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A Sequence-Specific RNA Binding Complex Expressed in Murine Germ Cells Contains MSY2 and MSY4

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The protamine mRNAs are stored for up to 8 days as translationally repressed ribonucleoprotein particles during murine spermatogenesis. Translational repression of the protamine 1, *Prm1,* mRNA is controlled by sequences in its 3′-untranslated region (UTR). In this study we used the yeast three-hybrid system to clone *Msy4,* which encodes a novel member of the Y box family of nucleic acid binding proteins. MSY4 specifically binds to a site within the 5′ most 37 nucleotides in the *Prm1* 3′ UTR. *Msy4* is highly expressed in the testis, and the protein is detected in the cytoplasm of germ cells in both the testis and the ovary, where repressed messages are stored. Analysis of a previously described 48/50-kDa binding activity in testis extracts by electrophoretic mobility shift assays and immunoprecipitation indicates the activity is composed of MSY4 and MSY2, another mouse Y box protein. Polysome analysis demonstrates MSY4 is associated with mRNPs, consistent with MSY4 having a role in storing repressed messages. © 2000 Academic Press

Key Words: **three-hybrid system; Y box proteins; translational control; mRNP; protamine.**

INTRODUCTION

Translational control is an important form of gene regulation, especially during gametogenesis and embryogenesis, in which there are periods of no transcription (Curtis *et al.,* 1995; Stebbins Boaz and Richter, 1997). During both oogenesis and spermatogenesis, repressed mRNAs are stored in ribonucleic protein (RNP) particles. In growing oocytes mRNA is synthesized and stored in mRNP particles in preparation for the suspension of transcription during oocyte maturation. These preexisting mRNAs are translated to make the proteins necessary for the completion of oogenesis and the beginning of embryogenesis. Maternal mRNAs from the oocyte are needed at least until zygotic transcription begins. Transcription ceases in spermatogenesis during the third and final stage, spermiogenesis, in which haploid round spermatids differentiate into mature spermatozoa. Translation of repressed messages is required for the synthesis of numerous proteins required for flagellar and nuclear morphogenesis.

Among the genes that are regulated by translational control during spermatogenesis are protamine 1 (*Prm1*), protamine 2 (*Prm2*), and the transition proteins *Tnp1* and *Tnp2* (Kleene, 1996). The transition proteins and the protamines compact the chromatin during the terminal stage of spermatid differentiation. The protamines are transcribed in round spermatids, and their mRNA is stored in mRNP particles in the cytoplasm for 2–8 days, until translated in elongated spermatids (Kleene *et al.,* 1984). Failure to delay the translation of the *Prm1* mRNA leads to precocious nuclear condensation and male sterility (Lee *et al.,* 1995). The mechanism of translational control of the protamines is unknown, but the *cis*-acting regulatory elements have been identified within the 3′-untranslated region (UTR) (Braun, 1990; Braun *et al.,* 1989). Two separate regions of the *Prm1* 3′ UTR, the 3′-most 62 nucleotides (nt) (Braun, 1990) and the 5′-most 37 nt ($Prm1_{1\text{-}37(wt)}$) (Fig. 1, Fajardo *et al.,* 1997) are sufficient for *Prm1*-like translational delay.

Several proteins that bind to the *Prm1* 3′ UTR have been described. Among these are the Y box protein MSY2 (Gu *et al.,* 1998), a single-stranded DNA binding protein TB-RBP

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(Kwon and Hecht, 1993), a double-stranded RNA binding protein PRBP (Lee *et al.,* 1996), and a sequence-specific RNA binding activity of 48/50 kDa (Fajardo *et al.,* 1994). The molecular function of most of these proteins is unknown, although mice nullizygous for the *Tarbp2* locus, which encodes PRBP, are sterile and have defects in the translational activation of the protamine mRNPs (Zhong *et al.,* 1999).

The 48/50-kDa sequence-specific RNA binding proteins are likely components of the protamine mRNPs. The proteins bind to a highly conserved sequence in the 5′-most region of *Prm1* and *Prm2* 3′ UTRs (Fajardo *et al.,* 1994; F. Giorgini, unpublished), and a transgenic mRNA that contains the binding site is translationally repressed *in vivo* (Fajardo *et al.,* 1997). The binding activity is present in the cytoplasm of round spermatids, where protamine mRNAs are repressed, and is absent in elongated spermatids, where protamine mRNAs are translated.

In this paper we describe the cloning of the gene that encodes the 48/50-kDa proteins and show that the RNA binding activity present in testis extracts consists of two Y box proteins.

METHODS

Three-Hybrid Selection and Screening

A derivative of yeast strain L40 (*MATa, ura3-52, leu2-3, 112, his∆200, trp1∆1, ade2, LYS2::(LexAop)-HIS3, ura3::(LexAop)-LacZ*), containing plasmids encoding the LexA DNA binding domain-MS2 coat fusion protein and the hybrid RNA pIII/MS2-2-$Prm1_{1\text{-}37(wt)}$ (SenGupta *et al.,* 1996), was transformed with a mouse testis cDNA Matchmaker library (Clontech, Palo Alto, CA). Transformants were plated onto synthetic medium lacking leucine, uracil, tryptophan, and histidine. A quantity of 5 mM 3-aminotriazole was used to select for higher levels of activation of *HIS3.* Approximately 7.5×10^6 transformants were screened. $HIS3^+$ prototrophs were assayed for β-galactosidase activity using 5-bromo-4-chloro-3-indolyl-β-D-galactoside as a substrate in Z buffer in a filter assay (Breeden and Nasmyth, 1985).

Colonies were tested for RNA dependence by assaying for β-galactosidase expression in cells cured of the plasmid that encodes the hybrid RNA, pIII/MS2-2-$Prm1_{1\text{-}37(wt)}$. Transformants were plated on synthetic medium lacking leucine and tryptophan and containing 0.15% 5-fluoroorotic acid to select for the loss of *URA3,* and thus the loss of the hybrid RNA, and then retested for β-galactosidase activity. Candidates that failed to activate *lacZ* following plasmid loss were tested for binding specificity by reintroducing hybrid RNA plasmids encoding $Prm1_{1\text{-}37(wt)}$ $Prm1_{1\text{-}37(mut)}$ or $Prm1_{134\text{-}156}$ (Fig. 1). Of 113 $HIS3^+$ transformants, 10 had β-galactosidase activity, and 5 were RNA-dependent. One transformant specifically bound the $Prm1_{1\text{-}37(wt)}$ RNA and not the $Prm1_{1\text{-}37(mut)}$ or $Prm1_{134\text{-}156}$.

Plasmids

The vector encoding the hybrid RNA, pIII/MS2-2, carries the *URA3* marker and a unique *Sma*I site between an RNA polymerase III promoter and the sequence encoding the MS2 target RNA. To construct the hybrid RNAs used in this study complementary oligonucleotides encoding different regions of the *Prm1* 3′ UTR were annealed and ligated into the *Sma*I site. *Msy2* was cloned via PCR with the Matchmaker library as the template, using the primers 5′ CGCGGATCCCAAGCCGGTGCTGGCAATCC 3′ and 5′ CGCGGATCCGAATCACTCCAGTATGGTG 3′, and then inserted into the *Bam*HI site of pACT (Clontech) to be expressed as a fusion protein with the GAL4 activation domain. Constructs were verified by ^{35}S sequencing using Sequenase (USB, Cleveland, OH) or dye-terminator cycle sequencing (Perkin–Elmer, Norwalk, CT). The LexA DNA binding domain-MS2 coat fusion protein is encoded by the plasmid pLexA-MS2, bearing the *TRP1* marker. The activation domain library was purchased from Clontech. The library plasmids carry the *LEU2* marker.

Phylogeny

The amino acid sequences were aligned using ClustalW (Thompson *et al.,* 1994). Phylogenies were estimated using a distance method, PROTDIST. The tree was built using a neighbor joining program, NEIGHBOR, and analyzed with SEQBOOT, all from the PHYLIP package of phylogeny programs (Felsenstein, 1993). Bootstrap analysis shows this tree is very highly supported. The use of bootstrapping in phylogeny estimation was introduced by Felsenstein (1985). It involves creating a new data set by sampling *N* characters randomly with replacement, so the new data set is the same size as the original, but some characters have been left out and others have been duplicated. One-hundred new data sets were generated and analyzed by PROTDIST and NEIGHBOR. The tree shown is the consensus tree.

Isolation of Full-Length cDNAs

Overlapping cDNAs were isolated from two libraries, a mouse germ cell cDNA library in λgt11 (M. Eddy, NIEHS) and a mouse testis cDNA Matchmaker library (Clontech), by plaque and colony hybridization, respectively, using the clone 3 partial cDNA as a probe (Sambrook *et al.,* 1989).

RNA Analysis

Total RNA was isolated from dissected mouse tissues as previously described (Cathala *et al.,* 1983). RNA samples were electrophoresed in agarose–formaldehyde gels, transferred to nylon (Hybond-N Amersham Life Sciences), and hybridized 15–20 h with radioactive α-^{32}P-DNA probes prepared by random oligonucleotide-primed synthesis. The nylon membrane was washed at a final stringency of 0.1× SSC and 0.5% SDS at 60°C and exposed to X-ray film.

Antibodies

An antibody was raised against N14, a peptide in the amino-terminus of MSY4, whose amino acid sequence is KTTGGT-TLPQAAADA. N14 was synthesized, purified by HPLC, and conjugated to BSA by the University of Washington Department of Pharmacology Protein Core. Antisera was raised by immunizing rabbits with N14. All rabbit work was done by R&R Rabbitry (Stanwood, WA). Affinity-purified antibody to the BA2 region of p54 (Murray, 1994) and antibody to the purified p54/p56 proteins (Murray *et al.,* 1991) were kindly given to us by M. Murray and affinity-purified antibody to recombinant FRGY2 (Tafuri and

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Wolffe, 1992) was provided by A. Wolffe. The α-BA2, α-p54/p56, and α-FRGY2 antibodies all crossreact specifically with MSY2.

Immunohistochemistry

Immunohistochemistry was performed as previously described (Braun *et al.,* 1989). Briefly, tissues were dissected from adult mice and fixed in Bouin's (testes) or Carnoy's (ovaries) overnight and embedded in paraffin. Sections were deparaffinized with xylene and rehydrated using standard procedures. Tissue sections were treated with primary antibody at 1:4000 or 1:5000 overnight at 4°C or 2–3 h at room temperature. Biotinylated goat anti-rabbit IgG and streptavidin conjugated to horseradish peroxidase were used as recommended by the manufacturer (Zymed Laboratories, San Francisco, CA). Peroxidase activity was visualized with the chromogen aminoethyl carbozole. Tissue sections were counterstained with hematoxylin.

Tissue Extracts

Testis extracts were prepared as described (Dignam *et al.,* 1983) with the following modifications. Testes were dissected from mature mice and the tissue was put in 1 ml of buffer containing 10 mM KOAc, 10 mM Popso, pH 7.5, 1.5 mM MgOAc, 1 mm DTT, and supplemented with protease inhibitors (1 mM PMSF, 1 μg/μl pepstatin A, 1 μg/μl leupeptin) per gram of tissue. Cells were dounced using 25 strokes in a tight pestle. The lysate was pelleted at 3000*g* for 15 min in a fixed angle rotor. To the supernatant was added 1/10 vol of 0.3 M Ropso, 1.4 M KOAc, 1.5 mM MgOAc, and glycerol to 20%.

Recombinant MSY4 was expressed in the BL21 strain of *Escherichia coli* using the pET15-b/pLysS expression system (Novagen, Madison, WI). A cDNA clone encoding MSY4 was cloned into the pET15-b expression vector and transformed into BL21. A single positive colony was used to inoculate 50 ml LB-ampicillin (100 μg/ml). At an OD_{600} of approximately 0.50 the culture was induced with a final concentration of 10 mM isopropyl-β-D-thiogalactopyranoside for 2 h. Cells were harvested by centrifugation at 10,000 rpm for 10 min at 4°C. The cells were resuspended in testis extract buffer (10 mM KOAc, 10 mM Popso, pH 7.5, 1.5 mM MgOAc, 1 mm DTT, and supplemented with protease inhibitors (1 mM PMSF, 1 μg/μl pepstatin A, 1 μg/μl leupeptin) to approximately 5 mg/ml final concentration of protein extract.

Immunoprecipitation

Coimmunoprecipitation of RNA was done with 50 μl of testis extract (approximately 12 mg/ml) incubated with 10 μl of rabbit sera and 200 μl of a 1:1 slurry of protein A–agarose (Gibco-BRL) and lysis buffer (20 mM Hepes, 100 mM NaCl, 1.5 mM $MgCl_2$, and 0.5% NP-40) for 2 h rocking at 4°C. The suspension was spun at 3000*g* for 4 min and the pellet was washed three times in 1 ml of cold lysis buffer. The final pellet was resuspended in 500 μl of solution D (20 mM Hepes, 20% v/v glycerol, 0.1 M KCl, 0.2 mM EDTA, and 0.5 mM DTT). The solution was treated with proteinase K at 0.2 μg/ml for 1 h at 42°C and then the RNA was isolated and analyzed as described under "RNA Analysis."

Coimmunoprecipitation of proteins was done as described above with the following modifications. Fifteen microliters of testis extract was mixed with 1 μl of rabbit sera and 25 μl of slurry for 1 h of rocking at 4°C. The RNase-treated testis extract was treated with RNase A at 0.5 μg/ml for 1 h at room temperature prior to immunoprecipitation. The proteins were analyzed as under "Immunoblotting."

RNase H Treatment

RNA was treated with RNase H and 100 pmol oligo(dT) in 20 mM Hepes (pH 7.6), 50 mM KCl, 4 mM $MgCl_2$, 1 mM DTT, and 0.5 μg BSA for 20 min at 37°C. The RNA was isolated and analyzed as described under "RNA Analysis."

RNA Probe Preparation

RNA probes were synthesized using SP6 bacteriophage polymerase using protocols suggested by the supplier (Promega, Madison, WI). *In vitro* transcription reactions were performed in 20-μl reaction volumes using 1 μg of linearized plasmid template; 500 μM ATP, UTP, and GTP; 25 μM CTP; and 50 μCi [α-^{32}P]CTP at 3000 ci/mmol (NEN Dupont, Boston, MA) for approximately 60 min. Samples were phenol–chloroform extracted, precipitated with ethanol, collected by microcentrifugation, and resuspended in 50 μl of H_2O.

RNA Binding Assays

RNA electrophoretic mobility shift assays (EMSA) were performed as described previously (Fajardo *et al.,* 1994) with the following modifications. RNA probes (1 μl, ~10^6 cpm) were combined with 1 μl of testis extract, in RNA binding buffer (20 mM Hepes (pH 7.6), 3 mM $MgCl_2$, 40 mM KCl, and 2 mM DTT) and incubated at room temperature for 20 min. The samples were then treated with 1 μl of RNase T1 (Calbiochem, La Jolla, CA) for 10 min at room temperature and then 2 μl of heparin (Sigma, St. Louis, MO) for an additional 10 min at room temperature. For the supershift analysis 1 μl of antibody was added after the RNA binding reaction and allowed to bind for 20 min at room temperature. The samples were combined with 5 μl of 50% glycerol and electrophoresed through a 4% polyacrylamide gel (60:1) for approximately 3 hat 4°C and 180 V in a running buffer of 45 mM Tris (pH 8.3), 50 mM boric acid, and 1 mM EDTA. Gels were dried and visualized by autoradiography.

Ultraviolet Crosslinking

RNA binding was performed as described under "RNA Binding Assays." Following the heparin treatment, the samples were placed on ice, with the tops of the microcentrifuge tubes open, and irradiated with ultraviolet light from a source located approximately 0.3 m away for 30 min. The irradiated samples were boiled in Laemmli buffer and analyzed by SDS–polyacrylamide gel electrophoresis using a 5% stacking gel and 10% resolving gel. Prestained molecular markers (Gibco-BRL Life Technologies, Rockville, MD) were used for size standards. The acrylamide gels were dried and visualized by autoradiography.

Immunoblotting

Protein extracts were mixed with Laemmli buffer, boiled and electrophoresed in 8% SDS–polyacrylamide gels. The proteins were transferred to nitrocellulose (Gibco-BRL Life Technologies). After transfer the membrane was blocked for 30 min to several h at room temperature in 5% nonfat dry milk and phosphate-buffered

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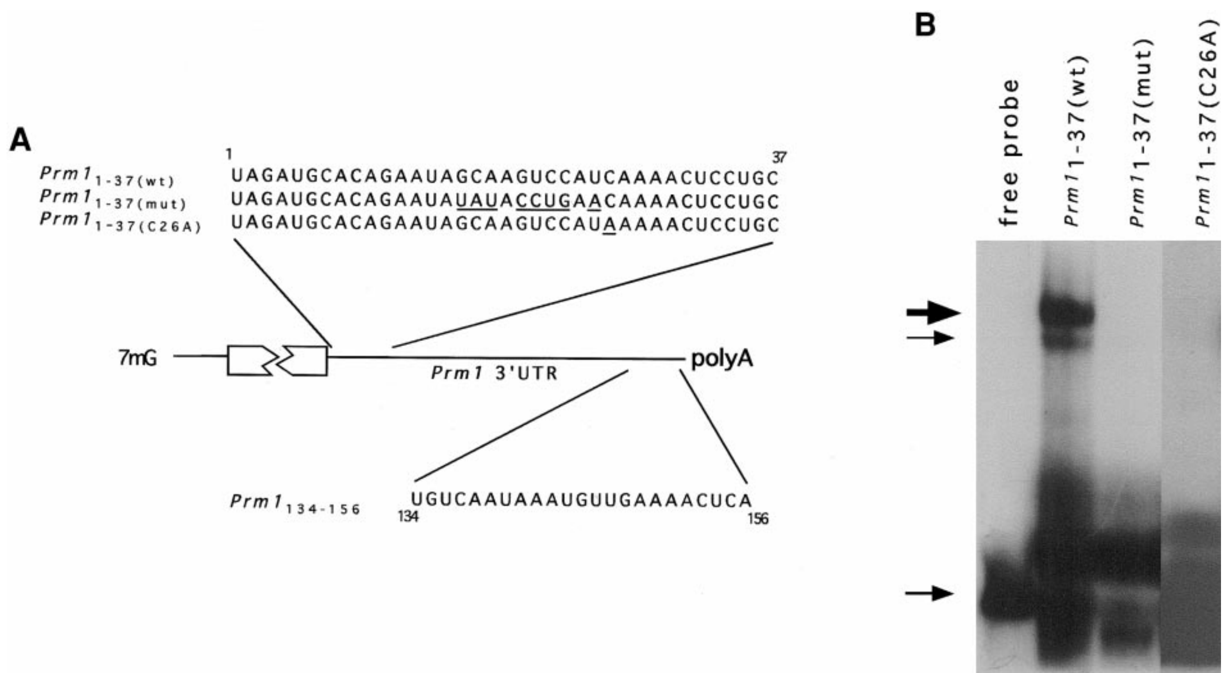

FIG. 1. (A) Sequences in the *Prm1* 3′ untranslated region (UTR) used in this study. $Prm1_{1\text{-}37(wt)}$ is the 5′-most 37 nt of the *Prm1* 3′ UTR. The altered nucleotide(s) in $Prm1_{1\text{-}37(mut)}$ and $Prm1_{1\text{-}37(C26A)}$ are underlined. $Prm1_{1\text{-}37(mut)}$ consists of mutations in 8 nt that are conserved between *Prm1* and *Prm2* in that region. $Prm1_{1\text{-}37(C26A)}$ contains a point mutation in which the C in position 22 has been changed to an A. The 3′-most 23 nt, $Prm1_{134\text{-}156}$, includes the polyadenylation signal. (B) Electrophoretic mobility shift analysis (EMSA) of the *Prm1* 3′UTR binding activity in mouse testis extracts. The probes are as described in (A). The top arrows indicate the sequence-specific 48/50-kDa binding activity. The bottom arrow indicates free probe.

saline (BPBS) and then incubated overnight at 4°C with primary antibody at a 1:10,000 dilution. The membrane was washed once in BPBS with 0.05% Tween 20 and twice in BPBS and then incubated with secondary antibody conjugated to horseradish peroxidase (HRP) for several hours at room temperature. After washing again as above, the HRP activity was detected using enhanced chemiluminescence (ECL) as described (Schneppenheim and Rautenberg, 1987). ECL reagent was prepared immediately prior to use by dissolving 40 mg of luminol (5-amino-2,3-dihydro-1,4-phthalazinedione) and 10 mg of 4-iodophenol in 1 ml of DMSO. Following the addition of 10 ml of 0.1 M Tris (pH 8.5), 5 ml of 5 M NaCl, 17 ml H_2O, and 125 μl H_2O_2, the membrane was incubated for 2 min and exposed to X-ray film.

Polysome Analysis

Each testis was dissected from an adult mouse and homogenized in 1 ml homogenization buffer (100 mM NaCl, 1.5 mM $MgCl_2$, 20 mM Popso (pH 7.5), and 1 mM PMSF). The nuclei and mitochondria were collected by centrifugation for 2 min at 12,000*g*, and the supernatant was layered over a 11-ml linear 15–50% (w/w) sucrose gradient in lysis buffer and centrifuged in a Beckman SW40 rotor for 110 min at 36,000 rpm. The gradients were fractionated into twelve 1-ml fractions using an Isco density gradient fractionator (Model 185), while monitoring ultraviolet absorbance at 254 nm. As a control to verify mRNA association with polysomes, equivalent supernatants were prepared and centrifuged in sucrose gradients in buffer in which the $MgCl_2$ was replaced by 20 mM EDTA. The presence of EDTA causes mRNA and ribosomes to disassociate. Northern and Western analysis was performed on each fraction. Two-hundred microliters of each fraction was concentrated and analyzed as described under "Immunoblotting." Five-hundred microliters of each fraction was treated with proteinase K at 0.2 μg/ml for 90 min at 55°C, and 100 μl was analyzed as described under "RNA Analysis."

RESULTS

Three-Hybrid Cloning of Msy4

In hopes of cloning the gene(s) that encodes the previously described 48/50-kDa proteins that bind to the $Prm1_{1\text{-}37(wt)}$ 3′ UTR RNA (Fig. 1), we screened a library of mouse testis cDNAs linked to the GAL4 transcriptional activation domain using the yeast three-hybrid system (SenGupta *et al.,* 1996). In this assay, interaction of an RNA-binding protein with an RNA leads to transcriptional activation of a reporter gene in yeast (Fig. 2). As bait we used a hybrid RNA, which included the first 37 nt of the *Prm1* 3′ UTR, $Prm1_{1\text{-}37(wt)}$, and two copies of the MS2 coat protein recognition site. Transformants were initially selected for HIS3 protrophy and positives were subsequently screened for β-galactosidase activity.

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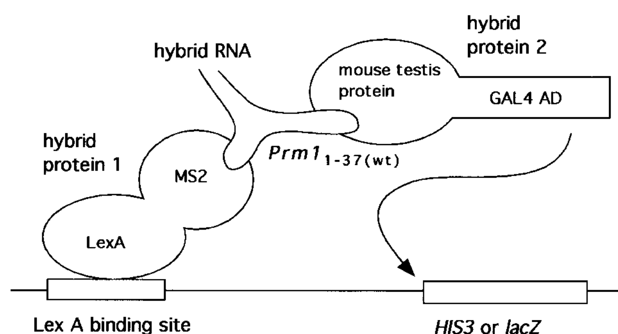
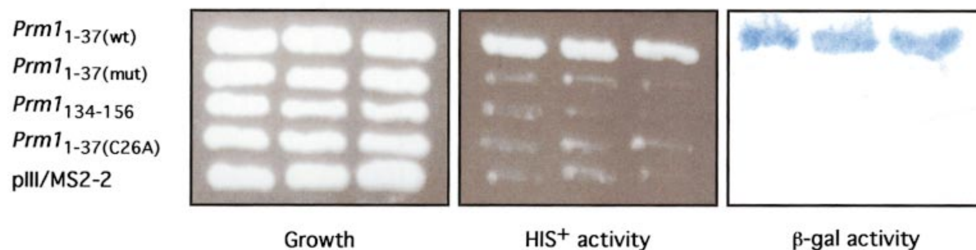


FIG. 2. The three-hybrid screen. *Prm1*$_{1\text{-}37(\text{wt})}$ was used as bait in a hybrid RNA with the bacteriophage MS2 coat protein RNA binding site to screen a mouse testis cDNA-GAL4 activation domain fusion library. Binding of a mouse testis protein to the *Prm1*$_{1\text{-}37(\text{wt})}$ results in the transcriptional activation of *HIS3* and *lacZ* reporter genes.

To identify RNA-dependent candidates we selected for loss of the plasmid encoding the hybrid RNA and rescreened for β-galactosidase activity. RNA-dependent candidates only had β-galactosidase activity when the hybrid RNA was present. Candidates that failed to activate *lacZ* after plasmid loss were tested for RNA binding specificity by reintroducing plasmids encoding hybrid RNAs with *Prm1*$_{1\text{-}37(\text{wt})}$ and two negative controls, *Prm1*$_{1\text{-}37(\text{mut})}$ or *Prm1*$_{134\text{-}156}$. The 48/50-kDa binding activity does not to bind to *Prm1*$_{1\text{-}37(\text{mut})}$, an RNA that contains mutations in 8 of the nucleotides conserved between *Prm1* and *Prm2* in that region (Fig. 1) or *Prm1*$_{134\text{-}156}$, an RNA consisting of the 3′-most 23 nt of the *Prm1* 3′ UTR (data not shown). One transformant, clone 3, satisfied all criteria.

Figure 3A shows the binding specificity of clone 3 for several RNAs. *HIS3*/*lacZ* expression was activated when the *Prm1*$_{1\text{-}37(\text{wt})}$ was present; however, *Prm1*$_{1\text{-}37(\text{mut})}$ and *Prm1*$_{1\text{-}37(\text{C26A})}$, which contains a single nucleotide change within the binding site that abolishes protein binding (Fig. 1), both eliminated activation. The plasmids encoding *Prm1*$_{134\text{-}156}$ or the MS2 binding sites alone (pIII/MS2-2) also failed to activate. The iron response element with its binding protein, the iron response protein, is shown as a positive control (SenGupta *et al.,* 1996).

Clone 3 was sequenced and determined to be incomplete at both its 5′ and its 3′ ends. To clone a full-length cDNA, clone 3 was used to screen a λgt11 mouse germ cell and Matchmaker testis cDNA libraries. Several overlapping clones containing the 5′ UTR, open reading frame (ORF)



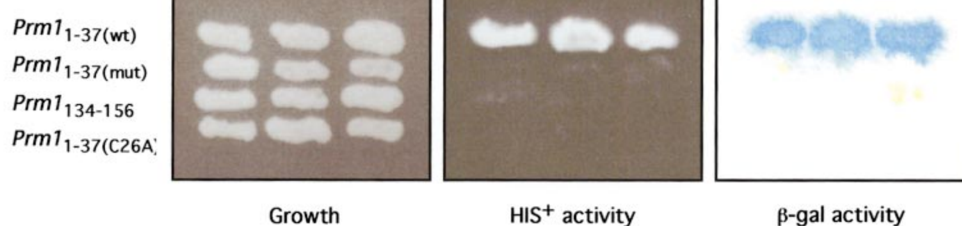


FIG. 3. Sequence-specific RNA binding of MSY4 (A) and MSY2 (B) in the three-hybrid system. The yeast strains are able to grow on media lacking leucine, tryptophan, and uracil, selecting for the three plasmids used in the three-hybrid assay (left panels). MSY4 and MSY2 specifically bind only the *Prm1*$_{1\text{-}37(\text{wt})}$, as shown by growth on media also lacking histidine (with 5 mM 3AT) (center panels), and by β-galactosidase activity in a filter assay (right panels). *Prm1*$_{1\text{-}37(\text{wt})}$, *Prm1*$_{1\text{-}37(\text{mut})}$, *Prm1*$_{134\text{-}156}$, and *Prm1*$_{1\text{-}37(\text{C26A})}$ are as previously described. pIII/MS2-2 encodes the MS2 RNA binding site alone. The iron response element and its binding protein were used as a positive control.

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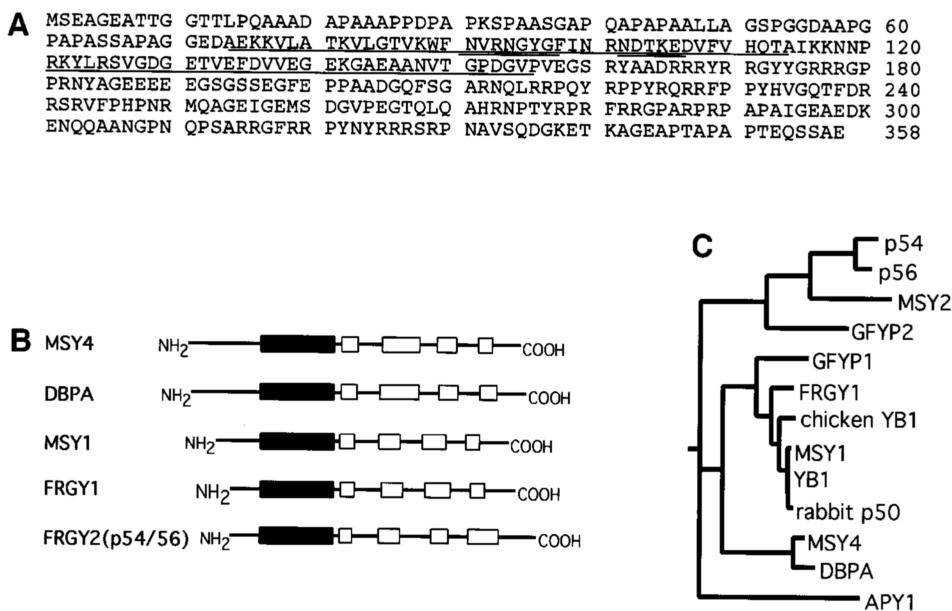

FIG. 4. (A) The predicted amino acid sequence of MSY4. The cold shock domain (CSD) is underlined. The RNA binding domains within the CSD, RNP-1 and RNP-2, are double underlined. (B) Structure of MSY4 and other family members: human DBPA, mouse MSY1, and *Xenopus* FRGY1 and FRGY2. All contain the variable N-terminus, the highly conserved CSD (closed box), and the tail of alternating basic and acidic regions (open boxes and lines). Although the identity of the amino acids in the tail is not conserved, the basic structure of four alternating basic and acidic regions is conserved. (C) Phylogeny of Y box proteins from frog (FRGY1 and FRGY2), goldfish (GFYP1 and GFYP2), chicken (chicken YB1), mouse (MSY1 and MSY2), rabbit (p50), and human (YB1 and DBPA). *Aplysia* APY1was used as an outgroup. The tree was estimated from amino acid sequences using a distance method; thus the branch lengths are meaningful.

and 3′ UTR were characterized. The composite cDNA is 1693 bp long and has an open reading frame which encodes a predicted protein of 358 amino acids (Fig. 4A).

A Blast search of the NIH nonredundant database revealed that we cloned a new member of the Y box family of nucleic acid binding proteins. Members of this family include DBPA (Coles *et al.,* 1996; Sakura *et al.,* 1988) and YB-1 (Didier *et al.,* 1988) in humans, FRGY1 and FRGY2 (Deschamps *et al.,* 1992) in frogs, and MSY1 (Tafuri *et al.,* 1993) and MSY2 (Gu *et al.,* 1998) in mice (reviewed by Matsumoto and Wolffe, 1998). We refer to this new Y box member as MSY4.

All family members contain the highly conserved cold shock domain (CSD) that is 43% identical from *E. coli* to humans (Fig. 4A, underlined). The structure of the CSD has been solved for bacterial members and is a five-stranded β-barrel (Schindelin *et al.,* 1993, 1994). Within the CSD are the RNA binding motifs RNP-1 and RNP-2, present on the $\beta 2$ and $\beta 3$ strands, respectively (Fig. 4A, double underlined). MSY4, and the other Y box proteins, contain a variable amino-terminus, the CSD, and then a tail with four alternating basic and acidic regions. The amino acids in the tail are not highly conserved among family members, but the structure of alternating basic and acidic regions is conserved. The structure of several family members is shown in Fig. 4B.

A phylogeny of Y box family members is shown in Fig. 4C. MSY4 is closest to human DBPA and is its mouse ortholog. MSY1 clusters with GFYP1, FRGY1, chicken YB-1, human YB-1, and rabbit p50, while MSY2 clusters with GFYP2 and FRGY2 (p54/p56).

MSY4 Is Localized to the Cytoplasm of Germ Cells

By Northern analysis, *Msy4* mRNA was most abundant in the testis (Fig. 5, top). Lower levels were detected in skeletal muscle and upon longer exposure low levels could be detected in all other tissues (data not shown). The membrane was reprobed with *Gapdh* as a control for levels of RNA in each lane (Fig. 5, bottom).

To determine if MSY4 is found in the cytoplasm of round spermatids during the time *Prm1* mRNA is translationally repressed, we raised an antibody to a peptide in the N-terminus of MSY4 (N14) and performed immunohistochemistry on sections of adult mouse testis. MSY4 protein was first detected in the cytoplasm of midpachytene cells (Fig. 6B, stage VIII tubule). The signal was strongest in late pachytene spermatocytes (Figs. 6D–6F) and early round spermatids (Figs. 6B, stages IV and V, and 6C) and persisted through nuclear elongation (Figs. 6D and 6B, stage XI). MSY4 was not detected in elongated spermatids (Figs. 6B, stages IV and V, and 6C). This expression of MSY4 is

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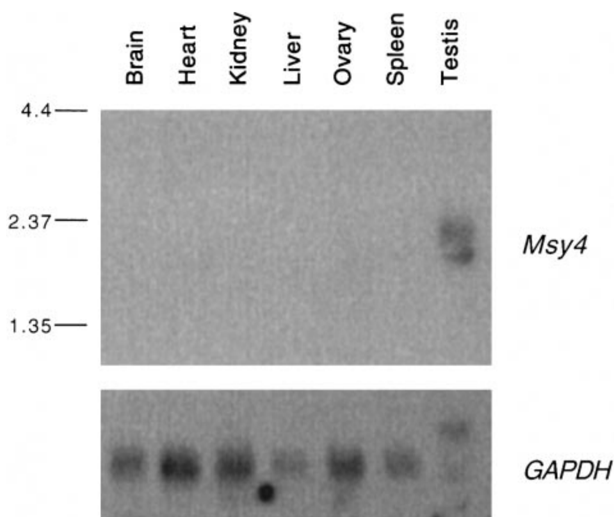

FIG. 5. Northern analysis of *Msy4* expression in adult mouse tissues. Ten micrograms of RNA from each tissue was resolved on a 1.5% agarose–formaldehyde gel and transferred to a nylon membrane. The ^{32}P-labeled 1.2-kilobase (kb) clone 3 cDNA was used as a probe for *Msy4.* The size standards, in kb, are on the left (Gibco-BRL Life Technologies). The ^{32}P-labeled 1.3-kb glyceraldehyde-3-phosphate dehydrogenase cDNA from chicken was used as a control (Dugaiczyk *et al.,* 1983). GAPDH is not detected at equal levels in all tissues and is detected at higher levels in the brain, heart, and kidney (Ambion, Austin, TX). Alternative forms are found in the testis (Mezquita *et al.,* 1998).

consistent with it functioning in the translational repression of *Prm1* mRNA in round spermatids.

The predominant proteins associated with masked messages in *Xenopus* oocytes, mRNP$_{3+4}$, are Y box proteins (Darnbrough and Ford, 1981). Despite the low level of *Msy4* message in ovaries by Northern analysis, α-MSY4 antibody specifically labeled the cytoplasmic compartment of both preantral and antral follicles in mouse ovaries (Fig. 6H). The presence of MSY4 in the cytoplasm of male and female germ cells, which both contain an abundance of masked messages, suggests that MSY4 is involved in mRNA storage.

The 48/50-kDa Activity Contains MSY4 and MSY2

To determine if the previously described 48/50-kDa binding activity contains MSY4 we performed several experiments utilizing MSY4 antibody. The molecular weight of the 48/50-kDa activity was originally determined by crosslinking a radiolabeled *Prm1* 3′ UTR RNA to the binding activity present in testis extracts, sizing the complexes on a denaturing polyacrylamide gel, and subtracting the molecular weight of the RNA component from the complexes (Fajardo *et al.,* 1994). The MSY4 polyclonal antibody specifically recognized proteins of 55/58-kDa in testis extract (Fig. 7A, lane 1) and a protein of 55 kDa in a bacterial extract in which MSY4 had been expressed (Fig. 7A, lane 2). Although the predicted size of the protein encoded by the ORF is 38 kDa, it is usual for Y box proteins to run anomalously on SDS–PAGE gels (Deschamps *et al.,* 1992). The MSY4 antibody also recognized a protein of 60 kDa present in extracts prepared from transformed as well as untransformed bacterial cells.

The 48/50-kDa proteins in testis extract migrated at 53/55 kDa when UV crosslinked to a radiolabeled *Prm1*$_{1\text{-}37(wt)}$ RNA (Fig. 7C, lane 1). Recombinant MSY4 expressed in bacterial cells was also UV crosslinked to radiolabeled *Prm1*$_{1\text{-}37(wt)}$ RNA (Fig. 7B, lanes 2 and 3), but not to radiolabeled RNA with a point mutation, *Prm1*$_{1\text{-}37(C26A)}$ (Fig. 7B, lanes 5 and 6). Both probes were seen to be intact on an acrylamide gel (data not shown). The UV-crosslinked species from testis extract were dependent on the MSY4 Y box recognition sequence (YRS) within the *Prm1*$_{1\text{-}37(wt)}$ RNA, as the *Prm1*$_{1\text{-}37(mut)}$ did not form either complex (Fig. 7C, lane 2). Antibody against MSY4 immunoprecipitated a portion of the UV-crosslinked complexes of 53/55 kDa from testis extract (Fig. 7C, lane 5). Preimmune sera did not precipitate either complex (Fig. 7C, lane 7).

The 48/50-kDa complex can also be detected by an EMSA. We attempted to supershift the EMS complex with MSY4 antibody, but were unable to do so when using an RNA probe containing a single copy of the binding site (Fig. 7D, lane 3 arrow). However, an RNA containing two copies of the MSY4 YRS also formed an EMS complex and was supershifted by MSY4 antibody (Fig. 7D, lane 6), but not by preimmune sera (lane 5). The ability to immunoprecipitate the UV-crosslinked complex, and supershift the EMS complex, strongly suggests that MSY4 is a component of the 48/50-kDa binding activity.

The failure to supershift all of the 48/50-kDa EMS complex (Fig. 7D, lane 6), suggested that the complex contained protein(s) in addition to MSY4. Mouse testis extracts also contain MSY2, the ortholog of the *Xenopus* Y box protein FRGY2 (mRNP$_{3+4}$), the major mRNP protein in oocytes. The similarity in the MSY2 and MSY4 cold shock domains suggested that MSY2 might also bind to the MSY4 YRS and be contained in the 48/50-kDa complex. To test this possibility antibody against FRGY2, which crossreacts with MSY2 (Kwon *et al.,* 1993), was used to examine the 48/50-kDa complex. Three different antibodies to FRGY2, all of which specifically crossreact with MSY2, were used (Tafuri and Wolffe, 1992; Murray, 1994; Murray *et al.,* 1991). α-FRGY2 detected a single band in mouse testis extract that migrated slightly lower than MSY4 (Fig. 7A, lane 4). This antibody immunoprecipitated a portion of the UV-crosslinked complexes of 53/55 kDa from mouse testis extract (Fig. 7C, lane 3). FRGY2 antibody was also able to supershift the EMS complex with both the *Prm1*$_{1\text{-}37(wt)}$ probe (Fig. 7D, lane 7) and the longer probe with the additional MSY4 YRS (Fig. 7D, lane 8). Antibody that had been heated to destroy the antibody did not supershift the EMS (data not shown).

Since both α-MSY4 and α-MSY2 antibodies were able to

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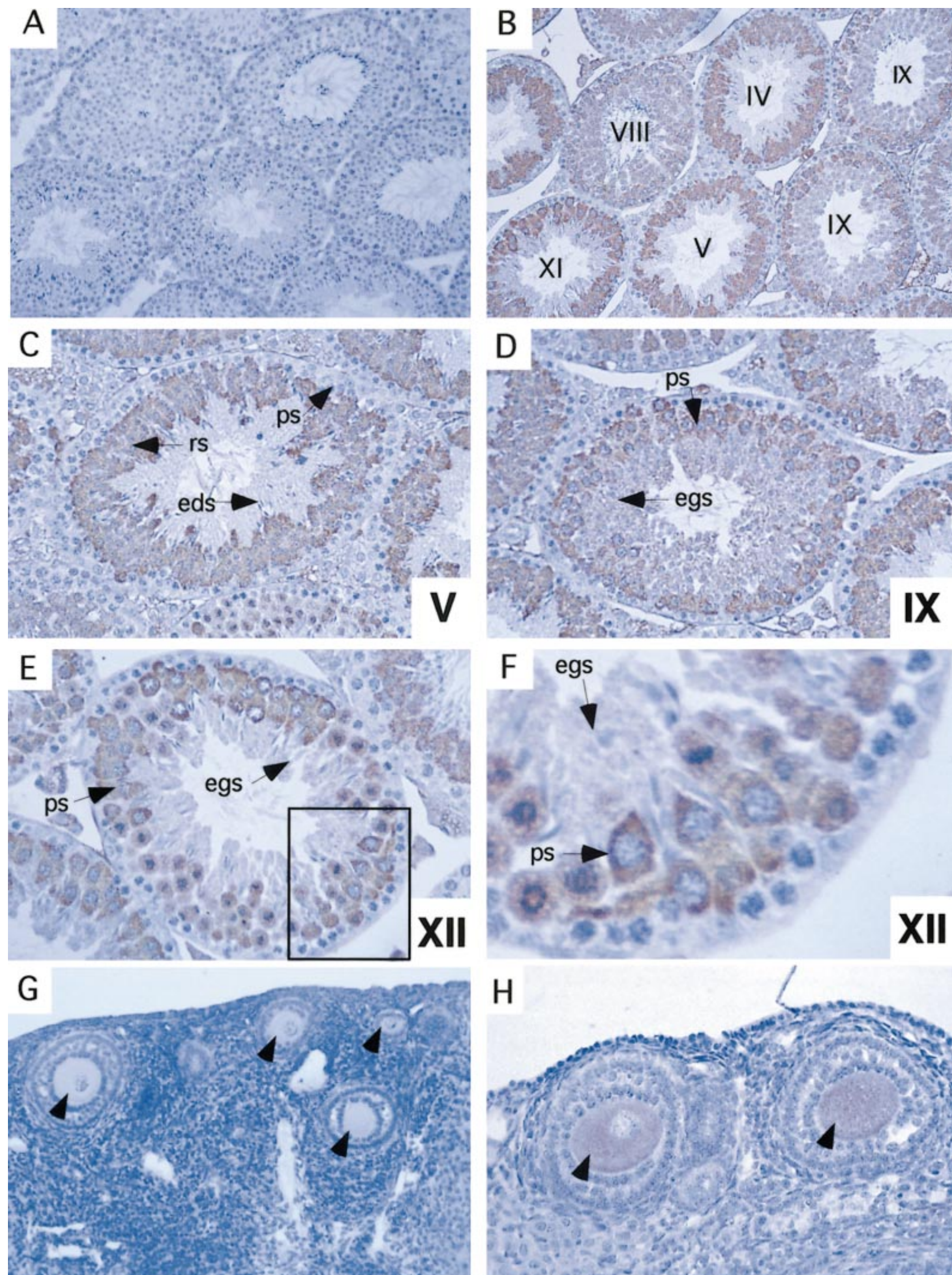

FIG. 6. Immunohistochemical localization of MSY4 in adult mouse testis and ovary. The sections were counterstained with hematoxylin. (A–F) Testis sections stained with (A) preimmune sera and (B–F) α-MSY4. (F) Enlargement of the boxed area in (E). Roman numerals indicate the stage of the tubule (Russell *et al.,* 1990). The germ cells are indicated as pachytene spermatocytes (ps), round spermatids (rs), elongating spermatids (egs), and elongated spermatids (eds). (G and H) Ovary sections stained with (G) preimmune sera and (H) α-MSY4. The arrows denote the oocytes within the follicles.

immunoprecipitate a portion of the UV-crosslinked complexes and supershift a portion of the EMS complex, it suggests that the 48/50-kDa binding activity contains both MSY4 and MSY2. It further suggests that MSY4 and MSY2 may be part of the same complex. To test this possibility, coprecipitation experiments were performed. MSY4 anti-

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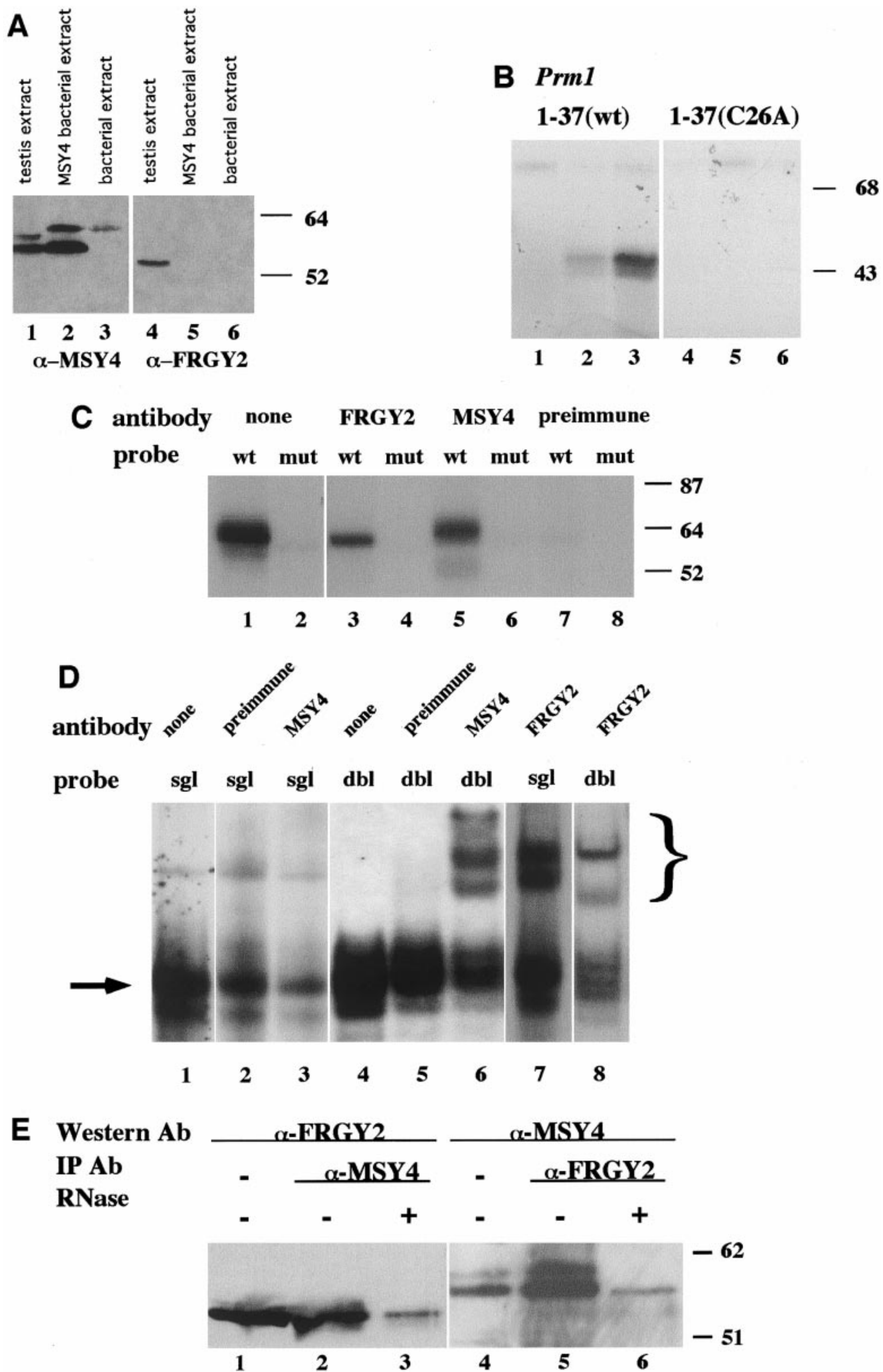

FIG. 7. The 48/50-kDa binding activity contains both MSY4 and MSY2. (A) Immunoblot of protein extracts from adult mouse testis, bacteria expressing MSY4, and untransformed bacteria. Aliquots of approximately 30 μg of each extract were resolved on a 8% SDS–PAGE gel, transferred to nitrocellulose, and probed with antibody to MSY4 or FRGY2 (Murray, 1994). Both antibodies specifically recognize either

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body immunoprecipitated MSY2 from testis extract (Fig. 7E, lane 2) and FRGY2 antibody immunoprecipitated MSY4 (lane 5). Both interactions were RNA-dependent, as the treatment of the extract with RNase A abolished most of the interaction (Fig. 7E, lanes 3 and 6). MSY4 and MSY2 antibodies were also able to immunoprecipitate MSY4 and MSY2, respectively, both with and without the RNase A treatment (data not shown). Preimmune sera did not immunoprecipitate either MSY2 or MSY4 (data not shown).

The ability of MSY2 antibody to supershift the 48/50-kDa EMS complex suggests that MSY2 is contained within the complex, but does not necessarily show that MSY2 can bind the YRS sequence directly. To determine if MSY2 binds the YRS we cloned a cDNA encoding MSY2 and expressed it as a fusion protein with the GAL4 activation domain in the yeast three-hybrid system. Figure 3B shows the binding specificity of MSY2 for several RNAs. *HIS3/lacZ* expression was activated when the $Prm1_{1\text{-}37(wt)}$ was present; however, $Prm1_{1\text{-}37(mut)}$ and $Prm1_{1\text{-}37(C26A)}$, which contains a single nucleotide change within the binding site that abolishes protein binding (Figs. 1 and 3A), both eliminated activation. The plasmid encoding $Prm1_{134\text{-}156}$ also failed to activate. Therefore, like MSY4, MSY2 is a sequence-specific RNA binding protein.

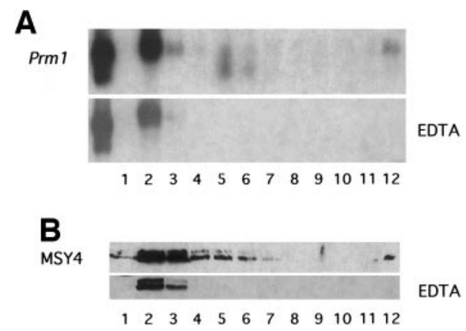

FIG. 8. Distribution of *Prm1* mRNA and MSY4 in sucrose gradients. Testis extract from an adult mouse was sedimented on a 15–50% (w/w) sucrose gradient and 12 fractions were collected. Sample number 1 is the top of the gradient. Each fraction was divided for Northern and Western analysis. (A) Northern analysis on total RNA isolated from each fraction and hybridized with a probe specific to *Prm1.* (B) Western analysis on protein in each fraction, probed with MSY4 antibody. As a control for polysome association, the analysis was also performed on lysate prepared and sedimented in the presence of EDTA, which dissociates polysomes.

MSY4 Associates with Stored and Unstored mRNA in Vivo

To ascertain if MSY4 is associated with mRNPs *in vivo* we fractionated testis extract on a sucrose gradient and performed Northern (Fig. 8A) and Western (Fig. 8B) analysis on each fraction. The location of *Prm1* mRNA was used to define the fractions. *Prm1* was detected as a larger message in the mRNP fractions (Fig. 8, lanes 2 and 3) and as a mixture of this larger message and shorter messages in the polysome fractions due to deadenylation (Fig. 8A, lanes 5 and 6). The majority of MSY4 protein was detected in the mRNP fractions (Fig. 8B, fractions 2 and 3), with a smaller amount in the monosomes (fraction 4), disomes (fraction 5), and polysomes (fractions 6 and 7). Equivalent gradients were run in the presence of EDTA, which disassociates RNA from ribosomes. In the presence of EDTA all the *Prm1* mRNA and MSY4 protein were detected in the mRNP fractions (Figs. 8A and 8B, bottom panels).

To determine if MSY4 associates with *Prm1* mRNA *in vivo,* we immunoprecipitated MSY4 from testis extract, isolated RNA, and probed for the presence of *Prm1* mRNA by Northern analysis. *Prm1* RNA coprecipitated with MSY4 antibody (Fig. 9A, lane 1), but not with preimmune sera (Fig. 9A, lane 2). *Prm1* mRNA is detected as a polyadenylated message in the repressed mRNP and as a deadenylated message when it is being actively translated on polysomes (Fig. 8A; Kleene, 1989). To determine if MSY4 is associated with the long or short form of the *Prm1* mRNA,

MSY4 or MSY2, respectively. The size standards, in kilodaltons (kDa), are indicated on the right (Gibco-BRL Life Technologies). (B) Specific binding of recombinant MSY4 to ^{32}P-labeled $Prm1_{1\text{-}37(wt)}$ RNA in lanes 2 and 3 compared to ^{32}P-labeled $Prm1_{1\text{-}37(C26A)}$ RNA in lanes 5 and 6. Lanes 1 and 4, uninduced extracts; lanes 2 and 5, extracts induced for 1 h; and lanes 3 and 6, extracts induced for 2 h. Twenty micrograms of each extract was resolved on a 10% SDS–PAGE gel and visualized by autoradiography. (C) Immunoprecipitation of testis proteins UV crosslinked to ^{32}P-labeled RNA and resolved on a 10% SDS–PAGE gel. The complexes were visualized by autoradiography. Lanes 1 and 2, the 48/50-kDa binding activity; lanes 3 and 4, immunoprecipitated with antibody to FRGY2 (Tafuri and Wolffe, 1992); lanes 5 and 6, immunoprecipitated with antibody to MSY4; and lanes 7 and 8, immunoprecipitated with preimmune sera. The $Prm1_{1\text{-}37(wt)}$ was used as the labeled probe in lanes 1, 3, 5, and 7. The $Prm1_{1\text{-}37(mut)}$ was used in lanes 2, 4, 6, and 8. (D) Supershift analysis of the 48/50-kDa binding activity bound to ^{32}P-labeled RNA. The arrow points to the EMS and the bracket indicates the supershift. MSY4 or MSY2 (Tafuri and Wolffe, 1992) antibody was added after the RNA binding reaction, and the complexes were resolved on a 4% native acrylamide gel and visualized by autoradiography. $Prm1_{1\text{-}37(wt)}$ was used as the labeled probe (sgl, single binding site) in lanes 1–3 and 7. A probe with an additional YRS (dbl, double binding site) was used in lanes 4–8. (E) Coimmunoprecipitation of MSY2 and MSY4 from testis extracts of adult mice. Proteins were immunoprecipitated, resolved on an 8% SDS–PAGE gel, transferred to nitrocellulose, and probed with antibody to MSY2 (left panel (Murray *et al.,* 1991) or MSY4 (right panel). Extracts in lanes 3 and 6 had been treated with RNase A prior to immunoprecipitation. Lanes 1 and 4, testis extracts that have not been immunoprecipitated. Size standards in kDa are shown on the right (Gibco-BRL Life Technologies).

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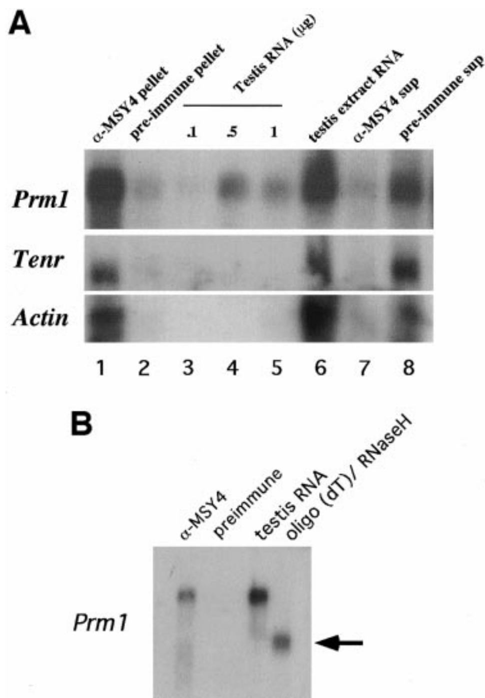

FIG. 9. Coimmunoprecipitation of RNA with antibody to MSY4. (A) Testis extract from adult mice was incubated with MSY4 antibody or preimmune sera. RNA was isolated from the immunoprecipitate and the supernatant, resolved on a 2% agarose–formaldehyde gel, transferred to a nylon membrane, and probed with ^{32}P-labeled *Prm1* cDNA (top), stripped, and then reprobed with ^{32}P-labeled *Tenr* and *actin* cDNAs (bottom). RNA isolated from testis extract and dilutions of total testis RNA was used as a positive control. (B) RNase H treatment of the coimmunoprecipitated RNA. The analysis was as in (A), with the following addition. RNA was isolated from testis extract and treated with oligo(dT) and RNase H to remove poly(A) tails. The arrow points to the deadenylated form of *Prm1.*

the RNA that was coprecipitated with the MSY4 antibody was analyzed by Northern blotting along with total testis RNA that had been treated with oligo(dT) and RNase H to remove the poly(A) tails. As shown in Fig. 9B, the α-MSY4 antibody coprecipitated the fully adenylated form of the *Prm1* message, strongly suggesting that MSY4 is bound to the repressed form of the *Prm1* message found in mRNP particles.

To determine if MSY4 was specifically bound to *Prm1* mRNA, we also probed for the presence of *Tenr* mRNA, an abundant message that is also under translational control (Schumacher *et al.,* 1995). We detected *Tenr* message in MSY4 immunoprecipitate (Fig. 9B, bottom, lane 2), demonstrating that MSY4 binds *Tenr* and *Prm1* mRNAs *in vivo.* To determine if other RNAs were also coprecipitated with α-MSY4 we probed for *Gapdh* and *Actin,* two mRNAs that are not known to be under translational control during spermatogenesis. Surprisingly, both mRNAs were coprecipitated (Fig. 9A and data not shown). While no specificity in which mRNAs were immunoprecipitated was observed, the mRNA immunoprecipitation was specific to the MSY4 antibody compared to preimmune sera. These data suggest that MSY4 may associate with many mRNAs *in vivo* (see Discussion) or that the immunoprecipitation conditions did not permit us to detect specific interactions.

DISCUSSION

We have used the yeast three-hybrid assay to clone a cDNA encoding a new member of the Y box family of RNA-binding proteins. MSY4 binds to a conserved 7-nt sequence in the *Prm1* 3′ UTR in the yeast three-hybrid system and *in vitro.* Using an antibody raised against a unique peptide from its amino-terminus, we showed that MSY4 coprecipitates with *Prm1* mRNA and that it constitutes part of a 48/50-kDa sequence-specific RNA binding activity present in murine testis extracts. The localization of MSY4 to the cytoplasm of round and elongating spermatids, the association of MSY4 with the fully adenylated form of the *Prm1* mRNA in ribonucleoprotein particles, and the ability of the binding site for MSY4 to inhibit translation of a transgenic mRNA *in vivo* (Fajardo *et al.,* 1997) strongly imply that MSY4 interacts with the *Prm1* mRNA *in vivo.* The localization of MSY4 in growing oocytes suggests the possibility that it also functions in maternal mRNA storage.

The Y box family of nucleic acid binding proteins were first identified as transcriptional activators that bind the Y box DNA sequence (CTGATTGGC/TC/TAA) (Deschamps *et al.,* 1992; Sakura *et al.,* 1988). Y box proteins were later shown to also bind RNA nonspecifically (Murray, 1994; Tafuri and Wolffe, 1993). The *Xenopus* Y box protein FRGY2 is the major component of mRNPs in oocytes (Darnbrough and Ford, 1981) and at high concentrations is able to repress translation *in vitro* (Bouvet and Wolffe, 1994; Ranjan *et al.,* 1993; Richter and Smith, 1984). All Y box proteins contain the highly conserved CSD, which is 43% identical from bacteria to humans. The structure of the CSD in bacterial proteins has been solved (Schindelin *et al.,* 1993, 1994) and consists of a five-stranded β-barrel. The RNA binding motifs, RNP1 and RNP2, are located on β-2 and β-3, respectively. The CSD has a different fold than the RNA recognition motif (RRM), within which the RNP1 and RNP2 motifs are usually found (Burd and Dreyfuss, 1994). RNP1 and RNP2 are thought to contact RNA as an open platform in a sequence-nonspecific manner with RNA specificity imparted by other sequences within the RRM (Burd and Dreyfuss, 1994). Within vertebrate family members the 90-amino-acid CSD domain is highly conserved, with any two members having greater than 95% amino acid identity. Vertebrate family members also contain a variable amino-terminus and a carboxyl-terminus consisting of four alternating basic and acidic regions. The carboxyl-terminus interacts with RNA nonspecifically (Bouvet *et al.,* 1995),

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mediates protein–protein interaction (Tafuri and Wolffe, 1992), and is necessary for the incorporation of FRGY2 into mRNPs *in vivo* (Matsumoto *et al.,* 1996).

MSY4 is most similar to human DBPA (92% at the amino acid level and 85% identical at the nucleotide level). *Msy4* is expressed in skeletal muscle as *DBPA* is, but unlike *DBPA, Msy4* is not highly expressed in heart (Kudo *et al.,* 1995). MSY4 shares 55 and 47% amino acid identity with mouse MSY1 and MSY2, respectively. The different murine Y box proteins contain unique amino-termini. They have their greatest conservation within the CSD and share a common structure within their carboxyl-termini consisting of four alternating basic and acidic regions.

Bouvet *et al.* (1995) used an *in vitro* selection amplification technique, SELEX (Tsai *et al.,* 1991), to show that FRGY1 and FRGY2 prefer to bind the 6-nt sequence 5′ ACCAUC 3′; however, specific *Xenopus* mRNAs containing this sequence have not been identified. While both the N-terminus and the carboxyl-tail were found to contribute to the interaction with RNA, specific RNA binding was mapped to the RNP1 motif within the CSD. The similarity of the MSY4 binding site to the SELEX-defined binding site of the *Xenopus* FRGY1 and FRGY2 Y box proteins led us to determine if the murine ortholog of FRGY2, MSY2 (Gu *et al.,* 1998), is also a part of the testis 48/50-kDa binding activity. Using an antibody specific to MSY2, we showed that it is. Previous reports had shown that MSY2 binds RNA nonspecifically (Kwon *et al.,* 1993). However, the three-hybrid data shown here, and an extensive mutational analysis of the binding site *in vitro* and in the three-hybrid assay (F. Giorgini and H. Davies, unpublished), strongly support the conclusion that MSY2 and MSY4 bind a specific 7-nt RNA sequence, 5′ UCCAUCA 3′, perhaps through the RNP1 and RNP2 motifs in its CSD. Furthermore, bacterially expressed MSY4 is able to bind the $Prm1_{1-37}$ RNA in a sequence-specific manner *in vitro.* We refer to the binding site within the *Prm1* 3′ UTR as the MSY2/MSY4 YRS. The similarity of the FRGY1/FRGY2 and MSY2/MSY4 binding sites, and their cold shock domains, suggests the possibility that all Y box proteins bind a similar RNA sequence.

Although MSY4 binds the YRS specifically *in vitro* and in the three-hybrid assay, we did not detect association of MSY4 to specific mRNAs in testis extracts by immunoprecipitation. In addition to probes for specific mRNAs, we probed for poly(A)-containing RNA and determined by phosphoimaging that 75% of polyadenylated RNA was coprecipitated with anti-MSY4, compared to 25% with preimmune sera (H. Davies, unpublished). Some of the coimmunoprecipitation may be caused by association of protein with RNA during the preparation of the extracts and may not represent physiological interactions. However, the association of MSY4 with non-YRS-containing RNAs may also result from the nonspecific RNA binding properties of the Y box proteins *in vivo.* In addition to numerous translationally regulated messages, a surprisingly large fraction of mRNAs that are not under translational control are found in mRNPs in spermatogenic cells (Kleene, 1996; Schmidt *et al.,* 1999). The large percentage of mRNA in mRNPs may reflect constraints on the translational machinery in spermatogenic cells.

Y box proteins activate transcription from promoters that contain Y box sequences (Bienz, 1986; Graves *et al.,* 1986). The presence of Y box elements in the promoter regions of *Prm1* (Zambrowicz *et al.,* 1993) and *Prm2* (Nikolajczyk *et al.,* 1995) suggest the possibility that MSY2 and MSY4 may also function in the transcriptional regulation of the protamine genes. MSY2 has been shown to bind to the *Prm2* Y box DNA sequence *in vitro* (Nikolajczyk *et al.,* 1995). The archetype of proteins which function in regulating transcription and translation is *Xenopus* TFIIIA (Pelham and Brown, 1980). TFIIIA stimulates transcription of the 5S RNA gene in growing oocytes and associates with the nascent 5S RNA to repress its translation in mature oocytes (Guddat *et al.,* 1990). TFIIIA interacts with DNA and RNA via separate zinc finger domains (Theunissen *et al.,* 1992). The function of TFIIIA, and perhaps some Y box proteins, as transcriptional activators, may enhance their association with particular mRNAs, via target sequences in the RNA, *in vivo.*

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