Nucleic Acids Research, 1999, Vol. 27, No. 7 **1747–1753**

The mouse Y-box protein, MSY2, is associated with a kinase on non-polysomal mouse testicular mRNAs

Terence P. Herbert[+] and Norman B. Hecht*

Center for Research on Reproduction and Women's Health and Department of Obstetrics and Gynecology, University of Pennsylvania Medical Center, Philadelphia, PA 19104, USA

Received October 23, 1998; Revised January 25, 1999; Accepted February 15, 1999

ABSTRACT

In male germ cells many mRNAs are sequestered by proteins into translationally silent messenger ribonucleoprotein (mRNP) particles. These masked paternal mRNAs are stored and translated at specific times of germ cell development. Little is known about the mammalian testicular mRNA masking proteins bound to non-polysomal mRNAs. In this report, the major proteins binding to non-polysomal testicular mRNAs were isolated and analyzed. The two predominant proteins identified were: a Y-box protein (MSY2), the mammalian homolog to the *Xenopus* oocyte masking protein FRGY2/mRNP$_{3+4}$, and a poly(A) binding protein. A kinase activity was also found associated with these non-polysomal RNAs. The kinase co-immunoprecipitates with MSY2 and phosphorylates MSY2 *in vitro*. The MSY2 associated kinase is not casein kinase 2, the kinase believed to phosphorylate mRNP$_{3+4}$ in oocytes, but a yet unidentified kinase. MSY2 was found to be phosphorylated *in vivo* and MSY2 dephosphorylation led to a decrease in its affinity to bind RNA as judged by northwestern blotting. Therefore, testicular masked mRNAs may be regulated by the phosphorylation state of MSY2. Reconstitution experiments in which non-polysomal mRNA-binding proteins are dissociated from their RNAs and allowed to bind to exogenous mRNAs suggest that MSY2 binds RNA in a sequence-independent fashion. Furthermore, association of the non-polysomal derived proteins to exogenous non-specific mRNAs led to their translational repression *in vitro*.

INTRODUCTION

Spermatogenesis is the process of cellular differentiation in which a population of diploid progenitor cells in the testis terminally differentiate into haploid spermatozoa (1). During mammalian spermatogenesis, the genome is repackaged and transcriptionally silenced as histones are replaced with transition proteins and protamines. However, ongoing protein synthesis is required for the extensive differentiation of germ cells into mature gametes. The regulated translation of stored RNAs, therefore, plays a prominent role in the later stages of male germ cell development. A number of paternal mRNAs which are synthesized early in spermiogenesis, e.g., the transition proteins 1 and 2 and the protamines 1 and 2, are stored within the cytoplasm. These RNAs are translationally silent and are present in the non-polysomal messenger ribonucleoprotein (mRNP) fraction presumably in protein–RNA complexes termed mRNP particles or masked mRNAs (2,3). At an appropriate time in development, these stored RNAs become translationally competent. Although proteins such as testis/brain-RNA binding protein (TB-RBP) and protamine binding protein (Prbp) are believed to modulate expression of specific mRNAs encoding proteins such as protamines (4,5), the identity and function of the major proteins in the paternal mRNP masking complexes of mammals is poorly understood.

Much is known on RNA masking in gametes from investigations in *Xenopus* oocytes. During oogenesis in the frog, an analogous situation occurs as in spermatogenesis. A number of RNAs synthesized early in oogenesis are sequestered by proteins to form mRNP particles. During the latter stages of oogenesis, these masked RNAs are temporally activated to ensure terminal differentiation of the oocyte into the mature egg.

Proteins associated with masked maternal mRNAs have been identified in mature *Xenopus* oocytes. Two of these proteins were found to be members of a family of Y-box transcription factors named FRGY2 (mRNP4) and mRNP3 (6–10). A role for mRNP$_{3+4}$ in masking mRNA is suggested from several lines of evidence. Reconstitution of mRNP$_{3+4}$ with mRNA *in vitro* represses translation (11,12) and expression of mRNP$_{3+4}$ in somatic cells leads to an increase in mRNA accumulation from promoters carrying the Y-box consensus sequence and to translational silencing of that RNA (13). It has also been demonstrated that overexpression of mRNP$_{3+4}$ in *Xenopus* oocytes facilitates the silencing of RNA synthesized *in vivo* (14). Antibodies recognizing the mRNP$_{3+4}$ family of proteins relieve the inhibition of translation when introduced into *Xenopus* oocytes (15,16).

In *Xenopus* oocytes, the Y-box proteins are phosphorylated leading to an increased affinity for RNA (12,17–20). Moreover, phosphorylation of mRNP$_{3+4}$ inhibits translation of its bound RNA both *in vitro* and *in vivo* (12,15,21). It has been suggested that phosphorylation of the Y-box proteins is important in the

*To whom correspondence should be addressed. Tel: +1 215 898 0144; Fax: +1 215 573 5408; Email: nhecht@mail.med.upenn.edu

[+]Present address: Department of Anatomy and Physiology, University of Dundee, Dundee DD1 4HN, UK

formation and disassembly of the mRNP particles (8,12,15,17). The kinase believed responsible for the phosphorylation of $mRNP_{3+4}$ is casein kinase 2 (CK2) (15,18).

In *Xenopus* oocytes, other proteins including nucleolin (21), nucleoplasmin (22) and Xp54 helicase (23) have also been found associated with the mRNP fraction. Xp54 helicase has been suggested to play a role in the unwinding of RNA secondary structure leading to the initiation of translation (23), whereas nucleoplasmin association has been shown to partially relieve the translational repression of histone H4, an mRNA known to be masked (22).

Y-box proteins have also been identified in mammalian testes (24–27). From immunocytochemical studies, the mammalian homologs are found to be abundant in the cytoplasm of meiotic and post-meiotic male mouse germ cells, cell types containing most of the paternal stored mRNAs (26). The Y-box proteins also function in nuclear extracts where they can activate protamine 2 transcription in *in vitro* assays (28). Recent cloning and sequencing of mouse testicular Y-box proteins has demonstrated the existence of two spliced variants, MSY2 and MSY2a, the mammalian germ cell homologs of FRGY2/$mRNP_{3+4}$ (27). MSY1, a homolog of the somatic *Xenopus* Y-box masking protein FRGY1, has also been characterized (25).

In this study, we identify two major proteins, MSY2 and PABP, and a kinase activity associated with mouse non-polysomal mRNAs. Evidence is provided that binding and subsequent translational repression by MSY2 is independent of RNA sequence. The associated kinase phosphorylates MSY2 *in vitro* and thereby likely influences its affinity to RNA. This testicular mRNP associated kinase appears to differ from CK2, the kinase found associated with the mRNP particles of *Xenopus* oocytes (15,18).

MATERIALS AND METHODS

mRNP particle isolation from mouse testis

Decapsulated adult CD1 mouse testes were disrupted in 1× gradient buffer (0.2 M KCl, 2 mM $MgCl_2$, 20 mM Tris–HCl, pH 7.4, 0.5% NP-40, 1 mM PMSF, 1 mg/l leupeptin, 1 mg/l aprotinin, 1 mg/l pepstatin, 100 mM NaFl and 100 U/ml RNAsin) using a glass–teflon homogenizer. After the homogenate was spun at 10 000 *g* for 10 min at 4°C to remove nuclei and cellular debris, 250 µl (1.2 mg of protein/ml) of the clarified post-nuclear supernatant was layered over a 4.75 ml 20–60% Nycodenz gradient (Accurate Chemical and Scientific Corp.) and centrifuged at 150 000 *g* for 20 h in a SW50 rotor (Beckman). The gradient was divided into 18 fractions which were then further divided for protein assays and northern and western blot analyses (described below). The protein content of each fraction was assayed using the Bradford protein assay (BioRad). For purification of mRNA binding proteins, fractions sedimenting as mRNPs in Nycodenz gradients (see Results) were pooled and oligo(dT) selected using the PolyATtract mRNA isolation system (Promega). Briefly, total mRNPs were annealed to biotinylated oligo(dT) in a 1× gradient buffer, duplexes were collected on Streptavidin-coupled paramagnetic beads, washed three times in 1× gradient buffer, and once in 0.1× SSC. After elution in ddH_2O, the eluent contained an enriched population of mRNA–protein complexes. Proteins were detected by silver staining using the Diachii silver stain kit as described by the manufacturer.

Western blotting and immunoprecipitations

Western blotting was performed as described by Murray *et al.* (8). Proteins were separated on an SDS–polyacrylamide gel and electroblotted onto nitrocellulose. The nitrocellulose filters were blocked with 1× TBS (20 mM Tris, 50 mM NaCl, pH 7.4)–5% milk for 1 h and incubated with guinea pig anti-$mRNP_{3+4}$ antibody at a 1/1000 dilution overnight at 4°C. The blots were then washed in 1× TBS, three times for 20 min. Antigen–antibody complexes were detected using a chemiluminescence system (Amersham International). For immunoprecipitations, the mRNA–protein complexes were suspended in RIPA buffer [50 mM Tris–HCl pH 7.5, 150 mM NaCl, 0.1% SDS, 1% NP-40, 0.5% deoxycholate (DOC)] and pre-cleared by incubation with IgG Sepharose beads (4°C for 1 h). The supernatants were then incubated for 12 h at 4°C with anti-$mRNP_{3+4}$ IgG Sepharose beads. The precipitated complexes were washed five times in RIPA buffer and resuspended in gel loading buffer (0.1 M DTT, 2% SDS, 0.625 M Tris, pH 6.8, 20% glycerol, bromophenol blue). After boiling, the samples were electrophoresed through a 10% SDS–polyacrylamide gel and the products visualized by autoradiography.

Northern blotting

Samples fractionated on a 20–60% Nycodenz gradient were phenol–chloroform extracted twice, ethanol precipitated and resuspended in RNA loading buffer for electrophoresis through 1.5% agarose formaldehyde gels. The RNAs were transferred to nylon membranes and hybridizations were performed overnight at 65°C in hybridization buffer with randomly primed ^{32}P-labeled cDNAs. The filters were washed four times in 0.5× SSC–5% SDS for 20 min at 65°C, dried, and exposed to autoradiographic film.

Reconstitution of mRNA–protein complexes and their *'in vitro'* translation

Dissociation/reconstitution experiments were performed using a modification of the procedure of Pullman and Martin (29). Briefly, protein was dissociated from purified oligo(dT) selected mRNPs (4 µg of protein) with 0.5% DOC and RNA was digested with micrococcal nuclease (300 µg/ml) in 1 mM $CaCl_2$ at 23°C for 20 min. The digestion was stopped by the addition of 10 mM EGTA. Reconstitution of the dissociated proteins with either luciferase RNA or brome mosaic virus RNA (1 µg) was achieved by the addition of 0.5% Triton X-100. Oligo(dT) selected mRNPs, dissociated mRNPs incubated with 0.5% Triton X-100 and reconstituted mRNP particles were analyzed on a 20–60% Nycodenz gradient. Reconstituted mRNP particles were translated in a rabbit reticulocyte *in vitro* translation system in the presence of [^{35}S]methionine using the protocol of the manufacturer (Promega). As a control, luciferase RNA was incubated in a mixture of DOC, micrococcal nuclease, EDTA and Triton X-100 at concentrations indicated above prior to *in vitro* translation.

'In vivo' labeling of testicular proteins

Decapsulated mouse testes were digested with 500 µg/ml collagenase–dispase (20 min, 32°C), washed twice, and then preincubated for 2 h at 32°C in phosphate-free RPMI (Gibco). The cells were incubated for an additional 4 h with [^{32}P]orthophosphate (250 µCi/ml), washed twice with RPMI, and resuspended in 1× gradient buffer. The radiolabeled mRNPs were then isolated as described above.

Assay of kinase activity

Approximately 0.1 µg of oligo(dT) selected mRNP particles were incubated for 30 min at 30°C in Kinase Buffer (KB: 100 mM Tris–HCl pH 7.4, 10 mM $MgCl_2$, 200 mM NaCl) in the presence of [γ-^{32}P]ATP (2 µCi) and 0.1 mM ATP. Inhibitors, activators and/or substrates were added to the kinase reactions at the concentrations indicated in Results.

Kinase assay of the mRNP complex of MSY2 and its associated proteins

Isolated mRNPs were digested with RNases A and T1 for 30 min at 37°C, suspended in Immunoabsorption Buffer (IB: 20 mM Tris–HCl pH 7.6, 150 mM KCl, 2 mM $MgCl_2$, 2 mM DTT, 1% Triton X-100, 1 mM PMSF, 1 mg/l leupeptin, 1 mg/l aprotinin, 1 mg/l pepstatin), and precleared by incubation with IgG Sepharose beads (4°C, 1 h). The supernatants were then incubated for 12 h at 4°C with anti-$mRNP_{3+4}$ IgG Sepharose beads. The immune complexes were washed five times with IB, twice with KB, and *in vitro* kinase assays were performed as described above.

Northwestern blot analysis

Northwestern blotting was performed essentially as described by Houman *et al*. (30). mRNPs were incubated at 37°C for 30 min with or without calf intestinal phosphatase (CIP; 25 U/ml) (Boehringer Mannheim). After separation on a 10% SDS–polyacrylamide gel, the mRNP-associated proteins were transferred to PVDF membranes. The membranes were prehybridized in renaturation buffer (50 mM Tris–HCl pH 7.6, 50 mM NaCl, 1 mM EDTA, 1 mM DTT, 1% BSA) overnight at 4°C and incubated with *in vitro* transcribed ^{32}P-labeled protamine RNA (transcript c) (4) (10^5 c.p.m./ml) for 1 h at 23°C in binding buffer (10 mM Tris–HCl pH 7.6, 50 mM NaCl, 1 mM EDTA, 1 mM DTT). The membrane was washed four times in binding buffer for 20 min at 23°C, dried, and exposed to X-ray film.

RESULTS

Identification and isolation of mouse paternal mRNPs

In order to identify and isolate proteins associated with mouse testicular non-polysomal mRNAs, post-nuclear testicular extracts were sedimented through a 20–60% Nycodenz gradient; a gradient that resolves free proteins and polysomes from mRNPs (25). To calibrate the gradient, RNA was purified from each gradient fraction, electrophoresed in a 1% agarose formaldehyde denaturing gel and stained with ethidium bromide. The position of the polysomal RNA within the gradient was identified by the electrophoretic mobilities of the 18S and 28S ribosomal RNAs (Fig. 1a, fractions 11–15). The low molecular weight 4–5S RNAs are seen in fractions 4 and 5.

To determine the location of non-polysomal mRNAs, purified RNA aliquots were transferred to nitrocellulose for northern blot analysis and probed with a ^{32}P-labeled protamine 2 cDNA, a gene known to be translationally repressed during spermiogenesis (31). Most of the protamine 2 mRNA was detected in fractions 8–11 (Fig. 1b). These data indicate that mRNPs sediment in fractions 8–11, between the 4–5S and ribosomal RNAs.

When protein content was assayed throughout the Nycodenz gradient, the majority of protein was detected as free protein in

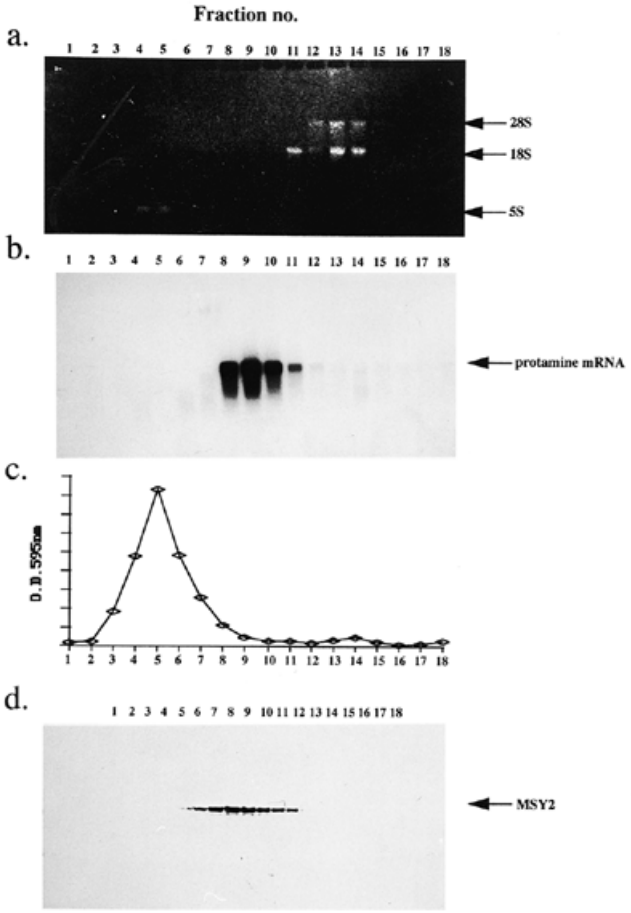

Figure 1. Calibration of Nycodenz gradient and identification and isolation of mRNPs. Post-nuclear testicular extracts were sedimented on a 20–60% Nycodenz gradient. The gradient was then divided into 18 fractions for several analyses. (**a**) Deproteinized RNA was electrophoresed through a denaturing formaldehyde–1% agarose gel and stained with ethidium bromide. (**b**) Northern blot of the gel in the top panel probed with a ^{32}P-labeled protamine 2 cDNA (10^8 c.p.m./ml). (**c**) Protein content of each fraction as determined by Bradford assay. (**d**) Western blot of protein electrophoresed through a 15% SDS–polyacylamide gel and detected with antibody to $mRNP_{3+4}$.

fractions 3–7 (Fig. 1c). Western blot analysis of the Nycodenz gradient fractions using an antibody which recognizes the mouse homolog, MSY2 (24), revealed that MSY2 sedimented in fractions 7–12, with a concentration in fractions 8–10. Since free proteins sediment slowly in Nycodenz gradients, the more rapid sedimentation of MSY2 suggests that MSY2 co-fractionates with mRNAs (7,25,32). Based on the results presented in Figure 1, fractions 8–10 were considered to contain an enriched population of mRNPs and were pooled for further purification.

Characterization of mouse mRNP particles

The mRNP fraction (tubes 8–10), containing an enriched population of proteins that bind to non-polysomal mRNAs, was further purified from any co-sedimenting contaminating free protein by selection with oligo(dT). The purification of the

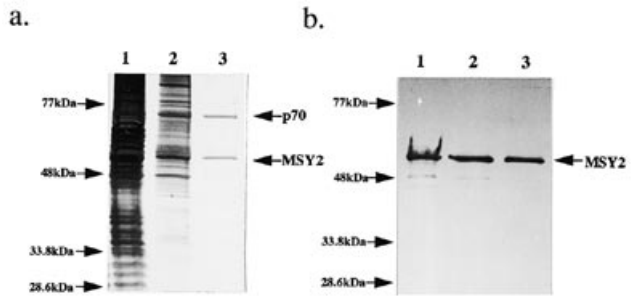

Figure 2. Purification of mRNPs and identification of major masking proteins. Following oligo(dT) selection, proteins were separated on a 10% SDS–PAGE gel and monitored by (**a**) silver staining and (**b**) western blotting using anti-mRNP$_{3+4}$ antibody. Lane 1, aliquot (5 µg) of Nycodenz gradient fractions 8–10; lane 2, proteins (1 µg protein) associated with biotinylated oligo(dT)-bound Streptavidin beads after washing; lane 3, proteins (0.1 µg protein) eluted from oligo(dT) selected mRNPs.

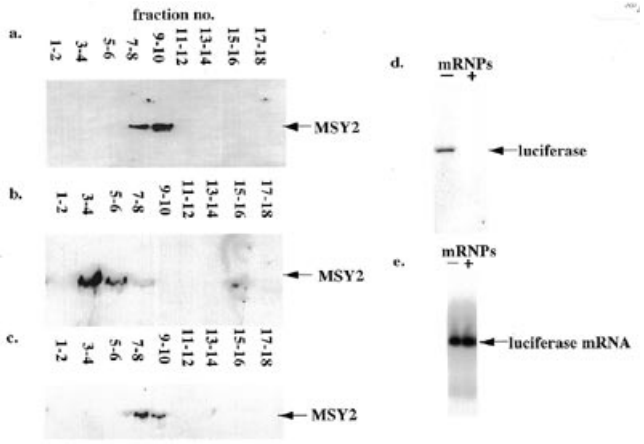

Figure 3. mRNPs reconstituted with an exogenous RNA and its subsequent translational repression *in vitro*. Western blot using anti-mRNP$_{3+4}$ antibody of samples separated on a 20–60% Nycodenz gradient of (**a**) native mRNPs (4 µg protein). (**b**) Dissociated mRNPs (4 µg protein) pre-treated with micrococcal nuclease and Triton X-100. (**c**) mRNPs (4 µg protein) reconstituted with luciferase mRNA (1 µg). (**d**) Cell-free translation of luciferase mRNA (1 µg) pre-incubated in a solution of DOC, $CaCl_2$, micrococcal nuclease, EGTA and Triton X-100 at concentrations indicated in methods. –, free luciferase RNA; +, reconstituted luciferase mRNPs. (**e**) Northern blot of total RNA after cell-free translation (–) and total RNA purified from reconstituted mRNPs after cell-free translation (+).

proteins was monitored by silver staining (Fig. 2a) and by western blotting (Fig. 2b) using antibody against mRNP$_{3+4}$ which recognizes the mouse homolog, MSY2 (24,26,27). Two major proteins with estimated molecular weights of 52 and 70 kDa and a number of minor proteins were found associated with the oligo(dT) selected RNA (Fig. 1a, lane 3). When identical samples were western-blotted using antibody against mRNP$_{3+4}$, proteins of 52 and 48 kDa were detected (Fig. 2b).

In order to identify the second major mRNP protein migrating with an apparent molecular weight of 70 kDa, the protein was purified from the gel and after an ingel tryptic digestion and peptide separation by HPLC, a peptide of 12 amino acids was sequenced. This sequence, EFSPFGTITSAK, was found to have complete identity with an amino acid sequence in mouse testicular poly(A) binding protein (PABP; amino acid positions 313–324; accession no. P29341) (33). We have previously demonstrated that this 70 kDa PABP binds to both polysomal and non-polysomal testicular mRNAs (34).

mRNPs reconstituted with an exogenous RNA show translational repression '*in vitro*'

To investigate the ability of MSY2 and associated proteins to bind and influence mRNA translation, mRNP proteins were dissociated from RNA–protein complexes purified by oligo(dT) selection and reconstituted with exogenous mRNAs such as luciferase mRNA, which were then translated *in vitro*. Untreated, dissociated and reconstituted mRNP particles were centrifuged through a 20–60% Nycodenz gradient, fractionated and assayed for MSY2 by western blotting. The control, i.e. untreated purified mRNP particles, sedimented in fractions 7–10 (Fig. 3a). Dissociated MSY2 was found predominantly in the free protein fractions 3–6 (Fig. 3b). When mRNPs were reconstituted using luciferase RNA, the mRNP particles had identical sedimentation properties to those of native untreated mRNP particles (Fig. 3c). Similar results were obtained when brome mosaic viral RNA was used in the reconstitution experiments, suggesting that MSY2 binds RNA *in vitro* in a sequence-independent fashion (data not shown).

To determine whether the proteins dissociated from the testicular mRNPs were able to inhibit the *in vitro* translation of luciferase RNA upon reconstitution, reassembled particles were translated in a rabbit reticulocyte lysate. Protein binding to luciferase RNA resulted in the complete inhibition of its translation (Fig. 3d). To rule out the possibility that RNA degradation was the reason translation was abolished, an aliquot of the post-translational mixture was phenol–chloroform extracted twice and the RNA assayed by northern blotting with a ^{32}P-labeled luciferase cDNA (Fig. 3e). No RNA degradation was observed, suggesting that the translational repression resulted from binding of the mRNP derived protein to the exogenous RNA.

MSY2 is phosphorylated *in vivo* and *in vitro*

CK2 co-isolates with *Xenopus* oocyte mRNP particles and is believed to be responsible for the phosphorylation of mRNP$_{3+4}$ within mRNP particles (15,18–20).

To test mouse testis mRNP particles for intrinsic protein kinase activity, oligo(dT) selected mRNPs particles were incubated with [γ-^{32}P]ATP in kinase buffer. Radiolabeled MSY2 and a likely degradation product of 33 kDa (24) were detected after immunoprecipitation with anti-mRNP$_{3+4}$ antibody (Fig. 4, lane 1), but not after immunoprecipitation with control guinea pig serum (Fig. 4, lane 2).

To determine whether MSY2 is also phosphorylated *in vivo*, parallel studies were performed with enzymatically dispersed mouse testicular cells incubated in [^{32}P]orthophosphate (Fig. 4). When the mRNP fractions (tubes 8–10) were immunoprecipitated with anti-mRNP$_{3+4}$ antibody, MSY2 was detected (Fig. 4, lane 3). No radiolabeled protein was detected with control guinea pig serum (Fig. 4, lane 4). These data demonstrate that MSY2 is phosphorylated *in vitro* and *in vivo* and the kinase responsible for its phosphorylation is present in the purified mRNP fraction.

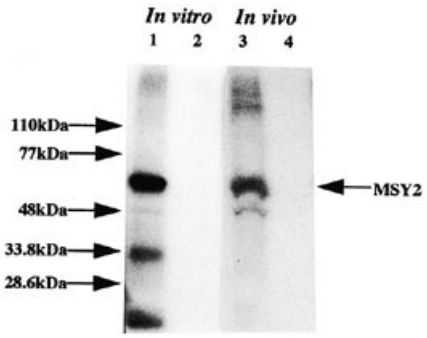

Figure 4. *In vitro* and *in vivo* phosphorylation of MSY2. Lane 1, *in vitro* phosphorylated mRNPs (0.1 µg) immunoprecipitated with anti-mRNP$_{3+4}$ antibody; lane 2, *in vitro* phosphorylated mRNPs (0.1 µg) incubated with guinea pig serum; lane 3, *in vivo* phosphorylated mRNPs (0.1 µg) immunoprecipitated with anti-mRNP$_{3+4}$ antibody; lane 4, *in vivo* phosphorylated mRNPs (0.1 µg) incubated with guinea pig serum. Proteins were separated by SDS–PAGE and visualized by autoradiography.

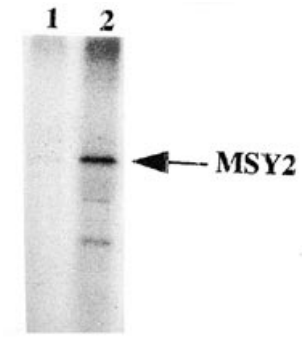

Figure 5. MSY2 kinase is associated with MSY2 in a complex. Purified oligo(dT) selected mRNPs (0.1 µg) were digested with RNase followed by immunoprecipitation with anti-mRNP$_{3+4}$ antibody or incubation with guinea pig serum. The precipitated complexes were phosphorylated *in vitro* and the products separated on 10% SDS–polyacrylamide gels. Lane 1, mRNPs incubated with guinea pig serum; lane 2, mRNPs incubated with anti-mRNP$_{3+4}$ antibody. Proteins were separated by SDS–PAGE and visualized by autoradiography.

MSY2 kinase is associated with MSY2 in a complex

To determine whether the immunoprecipitated kinase is physically associated with MSY2 in a complex, oligo(dT) selected mRNPs were digested with RNases A and T1, immunoprecipitated with anti-mRNP$_{3+4}$ antibody, and assayed for phosphorylation (Fig. 5). One major phosphoprotein, MSY2, and the putative 33 kDa degradation product of MSY2 were detected (Fig. 5, lane 2). No radiolabeled proteins were immunoprecipitated with control guinea pig serum (Fig. 5, lane 1). These data indicate the MSY2 kinase is associated with MSY2 within a protein complex.

The MSY2 associated kinase is not CK2

CK2 has been reported to phosphorylate mRNP$_{3+4}$ in *Xenopus* oocytes (15,18). To determine whether CK2 also functions in mammalian testes to phosphorylate MSY2, *in vitro* kinase assays were performed with testicular extracts in the presence of two

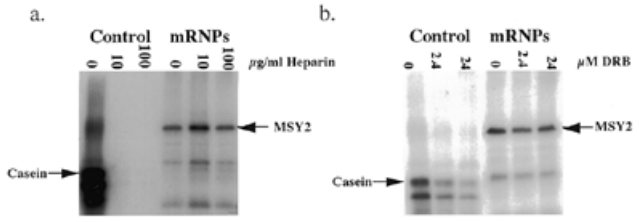

Figure 6. The MSY2 associated kinase is not CK2. (**a**) Control, phosphorylation of casein (50 ng) by CK2 (5 U) in the presence of increasing amounts of heparin; mRNPs, *in vitro* phosphorylation of mRNPs (0.1 µg per reaction) in the presence of increasing amounts of heparin. (**b**) Control, phosphorylation of casein (50 ng) by CK2 (5 U) in the presence of increasing amounts of DRB; mRNPs, *in vitro* phosphorylation of mRNPs (0.1 µg per reaction) in the presence of increasing amounts of DRB. Proteins were separated by SDS–PAGE and visualized by autoradiography.

inhibitors of CK2: the general inhibitor, heparin (35), and a specific CK2 inhibitor, 5,6-dichloro-1-β-D-ribofuranosylbenzimidazole (DRB) (36). As a control, the ability of purified CK2 to phosphorylate casein was also determined in the presence of these inhibitors. At heparin concentrations of 10 µg/ml, the phosphorylation of casein by purified CK2 was completely inhibited (Fig. 6a). However, heparin concentrations up to 100 µg/ml had no effect on the phosphorylation of MSY2 by its associated kinase (Fig. 6a). Since certain CK2 substrates such as nucleoplasmin are resistant to inhibition by heparin (37), a more specific CK2 inhibitor, DRB, was employed (Fig. 6b). Although the phosphorylation of casein by purified CK2 was reduced dramatically by 2.4 µM DRB, the phosphorylation of MSY2 by its associated kinase was unaffected at concentrations of DRB up to 24 µM (Fig. 6b). These data suggest that the kinase we detect associated with MSY2 differs from CK2.

Dephosphorylation of MSY2 results in decreased affinity for RNA

Phosphorylation of mRNP$_{3+4}$ has been reported to either increase or not change its affinity to bind RNA (12,38). To determine whether phosphorylation affects the binding of MSY2 to RNA, oligo(dT) selected mRNPs were incubated with or without CIP, separated by SDS–PAGE and analyzed by northwestern and western blotting (Fig. 7). Northwestern blot analyses using radiolabeled protamine mRNA as a probe revealed that dephosphorylation leads to a significant decrease in the affinity of two mRNP proteins of 70 and 52 kDa to bind protamine mRNA (Fig. 7i). The prominent protein band of ~55 kDa seen after CIP treatment is CIP. Western blot analysis using anti mRNP$_{3+4}$ antibody of identical samples as seen in Figure 7i, run on the same gel revealed that MSY2 migrated at the position of the 52 kDa protein seen in Figure 7i (Fig. 7ii).

DISCUSSION

In this report we describe the isolation of mRNA–protein particles from mouse male germ cells and the identification of the major proteins associated with testicular non-polysomal mRNAs: MSY2, the mammalian homolog to the *Xenopus* oocyte masking protein, mRNP$_{3+4}$, and a PABP (Fig. 2). We also detect a kinase activity bound to non-polysomal mRNAs which co-immuno-

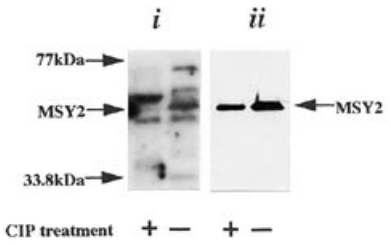

Figure 7. Dephosphorylation of MSY2 results in a decrease in its affinity for RNA. (**i**) Northwestern blot using ^{32}P-labeled protamine 2 RNA (10^5 c.p.m./ml) and (**ii**) western blot using anti mRNP$_{3+4}$ antiserum of purified oligo(dT) selected mRNPs (0.1 µg) pre-incubated in the presence (+) or absence (–) of CIP (25 U/ml).

precipitates with MSY2 and can phosphorylate MSY2 *in vitro* (Fig. 5). The MSY2 associated kinase appears not to be CK2, the kinase believed to phosphorylate mRNP$_{3+4}$ in oocytes (Fig. 6) (15,18). Since dephosphorylation of MSY2 leads to a decrease in its affinity to bind RNA (Fig. 7) and MSY2 is phosphorylated *in vivo* (Fig. 4), mRNA masking in the testes may be regulated by the phosphorylation states of MSY2. MSY2 can be dissociated from its endogenous RNAs and reassociated with non-testicular RNAs such as luciferase mRNA or brome mosaic virus RNA. Association of the non-polysomal derived proteins to non-specific mRNAs led to their translational repression *in vitro* (Fig. 3), indicating that MSY2 binds RNA in a sequence-independent fashion and its binding to mRNAs leads to their translational repression.

MSY2 has recently been cloned and sequenced and was found to be highly homologous to its *Xenopus* homolog (27). In the mouse testis, MSY2 is believed to play a similar role in mRNA masking as that of mRNP$_{3+4}$ in *Xenopus* oocytes. In *Xenopus* oocytes, both translationally competent and repressed RNAs are packaged into mRNP particles; however, only specific RNAs are recruited from these for translation (32). This suggests that mRNP$_{3+4}$ helps package a broad spectrum of RNAs. Additional RNA binding proteins likely provide specificity of translational repression and may regulate the recruitment of specific RNAs to ribosomes (4,5).

In mammalian spermatogenesis many mRNAs are under translational control and specific RNAs are temporally selected for translation (4,5). Here we show that MSY2 can be dissociated from its endogenous mRNAs and reassociated with non-testicular mRNAs such as luciferase mRNA or brome mosaic virus mRNA. Association of the non-polysomal-derived proteins to non-specific mRNAs lead to their translational repression *in vitro* (Fig. 3), at a protein to RNA mass ratio of ~4:1. The translational repression of luciferase mRNA following reconstitution with mRNP protein may either be through the binding of MSY2 or MSY2 binding in concert with other mRNP proteins (Fig. 3). Indeed, a number of specific RNA-binding proteins, including TB-RBP (4) and Prbp (5), bind to the 3′ untranslated region (UTR) of protamine and other mRNAs and have been found associated with masked paternal RNAs.

MSY1, an FRGY1 homolog, has also been shown to be associated with mouse non-testicular RNAs (25). MSY1 appears not to be one of the major mRNPs associated with non-polysomal testicular RNAs, but is probably one of a number of less abundant mRNA associated proteins (Fig. 2). As MSY2 is primarily associated with non-polysomal mRNA, a major role for MSY2 *in vivo* is probably to repress mRNA translation (Fig. 1). Therefore, in this study we focus on the ability of MSY2 to inhibit translation of mRNAs. Indeed, earlier work has shown that Y-box proteins have the capacity to direct the translational repression of mRNA in a rabbit reticulocyte lysate (11,39–42). This translational repression is dependent on the protein to RNA mass ratio (40,41). FRGY2, the homolog of MSY2, was shown to repress translation of reconstituted mRNPs *in vitro* and *in vivo* (11,41). However, at low protein to mRNA mass ratios (<2:1) a number of Y-box proteins have been shown to enhance translation (39–41). This enhancement may reflect the ability of mRNPs to destabilize secondary structure within the 5′UTR which is inhibitory to translational initiation (40). It is an intriguing possibility that MSY2 may enhance translation at low MSY2 to mRNA ratios and that Y-box proteins may play a dual role in translation. More detailed studies using recombinant MSY2 will lead to a better understanding of the role of MSY2 in translation.

MSY2 can bind RNA in an apparent sequence-independent fashion as MSY2 binds to non-testicular mRNAs such as brome mosaic virus mRNA and luciferase mRNA (Fig. 3). MSY2, like FRGY2, may bind preferentially to specific sequences within the mRNA (41,43). However, this selectability is not essential for the translational repression of mRNAs by FRGY2, at least at high FRGY2 to mRNA ratios (11–14,41).

PABP binds to the poly(A) tails of both mRNPs and polysomal mRNAs in mouse testes (34). The highly conserved 70 kDa PABP is an essential component in regulating poly(A)-dependent events in translation initiation and mRNA turnover (44,45). In mammalian cells, PABP helps protect RNAs from deadenylation/degradation (46,47). *Xenopus* oocytes have low levels of PABP which is believed to facilitate maturation-specific deadenylation and translational inactivation of maternal mRNAs (48). The high ratio of PABP to the major masking protein MSY2 (~1:1) reported here is in contrast to that ratio of PABP to mRNP$_{3+4}$ associated with purified *Xenopus* oocyte mRNPs (19). Although the functional significance of this is unclear, the high ratio of PABP to MSY2 associated with paternal mRNAs may reflect the relative lengths of the poly(A) tails of paternal and maternal mRNAs. Most maternal mRNAs have relatively short poly(A) tails of 15–80 nt (48,49) compared to the poly(A) tails of ~160 nt on many non-polysomal male germ cell mRNAs (31). PABP may confer increased stability on the stored paternal mRNAs and/or help in the masking process itself.

In addition to MSY2 and PABP, we have identified a kinase activity associated with testicular non-polysomal mRNAs. This kinase phosphorylates MSY2 *in vitro*. Dephosphorylation of MSY2 leads to a decrease in its affinity to bind RNA, suggesting the intriguing possibility that activation/inhibition of the MSY2 associated kinase and its phosphorylation of MSY2 may facilitate masking and unmasking of translationally repressed paternal mRNAs. However, we cannot exclude the possibility that another kinase not associated with the mRNA particles phosphorylates MSY2 *in vivo*.

mRNP$_{3+4}$ is also phosphorylated *in vivo* and *in vitro* by a CK2 found associated with the mRNP particles of *Xenopus* oocytes (18). In *Xenopus* oocytes, the role of phosphorylation in masking has been examined by microinjection. Masking is blocked by treating the oocytes with specific inhibitors of CK2 and by injecting antibodies against the kinase subunits (15). Progesterone

acts *in vivo* to promote the maturation of oocytes, coinciding with the recruitment of various masked mRNAs for translation. Progesterone-mediated unmasking is blocked by the specific phosphatase inhibitor okadaic acid (15). This suggests that phosphorylation of $mRNP_{3+4}$ is required to maintain translational repression. These masking and unmasking effects appear to be independent of the mRNA polyadenylation status, as the addition of cordycepin, which blocks polyadenylation, does not affect the masking and unmasking of microinjected reporter mRNAs (15).

Although the proteins and mechanisms involved in masking and unmasking of the germ cell stored mRNAs are probably highly conserved among species, specific differences such as different kinases appear to exist. Identification of the MSY2 associated kinase, its transduction pathway and specific regulatory proteins may help to define the mechanisms by which masking and unmasking of paternal mRNAs are regulated in the mammalian testis.

ACKNOWLEDGEMENT

This research was supported by NIH grant HD 29125.

REFERENCES

1 Hecht,N.B. (1998) *Bioessays*, **20**, 555–561.
2 Gold,B. and Hecht,N.B. (1981) *Biochemistry*, **20**, 4871–4877.
3 Gold,B., Stern,L., Bradley,F.M. and Hecht,N.B. (1983) *J. Exp. Zool*., **225**, 123–134.
4 Kwon,Y.K. and Hecht,N.B. (1993) *Mol. Cell. Biol*., **13**, 6547–6557.
5 Lee,K., Fajardo,M.A. and Braun,R.E. (1996) *Mol. Cell. Biol*., **16**, 3023–3034.
6 Deschamps,S., Viel,A., Garrigos,M., Denis,H. and Le Maire,M. (1992) *J. Biol. Chem*., **267**, 13799–13802.
7 Darnbrough,C.H. and Ford,P.J. (1981) *Eur. J. Biochem*., **113**, 415–424.
8 Murray,M.T., Krohne,G. and Franke,W.W. (1991) *J. Cell. Biol*., **112**, 1–11.
9 Tafuri,S.R. and Wolffe,A.P. (1990) *Proc. Natl Acad. Sci. USA*, **87**, 9028–9032.
10 Murray,M.T., Schiller,D.I. and Franke,W.W. (1992) *Proc. Natl Acad. Sci. USA*, **89**, 11–15.
11 Richter,J.D. and Smith,L.D. (1983) *Nature*, **309**, 378–380.
12 Kick,D., Barrett,P., Cummings,A. and Sommerville,J. (1987) *Nucleic Acids Res*., **15**, 4099–4109.
13 Ranjan,M., Tafuri,S.R. and Wolffe,A.P. (1993) *Genes Dev*., **7**, 1725–1736.
14 Bouvet,P. and Wolffe,A.P. (1994) *Cell*, **77**, 931–941.
15 Braddock,M., Muckenthaler,M., White,M.R.H., Thorburn,A.M., Sommerville,J., Kingsman,A.J. and Kingsman,S.M. (1994) *Nucleic Acids Res*., **22**, 5255–5264.
16 Gunkel,N., Braddock,M., Thorburn,A.M., Muckenthaler,M., Kingsman,A.J. and Kingsman,S.M. (1995) *Nucleic Acids Res*., **23**, 405–412.
17 Dearsley,A.L., Johnson,R.M., Barrett,P. and Sommerville,J. (1985) *Eur. J. Biochem*., **150**, 95–103.
18 Cummings,A. and Sommerville,J. (1988) *J. Cell. Biol*., **107**, 45–56.
19 Marello,K., LaRovere,J. and Sommerville,J. (1992) *Nucleic Acids Res*., **20**, 5593–5600.
20 Murray,M.T. (1994) *Biochemistry*, **33**, 13910–13916.
21 Yurkova,M.S. and Murray,M.T. (1997) *J. Biol. Chem*., **272**, 10870–10876.
22 Meric,F., Matsumoto,K. and Wolffe,A.P. (1997) *J. Biol. Chem*., **272**, 12840–12846.
23 Ladomery,M., Wade,E. and Sommerville,J. (1997) *Nucleic Acids Res*., **25**, 965–973.
24 Kwon,Y.K., Murray,M.T. and Hecht,N.B. (1993) *Dev. Biol*., **15**, 90–100.
25 Tafuri,S.R., Familari,M. and Wolffe,A.P. (1993) *J. Biol. Chem*., **268**, 12213–12220.
26 Oko,R., Korley,R., Murray,M.T., Hecht,N.B. and Hermo,L. (1996) *Mol. Reprod. Dev*., **44**, 1–13.
27 Gu,W., Tekur,S., Reinbold,R., Eppig,J.J., Choi,Y.C., Zheng,J.Z., Murray,M.T. and Hecht,N.B. (1998) *Biol. Reprod*., **59**, 1266–1274.
28 Yiu,G.K. and Hecht,N.B. (1997) *J. Biol. Chem*., **272**, 26926–26933.
29 Pullman,J.M. and Martin,T.E. (1983) *J. Cell. Biol*., **97**, 99–111.
30 Houman,F., Diaz-Torres,M.R. and Wright,A. (1990) *Cell*, **62**, 1153–1163.
31 Kleene,K.C., Distel,R.J. and Hecht,N.B. (1984) *Dev. Biol*., **105**, 71–79.
32 Tafuri,S.R. and Wolffe,A.P. (1993) *J. Biol. Chem*., **268**, 24255–24261.
33 Wang,M.Y., Cutler,M., Karimpour,I. and Kleene,K.C. (1992) *Nucleic Acids Res*., **20**, 3519.
34 Gu,W., Kwon,Y., Oko,R., Hermo,L. and Hecht,N.B. (1995) *Mol. Reprod. Dev*., **40**, 273–285.
35 Hathway,G.M., Lubben,T.H. and Traugh,J.A. (1980) *J. Biol. Chem*., **255**, 8038–8041.
36 Zandomeni,R. and Weinmann,R. (1984) *J. Biol. Chem*., **259**, 14804–14811.
37 Taylor,A., Allende,C.C., Weinmann,R. and Allende,J.E. (1987) *FEBS Lett*., **226**, 109–114.
38 Deschamps,S., Jacquemin-Sablon,H., Triqueneaux,G., Mulner-Lorillon,O., Potier,M., Le Caer,J.P., Dautry,F. and le Maire,M. (1997) *FEBS Lett*., **412**, 495–500.
39 Minich,W.B. and Ovchinnikov,L.P. (1992) *Biochimie*, **74**, 477–483.
40 Evdokimova,V.M., Kovrigina,E.A., Nashchekin,D.V., Dayydova,E.K., Hershey,J.W.B. and Ovchinnikov,L.P. (1998) *J. Biol. Chem*., **273**, 3574–3581.
41 Matsumoto,K., Meric,F. and Wolffe,A.P. (1996) *J. Biol. Chem*., **271**, 22706–22712.
42 Davydova,E.K., Evdokimova,V.M., Ovchinnikov,L.P. and Hershey,J.W.B. (1997) *Nucleic Acids Res*., **25**, 2911–2916.
43 Bouvet,P., Matsumoto,K. and Wolffe,A.P. (1995) *J. Biol. Chem*., **270**, 28297–28303.
44 Sachs,A. and Wahle,E. (1993) *J. Biol. Chem*., **268**, 22955–22958.
45 Standart,N. (1993) *Mol. Biol. Reports*, **18**, 135–142.
46 Bernstein,P., Peltz,S.W. and Ross,J. (1989) *Mol. Cell. Biol*., **9**, 659–670.
47 Caruccio,N. and Ross,J. (1994) *J. Biol. Chem*., **269**, 31814–31821.
48 Wormington,M., Searfoss,A.M. and Hurney,C.A. (1996) *EMBO J*., **15**, 900–909.
49 Cabada,M.O., Darnbrough,C., Ford,P.J. and Turner,P.C. (1977) *Dev. Biol*., **57**, 427–439.