BIOLOGY OF REPRODUCTION **59,** 1266–1274 (1998)

Mammalian Male and Female Germ Cells Express a Germ Cell-Specific Y-Box Protein, MSY2[1]

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ABSTRACT

Here we report the isolation and characterization of mouse testicular cDNAs encoding the mammalian homologue of the *Xenopus* germ cell-specific nucleic acid-binding protein FRGY2 (mRNP3+4), hereafter designated MSY2. MSY2 is a member of the Y box multigene family of proteins; it contains the cold shock domain that is highly conserved among all Y box proteins and four basic/aromatic islands that are closely related to the other known germline Y box proteins from *Xenopus*, FRGY2, and goldfish, GFYP2. *Msy2* undergoes alternative splicing to yield alternate N-terminal regions upstream of the cold shock domain. Although MSY2 is a member of a large family of nucleic acid-binding proteins, Southern blotting detects only a limited number of genomic DNA fragments, suggesting that *Msy2* is a single copy gene. By Northern blotting and immunoblotting, MSY2 appears to be a germ cell-specific protein in the testis. Analysis of *Msy2* mRNA expression in prepubertal and adult mouse testes, and in isolated populations of germ cells, reveals maximal expression in postmeiotic round spermatids, a cell type with abundant amounts of stored messenger ribonucleoproteins. In the ovary, MSY2 is present exclusively in diplotene-stage and mature oocytes. MSY2 is maternally inherited in the one-cell-stage embryo but is not detected in the late two-cell-stage embryo. This loss of MSY2 is coincident with the bulk degradation of maternal mRNAs in the two-cell embryo.

INTRODUCTION

Translational control represents a major mechanism of gene regulation in germ cell differentiation and early embryogenesis that is widely used by both vertebrates and invertebrates. Meiotic progression of oocytes to eggs and early embryogenesis depends on the translational control of maternally masked mRNAs that become recruited for translation during maturation of the egg, or subsequentially in the embryo before the accumulation of embryonic transcripts [1]. The differentiation of male gametes requires the expression of many germ cell-specific proteins at specific stages of development [2, 3]. Although the expression of many genes during spermatogenesis is regulated at the level of transcription [4], during the later phases of spermatogenesis, transcription ceases and stored mRNAs become activated to synthesize proteins such as the protamines and transition proteins.

Although little is known about the mechanisms inactivating and activating ''paternal'' mRNAs in germ cells, a number of RNA-binding proteins (RBPs) likely to be involved in mRNA translational control have been identified in the mammalian testis. Testis/brain (TB)-RBP, the mouse homologue of the human single-stranded DNA-binding protein translin, binds to the 3′ untranslated regions (UTRs) of many testicular mRNAs, serving as a repressor of translation in in vitro assays [5, 6]. Other proteins that bind to the 3′ UTRs of testicular mRNAs include two proteins of 53 kDa and 55 kDa that bind to specific 22- and 20-nucleotide (nt) sequences in the 3′ UTRs of protamine mRNAs [7], another protein with properties of a translational inhibitor of the protamines [8], and SPNR, a 71-kDa protein that localizes to cytoplasmic microtubules of the testis [9].

Multiple poly(A) binding proteins are also expressed in the testis [10], one of which, a 70-kDa protein, binds to both translated and stored mRNAs in male germ cells [11]. Another germ cell-specific RNA-binding protein, SOD-RBP, binds to the 5′ UTR of a testis-specific SOD-1 mRNA and can represses SOD-1 mRNA translation in vitro [12]. Mammalian homologues of the *Xenopus* germ cell-specific Y-box binding proteins are also abundant in rodent testes [13, 14]. Thus, a growing number of testicular RNA-binding proteins have been identified and are likely to facilitate the repression and activation of mRNA translation in male germ cells.

The vertebrate Y box proteins represent a family of nucleic acid-binding proteins containing an ~100 amino acid domain termed the cold shock domain (CSD), which contains sequences that are over 40% identical to the small bacterial cold shock proteins [15, 16] and binds single-stranded nucleic acids by their ribonucleoprotein (RNP)-1 and RNP-2-like motifs [17, 18]. The bacterial cold shock proteins act as transcription factors [19], a property conserved in the Y box proteins that bind the specialized CCAAT box elements (the Y box CTGATTGG^{C}/T^{C}/TAA) to either promote or repress transcription of a variety of genes [20–23]. The bacterial cold shock proteins also act as RNA chaperones, destabilizing RNA secondary structure [24]. In addition to the CSD, all vertebrate Y box proteins contain in their C-terminal region four basic/aromatic (B/A) islands that bind RNA [25, 26]. In *Xenopus* oocytes, the Y box protein FRGY2 (mRNP3+4) is abundant and found in cytoplasmic translationally repressed maternal mRNPs [27]. Immunologically related proteins have been identified in both the nuclei and cytoplasm of mammalian testes [13, 22, 23, 28, 29].

Many Y-box proteins have been cloned, including mouse MSY1, a protein sharing 85% identity with *Xenopus*

Accepted July 10, 1998.
Received May 28, 1998.
[1]Supported by NIH grant HD 29125 (N.B.H.), NSF grant MCB-9513751 (M.T.M.), and NIH grant CA 62392 (J.J.E.).
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FRGY1 [14]. MSY1 is most highly expressed in the testis but is restricted to neither the testis nor the germ cell [14]. No homologue of *Xenopus* FRGY2 had previously been isolated, leading to speculation that a direct mammalian homologue of FRGY2 may not exist. Here, we report the isolation of cDNAs encoding a germ cell-specific mouse testicular homologue of *Xenopus* FRGY2, which we name MSY2. We demonstrate that MSY2, like its *Xenopus* counterpart, is expressed in both male and female germ cells and is maternally inherited in the early embryo. However, unlike its *Xenopus* counterpart, MSY2 undergoes bulk degradation in the two-cell-stage embryo that is coincident with the loss of maternal mRNAs. In the testis, *Msy2* is alternatively spliced, giving rise to polypeptides that differ in the amino-terminal region upstream of the CSD. In addition to stimulating the transcription of testis-specific genes [30], the abundant cytoplasmic localization and temporal expression pattern of MSY2 suggests that it functions in the storage and translation of mRNAs in both male and female mammalian germ cells.

MATERIALS AND METHODS

Isolation and Sequence Analysis of cDNA and Genomic Clones of Mouse MSY2

A lambda gt 11 CD-1 mouse testis cDNA expression library (Clontech, Palo Alto, CA) was screened using antibodies to *Xenopus* mRNP3+4 ([22, 27, 31]; mRNP4 is identical to FRGY2) at a 1:5000 dilution. Complementary DNA clones encoding MSY2 were detected with horseradish peroxidase-conjugated secondary antibodies with diaminobenzidine (DAB)-H_2O_2 as substrate. To obtain the complete open reading frame, a mouse testis lambda Zap II cDNA library (kindly provided by Dr. E.M. Eddy, NIEHS, Research Triangle Park, NC) and a mouse genomic DNA library (Stratagene, La Jolla, CA) were screened with the *Msy2* cDNAs. The nucleotide sequences of the *Msy2* cDNAs and genomic DNA were analyzed using the GCG-Unix program (Genetics Computer Group, Madison, WI) and the Basic Local Alignment Search Tool (BLAST) from the National Center for Biological Information.

The 5′ RACE (rapid amplification of cDNA ends) system (Life Technologies Inc., Gaithersburg, MD) was employed according to the manufacturer's specifications. Oligonucleotide primers located either in the 3′ UTR (nt 1222–1204 and nt 1157–1139 of *Msy2*) or the CSD (nt 521–503 and nt 319–297) were used for reverse transcription and amplification using ELONGase (Life Technologies Inc.), and the products were cloned into the pCR 2.1 TA vector (Invitrogen Corp., Carlsbad, CA). The longest clones were selected by colony polymerase chain reaction (PCR) and found to contain two alternative 5′ ends. Clones containing each of these 5′ ends were isolated from 5′ RACE performed with both primer sets. In addition, multiple clones derived from unprocessed pre-mRNAs were isolated, thereby providing definitive assignment of the alternate splice site.

Southern Blot Analysis

Aliquots of high-molecular weight genomic DNA (10 μg), prepared from spleens of CD-1 mice, were digested with the restriction enzymes *Bam*HI, *Eco*RI, *Hin*dIII, or *Sac*I. After electrophoresis on 0.7% agarose gels, the digested DNA fragments were transferred to nylon membranes (NEN; Dupont, Boston, MA) and hybridized to a ^{32}P-labeled mouse *Msy2* cDNA (nt 253–1533).

Isolation of Meiotic and Postmeiotic Germ Cells

Germ cell suspensions were prepared from 10 adult CD-1 mice by enzymatic dissociation of 10 pairs of testes with collagenase (1 mg/ml) at 34°C for 12 min, followed by digestion with trypsin (0.5 mg/ml) for 10 min at 34°C. The cell suspensions were fractionated by unit gravity sedimentation on a BSA gradient as previously described [11]. The purity of the enriched populations of pachytene spermatocytes, round spermatids, and elongated spermatids, determined by Nomarski microscopy, was between 85% and 90%.

RNA Isolation and Northern Blotting

Total RNA was isolated from prepubertal and adult testes of CD-1 mice, from enriched populations of meiotic and postmeiotic germ cells, and from brain, spleen, liver, heart, and kidney as previously described [32]. Northern blot hybridization was performed as described by Gu et al. [11] with a 1533-nt *Msy2* cDNA.

Preparation of Tissue Extracts and Western Blot Analysis

Protein extracts from the testes, liver, brain, and kidneys of CD-1 mice were prepared as previously described [33]. For immunoblots, aliquots of protein extracts were resolved in 12% SDS-PAGE and transferred to polyvinylidene fluoride (PVDF) membranes. The membranes, after preincubation in 20 mM Tris-buffered saline (pH 7.5) containing 1% BSA and 0.05% Tween-20 (TTBS), were incubated with polyclonal antibodies against *Xenopus* mRNP3+4 in TTBS at a dilution of 1:20 000. MSY2 was detected using an enhanced chemiluminescence (ECL) Western blotting detection system (Amersham, Chicago, IL) according to the manufacturer's instructions.

Immunocytochemistry

The ovaries of 22-day-old C57BL/6J mice were fixed in Bouin's fixative. In addition, the ovaries of fetal mice were fixed on Days 16, 17, and 18 postcoitum (pc). Sections were prepared for standard double-antibody immunocytochemistry with peroxidase-conjugated antibody as the second antibody. The primary antibody against *Xenopus* mRNP3+4 was diluted 1:20 000 before application.

RESULTS

Sequence Analysis of MSY2 Established it as a Member of the Y-Box Family of Proteins

Initial attempts to isolate a murine homologue of FRGY2 by nucleic acid screening with FRGY2 failed; thus antibodies against *Xenopus* mRNP3+4 (FRGY2a+b) were used to isolate cDNAs from a mouse testis lambda gt11 expression library. The initial screening yielded a 900-base pair cDNA that was used to rescreen a second mouse testis cDNA library and a mouse genomic library. Overlapping cDNA clones extended the 5′ sequence of the initial clone. Analysis of the genomic clone and 5′ RACE products allowed final determination of the complete open reading frame of MSY2. The final sequence was derived from multiple PCR products compared to the genomic sequence and is shown in Figure 1A.

5′ RACE yielded clones of MSY2 and an alternatively spliced form, MSY2a, that differs from MSY2 in its amino terminus upstream of the CSD (Fig. 1B). MSY2 is derived

A.

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1    atgagcgaggcggaggcgtcagtggtggcgacagcggcccccgcggcgacggtgcccgcg  60
       M  S* E  A  E  A  S  V  V  A  T  A  A  P  A  A  T  V  P  A

61   acggcggcgggggtggtagcggtggtggtaccggtgcccgcgggagagccgcagaaagca  120
       T  A  A  G  V  V  A  V  V  V  P  V  P  A  G  E  P  Q  K  A

121  ggcggcggggcgggcggcgggggcggagccgcctctggccccgctgctgggacccccctc  180
       G  G  G  A  G  G  G  G  G  A  A  S  G  P  A  A  G  T  P  L

181  cacgcgcccggccctcgcacccctggcaaccaggcgacggcggcctcggggactcccgcg  240
       H  A  P  G  P  R  T  P  G  N  Q  A  T  A  A  S  G  T  P  A

241  ccccggcccggagtcaggcggacaagccggtgctggcaatccaagtcctgggcacagtc   300
       P  P  A  R  S* Q  A  D  K  P  V  L  A  I  Q  V  L  G →T  V

301  aaatggttcaacgtccggaatggttacggattcatcaacaggaatgacaccaaggaggat  360
       K  W  F  N  V  R  N  G  Y  G  F  I  N  R  N  D  T* K  E  D

361  gtctttgttcaccagacagctattaagagaaacaacccccaggaagtttctgcggagtgtt  420
       V  F  V  H  Q  T  A  I  K  R  N  N  P  R  K  F  L  R  S* V

421  ggagatggggagactgtggagtttgatgtcgtggaaggagagaagggtgcaagagctgct  480
       G  D  G  E  T  V  E  F  D  V  V  E  G  E  K  G  A  R  A  A

481  aatgtcactgggcctgggggggtaccagtgaaggggagccgctatgcccctaatcgacgt  540
       N  V  T  G  P  G  G  V  P  V  K  G  S  R  Y  A  P  N  R  R

541  aggttccggaggttcattcctcggcctcgcccagctgccccaccacctatggtggcagag  600
       R  F  R  R  F  I  P  R  P  R  P  A  A  P  P  P  M  V  A  E

601  gctccctcaggtgggacagaacctggcagcgaaggggagcgggcagaggactcggggcag  660
       A  P  S  G  G  T  E  P  G  S* E  G  E  R  A  E  D  S  G  Q

661  cgacctagacgacggcgcccaccaccctcttctatcgaaggcggtttgtgcgaggccct  720
       R  P  R  R  R  R  P  P  P  F  F  Y  R  R  R  F  V  R  G  P

721  cggccccccaaccagcaacagcccatagagggctctgatggggtagaacccaaggagaca  780
       R  P  P  N  Q  Q  Q  P  I  E  G  S  D  G  V  E  P  K  E  T

781  gcaccattggagggtgatcaacagcagggagatgaacgggtgcccccacccagattccgg  840
       A  P  L  E  G  D  Q  Q  Q  G  D  E  R  V  P  P  P  R  F  R

841  cccaggtaccgaaggcctttccgccccaggccaccccaacagcctaccacagaaggtggg  900
       P  R  Y  R  R  P  F  R  P  R  P  P  Q  Q  P  T  T  E  G  G

901  gatggagagaccaagcccagccaaggccccactgatggctcccggcctgagccccagcgt  961
       D  G  E  T  K  P  S  Q  G  P  T  D  G  S* R  P  E  P  Q  R

961  ccacgaaaccgtccctacttccagcggcgtcggcagcagccccctggccccccggcagcct 1020
       P  R  N  R  P  Y  F  Q  R  R  R  Q  Q  P  P  G  P  R  Q  P

1021 atagccgcagagacctccgcgcctatcaacagtggggacccccccaccaccatactggag 1080
       I  A  A  E  T  S  A  P  I  N  S  G  D  P  P  T  T* I  L  E

1081 tgattccaactcagaggacacccagagctaccatctggtatctgccagtttccaactgac 1140

1141 tctacccagcatcccctcccccacttttcccataattcatgacatcaaaacatcggctttt 1200
1201 cccttgagactcaggagggccaaagcaccagcctttggctttttctctttttttccct  1260
1261 ctcccctagcatgggttgaaggaagggatccatccttattgttccgaggcatcacctccc 1320
1321 tccctagtcaggctgagaaggaaccagccagctcatacctcctcctgattgttttcttc  1380
1381 cccacccatttatttttttgtttcccttcgacctcccccccaacacctctgaagccatttta 1440
1441 tgaactgtcatgtgccacctgagcctccagtaaaaacaaaagcaggcttaaaaaaaaaaa  1500
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B.

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1    gtaggcctggccccgaaggaacttattcataacttagtccccggagctgccgaataaact  60

61   atttagtggattccgctggagggaaatcttgccgcaccttcatgagccggagggcggggc  120
                                                  M  S  R  R  A  G

121  aggcaggttcagctaaggctttggcaatccaagtcctgggcacagtcaaatggttcaacg  181
       Q  A  G  S  A  K  A  L  A  I  Q  V  L  G  T  V  K  W  F  N
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FIG. 1. **A**) Nucleotide and predicted amino acid sequence of mouse MSY2. The conserved CSD is bold. Putative nuclear localization signals are underlined. A putative poly adenylation signal is double-underlined. Potential casein kinase 2 phosphorylation sites are marked with an asterisk, and a protein kinase C site is maraked with an arrow. The nucleotide sequence of the alternatively spliced region of MSY2a is shown along with its predicted amino terminal end. **B**) The nucleotide sequence of the alternative 5′ end is underlined, and the amino acid sequence of the conserved residues of the CSD are shown in bold. The sequence data of MSY2 and MSY2a are available from GenBank/EMBL/DDBJ under accession numbers AFO73954 and AFO73955, respectively.

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MSY2   MSEAEASVVA ..(58aa).. QATAASGTPA PPARSQADKP VLAIQVLGTV
MSY2a                              MS RRAGQAGSAK ALAIQVLGTV
FRGY2  MSEAEAQEPE ....PVPQPE SEPEIQKPGI AAARNQANKK VLATQVQGTV
GFYP2                           MSDIE ASEQPQPERK VIATGVEGTV
MSY1   MSSEAETQQP PA.(19aa). AGSGGPGGLT SAAPAGGDKK VIATKVLGTV
FRGY1  MSSEVETQQQ QPD..... ALEG  KAGQ EPAATVGDKK VIATKVLGTV
GFYP1  MSSEAETQQP PQPA..... ADAESPSSPS SPAATAGDKK VIATKVLGTV

MSY2   KWFNVRNGYG FINRNDTKED VFVHQTAIKR NNPRKFLRSV GDGETVEFDV
FRGY2  KWFNVRNGYG FINRNDTKED VFVHQTAIKK NNPRKFLRSV GDGETVEFDV
GFYP2  KWFNVRNGYG FINRNDTKED VFVHQTAIKK NNPRKFLRSV GDGEVVEFDV
MSY1   KWFNVRNGYG FINRNDTKED VFVHQTAIKK NNPRKYLRSV GDGETVEFDV
FRGY1  KWFNVRNGYG FINRNDTKED VFVHQTAIKK NNPRKYLRSV GDGETVEFDV
GFYP1  KWFNVRNGYG FINRNDTKED VFVHQTAIKK NNPRKYLRSV GDGETVEFDV

MSY2   VEGEKGARAA NVTGPGGVPV KGSRYAPNRR RFRRFIPRPR PAAPPPMVAE
FRGY2  VEGEKGAEAA NVTGPGGVPV KGSRFAPNRR RFRRRFYRPR ADTAGESGGE
GFYP2  VEAAKGSEAA NVTGPGGIPV KGSRYAPNKR RFRRRFY.PR NAQPAEGEEK
MSY1   VEGEKGAEAA NVTGPGGVPV QGSKYAADRN HYRRY...PR RRGPPRNYQQ
FRGY1  VEGEKGAEAA NVTGPEGVPV QGSKYAADRN HYRRY...PR RRGPPRNYQQ
GFYP1  VEGEKGAEAA NVTGPGGVPV QGSKYAADRN RYRRY...PR RRGPPRDYQD

MSY2   APSGGTEPGS EGERAEDSG. .QRPRRRRPP PFFYRRRF.. VRGPRPPNQQ
FRGY2  GVS..PEQMS EGERGEETSP QQRPQRRRPP PFFYRRRF.. RRGPRPNNQQ
GFYP2  SGAAPAGPIS EGEGSEEKGA PLPHPRRQRP   ..RRR... ........PVQ
MSY1   NYQNS.ESGE KNEGSESAPE G.QAQQRR.P YR..RRRFPP YYMRRPYARR
FRGY1  NYQNN.ESGE KAEENESAPE GDDSNQQR.P YH..RRRFPP YYSRRPYGRR
GFYP1  NYQSDGETRE KREEEENAPE GEMQPQQRIP TYPGRRRYPP YFVQRRYGRR

MSY2   .QPIEGSDGV EPKE.TAPLE   GDQQQGDE RVPPPRFRPR YRRPFRPRPP
FRGY2  NQGAEVTEQS ENKDPVAPTS EALASGDDPQ RPPPRRFRQR FRRPFRPRPA
GFYP2  PQGEGEAG.. .......... EKEEVEGAQQ RPLPRRFRPR FRRPLRPRPP
MSY1   PQYSNPPVQG EVMEGA...D NQGAGEQG.R PVRQNMYRGY RPRFRRGPPR
FRGY1  PQYSNAPVQG EEAEGA...D SQGTDEQG.R PARQNMYRGF RPRFRRGPPR
GFYP1  PPYSNAPQRG EMPEGGEGDE NQGVPDQGNK PMRQNYYRGF GP...RGLPR

MSY2   QQPTTEGGDG ETKPSQGPTD GSRPEPQRPR NRPYFQRRRQ QPPGPRQPIA
FRGY2  PQQTPEGGDG ETKAESG..E DPRPEPQRQR NRPYVQRRRR QGATQVAATA
GFYP2  PGEAQPTVVP AEEQESPPKG SETNREQTEG QPQKPRRQFS RRRQTKSESG
MSY1   QRQPREDGNE EDKENQGDET QGQQPPQRRY RRNFNYRRRR PENPKPQDGK
FRGY1  QRQPREEGNE EDKENQGDET QSQPPPQRRY RRNFNYRRRR PENPKSQDGK
GFYP1  PRPVREGEEE EEKENQGEGG QNQEPRQCRY RRNFNYRRRR PQTTKLQDGK

MSY2   AETSAPINSG DPPTTILE
FRGY2  QGEGKAEPTQ HPASEEGTP SDSPTDDGAP VQSSAPDPGI ADTPAPE
GFYP2  ASKDGDEPEK PESPAQASK PADVKSSSEA SPKQSLKMDA LPPTASKSSE
MSY1   ETKAADPPAE NSSAPEAEQ GGAE
FRGY1  ETKAAETSAE NTSTPEAEQ GGAE
GFYP1  DSKAADASAD KPAAPEAEQ GGAE
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FIG. 2. The predicted amino acid sequence of mouse MSY2 is aligned with *Xenopus* FRGY2, goldfish GFYP2, mouse MSY1, *Xenopus* FRGY1, and goldfish GFYP1. Residues 95 and 136 of MSY2, which are substituted for normally invariant CSD residues, are indicated by solid arrow heads. The amino terminal end of the alternatively spliced form MSY2a is also included. The CSD is shown in bold, and the B/A islands are underlined. Maximal alignment was achieved by inserting gaps (shown as dots). Accession numbers follow in parentheses: MSY1 (M62867), FRGY2 (M59454), FRGY1 (M59453), GFYP2 (AB003336), GFYP1 (AB003335).

from an open reading frame of 1080 nt that encodes a polypeptide of 360 amino acids (Fig. 1A), whereas the alternatively spliced form encodes a polypeptide of 282 amino acids. The predicted molecular weights of these polypeptides are 38 and 31 kDa, respectively. Immunoblot analysis of mouse testis extracts using antibodies against *Xenopus* mRNP3+4 (FRGY2), or a peptide of B/A island II of mRNP3 (FRGY2b), revealed two forms of the mouse germ cell Y box protein at 52 and 48 kDa, corresponding to MSY2 and its alternatively spliced form MSY2a, respectively [13, 29]. All known Y box proteins demonstrate significantly retarded mobility in SDS-PAGE, and in the case of FRGY2 this results from a highly extended conformation [31].

The predicted MSY2 protein was rich in arginine (11.9%) and proline residues (16%), indicating that an unmodified protein would be highly basic with an isoelectric point of 11.56. Computer analysis of the predicted MSY2 protein revealed seven putative phosphorylation sites for casein kinase II and one site for protein kinase C (Fig. 1A). Several of these are conserved from FRGY2, which is highly phosphorylated [22]. MSY2 also contained two putative nuclear localization signals imbedded in the second B/A island (Fig. 1A). These may facilitate shuttling of the protein between the nucleus and cytoplasm.

Sequence analysis of MSY2 confirmed it to be a member of the Y box family of proteins that include *Xenopus* FRGY1 and FRGY2, mouse MSY1, several human somatic

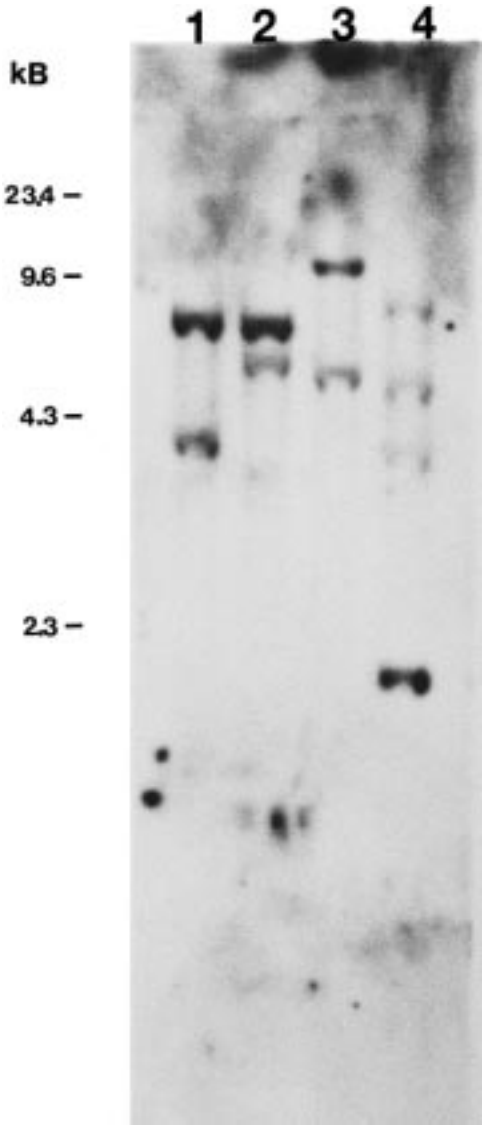

FIG. 3. Southern blot of mouse genomic DNA with mouse *Msy2* cDNA. Aliquots (10 μg) of CD-1 mouse genomic DNA were digested with restriction enzymes *Bam*HI (lane 1), *Eco*RI (lane 2), *Hin*dIII (lane 3), or *Sac*I (lane 4). The digested DNAs were resolved on a 0.7% agarose gel, transferred to nylon membranes, and hybridized with a ^{32}P-labeled cDNA probe.

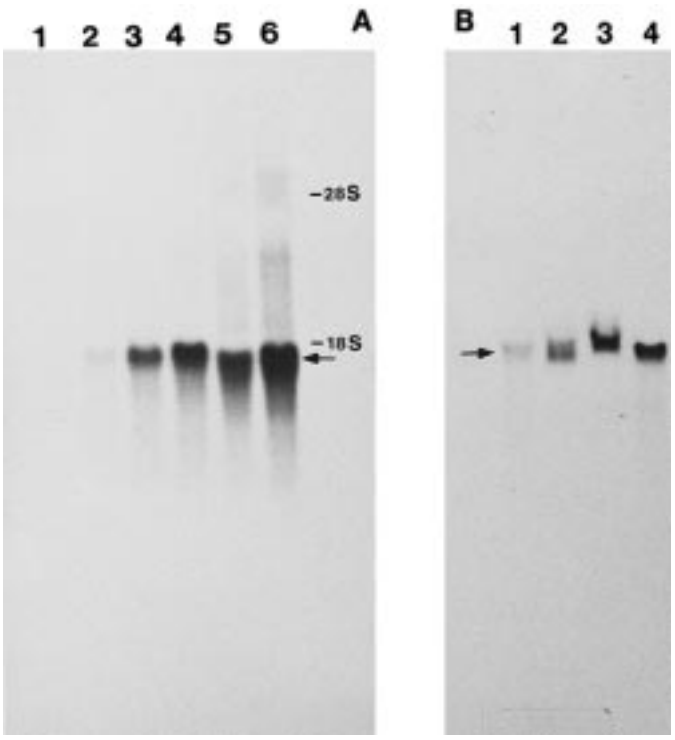

FIG. 4. Expression of *Msy2* mRNA in prepubertal and adult mouse testes and in meiotic and postmeiotic germ cell populations. **A**) RNA aliquots (10 μg) from testes of 6-, 12-, 17-, 22-, and 25-day-old and adult mice (lanes 1, 2, 3, 4, 5, and 6, respectively) were electrophoresed in a 1% agarose gel, transferred onto nylon membranes, and hybridized with a ^{32}P-labeled *Msy2* cDNA. The arrow indicates the position of *Msy2* mRNAs. **B**) RNA aliquots (10 μg) from pachytene spermatocytes (lane 1), round spermatids (lane 2), elongating spermatids (lane 3), and adult mouse testis (lane 4) were analyzed as in **A**.

homologues [16], and the recently reported goldfish Y box proteins, GFYP1 and GFYP2 [34]. Like all other Y box proteins, MSY2 contained the highly conserved N-terminal CSD (from amino acids 89–166; Fig. 1) with motifs related to RNP-1 and RNP-2 that bind single-stranded nucleic acids [25]. In addition, overall MSY2 shared 61% amino acid identity and 56% nucleotide identity with FRGY2. Therefore, the overall similarity of MSY2 to FRGY2 is relatively low as compared to the 85% amino acid identity shared by MSY1 and FRGY1. However, alignment of MSY2 and FRGY2 revealed, in addition to the CSD, a high degree of conservation of the C-terminal B/A islands that have been proven to bind RNA [22, 25, 26]. The conservation of the B/A islands was further emphasized by alignment of MSY2, FRGY2, GFY2, MSY1, FRGY1, and GFY1, revealing the B/A islands (Fig. 2, underlined) to be uniquely conserved for the two different Y box proteins.

MSY2 Was Encoded by a Single Copy Gene

To determine the size of the *Msy2* gene family, mouse genomic DNA was digested with a single restriction enzyme (*Bam*HI, *Eco*RI, *Hin*dIII, or *Sac*I), and the DNA fragments were analyzed by Southern blotting (Fig. 3). Two or three DNA fragments with sizes ranging from 2 to 10 kilobases (kb) were detected. Mapping of the *Msy2* genomic clone with *Bam*HI yielded similar DNA fragments, suggesting that MSY2 is transcribed from a single copy gene (data not shown).

Expression of Msy2 mRNA Was Developmentally Regulated during Spermatogenesis

To determine the temporal expression of *Msy2* mRNA in mouse testes, Northern blot hybridizations were performed with RNA samples from the testes of prepubertal and adult mice, and from enriched germ cell populations. *Msy2* mRNA was barely detected in testes from 6-day-old mice (Fig. 4A, lane 1). A low level of *Msy2* mRNA is seen in testes from 12-day-old mice, the age at which meiosis begins (Fig. 4A, lane 2). In 17-day-old mice, a developmental stage in which the testis is enriched with meiotic pachytene spermatocytes, the expression of *Msy2* mRNA increased significantly (Fig. 4A, lane 3). Maximal levels of *Msy2* mRNA were detected in testes from 22-day-old mice and persisted throughout adulthood (Fig. 4A, lanes 4–6). A similar pattern of mRNA expression has been observed for *Msy1* [14]. Analysis of the expression of *Msy2* mRNAs in enriched populations of meiotic and postmeiotic germ cells

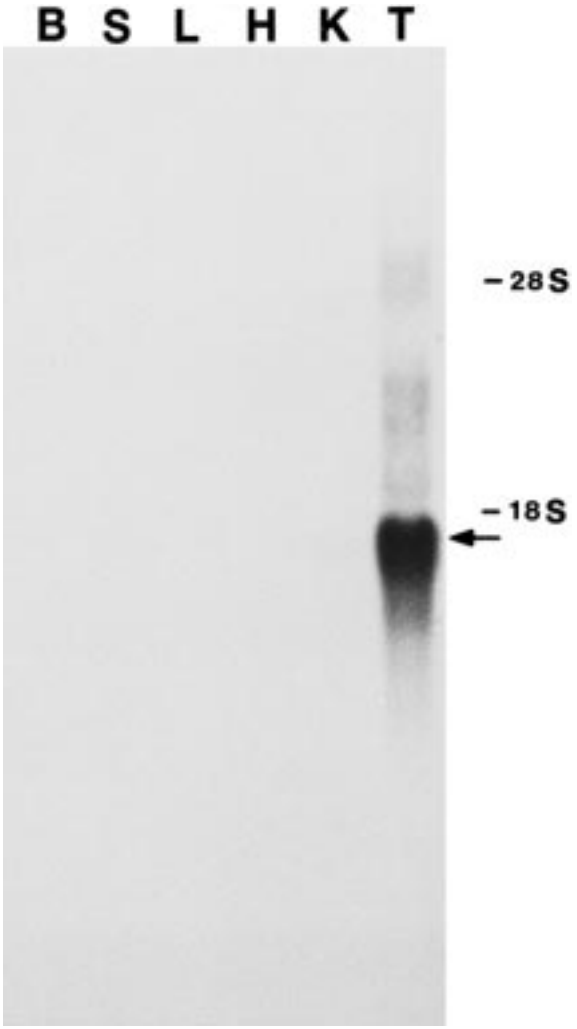

FIG. 5. Northern blot analysis of *Msy2* mRNA in somatic tissues and testes of adult mice. RNA aliquots (10 μg) from brain (lane B), spleen (lane S), liver (lane L), heart (lane H), kidney (lane K), and testis (lane T) were resolved on a 1% agarose gel, transferred onto a nylon membrane, and hybridized with a ^{32}P-labeled *Msy2* cDNA. The arrow indicates the position of *Msy2* mRNAs.

showed that pachytene spermatocytes contained lower levels of *Msy2* mRNA (Fig. 4B, lane 1) than did postmeiotic germ cells (Fig. 4B, lanes 2 and 3) or control adult testes (Fig. 4B, lane 4). The expression pattern of MSY2 mRNA in individual germ cell types (Fig. 4B) is consistent with that seen in extracts of total testes (Fig. 4A). The inability to detect *Msy2* mRNA or protein [13, 29] (Fig. 5) in the somatic tissues suggests that the expression of MSY2 is testis-specific in the male germ cell development.

In Male Mice, MSY2 Was Abundant in Testes

To determine the extent of MSY2 expression in adult mice, total RNA from somatic tissues and testis was analyzed by Northern blotting with a *Msy2* cDNA. An abundant and broad *Msy2* mRNA band of about 1.8 kb was detected in the testis (Fig. 5), but not in the brain, spleen, liver, heart, or kidney (Fig. 5). Similar results were observed when the hybridization was performed under reduced stringency or after overexposure of the gel. Although multiple Y box proteins were expressed in the tissues tested, the *Msy2* cDNA did not cross-hybridize to other Y box mRNAs such as *Msy1*. The inability to detect *Msy2* mRNA

FIG. 6. Western blot of MSY2 in protein extracts from mouse tissues probed with antibodies specific to *Xenopus* mRNP3+4(FRGY2). Aliquots of protein extracts from testes, brain, liver, and kidney of CD-1 mice were electrophoresed on a 12% SDS/PAGE gel, transferred onto PVDF membranes, and probed with an antiserum to *Xenopus* mRNP3+4 (FRGY2). Protein was detected with an ECL kit. Lanes 1, 3, 5, and 7 contain 20 μg of protein extracts from testes, brain, kidney, and liver, respectively. Lanes 2, 4, 6, and 8 contain 100 μg of protein extracts from testes, brain, kidney, and liver, respectively. Lane C contains 20 ng of purified p54/56 as control. The arrow indicates mouse MSY2.

in the somatic tissues suggests that the expression of *Msy2* mRNA is testis-specific.

Western blotting of extracts from brain, liver, and kidney were performed to further evaluate whether MSY2 protein could be detected in somatic tissues. Antibodies against *Xenopus* mRNP3+4 detected MSY2 in protein extracts of testes (Fig. 6, lanes 1–2), but not in extracts from brain (Fig. 6, lanes 3–4), kidney (Fig. 6, lanes 5–6), or liver (Fig. 6, lanes 7–8). The more slowly migrating *Xenopus* mRNP3+4 is also shown (Fig. 6, lane c). The Western blots were in agreement with the Northern blots and further indicated that MSY2 is a germ cell-specific protein.

In Mouse Ovaries, MSY2 Was Exclusively Expressed in Oocytes

The localization of MSY2 in mouse ovaries and early embryos was determined by immunocytochemistry. In the 22-day-old mouse ovary, all oocytes, regardless of stage of development, were strongly positive for MSY2 immunostaining. Figure 7 shows positively staining oocytes in graafian follicles, preantral follicles, and primordial follicles. No cells other than oocytes were immunopositive. Diplotene oocytes in the ovaries of 18-day pc fetuses were immunopositive, but pachytene oocytes in 17-day pc fetuses were not (Fig. 8). Metaphase II oocytes were immunopositive just before ovulation as were single cell embryos (not shown), but MSY2 levels decreased greatly in 2-cell-stage embryos in the oviduct (Fig. 7).

DISCUSSION

The Y box proteins are defined by their CSD, which is more than 40% identical to that of the small bacterial cold shock proteins [15]. Vertebrate Y box proteins also contain four B/A islands that participate in RNA binding [25, 26]; however, these are not found in Y box proteins from invertebrates [35] or plants [36]. Vertebrate Y box proteins have the potential to regulate gene expression either as a transcription factor or cytoplasmically as a modulator of

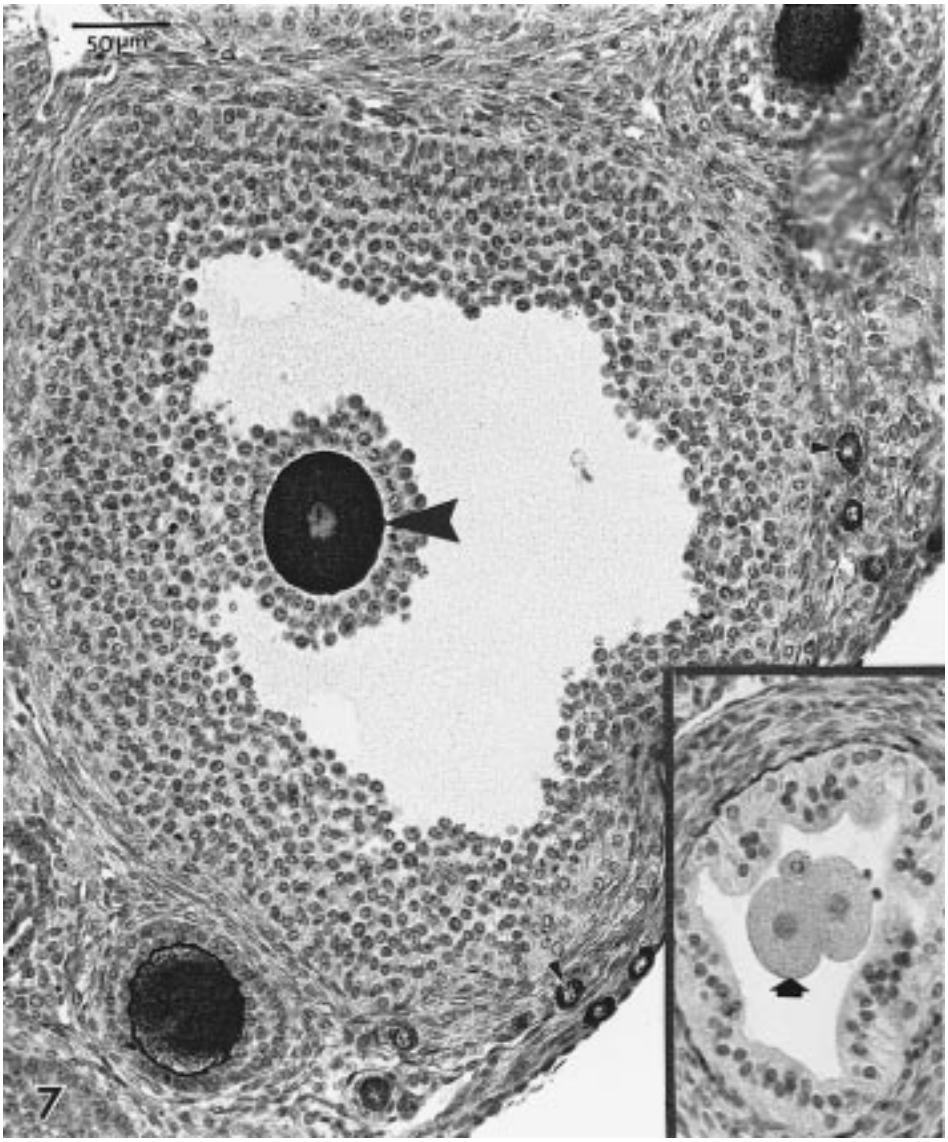

FIG. 7. Immunocytochemical localization of MSY2 in ovaries of 22-day-old mice. Immunostaining was observed only in oocytes (arrowheads). The small arrowheads indicate diplotene oocytes in primordial follicles, and the large arrowhead delineates a diplotene oocyte in a large preovulatory follicle. The inset shows a 2-cell-stage embryo within the oviduct.

translation [37]. Altered nuclear/cytoplasmic localization of the human protein YB-1 results in multidrug resistance and demonstrates that Y box proteins can significantly impact cell phenotype [38]. MSY1, the mouse homologue of YB-1, is normally expressed at its highest level in spermatocytes, primarily as a cytoplasmic protein found in nontranslated mRNPs [14]. The rabbit homologue of YB-1 and MSY1, p50, is primarily cytoplasmic and found associated with both translated and nontranslated globin mRNPs in reticulocytes [39]. In this study, we report the cloning of MSY2. MSY2 is identified as the murine homologue of *Xenopus* FRGY2 on the basis of their 1) shared amino acid substitutions of CSD residues, 2) conserved B/A islands, and 3) germ cell-restricted pattern of expression.

Highly specific antibodies to FRGY2 identify mouse testis polypeptides of 52 and 48 kDa in SDS-PAGE corresponding to MSY2 and its alternatively spliced form MSY2a [13, 29]; however, their cDNA-predicted molecular masses are 38 and 31 kDa, respectively. FRGY2 exhibits a similar disparity in migration relative to its true molecular mass because of its highly extended conformation [31]. Subcellular fractionation has demonstrated MSY2a to be primarily restricted to the cytoplasm, while MSY2 is found in both nuclear and cytoplasmic compartments [23], suggesting that the alternate N-terminal end may modify MSY2 localization and possibly its function.

Comparison of cDNA-derived sequences of Y box proteins from mice, frogs, and goldfish demonstrates the relationship of the germ cell-restricted Y box proteins MSY2, FRGY2, and GFY2 and their divergence from the non-germ cell-restricted forms (Fig. 2). Among vertebrate Y box proteins, the CSD is more than 90% conserved from *Xenopus* FRGY2 to human YB-1 across its ~100 amino acids [22]. However, divergence of the germ cell-restricted Y box proteins is apparent in MSY2, FRY2, and GFY2 at two positions that are invariant in all other known vertebrate Y box proteins (Fig. 2, Q95 and F136 of MSY2 found as K56 and Y97 in MSY2). The coincident substitution of these otherwise invariant CSD amino acids supports a close relationship between MSY2 and FRGY2. Stronger support for MSY2 as the homologue of FRGY2 is derived from the conservation of the B/A islands (Fig. 2, underlined). The position and sequence of the four B/A islands is conserved between MSY2, FRGY2, and GFY2 and divergent from those in MSY1, FRGY1, and GFY1. In particular, B/A islands II and IV of FRGY2 are each > 90% identical in MSY2 and FRGY2. Since these FRGY2 B/A islands bind

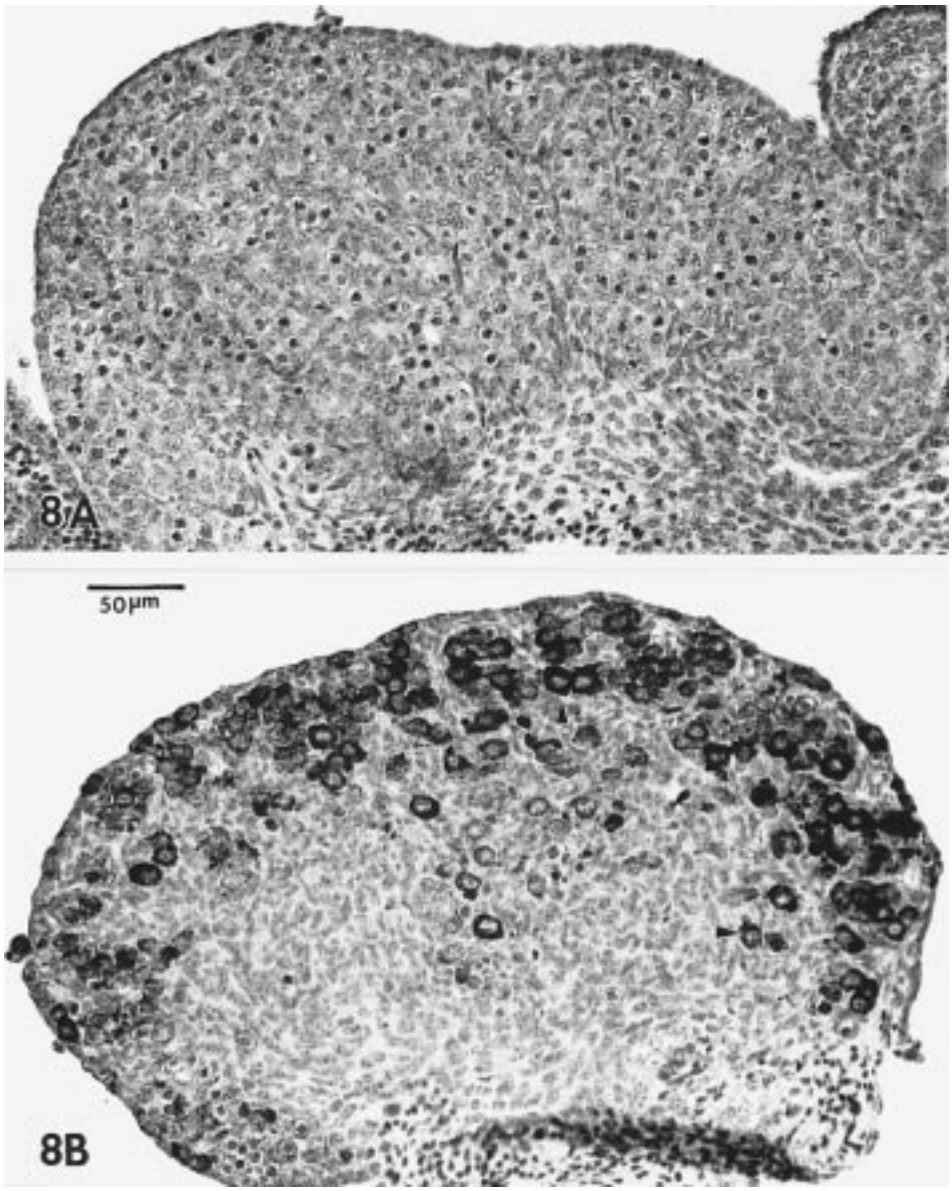

FIG. 8. Immunocytochemical localization of MSY2 in fetal ovaries. Pachytene oocytes in a 17-day pc fetus (**A**) are immunonegative while diplotene oocytes, indicated by arrowheads, in a 19-day pc fetus (**B**) are immunopositive.

[26], it is highly probably that these motifs also participate in MSY2 RNA binding.

Sequence analysis of MSY2 protein predicts seven putative sites for phosphorylation by casein kinase II and one site for protein kinase C, raising the likelihood that the protein could be phosphorylated in vivo, as is FRGY2 [27]. MSY2 also contains several predicted nuclear localization signals. This is not surprising since the transcription factor MSY2 is located both in the nucleus and the cytoplasm [23], functioning as an mRNA-binding storage protein in the cytoplasm and in the nucleus.

MSY2 and FRGY2 also share a pattern of germ cell-restricted expression in contrast to the widespread expression of MSY1 and FRGY1 in both somatic and germ cells [14, 40]. Northern blot analysis using *Msy2* cDNA detected a broad band of mRNAs of about 1.8 kb in the testis, but not in somatic RNAs (Fig. 5). Similarly, FRGY2 [40] was detected solely in male and female germ cells. Other vertebrate Y box genes such as FRGY1 and MSY1 are widely expressed in both germ cells and somatic tissues although not translated in all cases [30]. Detection of MSY2 exclusively in the germ cells of rodent testes and ovaries, but not in somatic tissues, and the absence of *Msy2* mRNA in testes from 6-day-old mice, in which most of the cell types are somatic, suggests that MSY2 expression is germ cell-specific.

The expression of MSY2 is developmentally regulated during spermatogenesis. Kwon et al. [13] demonstrated that the mammalian protein immunologically related to FRGY2 (i.e., MSY2) is present in meiotic pachytene spermatocytes and accumulates markedly in postmeiotic spermatids, the cell types in which most of the ''paternal'' mRNA is stored for translation in later stages. Immunocytochemical studies by Oko et al. [29] demonstrated that in mouse and rat testes, MSY2 (previously called p48/52) is a predominant germ cell cytoplasm protein with maximal amounts in postmeiotic round spermatids, further establishing its role as an mRNA-binding storage protein. Our Northern blot hybridization confirms these observations, since *Msy2* mRNA is detected in testes from 12-day-old mice and reaches a maximum in testes from 22-day-old mice (Fig. 4). Although *Msy2* mRNA is detected in testes from 12-day-old mice, immunocytochemical and Western blot analyses first detect MSY2 protein in meiotic pachytene spermatocytes [29].

This may result from the limited sensitivity of the protein detection methods or may be indicative of translational regulation. Both possibilities are likely since MSY2 can be detected in the nuclei of germ cells by gel shift assays but not by immunocytochemical methods; and preliminary data indicate that most of the total *Msy2* mRNA in mouse testes is nonpolysomal (unpublished results).

The abundance and temporal appearance of MSY2 in oocytes suggest a role in the storage of maternal mRNAs. MSY2 is developmentally regulated in mouse oocytes. MSY2 appears as oocytes enter the prolonged diplotene (dictyate) stage and persists until after fertilization in the single-stage embryo. MSY2 is not detected by our immunocytochemical methods in the two-cell embryo even though the antibody recognizes modified embryonic forms of FRGY2 [27]. This suggests that significant turnover of MSY2 occurs in the two-cell embryo. The timing of this event is coincident with the massive degradation of maternal mRNAs [41, 42], suggesting that degradation of MSY2 may trigger decay of maternal mRNAs. Western blot analyses indicate that testicular and oocyte MSY2 proteins have identical electrophoretic mobilities (data not shown). Any role MSY2 may play in transcription during oogenesis or after fertilization awaits analysis.

On the basis of the high levels of MSY2 in mouse male and female germ cells and its interactions with both DNA and RNA, we propose that MSY2 first functions in the earlier stages of germ cell development as a DNA-binding protein directing germ cell-specific gene transcription [23, 30], and it subsequently serves as a RNA-binding protein in the cytoplasm [13, 29], where it stores mRNAs and regulates their translation.

ACKNOWLEDGMENT

We thank Dr. E.M. Eddy of NIEHS for providing a mouse testis lambda ZAP II cDNA library.

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