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Translational repression by MSY4 inhibits spermatid differentiation in mice

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SUMMARY

In developing male germ cells, newly synthesized protamine mRNAs are stored for up to 7 days before translational activation. Translational repression of protamine 1 (*Prm1*) mRNA requires sequences present in its 3′ untranslated region (UTR) and substantial evidence suggests a role for the murine Y-box protein MSY4 in this process. To determine if MSY4 can mediate translational repression in vivo, we generated transgenic mice in which the temporal window of MSY4 expression was extended during spermatogenesis. Expression of MSY4 disrupted the normal completion of spermatogenesis and caused dominant sterility. Immunocytochemical analysis of several markers, including the protamines, indicated that MSY4 prevented normal activation of translation. mRNAs whose translation was inhibited contained at least one MSY4 RNA recognition site, suggesting sequence-dependent translational repression. Altered translational activation resulted in defective processing of protamine 2 and severe defects in sperm morphogenesis. These results suggest that MSY4 plays an active role in translational repression of several mRNAs in differentiating spermatids.

Key words: Translational control, mRNPs, Protamine, Spermatogenesis, RNA-binding, Mouse

INTRODUCTION

Post-transcriptional control is a major form of gene regulation during mammalian gametogenesis. In haploid spermatids, proteins required for sperm morphogenesis are synthesized from stores of polyadenylated mRNAs packaged as cytoplasmic ribonucleoprotein particles (RNPs). Unknown signaling events promote unmasking, translational activation, deadenylation and the eventual degradation of translationally repressed mRNAs. Several proteins have been proposed to function as translational regulators in spermatids; however, little is known about the mechanism of repression and activation.

Chromosome condensation in mammalian spermatids involves an initial displacement of the somatic histones with the testis-specific transition proteins TP1 and TP2, and their subsequent replacement by the protamines (Bellve, 1979). The protamine genes *Prm1* and *Prm2* encode small basic proteins first transcribed in haploid round spermatids (Mali et al., 1989). The protamine mRNAs are stored in translationally inert mRNP particles for up to 10 days, until translational activation in elongated spermatids (Balhorn et al., 1984; Kleene et al., 1984; Kleene and Flynn, 1987). Translational regulation of the protamine mRNAs is mediated by sequences in their 3′ untranslated region (3′ UTR) (Braun et al., 1989; Fajardo et al., 1997; Zhong et al., 2001). Mutation of the protamine 1 (*Prm1*) 3′ UTR causes premature nuclear condensation and dominant male sterility (Lee et al., 1995). Translation of the transition protein mRNAs, as well as that of other mRNAs required for spermatid differentiation, is also repressed during spermiogenesis (Balhorn et al., 1984; Mali et al., 1989). Translational repression occurs at a time when other mRNAs are actively being translated, indicating that repression is message specific (Braun, 1998).

Y-box proteins were first isolated based upon their ability to bind the DNA Y-box element present in many eukaryotic promoters (Wolffe and Meric, 1996). Y-box proteins have since been shown to bind dsDNA, ssDNA and RNA, both specifically and non-specifically (Matsumoto and Wolffe, 1998). The murine Y-box protein MSY4 is expressed in the testis and specifically binds RNA (Davies et al., 2000). MSY4 binds the Y-box recognition sequence (YRS), 5′ UCCAUCA 3′, found in the *Prm1* 3′ UTR (Giorgini et al., 2001). The YRS is conserved in vertebrate *Prm1* 3′ UTRs, and between the murine *Prm1* and *Prm2* 3′ UTRs. Mutation of the YRS in vivo relieves *Prm1*-like translational control of a reporter transgene, suggesting that the YRS can function as a translational control element in vivo.

Other Y-box proteins regulate translation of mRNAs via sites similar to the YRS. The *Xenopus* ortholog of MSY2, FRGY2, is a major component of stored maternal mRNAs in oocytes and plays an active role in masking of these mRNAs during oogenesis (Bouvet and Wolffe, 1994). In addition to binding mRNAs non-specifically during masking, FRGY2 also binds the hexanucleotide FRGY recognition sequence 5′ AACAUC 3′ (Bouvet et al., 1995). This site is very similar to the consensus YRS, and we have previously shown that MSY4 binds the FRGY2-binding site with high affinity (F. G. and R. E. B., unpublished). Selective translational repression of reconstituted mRNPs containing FRGY2 requires that the

FRGY recognition sequence be present in the repressed reporter RNA (Matsumoto et al., 1996). The chicken Y-box proteins chk-YB-1b and ckh-YB-2 are also able to repress translation via specific RNA binding (Swamynathan et al., 2000). A chk-YB RNA binding site contains a sequence very similar to the YRS, 5′UCCACCC3′, and is found in the 5′ Rous Sarcoma Virus (RSV) leader RNA. A reporter construct, with a region of the RSV leader containing this site, is specifically repressed by chk-YB-1b and 2 in a rabbit reticulocyte extract system.

Substantial evidence suggests an involvement of MSY4 in the translational regulation of the protamine mRNAs. MSY4, along with the murine Y box protein MSY2, is a component of a 48/50 kDa RNA-binding activity present in the cytoplasm of round spermatids (Davies et al., 2000). MSY4 binds to a conserved sequence in the *Prm1* and *Prm2* 3′ UTRs and is present in cytoplasmic mRNP particles that co-sediment with translationally repressed protamine mRNA (Davies et al., 2000; Giorgini et al., 2001). MSY4 could have a direct role as a repressor of translation, or alternatively, it could function to protect mRNA from degradation during storage. To test for a direct function in translational repression we expressed MSY4 in late-stage spermatids just prior to and during the period of protamine translation. Expression of MSY4 from these gain-of-function transgenes interfered with translational activation suggesting that MSY4 can function as a repressor in vivo.

MATERIALS AND METHODS

Mice

C57BL/6J male mice were purchased from Jackson Laboratory (Bar Harbor, ME) and sacrificed by carbon dioxide asphyxiation.

Transgenic mice were generated by microinjecting a purified DNA fragment at a concentration of 2 ng/μl in 10 mM Tris pH 7.5 and 0.25 mM EDTA into pronuclei of fertilized eggs derived from FVB/N × FVB/N (Taconic Laboratories, Germantown, NY) matings (Brinster et al., 1985). Pseudopregnant B6CBAF1/ (Taconic Labs) foster females were used for oviduct implantation of eggs that survived microinjection. Transgenic animals were identified by PCR.

Transgenic constructs

The *PMP* transgene contains 4.1 kb of upstream *Prm1* sequence, the 95 bp *Prm1* 5′ UTR and 270 bp *Prm1* 3′ sequence derived from a previously described transgene (Braun et al., 1989). *Msy4* was cloned into a *Bam*HI site by PCR using the primers 5′CGCGGATCC-ATGAGCGAGGCGGGCGAGGCC3′ and 5′CGCGGATCCTCAA-GCGTAGTCTGGGACGTCGTATGGGTACTCGGCACTGCTCTG-TTCGG 3′, which includes the sequence for the HA epitope in the 3′ PCR primer and *Bam*HI sites in both PCR primers. The PCR template was a cDNA with the complete *Msy4* ORF in pGAD10. The transgenic protein is missing 76 amino acids in the N terminus. The deletion begins after the first six amino acids and ends three amino acids N-terminal to the MSY4 cold shock domain (Davies et al., 2000).

The *PMH* construct is derived from the *PMP* construct. The *Bam*HI fragment from *PMP*, which contains the sequence for the HA epitope and the *Msy4* cDNA mentioned above, was blunted with T4 DNA polymerase by standard protocols. This fragment was cloned into blunted *Sma*I-*Bam*HI sites of a construct containing *Prm1* promoter sequences, the 95 bp *Prm1* 5′ UTR and the 105 bp *hGH* 3′ UTR (Braun et al., 1989).

RNA isolation and analysis

Total RNA was isolated from dissected mouse tissues as previously described (Cathala et al., 1983). RNA samples were electrophoresed in 1.5% agarose-formaldehyde gels, transferred to nylon (hybond-N; Pharmacia BioTeck, Peapack, NJ) and hybridized for 15-20 hours 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.

Sperm counts and analysis

Sperm counts were made on epididymal sperm. The epididymis was isolated from mice by dissection and placed in 1 ml of 1×phosphate buffer saline solution (PBS). The tissue was diced to release sperm and incubated at room temperature for 2 hours before counting. Samples were counted with a hemocytometer either undiluted or diluted 10-fold. All counts were made in duplicate and averaged.

Sperm morphology was analyzed using phase contrast microscopy. Samples were analyzed in PBS with a hemocytometer. Acridine Orange staining was as previously described (Kosower et al., 1992). Fluorescence microscopy was carried out using a Zeiss Axioscop microscope with Filterset 10.

Antibodies

The MSY4 antibody used is as previously described (Davies et al., 2000). Polyclonal antibody to the HA tag was purchased from Clontech (Palo Alto, CA). Mouse monoclonal antibodies to PRM1 and PRM2 are HUB1N and HUP2B, respectively (Stanker et al., 1993). TP1 and TP2 antibodies were kindly provided by Steven Kistler. Debbie O'Brien provided the GAPD-S antibody and Frans van der Hoorn provided the ODF2 antibody.

Immunocytochemical and histological analysis

Immunocytochemistry was performed as previously described (Braun et al., 1989). Testis were dissected from adult mice and fixed in Bouin's fixative overnight and embedded in paraffin wax. Sections were deparaffinized with xylene and rehydrated using standard procedures. Tissue sections were treated with primary antibody overnight at 4°C or 2-3 hours at room temperature. Either biotinylated goat anti-rabbit IgG or biotinylated goat anti-mouse IgG was used in conjunction with streptavidin conjugated to horseradish peroxidase as recommended by the manufacturer (Zymed Laboratories, San Francisco, CA). Peroxidase activity was visualized with the chromagen aminoethyl carbozole. Tissue sections were counterstained with Hematoxylin. Primary antibodies were used in the following dilutions: MSY4 (1:3000), HA (1:500), ODF2 (1:400), HUP1N (1:1500), HUP2B (1:2000), TP1 (1:1500), TP2 (1:1500) and GAPD-S (1:2000). The following antigen retrieval protocol was used with the TP1, TP2, HUP1N and HUP2B antibodies: testis sections were boiled in 100 mM sodium citrate for 10 minutes. Sections were counterstained with Hematoxylin and Periodic Acid/Schiff reagent.

Basic nuclear protein preparation

Isolation and analysis of spermatid nuclear basic proteins was performed as previously described (Lee et al., 1995; Platz et al., 1977), with the following modifications. The protease inhibitors used were leupeptin (0.5 μg/ml), phenylmethylsulfonyl fluoride (PMSF) (0.5 mM) and pepstatin A (1 μg/ml). One testis was homogenized in 0.8 ml of 20 mM Tris-HCl (pH 7.7), 40 mM KCl and 17 mM $MgCl_2$. Homogenates were not filtered through cheese cloth, unlike the method that has previously been described (Lee et al., 1995). Basic proteins were dissolved in 8 M urea/0.9 M acetic acid/0.1 M 2-mercaptoethanol/1% Methyl Green and separated in polyacrylamide slab gels containing 15% acrylamide, 0.1% bisacrylamide, 6.2 M urea and 0.9 M acetic acid. Gels were run and stained as previously described (Lee et al., 1995).

RNA probe synthesis, protein extracts and EMSA analysis

RNA probes were prepared as previously described (Giorgini et al., 2001). The wild-type YRS RNA contains two copies of the YRS (Davies et al., 2000). The C26A RNA contains a point mutation of the YRS that disrupts binding of MSY4 (Giorgini et al., 2001).

Testes were dissected from adult mice and placed in 1 mg/ml Buffer A [10 mM Hepes (pH 7.6), 1.5 mM $MgCl_2$, 10 mM KCl, 0.5 mM DTT] containing the following protease inhibitors: leupeptin, pepstatin A and PMSF. The cells were lysed with 20 strokes of a dounce homogenizer and cell debris was pelleted via centrifugation at 3000 ***g*** for 15 minutes at 4°C in a fixed angle rotor. 0.11 volumes of Buffer B [0.3 M Hepes (pH 7.6), 1.4 M KCl, 30 mM $MgCl_2$] was added to the supernatant, followed by addition of glycerol to 20% v/v final. Extracts were stored at –70°C following quick freezing in liquid nitrogen.

EMSA analysis was performed as previously described (Giorgini et al., 2001).

Polysome analysis

Each testis was dissected from an adult mouse and homogenized in 1 ml homogenization buffer [100 mM NaCl, 1.5 mM MgCl, 20 mM POPSO (pH 7.5) and 1 mM PMSF]. The nuclei and mitochondria were collected by centrifugation for 2 minutes 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 minutes at 205,000 ***g***. The gradients were fractionated into 12×1 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 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. For western analysis, 200 μl of each fraction was concentrated and analyzed as described in the section Immunoblotting. For northern analysis, 500 μl of each fraction was treated with proteinase K at 0.2 μg/ml for 90 minutes at 55°C, and 100 μl was analyzed as described in the section RNA isolation and analysis.

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 minutes to several hours at room temperature in 5% nonfat dry milk and phosphate buffered 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, 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 minutes and exposed to X-ray film.

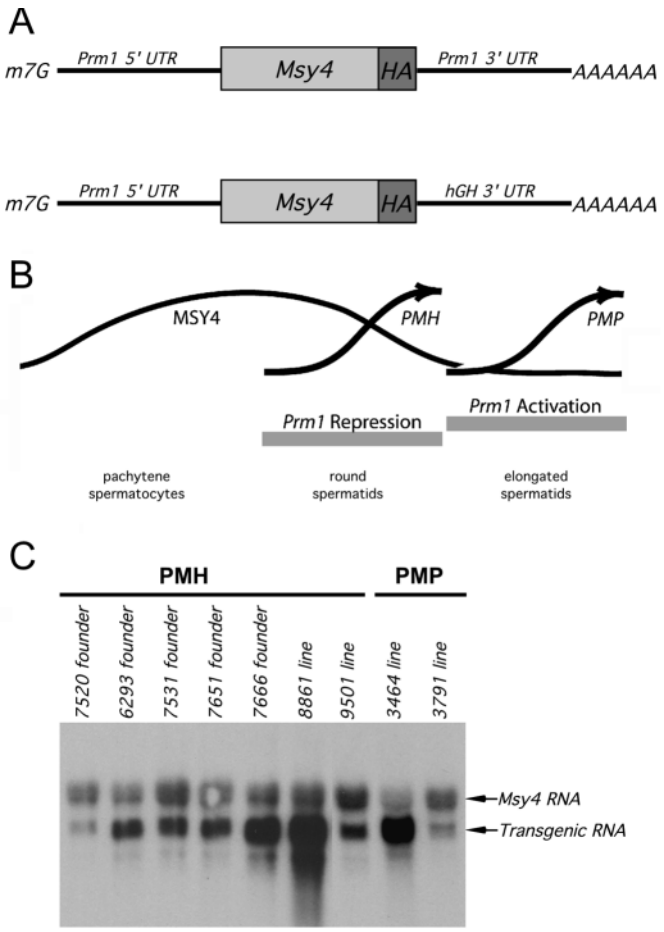

Fig. 1. MSY4 gain-of-function transgenes. (A) The upper construct contains the *Prm1* 5′ and 3′ UTRs, and a cDNA encoding an HA-tagged version of MSY4. This construct, *PMP*, is under *Prm1*-like translational control. The lower construct, *PMH*, is a variant of the upper transgene and substitutes the *hGH* 3′ UTR for the *Prm1* 3′ UTR. Both constructs are under the control of the *Prm1* promoter. (B) Schematic representation of endogenous and transgenic expression of MSY4. The curve labeled MSY4 represents expression of endogenous MSY4 in pachytene spermatocytes and round spermatids. The curve labeled *PMH* represents transgenic MSY4 expression from the *PMH* transgene, initiating in round spermatids and continuing in later stage spermatids. The curve labeled *PMP* represents transgenic MSY4 expression from the *PMP* transgene initiating in elongated spermatids. Repression and activation of the endogenous *Prm1* message during spermatogenesis is represented by the labeled gray boxes. (C) Northern blot analysis of RNA from transgenic *PMH* founder males and males derived from both the *PMH* and *PMP* transgenic lines. Both the endogenous *Msy4* mRNA (upper arrow) and the transgenic mRNAs (lower arrows) were detected with a probe prepared from a *Msy4* cDNA clone fragment. The endogenous *Msy4* mRNA levels serve as an internal loading control.

RESULTS

Expanded expression of MSY4 causes sterility

To determine if the temporal expression pattern of MSY4 is important for the translational repression and activation of developmentally regulated mRNAs, we extended its normal expression pattern in spermatogenesis. Endogenous MSY4 is detected in pachytene spermatocytes and round spermatids (Davies et al., 2000). By expressing MSY4 in later stage spermatids, and observing its effect on translational repression and activation of developmentally regulated messenger RNAs, we hoped to gain insight into the normal function of MSY4 in vivo. In order to express MSY4 in elongating and elongated

Table 1. Epididymal sperm counts of wild-type, *PMH* and *PMP* mice

Founders	Fertility	Transmission ratio (number of positives/ number of progeny)	Sperm count (number of sperm/ml)
Wild-type	Fertile	n/a	1.8×10^7 (n=4)
PMH 6293	Fertile	0/27	1.7×10^7
PMH 7520	Fertile	0/7	0.95×10^7
PMH 7531	Fertile	0/10	0.14×10^7
PMH 7651	Sterile (plugs n=2)	n/a	0.009×10^7
PMH 7666	Sterile (plugs n=2)	n/a	No sperm
PMH 8861*	Sterile (plugs n=6)	n/a	No sperm (n=6)
PMH 9501*	Sterile (plugs n=2)	n/a	0.54×10^7 (n=2)
PMP 3578	Sterile (plugs n=3)	n/a	0.021×10^7
PMP 3791*	Fertile	n/a	1.2×10^7 (n=5)
PMP 3464*	Sterile (plugs n=2)	n/a	0.062×10^7 (n=3)

n/a, no offspring born or offspring were not analyzed.
*Female founders; male progeny from these founders were analyzed.

spermatids, we constructed two gain-of-function transgenes (Fig. 1A). Both transgenes contain the *Prm1* promoter, *Prm1* 5′ untranslated region (UTR) and *Msy4* cDNA. The *PMP* transgene contains the *Prm1* 3′ UTR while the *PMH* transgene contains the *hGH* 3′ UTR. These control sequences have been previously used to drive expression of heterologous transgenes specifically in haploid spermatids (Braun et al., 1989). The presence of the *Prm1* 3′ UTR should confer *Prm1*-like translational control on the *PMP* transgene, and thus delay translation of MSY4 until the elongated spermatid stage (Fig. 1B). The presence of the *hGH* 3′ UTR in the *PMH* transgene is expected to relieve this repression and allow translation in round spermatids.

Our analysis of the *PMH* and *PMP* transgenic lines is summarized in Table 1. For the *PMH* transgene, we analyzed five transgenic founder males and the F_1 male offspring from two founder females. Two of the five *PMH* founder males were infertile and had either no sperm or very low sperm numbers by epididymal sperm counts. The remaining *PMH* founder males were fertile and had a range of sperm counts, from approximately 8% of wild-type levels (founder 7531) to wild-type amounts (founder 6293). However, although the founder males were fertile, transmission ratio distortion was observed: out of a combined 44 offspring, none carried the *PMH* transgene. Male progeny from the *PMH* 8861 line were sterile, with no detectable normal sperm by epididymal sperm counts. Male offspring of the *PMH* 9501 founder line were also sterile. Although sperm counts were 30% of wild-type, a high

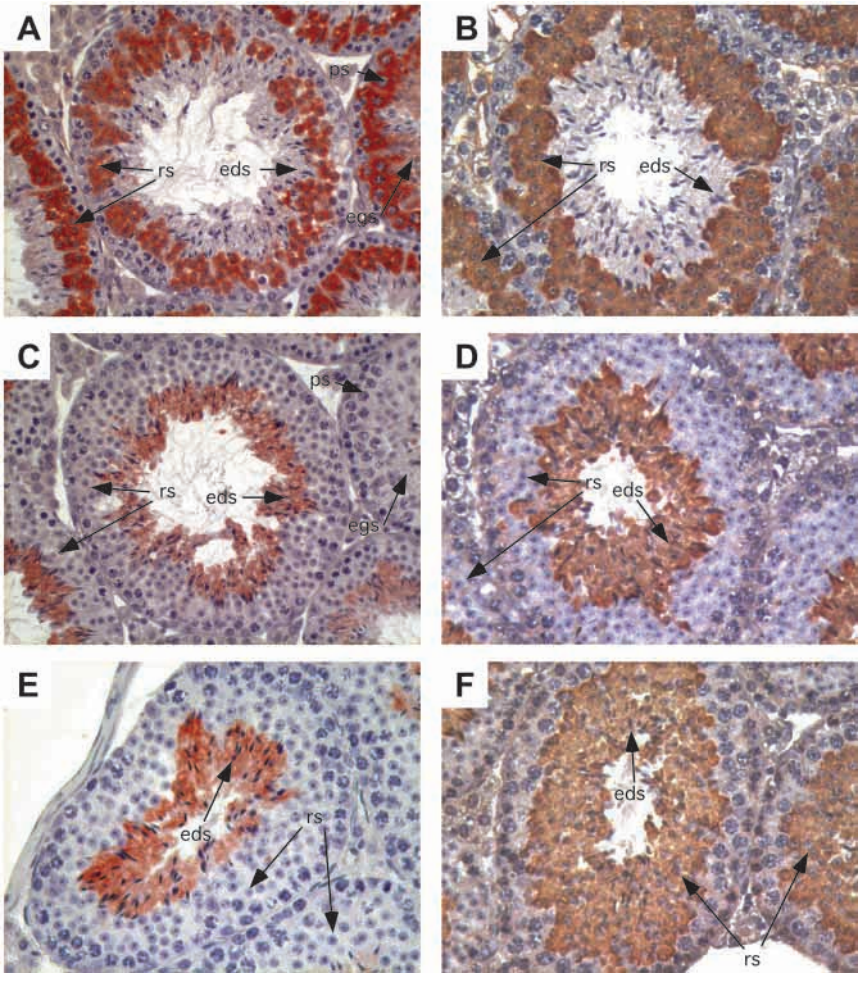

Fig. 2. Immunocytochemical detection of MSY4 and MSY4-HA. (A,C,E) Testis sections from *PMP* transgenic mice. (B,D,F) Testis sections from *PMH* transgenic mice. (A,C) Serial sections of a stage VI tubule from a *PMP* animal. (B,D) Serial sections of a stage VI tubule from a *PMH* animal. (A,B) Endogenous MSY4 detected with MSY4 antibody in pachytene spermatocytes (ps) and round spermatids (rs). The transgenic MSY4-HA proteins lack the N-terminal epitope recognized by the MSY4 antibody. (C,D) MSY4-HA detected with HA antibody in elongated spermatids (eds) of *PMP* and PMH animals, respectively. (E) Detection of MSY4-HA expression in elongated spermatids in a stage VII tubule of a *PMP* transgenic testis. Notice the absence of MSY4-HA in round spermatids. (F) Expression of MSY4-HA in round and elongated spermatids of a stage VII tubule from a *PMH* testis. Sections were counterstained with Hematoxylin.

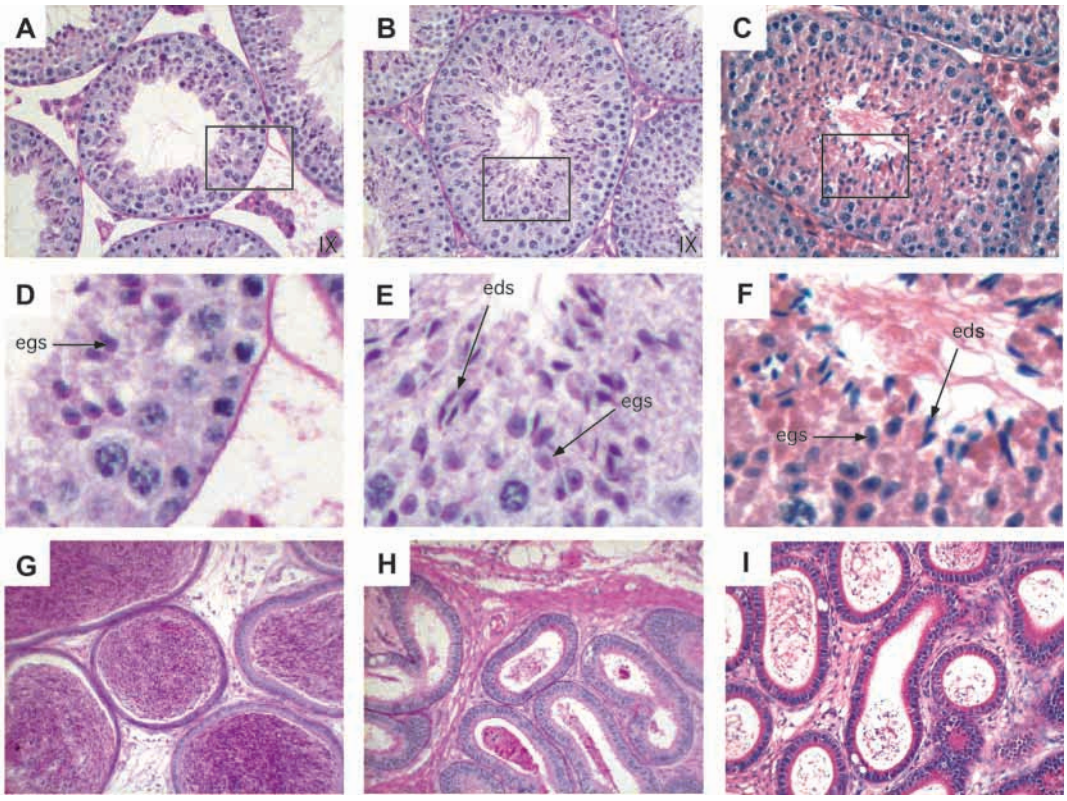

Fig. 3. Histological analysis of testes and cauda epididymides. (A-C) Sections of stage IX tubules from wild-type (A), *PMP* (B) and *PMH* (C) mice. (D-F) Enlargements of the boxed areas shown above. As expected, elongating spermatids (egs) are present in stage IX tubules from wild-type and transgenic animals. Elongated spermatids (eds) fail to release in the transgenic epithelium and are eventually engulfed by the Sertoli cells. (G-I) Cauda epididymis from wild-type (G), *PMP* (H) and *PMH* (I) mice. The sections were stained with PAS and Hematoxylin.

proportion of the sperm had abnormal morphology (described below).

The 3578 *PMP* founder male was sterile with sperm counts 1% of wild-type levels (Table 1). The male progeny from one of the two *PMP* founder females were sterile, with sperm counts less than 4% of wild-type levels. The male progeny from the second *PMP* founder female were fertile with epididymal sperm numbers at about 67% of wild-type levels.

To determine whether epididymal sperm counts and fertility correlated with transgene expression levels, we performed Northern blot analysis on RNA isolated from testes of transgenic mice (Fig. 1C). The *PMH* transgenic founder male with the lowest levels of transgenic mRNA (7520) correlated to near wild-type levels of sperm and fertility, while the highest expresser of the transgene among the *PMH* male founders (7666) had no visible sperm by epididymal sperm counts and was sterile. Male progeny of the *PMH* 9501 line expressed low levels of the transgenic mRNA and, though sterile, produced moderate levels of sperm. F_1 males from the *PMH* line generated by the 8861 founder female expressed transgenic mRNA at high levels and were sterile with no detectable sperm. Expression levels of transgenic mRNA in the *PMP* mice also correlated with sperm counts and fertility: males descended from the 3791 line expressed low levels of the transgenic mRNA, were fertile and had high sperms counts, while males of the 3464 line were infertile and oligospermic. It is clear that in both *PMH* and *PMP* transgenic males, increasing levels of transgene expression, and consequently increasing levels of MSY4, correlates with decreased sperm count and decreased fertility.

Expression of MSY4-HA

The MSY4 transgenic protein encoded by both *PMH* and *PMP* transgenes was tagged with the small hemaglutinin (HA) epitope tag on its C terminus for immunodetection. Additionally, 76 of the codons encoding the N terminus of MSY4 are deleted in this transgene. A MSY4 antibody was raised to a peptide present in the MSY4 N-terminal region, and thus specifically recognizes endogenous MSY4 and not MSY4-HA (Davies et al., 2000). Previous studies have shown that the N terminus is not necessary for the specific RNA-binding of MSY4 (Davies et al., 2000; Giorgini et al., 2001).

We performed immunocytochemistry on serial testis sections from *PMH* and *PMP* transgenic mice with antibodies to MSY4 and HA. In the *PMP* mice, normal expression of endogenous MSY4 was seen in the cytoplasm of pachytene spermatocytes and round spermatids (Fig. 2A). However, MSY4-HA was only detected in the cytoplasm of elongated spermatids, and was absent in pachytene spermatocytes and round spermatids (Fig. 2C,E). Thus, the *PMP* transgenic mRNA in under the same translational control as endogenous *Prm1* mRNA and expresses MSY4-HA in elongated spermatids. In stage VI tubules of *PMH* transgenic males, expression of endogenous MSY4 was again seen in the cytoplasm of round spermatids (Fig. 2B), while MSY4-HA was detected in the cytoplasm of elongated spermatids (Fig. 2D). However, MSY4-HA was also detected in round spermatids of stage VII tubules (Fig. 2F) demonstrating that the presence of the *hGH* 3′ UTR relieves *Prm1*-like translation control of the *PMH* transgenic mRNA. From this analysis we conclude that the two gain-of-function transgenes extend the normal temporal window of MSY4 expression as designed.

MSY4-HA disrupts spermatid differentiation

To investigate the consequences of expressing MSY4-HA in later stage spermatids, we analyzed testes from wild-type and transgenic mice using histological methods. In wild-type adult male mice, stage IX seminiferous tubules contain leptotene spermatocytes, pachytene spermatocytes, and step 9 elongating spermatids (egs) (Fig. 3A,D). Like the control, stage IX tubules from both *PMP* and *PMH* transgenic adult males contained leptotene spermatocytes, pachytene spermatocytes and step 9 elongating spermatids; however, they also contained elongated spermatids (Fig. 3B,C,E,F). Mature spermatids are normally released into the lumen in stage VIII tubules. As expected from the retained elongated spermatids in the tubules, very few spermatozoa were seen in the *PMP* cauda epididymis (Fig. 3H) or *PMH* cauda epididymis (Fig. 3I) when compared with a wild-type epididymis (Fig. 3G). The retention of elongated spermatids in transgenic tubules probably accounts for the low sperms counts seen in the *PMP* 3464, *PMH* 8861 and *PMH* 9501 lines.

Despite the presence of retained spermatids in stage IX tubules, some transgenic males contained sperm in their epididymis. To ascertain the morphology of the epididymal spermatozoa, we released sperm from the epididymis into phosphate-buffered saline and viewed them by phase contrast microscopy. The majority of epididymal sperm from wild-type mice had normal morphology, with less than 12% abnormal (Table 2). The normal sperm displayed the characteristic hook at the apex of their heads (Fig. 4A, Fig. 5A) and intact flagella with normal middle pieces (Fig. 4A). Epididymal sperm from *PMH* transgenic mice exhibited a high percentage of abnormal sperm. Line 8861 produced no normal looking sperm. Those that were produced had abnormal head morphology (Fig. 4C, Fig. 5A) and flagellum that were short and thin (Fig. 4C). Line 9501 had only 4% normal sperm, and nearly 71% of the sperm were found to have a bent head, a 14-fold increase over wild-type levels (Fig. 4D and Table 2). A fourfold increase in the number of kinked sperm was seen in these mice when compared with wild-type mice (Table 2, Fig. 4E). Additionally, a 10-fold increase in two-headed sperm was seen in *PMH* mice over wild type (Table 2, Fig. 4F). Despite sterility in the *PMP* mice, their epididymal sperm were very similar to wild type (Table 2, Fig. 5A).

To analyze chromatin compaction in wild-type and transgenic sperm heads, we performed Acridine Orange staining of sperm samples. Acridine Orange staining fluoresces from yellow to orange when chromatin is partially compacted and fluoresces green when chromatin is fully compacted (Kosower et al., 1992). When the integrity of the chromatin is highly compromised, absence of fluorescence occurs (Cho et al., 2001). Green fluorescence was observed from wild-type sperm heads (Fig. 5B, part i) as well as from morphologically normal heads from *PMP* and *PMH* samples (Fig. 5B, part iii and Fig. 5B, part iv, respectively). In our studies, morphologically abnormal heads did not fluoresce, indicating that chromatin integrity was severely disrupted. As a control, wild-type sperm were first treated with dithiothreitol to reduce disulfide bonds between cysteines in the protamines and then with 4-vinylpyridine to alkylate these cysteine residues. This treatment compromised the integrity of chromatin compaction in wild-type sperm, and produced yellow and orange fluorescence upon Acridine Orange staining (Fig. 5B, part ii).

We analyzed the nuclear basic proteins from normal wild-type testis and epididymis, as well from these tissues in *PMH* and *PMP* transgenic mice (Fig. 6). Total basic proteins from both sonication sensitive nuclei and sonication resistant spermatid nuclei were fractionated by acid-urea polyacrylamide gel electrophoresis and detected by napthol

Table 2. Abnormal epididymal sperm percentages in wild-type, *PMP*, and *PMH* mice

Morphology	Wild type*	PMP 3464 line†	PMH 9501 line‡
Normal	166/187 (88%)	140/160 (88%)	6/155 (4%)
Bent	10/187 (5%)	4/160 (3%)	110/155 (71%)
Kinked	3/187 (2%)	5/160 (3%)	12/155 (8%)
Two-headed	1/187 (0.5%)	6/160 (4%)	11/155 (7%)
Other	7/187 (4%)	5/160 (3%)	16/155 (10%)

Sperm were classified and counted using phase contrast microscopy.
*Sperm count: 1.8×10^7/ml.
†Sperm count: 0.062×10^7/ml.
‡Sperm count: 0.54×10^7/ml.

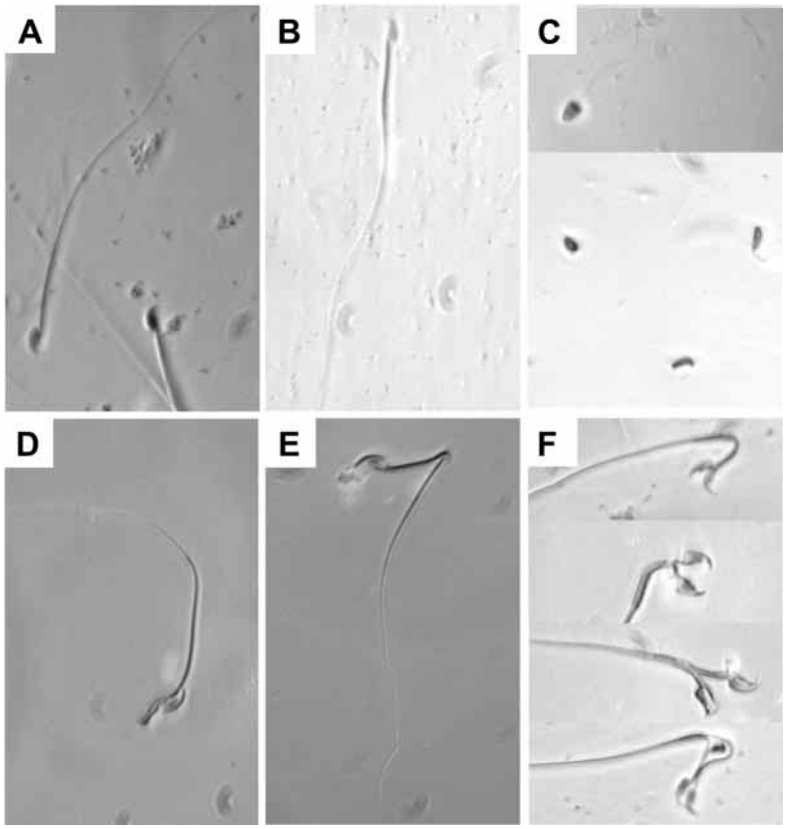

Fig. 4. Morphology of epididymal sperm. (A) Epididymal sperm from wild-type males. (B) Sperm from a male of the *PMP* 3464 line. (C) Upper and lower panels show sperm from *PMH* line 8861 males. Note abnormal head morphology and the extremely small and thin flagella. (D-F) Abnormal sperm from the *PMH* line 9501.

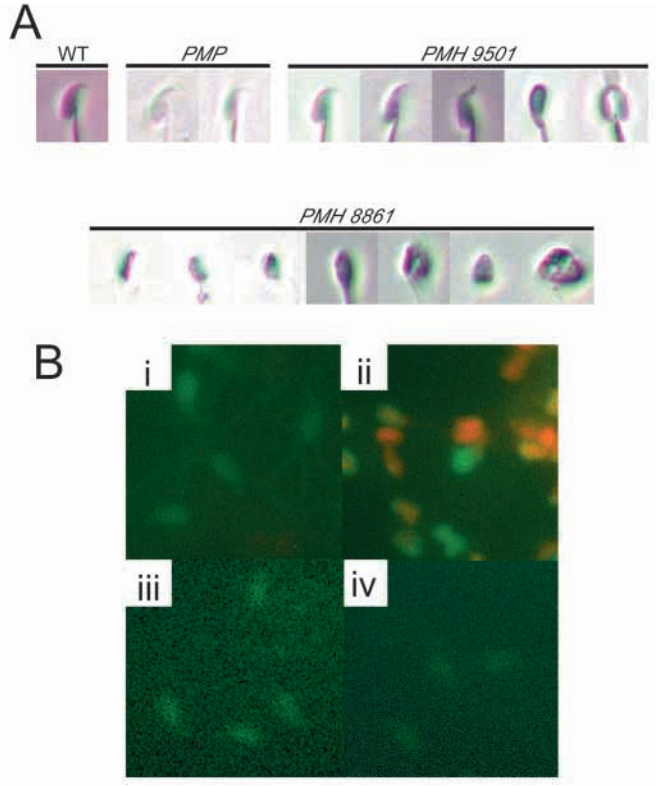

Fig. 5. Abnormal head morphology of transgenic epididymal sperm. (A) Phase-contrast microscopy of wild-type, *PMP* and *PMH* sperm heads. (B) Acridine Orange fluorescence of sperm heads. (i) Wild-type sperm, (ii) wild-type sperm treated with dithiothreitol and 4-vinylpyridine in order to disrupt chromatin integrity, (iii) sperm from the *PMP* 3464 line, and (iv) sperm from the *PMH* 9501 line. Sperm heads from *PMP* and *PMH* transgenic mice with normal morphology emitted the same green fluorescence as wild-type sperm. Abnormal sperm heads did not fluoresce.

Table 3. Presence or absence of Y-box recognition site (YRS) consensus sequences in candidate mRNAs

YRS	UCCAUCA[1]	*Gapd-s* mRNA
		UCCACCA[3]
Single	ACCAUCA[2]	UCCAUCU[4]
	CCCAUCA	AACAUCA[5]
	UACAUCA	ACCACCA[6]
	UCCACCA[3]	CCCACCA[7]
	UCCAUCC	UCCACCC[9]
	UCCAUCU[4]	UCCACCU[10]
Double	AACAUCA[5]	*Odf2* mRNA
	ACCACCA[6]	ACCAUCA[2]
	ACCAUCC	ACCACCA[6]
	ACCAUCU	
	CACAUCA	*Prm1* mRNA
	CCCACCA[7]	UCCAUCA[1]
	CCCAUCC	UACACCA[8] – two copies
	CCCAUCU	
	UACACCA[8]	*Prm2* mRNA
	UACAUCC	ACCAUCA[2]
	UACAUCU	ACCACCA[6]
	UCCACCC[9]	
	UCCACCU[10]	*Tp1* mRNA
		None
		Tp2 mRNA
		CCCACCA[7]

The left-hand column lists the YRS, and all single and double YRS nucleotide variants as defined by Giorgini et al. (Giorgini et al., 2001). The right-hand column lists YRS variants found in the candidate mRNAs. Superscript numbers on sites in the right-hand column correspond to the superscripts of possible YRS variants listed in the left-hand column. Consensus nucleotide changes to the YRS are underlined.

blue-black staining. As expected, the protamines (PRM1 and PRM2) were not seen in sonication sensitive fractions (Fig. 6, lanes 1-3 and 7-9). PRM2 is synthesized as a precursor of 106 amino acids, and through a series of proteolytic cleavages is processed to a mature size of 63 amino acids (Chauviere et al., 1991; Elsevier et al., 1991). In the sonication resistant nuclei preparations from the testes of *PMH* and *PMP* mice, processing of PRM2 to the mature 63 amino acid form did not occur (Fig. 6, lanes 4 and 5). In the epididymis, sperm nuclei from *PMH* mice lacked PRM2, but contained low levels of PRM1 (Fig. 6, lane 10). *PMP* sperm nuclei contained both PRM1 and correctly sized mature PRM2, albeit at much lower levels and at different ratios from wild type (Fig. 6, lanes 11 and 12). Thus, in both lines of mice MSY4-HA expression disrupted the normal displacement of the histones with the protamines, resulting in altered processing of PRM2 and incomplete nuclear condensation.

MSY4-HA retains sequence-specific binding in vivo

We have previously characterized a 48/50 kDa RNA-binding activity in testis extracts that contains MSY4 (Davies et al., 2000). This activity has been shown to specifically bind the YRS consensus sequence (Giorgini et al., 2001). In order to show that transgenic MSY4-HA protein retained its binding specificity, electrophoretic mobility shift assays (EMSAs) were performed (Fig. 7). Testis extracts from wild-type, *PMH*, and *PMP* mice were incubated with either a radiolabeled portion of the wild-type *Prm1* 3′ UTR (wt YRS) or a radiolabeled mutant version of this RNA (C26A). We have previously shown that the C26A point mutation disrupts binding by MSY4 (Giorgini et al., 2001). These binding complexes were resolved by non-denaturing polyacrylamide gel electrophoresis (PAGE). Extracts from the testis of all three strains produced an EMSA complex with wt YRS RNA (indicated by the lowest arrow in Fig. 7). Formation of this complex was completely disrupted by C26A RNA in both *PMH* and *PMP* testis extracts (Fig. 7, lanes 9 and 15). HA antibody supershifted a region of the complexes formed by wt YRS and either PMH or PMP testis extracts (Fig. 7, lanes 7 and 13, middle arrow), while failing to supershift the complex containing wt YRS in wild-type extracts (Fig. 7, lane 3). These results indicate that MSY4-HA can specifically bind the YRS. MSY4 antibody, which specifically recognizes endogenous MSY4, also supershifted a portion of the EMSA complex in PMH and PMP extracts (Fig. 7, lanes 6 and 12, upper arrow). Double-supershift experiments using both MSY4 and HA antibody (Fig. 7, lanes 8 and 14) did not produce double-supershift complexes, indicating either that endogenous MSY4 and transgenic MSY4-HA do not bind the same RNA molecule or that protein binding by one antibody excludes protein binding by the other antibody.

Altered translational regulation in PMH mice

If MSY4-HA plays a role in translational repression one would

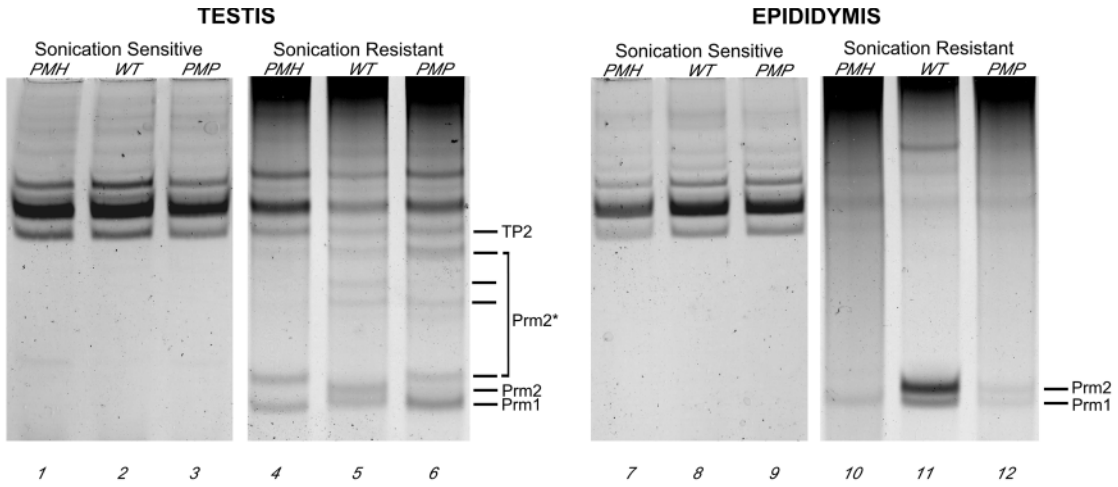

Fig. 6. Analysis of total testis basic proteins from sonication sensitive (SS) and sonication resistant (SR) spermatid nuclei from wild-type and transgenic animals. Total testis basic protein preparations were fractionated by urea-gel PAGE and detected with napthol blue-black staining. Though the total yield of protein was reduced in *PMH* and *PMP* basic protein preparations, an equivalent amount of protein was loaded in each lane (15 μg). The left-hand panel contains samples prepared from testis tissue, while the right-hand samples were prepared from epididymal tissue. Wild-type controls are shown in lanes 2, 5, 8 and 11. Lanes 1, 4, 7 and 10 contain protein isolated from *PMH* mice, while lanes 3, 6, 9 and 12 contain protein isolated from *PMP* mice. The positions of transition protein 2 (TP2), protamine 1 (PRM1) and mature protamine 2 (PRM2) are labeled. The PRM2 precursor, the partially processed forms of PRM2 and an abnormally processed form of PRM2 are all labeled by PRM2*.

expect to find MSY4-HA associated with translationally inert messenger ribonucleoprotein particles (mRNPs). Sucrose gradient sedimentation coupled with western analysis was used to evaluate the polysome distribution of MSY4 and MSY4-HA in transgenic testis extracts (Fig. 8). Testis extract was fractionated on a sucrose gradient while monitoring the optical density of the eluant. The optical density profile (top panel) shows noticeable peaks of ribosomal subunits, monosomes, disomes, trisomes and higher molecular weight polysomes. Transgenic MSY4-HA and endogenous MSY4 sedimented in the mRNP fraction suggesting that both proteins are complexed with repressed mRNAs.

Translationally regulated proteins expressed late in spermatogenesis were analyzed by immunocytochemistry of adult testis sections from wild-type and PMH animals to determine if expression of these proteins is perturbed by MSY4-HA expression in round and elongated spermatids (Fig. 9). In addition to the basic nuclear proteins TP1, TP2, PRM1 and PRM2, we analyzed expression of testis-specific glyceraldehyde 3-phosphate dehydrogenase (GAPD-S) and outer dense fiber protein 2 (ODF2). GAPD-S probably plays a role in regulation of glycolysis during spermatogenesis and regulation of energy production for sperm motility (Cooper, 1984; Fraser and Quinn, 1981; Jones, 1978; Mohri et al., 1975). ODF2 is one of three outer dense fiber proteins that assemble as nine fibers surrounding the axoneme of the sperm tail (reviewed by Kierszenbaum, 2002). All six of these proteins have been shown to be under translational control during murine spermatogenesis (Balhorn et al., 1984; Bunch et al., 1998; Kleene et al., 1984; Kleene and Flynn, 1987; Yelick et al., 1989). Translation of five of the six markers was dramatically reduced in PMH transgenic mice when compared with wild-type mice (Fig. 9). Only the expression of TP1 seemed unaffected in PMH mice (Fig. 8I,J). To determine if this specificity of MSY4-HA dependent repression was due to the presence of YRS consensus sites in the repressed messages, we searched the published full-length mRNA sequences for these MSY4 binding sites (Table 3). In addition to scanning the

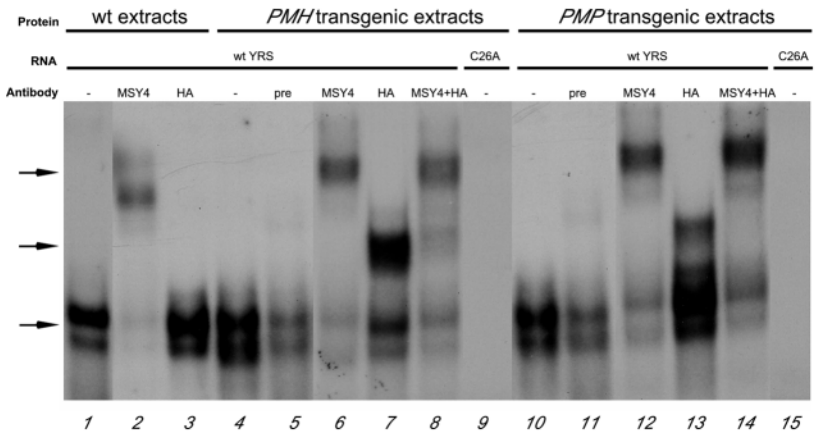

Fig. 7. Electrophoretic mobility shift assay analysis of wild-type and transgenic testis extracts. Lanes 1-3, wild-type testis extracts; lanes 4-9, *PMH* extracts; and lanes 10-15, *PMP* extracts. RNA containing the wild-type YRS (wt YRS) was used in lanes 1-8 and lanes 10-14. RNA containing a YRS mutation (C26A) that disrupts MSY4 binding (Giorgini et al., 2001) was used in lanes (9) and (15). Supershifts of the EMSA complex were carried out using either MSY4 antibody (lanes 2, 6, 12), HA antibody (lanes 3, 7, 13) or both (lanes 8 and 14). As a control, pre-immune sera was added to reactions in lanes 5 and 11. No sera was added to reactions in lanes 1, 4 and 10.

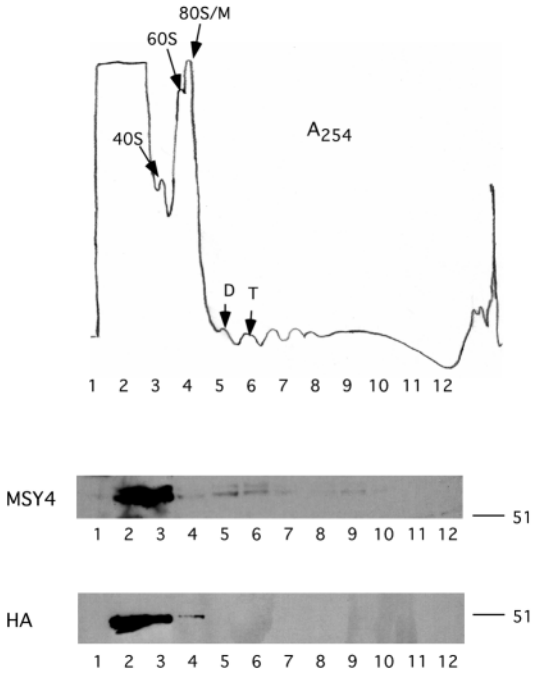

Fig. 8. Distribution of endogenous MSY4 and transgenic MSY4-HA in a sucrose gradient. Testis extract was sedimented on a 15% sucrose (w/w) gradient, the absorbance at 254 nm was recorded and 12 fractions collected. Sample 1 is the top of the gradient. Prominent peaks are labeled on the A_{254} graph. Single ribosome subunits are labeled 40S and 60S, monosomes as 80S/M, disomes as D and trisomes as T. Protein from each fraction was run on a 9% SDS denaturing gel, transferred to nitrocellulose and probed with MSY4 antibody (upper panel) and then HA antibody (lower panel). The location of the 51 kDa standard is indicated on the right.

sequences for the canonical YRS site found in the Prm1 3′UTR, we searched for all single and double variations of the defined consensus nucleotides (Table 3). Interestingly, the Tnp1 mRNA is the only one of the six sequences analyzed lacking a YRS consensus site. Thus, a strong correlation can be drawn between presence of a YRS site in an mRNA and MSY4-dependent repression of that message in vivo.

DISCUSSION

In this study, we have analyzed two gain-of-function transgenes. Using the *Prm1* promoter and different 3′ UTRs, we extended the temporal window in which MSY4 protein is normally expressed. Both transgenes severely disrupted late-stage spermatid differentiation and caused dominant sterility. Biochemical and immunocytochemical analyses suggest the primary cause of sterility was a failure to execute the normal developmental program of translational activation. These studies provide strong support for MSY4 as a translational regulator in spermatids.

PMH and *PMP* transgenic mice exhibit severe oligospermia. The most striking of the abnormalities seen in these mice is the large number of two-headed sperm. Approximately 7% of epididymal sperm from *PMH* 9501 mice were double-headed, compared with 0.5% in wild-type mice. Sperm from the *PMP* 3464 line exhibited approximately 4% two-headed sperm. High incidence of double-headed sperm have been previously characterized in heterozygous *In(5)9Rk* mice (Hugenholtz and Bruce, 1979). These mice carry one copy of a long paracentric inversion in chromosome 5, and were found to produce between 6.0 and 8.8% double-sized sperm (double-headed or with a double-sized head) dependent on genetic background. It is thought that the dicentric anaphase bridge of paracentric inversion heterozygotes impedes cytokinesis and causes double-sized sperm. It is possible that the increase in two-headed sperm in *PMH* and *PMP* mice is due to reduced synthesis of flagellar components and the failure to complete the process of sperm individualization that occurs during spermatid differentiation.

Mice lacking the casein kinase II α2 catalytic subunit, the *Csnk2a2* gene product, exhibit round-headed sperm (globozoospermia) similar to that in both the *PMP* 3464 and *PMP*8861 lines of mice (Xu et al., 1999). In addition, the epididymal sperm occasionally have bent and kinked flagella. Similar head abnormalities have been seen in human familial globozoospermia, making *Csnk2a2* a candidate gene for the syndrome. As *Csnk2a2* is translated during spermiogenesis, it is possible that the globozoospermia seen in *PMP* and *PMH* mice is due to repression of this message by MSY4-HA. Haploinsufficiency of either protamine 1 or protamine 2 in mice causes infertility due to disruption of spermatid nuclear formation (Cho et al., 2001). Sperm from these mice exhibit various abnormalities in head structure, including heads with a rounded appearance. Additionally, an infertile individual with 76-99% round-headed sperm in his ejaculate also expresses reduced amounts of protamine 1 and 2 (Carrell et al., 1999), suggesting that the repression of protamine translation in elongating and elongated spermatids of the *PMP* and *PMH* transgenic mice could contribute to the observed head defects.

Analysis of the spermatid nuclear basic proteins in the *PMH* and *PMP* transgenic mice demonstrated that these animals are defective in processing PRM2. Defects in PRM2 processing have been seen in mice deficient for either TP1 or TP2 (Yu et al., 2000; Zhao et al., 2001), in mice haploinsufficient for *Prm1* or *Prm2* (Cho et al., 2001), and mice that pre-maturely express PRM1 in round spermatids (Lee et al., 1995). Perturbation in the levels of these basic proteins in the spermatid nucleus could lead to an alteration in chromatin packaging required for normal PRM2 processing. This model would explain the processing defects seen in *PMH* and *PMP* mice, as three of these proteins exhibit reduced levels in these mice.

Expression of MSY4-HA in round spermatids produced a more severe phenotype than its expression in elongated spermatids. When comparing high expression lines, *PMH* mice had lower epididymal sperm counts, and the few sperm found had severely abnormal head and tail morphologies. In addition, the nuclear basic protein preparations described herein show that *PMP* epididymal sperm contain both mature PRM2 and PRM1, whereas *PMH* preparations contain only PRM1. Finally, *PMH* mice exhibit stronger repression of marker protein translation during spermiogenesis when compared with *PMP* mice (data not shown). The increased severity of the *PMH* phenotype is presumably due to its early expression in round spermatids and more severe effects on protein synthesis.

Transgenic experiments indicate that the YRS probably plays a role in *Prm1* translational control. Deletion mapping of the *Prm1* 3′ UTR has shown that the first 37 nucleotides of the

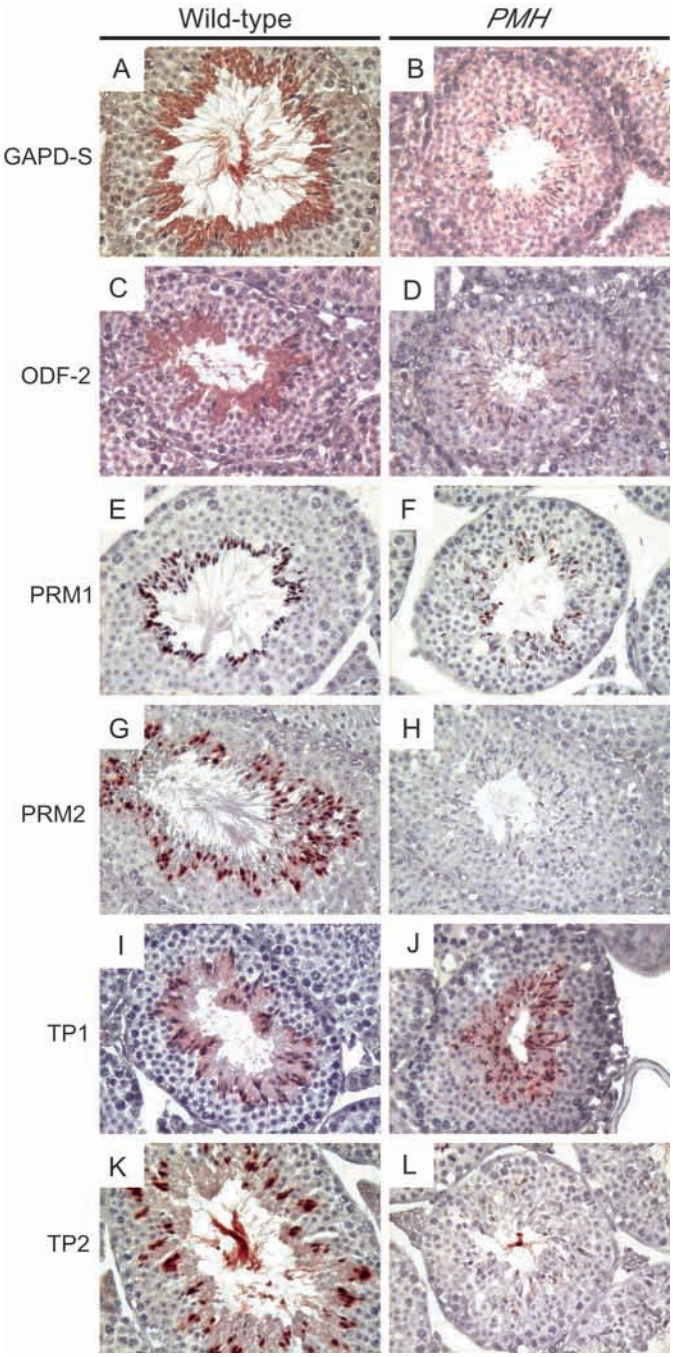

Fig. 9. Expression analysis of candidate proteins in wild-type and *PMH* transgenic mice by immunocytochemistry. (A,B) GAPD-S expression in a wild-type (A) and a *PMH* (B) stage 1 tubule detected with GAPD-S antibody. (C,D) ODF2 expression in a wild-type (C) and a *PMH* (D) stage 1 tubule detected with ODF2 antibody. (E,F) PRM1 expression in a wild-type (E) and a *PMH* (F) stage VII tubule detected with HUP1N antibody. (G,H) PRM2 expression in a wild-type (G) and a *PMH* (H) stage V tubule detected with HUP2B antibody. (I,J) TP1 expression in a wild-type (I) and a *PMH* (J) stage III tubule detected with TP1 antibody. (K,L) TP2 expression in a wild-type (K) and a *PMH* (L) stage 1 tubule detected with TP2 antibody. Sections were counterstained with Hematoxylin.

3′ UTR are sufficient to confer *Prm1*-like translational control on a reporter gene (Fajardo et al., 1997). A consensus YRS site maps to this region and mutation of this site relieves translational repression (Giorgini et al., 2001). Our data strongly suggest that the function of the YRS is mediated by the binding of MSY4 and probably the other major Y box protein MSY2. A second site within the *Prm1* 3′ UTR also appears to be involved in this translational control. This site, known as the translational control element (TCE), is found in the 3′ end of the *Prm1* 3′ UTR and is both necessary and sufficient for protamine-like translational control of a transgene (Zhong et al., 2001). A trans factor for the TCE has not yet been identified.

The YRS consensus sequences are likely to appear very frequently in the genome. One of the consensus sites appear randomly approximately once every 455 nucleotides. Thus, almost every mRNA would be predicted to contain a YRS consensus site. Does MSY4 actually bind all mRNAs containing one of these sites in vivo? Perhaps, but it is more likely that additional factors are involved in selection of mRNA targets by MSY4. It is possible that proteins which bind other regulatory sequences, such as the TCE in the *Prm1* 3′ UTR (Zhong et al., 2001), or the other murine Y-box proteins expressed in spermatids, MSY1 and MSY2, aid in target specificity. It is also possible that the number or position of YRS sites within the targeted mRNA is important for MSY4-dependent repression. We have seen that the presence of two adjacent copies of the YRS in an RNA dramatically increases EMSA binding by MSY2 and MSY4 (F. G. and R. E. B., unpublished). Proximity of nascent transcripts to Y-box DNA elements in the nucleus could also aid in selection of mRNA targets. Both the *Prm1* and *Prm2* promoters contain Y-box DNA elements, and MSY2 has been shown to interact with the *Prm2* promoter (Johnson et al., 1988; Nikolajczyk et al., 1995). Thus, it is possible that MSY4 first binds a Y-box DNA element in the *Prm1* promoter and then binds the YRS site in the 3′ UTR of the message, and that this complex is exported from the nucleus to the cytoplasm.

Our analysis of six different mRNAs showed that translation of five of these mRNAs was suppressed in *PMP* and *PMH* mice. Provocatively, the mRNAs that were affected all contained at least one YRS-binding site. The one transcript that was not affected, *Tnp1*, did not contain a YRS site. These data, as well as extensive three-hybrid and EMSA analysis of all possible single nucleotide sequence variants of the YRS (Davies et al., 2000; Giorgini et al., 2001), support our in vivo transgenic data that MSY4 binds specific RNAs via the YRS. In previously published studies (Davies et al., 2000), we found that several mRNAs co-precipitated with MSY4. A retrospective analysis of these mRNAs indicated that all contained at least one YRS, suggesting that these results may describe physiological interactions. However, one of these mRNAs, actin, is not known to be under translational control during spermatogenesis. This suggests that there are likely to be other factors that work together with MSY4 and MSY2 to mediate selective translational repression during spermatogenesis.

The mechanism by which MSY4 contributes to translational repression remains unknown. MSY4 could inhibit an early step in translational initiation by masking the entire mRNA, or alternatively, MSY4 (or proteins that interact with MSY4) could directly interfere with translation initiation through a

nonlinear mechanism that involves an interaction between the two ends of the message. Further identification of the proteins contained in the ribonucleoprotein particle should help distinguish between these two possibilities.

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