatocytes at a very low level. Prbp was also detected in all later stages of primary and secondary spermatocytes and in haploid spermatids through step 10. Immunostaining appeared to be greatest in round spermatids at step 2 to 3. In all cell types that expressed Prbp, the protein appeared to be localized to the cytoplasm, consistent with our earlier Western blot analysis (Fig. 6). In summary, the Prbp protein is detected in the cytoplasm of round spermatids through step 10, in which *Prm-1* mRNA is repressed, and is not detected in elongated spermatids (steps

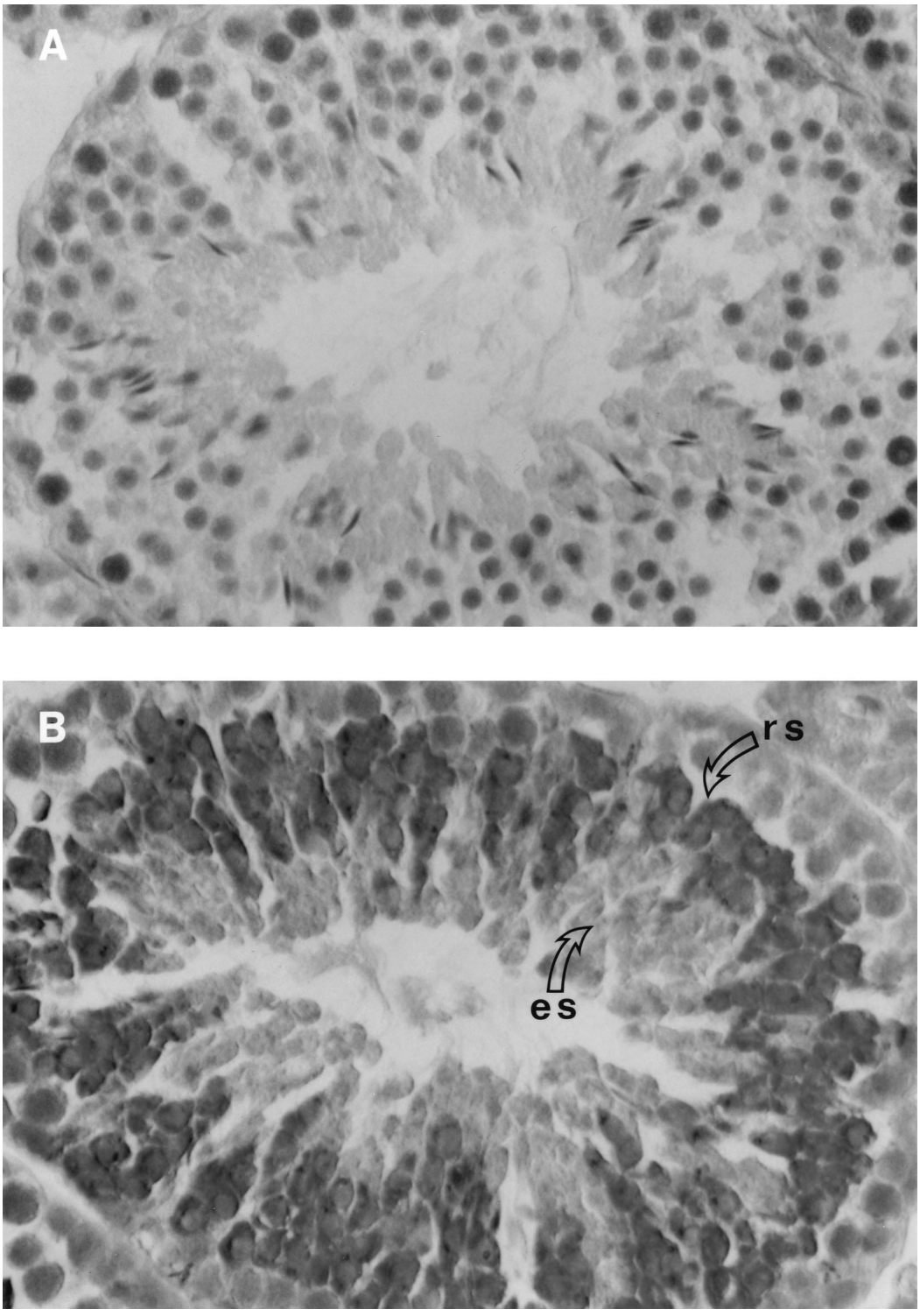

FIG. 7. Immunolocalization of Prbp to the cytoplasmic compartment of round spermatids. Immunocytochemistry was performed on paraffin-embedded testis sections by using an affinity-purified rabbit anti-MBP-Prbp antibody as described in Materials and Methods. Sections were counterstained with hematoxylin. (A) Control testis section treated with the secondary antibody only. (B) Testis section treated with primary and secondary antibodies. Immunostaining is detected in the cytoplasm of round spermatids (rs) and is absent in elongated spermatids (es). Magnification, ×540.

11 to 16), the cells in which *Prm-1* mRNA is translated (1, 24).

Prbp protein inhibits translation in vitro. The presence of Prbp in the cytoplasmic compartment of round spermatids, coupled with the ability of testis extracts that contain Prbp to bind to exogenously added *Prm-1* 3′ UTR RNA, suggests that Prbp interacts with *Prm-1* mRNA in vivo to inhibit its translation. To test the effect of Prbp on mRNA translation, the

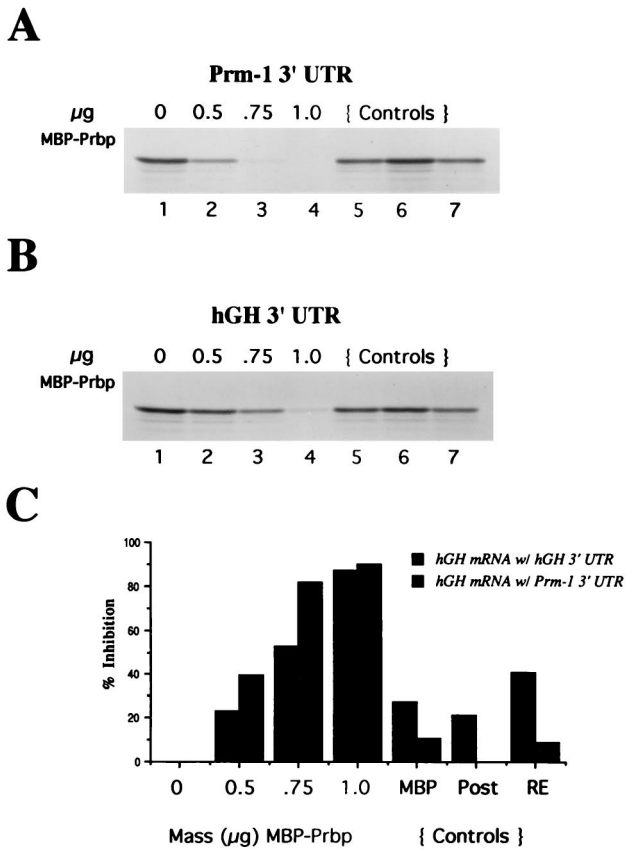

FIG. 8. Translational inhibition in wheat germ extracts supplemented with MBP-Prbp. Lanes 1 to 4, translation of in vitro-synthesized RNA containing the *hGH* mRNA, fused to either the *Prm-1* 3′ UTR (A) or the *hGH* 3′ UTR (B), with increasing amounts of the MBP-Prbp fusion protein. Lanes 5 and bars MBP, synthesis of the ^{35}S-labeled hGH product when 1.0 μg of MBP alone was used in the assay. The hGH protein was not degraded when incubated with 1.0 μg of MBP-Prbp following the synthesis reaction (lanes 6 and bar Post). Lanes 7 and bar RE, synthesis of hGH from mRNA preincubated with 1.0 μg of MBP-Prbp and then phenol-$CHCl_3$ extracted prior to the translation reaction as described in Materials and Methods. After 2.0 h at 30°C, the newly synthesized [^{35}S]methionine-labeled hGH protein was analyzed by SDS-PAGE and autoradiography. (C) Densitometry was performed by using Imagequant, with autoradiographic density plotted as percent inhibition {[(value of no-protein control/value of test sample) − 1] × 100%}.

MBP-Prbp fusion protein was preincubated with either a chimeric message containing the *hGH* coding sequence fused to the *Prm-1* 3′ UTR or an *hGH* mRNA containing its normal 3′ UTR and was translated in a wheat germ cell-free translation lysate. Dose-response experiments revealed that both messages, the *Prm-1* 3′ UTR-containing message and the *hGH* 3′ UTR-containing message, were sensitive to inhibition in the presence of MBP-Prbp (Fig. 8A and B, lanes 1 to 4, and Fig. 8C). This inhibitory effect was also observed in a reticulocyte lysate (data not shown). Translation was not significantly inhibited in the presence of MBP alone (Fig. 8A and B, lanes 5; Fig. 8C, MBP) or in protein storage buffer alone (data not shown). These results suggest that Prbp is capable of inhibiting translation in vitro but that it is not specific to mRNAs containing the *Prm-1* 3′ UTR. The stability of the newly synthesized hGH protein product was not markedly affected by incubation with the MBP-Prbp fusion protein following the translation reaction (Fig. 8A and B, lanes 6; Fig. 8C, Post), suggesting that the MBP-Prbp fusion protein does not cause protein proteolysis. Induced instability of the reporter mRNAs was not accountable for the reduced amount of hGH protein product observed in the presence of the MBP-Prbp fusion protein. Reporter mRNA extracted from a sample preincubated with MBP-Prbp produced nearly the same amounts of hGH protein product as untreated mRNA (Fig. 8A and B, lanes 7; Fig. 8C, RE). RNA losses sustained during the extraction protocol probably account for the reduced amount of hGH detected in this control. Thus, Prbp protein can inhibit translation in vitro, but it does so in a nonspecific manner.

Tissue distribution of Prbp mRNA and protein. Hybridization of a Northern blot containing total RNA prepared from various adult mouse tissues with the radiolabeled *Prbp* cDNA revealed a single transcript of approximately 1.6 kb that is present at high levels in the testis and at low but detectable levels in numerous other tissues (Fig. 9A). Surprisingly, hybridization of a Western blot containing total protein extracts prepared from adult mouse tissues with anti-MBP-Prbp antibody failed to detect the cluster of the four major Prbp bands around 40 kDa, and the minor band of approximately 33 kDa, in any tissue except the testis (Fig. 9B). We did, however, detect the cross-reacting band of 35 kDa in all of the extracts and a minor band of approximately 80 kDa in the spleen and ovary. These results suggest that, despite the presence of *Prbp* mRNA in all tissues tested, Prbp protein either is not expressed or is below our limit of detection in any tissue except the testis.

DISCUSSION

The properties of the Prbp protein described in this paper suggest that it has a role in the translational repression of *Prm-1* mRNA. First, the temporal and spatial patterns of Prbp expression are those expected for an inhibitor of *Prm-1* translation. Prbp protein is abundant in the testis and is localized in the cytoplasmic compartment of round spermatids, where translation of *Prm-1* mRNA is repressed, but it is not detected in elongated spermatids, where *Prm-1* mRNA is translated. Second, both recombinant and testis-derived Prbp proteins bind to a region contained in the 3′-most 62 nt of the *Prm-1* 3′ UTR mRNA. This region is sufficient for translational repression of a heterologous mRNA in transgenic mice (3) and is the region most highly conserved between the 3′ UTRs of mouse *Prm-1* and *Prm-2* (20). This sequence is also highly conserved in the bovine (27) and human (29) *Prm-1* 3′ UTRs. Third, translation of a reporter mRNA is inhibited in a cell-free lysate in the presence of Prbp protein, although inhibition is not dependent on the *Prm-1* 3′ UTR.

The numerous properties of Prbp just described are consistent with, but do not prove, the hypothesis that Prbp functions as a translational repressor of *Prm-1* mRNA in spermatids. Although Prbp binds to the most conserved region of the *Prm-1* 3′ UTR, it also binds to poly(I) · poly(C), suggesting that it may be recognizing a highly structured region within the *Prm-1* 3′ UTR and that it may interact with mRNAs other than *Prm-1* mRNA in vivo. Consistent with its ability to bind to poly(I) · poly(C), Prbp contains two copies of a previously described dsRNA-binding motif (14, 49). Despite the apparent nonspecific binding properties of these proteins in vitro, genetic studies suggest that at least some of the members of this family interact with selected mRNAs in vivo (11–13, 22, 48). We presume that, in vivo, additional factors may facilitate specific interactions between the Prbp protein and its target mRNAs, such as *Prm-1.*

Developmental expression of Prbp parallels that of *Prm-1* translational repression. The *Prbp* gene is transcribed at high levels in the testis, and by Western blotting analysis we were

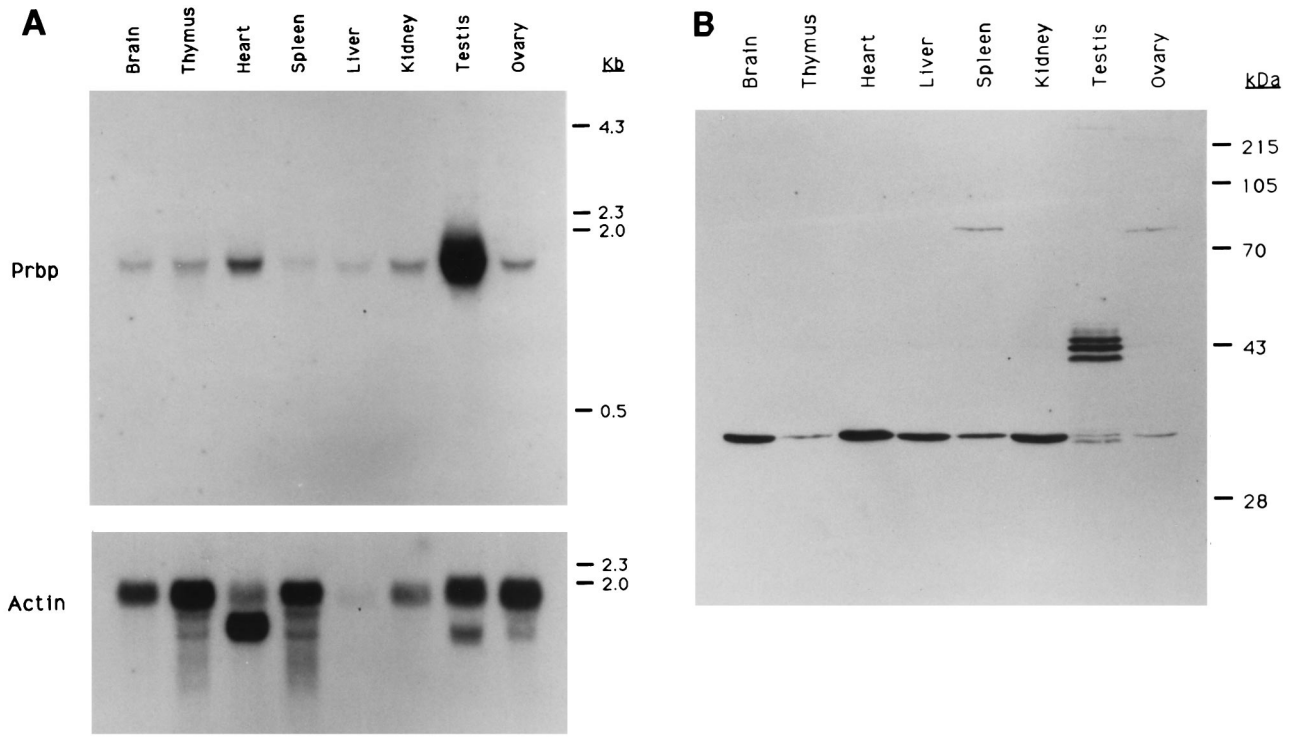

FIG. 9. Tissue distributions of *Prbp* mRNA and protein. (A) Northern blot analysis of *Prbp* mRNA levels in adult tissues. Each lane contains approximately 15 μg of total RNA. The blot was hybridized with a probe prepared from the entire *Prbp* cDNA by random hexamer labeling. To verify the quantity and integrity of the RNA present in each lane, the *Prbp* probe was stripped from the filter and then rehybridized with a radiolabeled mouse actin probe. DNA size markers are indicated on the right. (B) Western blot analysis of Prbp protein levels in adult tissues. Total proteins (approximately 150 μg) were resolved on SDS–10% polyacrylamide gels that were then electroblotted onto a nitrocellulose filter as described in Materials and Methods. The filter was incubated overnight at 4°C in the affinity-purified anti-MBP-Prbp antibody. After being washed, the blot was incubated with goat anti-rabbit immunoglobulin G antibody conjugated to horseradish peroxidase, and the presence of enzyme was revealed by chemiluminescence with luminol and 4-iodophenol as a luminogen as described previously (44). Protein size markers are indicated on the right.

able to detect a collection of proteins migrating at approximately 40 kDa. Analysis of the in vitro translation products of Prbp mRNA supports the conclusion that the different proteins are all translation products of *Prbp* mRNA. The different sizes of Prbp may result from posttranslational modifications of a single translation products, or they may be amino-terminal truncations derived from alternative translational start sites. The latter possibility seems most likely given the existence of one additional in-frame AUG codon, and two CUG codons, positioned slightly downstream of the putative initiator AUG (Fig. 1A).

Despite the fact that we were able to detect low levels of *Prbp* mRNA in various adult tissues analyzed, we were unable to detect any of the 40-kDa forms of Prbp protein by Western blotting in extracts prepared from those same tissues. These data suggest that expression of Prbp protein is restricted to germ cells or that the level of Prbp protein in somatic adult tissues is below our limit of detection. A common band of approximately 35 kDa was detected in tissues other than the testis; however, because we were unable to detect this band in vitro following translation of *Prbp* mRNA prepared from *Prbp* cDNA, we conclude that the 35-kDa protein is not encoded by *Prbp*.

Genetic, biochemical, and immunocytochemical evidence supports the conclusion that Prbp protein is expressed in late-stage pachytene spermatocytes and round spermatids. We were unable to detect Prbp protein by Western blot analysis in testis extracts prepared from mouse mutants that either lack germ cells entirely (W/W^v) or contain diploid germ cells but lack meiotic and postmeiotic germ cells (*Tfm*/*Y*), suggesting that Prbp is expressed in germ cells and not in the somatic cells of the testis. Furthermore, immunocytochemical localization of Prbp in testis sections from normal mice showed that Prbp is present at low levels in stage VII pachytene spermatocytes, is abundant in round spermatids, and remains detectable through step 10. Prbp is localized to the cytoplasm in all of these cell types, and many mRNAs are under translational control during this phase of spermiogenesis. The temporal expression and spatial localization of Prbp are consistent with it functioning as a general translational repressor during this period.

Prbp is the mouse homolog of human TRBP. Sequence analysis showed that Prbp is a member of a rapidly growing family of proteins which share a common RNA-binding motif (14, 49). This family includes human TRBP (15), human interferon-inducible and dsRNA-activated protein kinase (referred to as p68, DAI, and PKR) (16), *Drosophila* staufen (48), and the murine Tenr (45) and Spnr (46) proteins, the products of two other genes identified in our expression screen. The Prbp protein contains two copies of the RNA-binding motif that defines this family of proteins.

Prbp is striking in its similarity (93% identical at the amino acid level) to human TRBP (Fig. 1B) and is most likely its mouse homolog. The TRBP gene was originally cloned from a HeLa cell cDNA expression library on the basis of its ability to bind to the HIV-1 TAR RNA in vitro (15) and was cloned a second time on the basis of its ability to bind to the HIV-1 Rev-responsive element (39). The function of TRBP in noninfected cells is unknown, although it has been suggested that TRBP may act as an inhibitor of the interferon-induced protein kinase, PKR, and in doing so indirectly control protein

synthesis (39). In support of this, TRBP has been shown to interact with PKR in the two-hybrid system and by far-Western (protein-protein) blotting (7), although in both cases it may be doing so by binding to a common RNA. Our data do not address the possible function of Prbp as a regulator of the murine PKR. However, in vitro, the RNA-binding properties of Prbp and TRBP appear to be quite similar. One difference between our data on Prbp and those reported for TRBP is that we find that in the testis Prbp is cytoplasmic. This was shown by both Western blotting and immunocytochemistry. In contrast, in HeLa cells TRBP has been shown to be primarily nuclear (26). In the future it will be important to determine if TRBP is expressed during spermatogenesis in humans, if it is localized in the cytoplasm, and if TRBP interacts with the 3′ UTR of human *Prm-1* RNA.

RNA binding in vitro. In vitro RNA-binding studies showed that Prbp binds to a specific region of the *Prm-1* 3′ UTR. In the band shift assay that we used, Prbp either bound the substrate RNA completely or failed to bind at all, suggesting that a binding site is present within the 3′-most region of the *Prm-1* 3′ UTR. We do not know if sequence and/or structure is important for binding of Prbp to the *Prm-1* 3′ UTR. However, competition assays performed with poly(I) · poly(C) suggest that, in vitro, Prbp binds to dsRNA nonspecifically. Although there are no extensive regions of potential base pairing in the *Prm-1* 3′ UTR, there are two alternate stem-loop structures that could theoretically form and be stabilized by protein binding (Fig. 10A).

While deletion analyses suggest that sequences contained within a 24-nt region in the 3′-most region of the *Prm-1* 3′ UTR are important for Prbp binding (Fig. 3 and 10A, nt 107 to 130), attempts to detect binding to an RNA that contains only the 24-nt sequence were unsuccessful. This result can be explained in two different ways. First, the 24-nt RNA may contain all of the binding specificity for Prbp binding but may not be of sufficient length for binding. Support for this possibility comes from studies with PKR, which have shown that a minimum target of 30 bp of dsRNA is required for binding (35). Second, sequences flanking the 24-nt region may constitute part of the binding site. Sequences that flank the 24 nt that may be important are two nearly perfect 17-nt repeats (Fig. 10B). Each repeat is capable of base pairing with a sequence between the repeats (nt 105 to 115) to form a region of dsRNA. In this scenario, RNAs extending from nt 72 to 130 or from nt 107 to 156 can base pair to form similar stem-loop structures (Fig. 10A). In contrast, an RNA that contained only the 24-nt region defined by deletion analysis (nt 107 to 130) would not form either structure, nor would an RNA extending from nt 121 to 156 (Fig. 3). Clearly, additional mutational analysis is required to distinguish between the roles of sequence and structure in Prbp binding.

Another feature of Prbp binding is its tendency to form oligomers (Fig. 2B). This feature has also been reported for TRBP (14) and PKR (35), as well as for proteins of other families of RNA-binding proteins, including the Rev protein of HIV-1 (6, 17, 34, 53) and the *Xenopus* Y box protein (50). In the case of the Rev protein, oligomerization seems to be essential for RNA binding, although this has been challenged by others (6, 34). At high protein concentrations, Rev protein binds to RNA and nucleates the assembly of long filamentous ribonucleoprotein structures (17). The oligomerization of the Rev protein also seems to be important for in vivo Rev function because Rev mutants defective in oligomerization but not in RNA binding lack biological activity (34).

Given that Prbp is capable of protecting large regions of RNA from RNase digestion in vitro (29b) and that it can form oligomers in vitro, it is easy to imagine a simple model for translational repression in which Prbp first binds to a specific site in the 3′ UTR of *Prm-1* RNA and then oligomerizes to form a filamentous ribonucleoprotein particle that is translationally inactive.

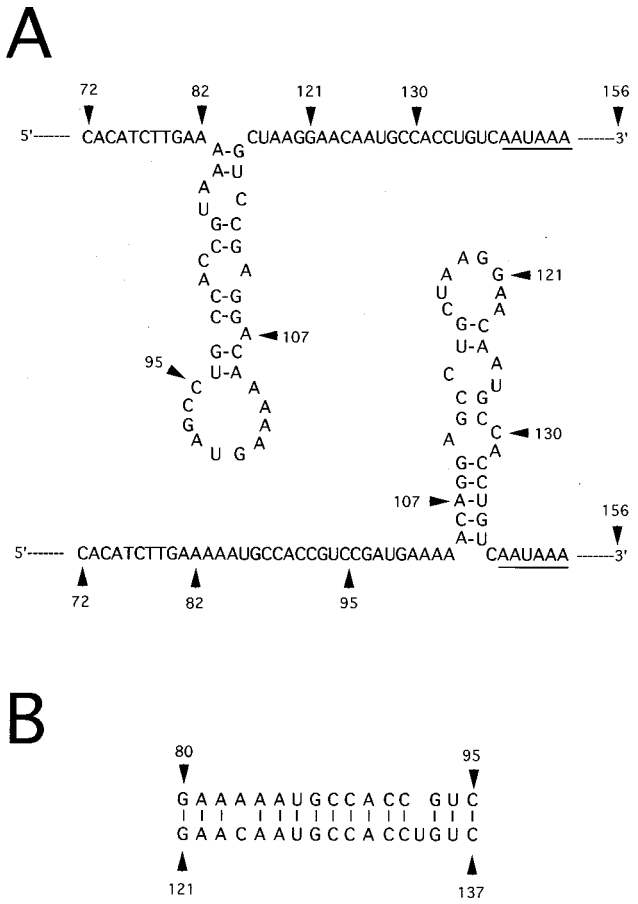

FIG. 10. Potential regions of secondary structure formed in the 3′-most region of the *Prm-1* 3′ UTR. (A) The full-length *Prm-1* 3′ UTR is 156 nt. Only the nucleotide sequence from position 72 through 143 is shown. Various nucleotide positions are indicated with arrowheads. Regions of potential base pairing are indicated by hatch marks between bases. Two potential alternative secondary structures are shown. The minimal region of the *Prm-1* 3′ UTR shown to be sufficient for translational repression in vivo is 62 nt and extends from position 95 through 156 (3). (B) Nucleotide sequence of two regions of the *Prm-1* 3′ UTR showing a nearly perfect direct repeat. The same sequence is highly conserved in the bovine (27) and human (28) *Prm-1* 3′ UTRs and is the region most highly conserved between the 3′ UTRs of mouse *Prm-1* and *Prm-2* (referred to as the Z box [20]). As shown in panel A, either repeat is able to base pair with another region of the *Prm-1* 3′ UTR (located between nt 105 and 115) and form a region of dsRNA with a similarly sized single-stranded loop.

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