

Germ Cell-Specific Proteins Interact with the 3' Untranslated Regions of *Prm-1* and *Prm-2* mRNA

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The testis-specific mouse protamine genes (*Prm-1* and *Prm-2*) are transcribed in haploid round spermatids, their mRNAs stored as cytoplasmic ribonucleoprotein particles and translated about 1 week later in elongating spermatids. We have compared the *in vitro* translational efficiencies of deproteinized *Prm-1* mRNA isolated from purified populations of germ cells and found that *Prm-1* mRNA from round spermatids translates as efficiently as *Prm-1* mRNA from elongating spermatids, suggesting that translation of *Prm-1* mRNA is normally repressed in round spermatids. Previous studies in transgenic mice have shown that the 3' UTR of *Prm-1* mRNA is necessary and sufficient for its translational control (Braun *et al.*, 1989). In this manuscript, we have used an RNA band shift assay to identify an activity, present in cytoplasmic fractions of meiotic spermatocytes and postmeiotic round spermatids, that binds the 3' UTRs of both *Prm-1* and *Prm-2* mRNA. We have used 3' UTR deletion variants to map the binding site to a 22-nt region within the *Prm-1* 3' UTR and to a 20-nt region within the *Prm-2* 3' UTR. uv cross-linking of the RNA band shift activities detected with the *Prm-1* and *Prm-2* 3' UTRs generated the same two RNA/protein complexes of 53 and 55 kDa. The presence of the binding activity in the cell type and subcellular compartment associated with *Prm-1* and *Prm-2* mRNA storage suggest that the activity may be actively engaged in translational repression of these mRNAs. © 1994

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INTRODUCTION

The protamines are small arginine-rich proteins that are found associated with the DNA in the mature condensed spermatozoon nucleus (Balhorn, 1982; Oliva and Dixon, 1991). In the mouse there are two protamine genes, *Prm-1* and *Prm-2* (Kleene *et al.*, 1985; Yelick *et al.*, 1987). Transcription of *Prm-1* and *Prm-2* initiates shortly after the completion of meiosis in round sper-

matids (Kleene *et al.*, 1983; Mali *et al.*, 1989) and ceases about a week later in early elongating spermatids when all transcription stops (Kierszenbaum and Tres, 1975; Monesi, 1964). During this time, the protamine mRNA is transported to the cytoplasm and stored as a translationally inert ribonucleoprotein particle until it is translated in elongating spermatids 2 to 8 days later (Balhorn *et al.*, 1984; Kleene *et al.*, 1984).

The *cis*-acting sequences that are required for the proper temporal translational control of *Prm-1* mRNA have been localized to its 3' untranslated region (UTR) through the study of gene fusions in transgenic mice (Braun *et al.*, 1989; Braun, 1990). A heterologous transgene that contained the *Prm-1* promoter, the human growth hormone reporter gene, and either the entire 156 nt 3' UTR of the *Prm-1* gene or only the 3'-most 62 nt of the 3' UTR was transcribed in round spermatids, but its mRNA not translated until up to a week later in elongating spermatids, demonstrating that sequences in the *Prm-1* 3' UTR mediate its translational regulation. Although it has not been demonstrated, it is likely that the 3' UTR of *Prm-2* also contains the necessary *cis*-acting sequences for its translational regulation. For both *Prm-1* and *Prm-2*, one assumes that there may be sequence-specific RNA binding proteins that bind to their 3' UTRs and in some way repress their translation. Identification of such RNA binding proteins is an important first step toward a molecular and genetic dissection of the translational control mechanism. Using an RNA band shift assay, Kwon and Hecht (1991) have identified two regions of the *Prm-2* 3' UTR that bind to proteins present in S100 extracts prepared from the testis. They have also shown that a protein of 18 kDa can be cross-linked to one of the sites with uv light.

In this paper we show that deproteinized *Prm-1* mRNA isolated from purified round spermatids is translatable *in vitro*, supporting the hypothesis that translation of *Prm-1* mRNA is normally inhibited in round spermatids. In addition, we describe two previously unidentified germ cell-specific proteins, of approximately

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48 and 50 kDa, that bind in a sequence-specific manner to the 3' UTRs of *Prm-1* and *Prm-2* mRNA.

MATERIALS AND METHODS

Mice

Swiss Webster males were purchased from Simonsen Laboratories Inc. (Gilroy, CA). *Tfm/Y*, *W/W^v*, and *qk/qk* males were all obtained from The Jackson Laboratory (Bar Harbor, ME).

Germ Cell Purification

Purified populations of pachytene spermatocytes, round spermatids, and elongating spermatids were obtained by unit gravity sedimentation of testis cell suspensions through 2–4% bovine serum albumin (BSA) gradients using a Staptut sedimentation chamber (O. H. Johns Scientific, Toronto, Canada) as described (Romrell *et al.*, 1976). The purity of individual fractions collected was determined by phase-contrast or differential interference contrast microscopy. Fractions containing highly enriched populations of pachytene spermatocytes (>80% pure) round spermatids (80–90% pure), and elongating spermatids plus cytoplasmic fragments (>80% pure) were pooled and concentrated by centrifugation. RNA and nuclear and cytoplasmic protein extracts were prepared from each cell pellet as described below.

Tissue RNA Purification

Total cytoplasmic RNA was prepared as described (Favaloro *et al.*, 1980) with the following modifications. Frozen spermatid pellets containing $1\text{--}2 \times 10^7$ cells were thawed and resuspended in four packed cell volumes of RNA extraction buffer containing 0.14 M NaCl, 1.5 mM MgCl₂, 10 mM Tris-HCl (pH 8.6), 0.5% NP-40, 1 mM DTT, and 1000 units/ml RNasin. Nuclei and cell debris were removed by centrifugation at 12,000g for 90 sec at 4°C. The supernatant was recovered, diluted with an equal volume of proteinase digestion buffer containing 0.2 M Tris-HCl (pH 8.0), 25 mM EDTA, 0.3 M NaCl, 2% SDS, and 50 µg/ml proteinase K, and incubated at 37°C for 30 min. The RNA was phenol extracted, precipitated with isopropanol, resuspended in ddH₂O, and quantitated by absorbance at A₂₆₀.

Protein Extracts

Extracts were prepared as described (Dignam *et al.*, 1983) with the following modifications. Testes were dissected from two sexually mature mice and suspended in 0.4 ml (50–100 µl/mg tissue) Buffer A [10 mM Hepes (pH 7.6), 1.5 mM MgCl₂, 10 mM KCl, 0.5 mM DTT] supple-

mented with protease inhibitors as follows: 0.5 µg/ml aprotinin, 0.5 µg/ml pepstatin A, 0.5 µg/ml leupeptin, 1 µg/ml PMSF, 5 µg/ml Na-T-boc-deacetyl-leupeptin (Sigma). Cells were lysed using 10–20 strokes of a hand-held Dounce homogenizer. Nuclei and cell debris were removed from the lysate by centrifugation at 300g for 10 min in a fixed-angle rotor. The pellet was set aside and the supernatant (cytoplasmic extract) was gently mixed with 0.1 vol Buffer B [0.3 M Hepes (pH 7.6), 1.4 M KCl, 0.03 M MgCl₂] and dialyzed for 5 hr against Buffer D [20 mM Hepes (pH 7.6) 20% v/v glycerol, 0.1 M KCl, 0.2 mM EDTA, 0.5 mM DTT]. The nuclear pellet was thoroughly resuspended and homogenized in 0.4 ml Buffer C [20 mM Hepes (7.6), 25% v/v glycerol, 0.42 M NaCl, 1.5 mM MgCl₂, 0.2 mM EDTA, 0.5 mM DTT] supplemented with protease inhibitors, stored on ice for 30 min, and centrifuged at 16,000g for 30 min. The supernatant (nuclear extract) was dialyzed against Buffer D for 5 hr and cleared by centrifugation at 16,000g for 20 min.

Fractionated germ cells ($10^7\text{--}10^8$ pachytene spermatocytes, round or elongating spermatids) were collected by centrifugation at 300g for 5 min, washed, resuspended in 0.8 ml Buffer A. Nuclear and cytoplasmic extracts were prepared as described above. Protein concentrations, measured against a BSA standard using a Bradford assay (Bradford, 1976), were 25–50 mg/ml for the cytoplasmic extracts and 1–2 mg/ml for the nuclear extracts.

In Vitro Translation

Total cytoplasmic RNA from round or elongating spermatids (0.8 µg) was denatured at 65°C for 2 min, chilled, and translated for 2 hr at 25°C in a 10-µl wheat germ reaction (Promega) containing 60 mM KCl and supplemented with 0.5 mCi/ml [³⁵S]cysteine (NEN-DuPont). Completed reactions were terminated by the addition of RNase A to 100 µg/ml followed by a 15 min incubation at 37°C and processed for gel electrophoresis.

Preparation of Translation Extracts for Electrophoresis

Translation extracts were prepared for electrophoresis through acid-urea polyacrylamide gels by a modification of a previously described method (Marushige and Marushige, 1974; Kleene and Flynn, 1987). In brief, a 10-µl wheat germ reaction was reduced and denatured by the addition of an equal volume of a solution containing 12 M urea, 0.1 M β-mercaptoethanol, 0.3 M Tris-HCl (pH 8.0), followed by a 1-hr incubation at 37°C. Alkylation (to improve electrophoretic resolution of protamines) was accomplished with the addition of 20 µl of a solution containing 0.5 M iodoacetamide, 6 M urea, 0.3 M Tris-HCl (pH 8.0) followed by a 1-hr incubation at 37°C protected from light. Samples were mixed with 50–100 µg

alkylated late stage spermatid basic nuclear proteins prepared as previously described (Meistrich *et al.*, 1976) and diluted to a final volume of 400 μ l adjusted to 0.25 *N* HCl. After a 1-hr incubation at 4°C, insoluble proteins were pelleted with a 15-min microfuge spin and discarded. Basic proteins were precipitated from the supernatant in 20% TCA, collected by centrifugation, and dissolved in acid-urea sample buffer containing 1 *M* urea, 0.9 *M* acetic acid, 1% methyl green, and 10% glycerol. The samples were boiled for 2 min and electrophoresed through slab gels cast from 15% acrylamide, 0.1% bis-acrylamide, 6.2 *M* urea, and 0.9 *M* acetic acid as previously described (Panyim and Chalkley, 1969).

Northern Analysis

Cytoplasmic RNA from round or elongated spermatids was denatured for 15 min at 68°C in a 50% formamide, 2.2 *M* formaldehyde buffer and electrophoresed through a 1% agarose gel cast in running buffer containing 0.02 *M* Mops (pH 7.0), 8 *mM* sodium acetate, 5 *mM* EDTA (pH 8.0), and 2.2 *M* formaldehyde (Goldberg, 1980; Lehrach *et al.*, 1977; Seed, 1982). RNA was electrophoresed at 3–4 V/cm for 3–4 hr, transferred to nitrocellulose (Thomas, 1980), and hybridized with a mouse *Prm-1* cDNA that was labeled by random oligonucleotide-primed synthesis (Feinberg and Vogelstein, 1984) with [α -³²P]dATP to a specific activity of 3×10^8 cpm/ μ g.

RNA Probe Preparation

RNA probes were synthesized using SP6 or T7 bacteriophage polymerases using protocols suggested by the supplier (Promega). *In vitro* transcription reactions were generally performed in 20- μ l reaction volumes using 1 μ g of linearized plasmid template, 500 μ M ATP, CTP, and GTP, 20 μ M UTP, and 50 μ Ci [α -³²P]UTP at 3000 Ci/mmol (NEN-Dupont) for approximately 60 min. Samples were heat denatured for 5 min at 70°C, mixed with 5 μ l 50% glycerol, and electrophoresed in a 5% polyacrylamide (30:1) and TBE buffer containing 89 *mM* Tris, 89 *mM* boric acid, and 2 *mM* EDTA. The full-length products were visualized by autoradiography, excised from the gel, and eluted overnight in 150 μ l 0.5 *M* ammonium acetate, 0.5 *M* EDTA, and 0.1% SDS at 37°C. The acrylamide slice was removed from the sample by microcentrifugation through mini-glass wool columns and the aqueous phase precipitated with 0.2 *M* NaCl, 40 μ g carrier yeast RNA, and 2.5 vol pure ethanol. RNA pellets were collected by microcentrifugation, washed in 70% ethanol, and resuspended in 100 μ l H₂O.

RNA Band Shift Analysis

RNA band shift assays were performed as described (Koeller *et al.*, 1989) with the following modifications.

RNA probes (1 μ l, $\sim 10^5$ cpm) purified as described above, were combined with 3 μ l H₂O, and 5 μ l 2 \times RNA binding buffer containing 40 *mM* Hepes (pH 7.6), 6 *mM* MgCl₂, 80 *mM* KCl, 4 *mM* DTT, and 10% glycerol, heat denatured at 70°C for 5 min, and slow cooled at room temperature for 30 min. Following renaturation, 1 μ l of protein extract (25–50 μ g/ μ l cytoplasmic or 1–2 μ g/ μ l nuclear) was added to the sample. The samples were incubated at room temperature for 20 min and treated sequentially with 1 μ l of RNase T1 (Calbiochem) at 0.5 units/ μ l, and 2 μ l of heparin (Sigma) at 50 μ g/ μ l, each for 10 min at room temperature. The samples were combined with 5 μ l of 50% glycerol and electrophoresed through a 4% polyacrylamide (60:1) gel for approximately 3 hr at 4°C and 180 V in a running buffer of 45 *mM* Tris (pH 8.3), 50 *mM* boric acid, and 1 *mM* EDTA. Gels were dried and visualized by autoradiography.

RNase T1 Mapping

RNA probes, synthesized as described above, were purified with the following modifications. Samples were heat denatured for 5 min at 70°C, mixed with 10 μ l of RNA sequencing buffer containing 20 *mM* sodium citrate (pH 5.0), 1 *mM* EDTA, 7 *M* urea, and 0.025% (w/v) of both xylene cyanol and bromophenol blue (Knapp, 1989), electrophoresed on a denaturing gel of 20% polyacrylamide and 7 *M* urea, and the RNA products were purified as described above. A preparative band shift was performed as described except that the gel was not dried before visualization by autoradiography. The RNA-protein complexes were excised from the gel, soaked overnight in 300 μ l of gel elution buffer, passed through a mini-glass wool column, extracted once with phenol, once with chloroform, and ethanol precipitated. RNA pellets were collected by microcentrifugation, washed with 70% ethanol, and resuspended in ddH₂O to a final concentration of 800–1000 cpm/ μ l. The RNA was incubated in RNA sequencing buffer containing 2 units of RNase T1, or 0.2 units RNase A, for 15 min at 50°C and then immediately put into an ice-slurry bath (Knapp, 1989). To confirm the identity of the protected RNase T1 fragment, complementary (5'-TAGTTT-GAGGA-3') and noncomplementary (5'-GACAGGTGG-CATTGTTCTTA-3') oligonucleotides were combined with the isolated RNA and incubated with 0.5 units of RNase H in 1 \times RNA binding buffer for 30 min at 37°C, mixed with 5 μ l RNA sequencing buffer, heat denatured for 5 min at 70°C, and put on ice. All samples were electrophoresed on a denaturing 20% polyacrylamide 7 *M* urea gel.

uv Cross-linking

RNA probes were heat denatured, renatured by slow cooling at room temperature, treated with protein ex-

tracts, RNase T1, and heparin exactly as described for the band shift assays. Following heparin treatment, the samples were placed on ice, with the tops of the microcentrifuge tubes open, and irradiated with an ultraviolet light source located a distance of approximately 0.3 m away for 30 min. The irradiated samples were boiled in Laemmli buffer and analyzed by SDS-polyacrylamide gel electrophoresis using a 5% stacking and a 9% separating gel (Laemmli, 1970). The acrylamide gels were dried and visualized by autoradiography. Prestained protein molecular weight markers were purchased from GIBCO-BRL.

RESULTS

Translational Efficiency of Prm-1 mRNA in Vitro

To distinguish between negative and positive regulatory mechanisms of translational control of *Prm-1*, we isolated total cytoplasmic RNA from purified populations of round and elongating spermatids and compared the translational efficiency of *Prm-1* mRNA present in each cell type *in vitro*. Total cytoplasmic RNA extracted from both cell populations directed the synthesis of numerous proteins encompassing a wide range of molecular weights in both wheat germ and reticulocyte systems (data not shown). To visualize the translation product of *Prm-1* mRNA from each population, we translated equivalent amounts of deproteinized cytoplasmic RNA in parallel wheat germ reactions containing [³⁵S]cysteine. The completed reactions were alkylated, acid extracted, electrophoresed through an acid urea gel, and autoradiographed (Fig. 1A, lanes 2 and 3). Three pieces of evidence support the assignment of the most rapidly migrating band as protamine 1 (P1). First, this protein comigrates precisely with the *in vitro* product specified by an SP6 RNA polymerase transcript produced from a mouse *Prm-1* cDNA (data not shown). Second, it comigrates with unlabeled P1 carrier protein, itself identified with a P1-specific monoclonal antibody on a Western blot (data not shown). Finally, hybridization of testis cytoplasmic RNA with an antisense oligonucleotide complementary to the 5' end of the *Prm-1* mRNA followed by RNase H digestion of the annealed RNA resulted in the specific loss of this protein band in subsequent *in vitro* translation reactions (data not shown).

To determine the translational efficiency of *Prm-1* *in vitro*, we compared the amount of P1 protein synthesized per unit mass of *Prm-1* mRNA present in total cytoplasmic RNA purified from round or elongating spermatids. Densitometry was used to quantitate the amount of P1 translated *in vitro*. The relative amount of *Prm-1* mRNA per microgram of total RNA in each cell population was determined by quantitative Northern blot analysis of RNA purified from each cell type (Fig. 1B).

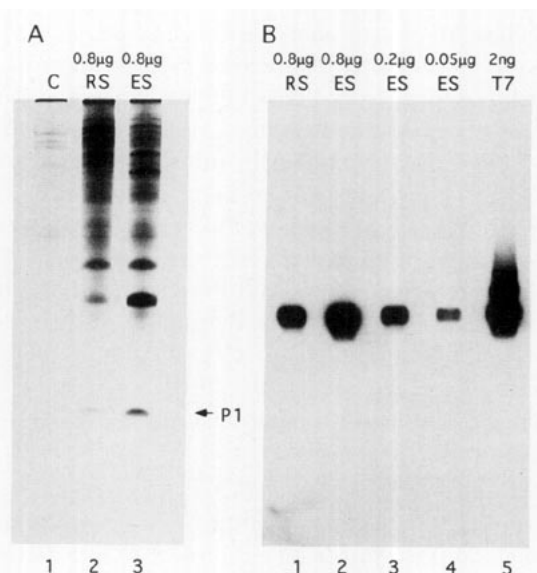


FIG. 1. Comparative *in vitro* translational efficiencies of *Prm-1* mRNA isolated from round versus elongating spermatids. Total cytoplasmic RNA isolated from purified populations of round and elongating spermatids was used in *in vitro* translation and Northern analyses. (A) *In vitro* translation. RNAs (lane 1, control with no RNA added; lane 2, 0.8 µg round spermatid (RS) RNA; lane 3, 0.8 µg elongating spermatid (ES) RNA) were translated in a 10-µl wheat germ reaction containing [³⁵S]cysteine. The position of the protamine 1 translation product is indicated to the right of the autoradiogram by the arrow labeled P1. (B) Northern blot. Lane 1, 0.8 µg RS RNA; lane 2, 0.8 µg ES RNA; lane 3, 0.2 µg ES RNA; lane 4, 0.05 µg ES RNA; lane 5, 2 ng of a T7 RNA polymerase runoff transcript of a cDNA clone encoding mouse *Prm-1*.

From this analysis we determined that the *Prm-1* mRNA isolated from round spermatids was translated with twice the efficiency as that isolated from elongated spermatids. These results demonstrate that the *Prm-1* mRNA present in round spermatids is translationally competent in a wheat germ translation extract and suggests that its failure to be translated in round spermatids *in vivo* is due to active repression.

RNA Band Shift Analysis

RNA band shift analysis of the full-length 3' UTRs of *Prm-1*, *Prm-2*, and human growth hormone (hGH) is shown in Fig. 2. Lanes 1, 5, and 9 contain the free RNA probes prior to incubation with extract. Treatment of the probes with RNase T1 reduced all of the free probes to a collection of smaller RNAs of which only the largest are present (lanes 2, 6, and 10). Incubation of the RNA probes with testis cytoplasmic extract resulted in all of the probes migrating as large smears (lanes 3, 7, and 11). Treatment of *Prm-1* 3' UTR RNA with testis protein extract, RNase T1, and heparin resolved the smear into two bands, an upper darker band and a lower lighter

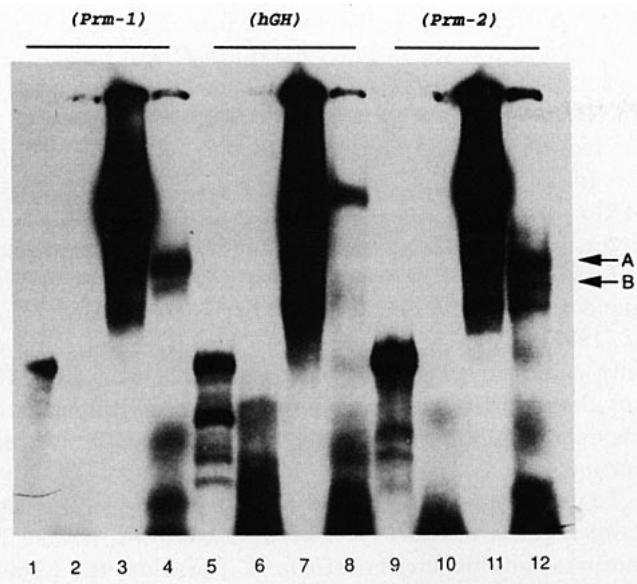


FIG. 2. RNA band shift analysis of *Prm-1*, *Prm-2*, and hGH 3' UTRs. Samples containing probe alone (2×10^4 cpm) were combined with RNA binding buffer and H_2O , incubated at room temperature for 40 min, combined with 50% glycerol, and heated at $68^\circ C$ for 5 min prior to loading onto a 4% polyacrylamide gel (lane 1, 156-nt *Prm-1* 3' UTR; lane 5, 144-nt hGH 3' UTR; and lane 9, 172-nt *Prm-2* 3' UTR). All other RNAs (1×10^5 cpm) were combined with RNA binding buffer and H_2O , heat denatured at $68^\circ C$ for 5 min, slow cooled at room temperature for 30 min, treated as indicated below, and combined with 50% glycerol prior to gel loading. RNA samples in lanes 2, 6, and 10 were treated with RNase T1 (0.5 units) alone for 10 min at room temperature. RNA samples in lanes 3, 7, and 11 were incubated with 1 μ l of testis cytoplasmic protein extract (25–50 μ g/ μ l) alone for 20 min at room temperature. Lanes 4, 8, and 12 contain samples that were treated sequentially at room temperature with 1 μ l of testis protein extract for 20 min, RNase T1 for 10 min, and heparin (10 mg/ml) for 10 min. Arrows labeled A and B refer to two bands that are seen with both the *Prm-1* and *Prm-2* RNAs.

band (lane 4), that migrate more slowly than the free *Prm-1* probe. Bands of similar mobility, labeled with arrows as A and B, are also seen with a *Prm-2* 3' UTR probe (lane 12), as is a third band with slightly faster mobility. None of the bands was detected with a radiolabeled hGH 3'-UTR probe, although a new band of even lesser mobility was detected (lane 8), suggesting that the bands labeled A and B are specific to *Prm-1* and *Prm-2*.

To confirm that the complexes are specific to *Prm-1* and *Prm-2*, RNA competition experiments were performed as shown in Fig. 3. Preincubation of testis cytoplasmic extracts with 25, 100, and 300 ng of *in vitro* synthesized unlabeled *Prm-1* 3' UTR RNA resulted in nearly complete elimination of both activities as detected with a 156-nt radiolabeled *Prm-1* 3' UTR probe, whereas preincubation of the extracts with equivalent amounts of unlabeled hGH competitor RNA did not deplete the activities. In addition, unlabeled *Prm-2* 3' UTR RNA served as a successful competitor against radiolabeled

Prm-1 3' UTR probe, and unlabeled *Prm-1* 3' UTR RNA competed for protein binding to a radiolabeled *Prm-2* 3' UTR probe (data not shown). Thus, the same proteins appear to be binding to the 3' UTRs of *Prm-1* and *Prm-2* RNA (see below).

Testis and Germ Cell Localization of RNA Binding Proteins

Prm-1 RNA band shift assays performed with cytoplasmic extracts prepared from the testis, heart, kidney, liver, and spleen demonstrated that the binding activities were detected only with testis extracts as shown in Fig. 4. To determine if the factors are present in germ cells, and if they are expressed at a specific time during spermatogenesis, extracts were prepared from testes isolated from mouse mutants that are blocked at different stages of germ cell development. Mice that are transheterozygous for the *W* and *W^v* mutations are devoid of germ cells (Mintz and Russell, 1957), while mice that carry the X-linked *Tfm* mutation contain germ cells but spermatogenesis is arrested at the beginning of meiosis I (Lyon and Hawkes, 1970). Testis extracts prepared from *W/W^v* and from *Tfm/Y* males lack both binding activities (Fig. 4), suggesting that the factors are present in the germ cell compartment of the testis and that they are synthesized after the initiation of meiosis I. Male mice that are homozygous for the pleiotropic mutation quaking, *qk/qk*, are sterile as a result of an arrest of spermatogenesis during spermatid differentiation

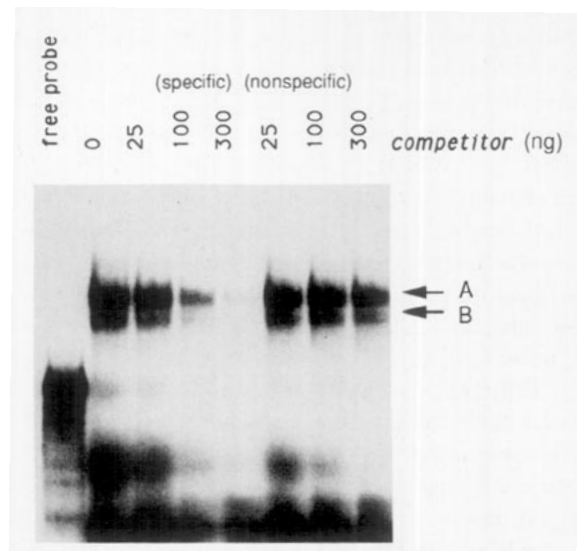


FIG. 3. RNA band shift competition analysis. Band shift assays were performed as indicated in Fig. 2 using a radiolabeled 156-nt *Prm-1* 3' UTR probe (10^5 cpm, 1 ng RNA) except that protein extracts were preincubated for 15 min with either unlabeled 156-nt *Prm-1* 3' UTR (specific) or 144-nt hGH 3' UTR (nonspecific) RNA. The arrows labeled A and B refer to the *Prm-1*-specific bands described in Fig. 2.

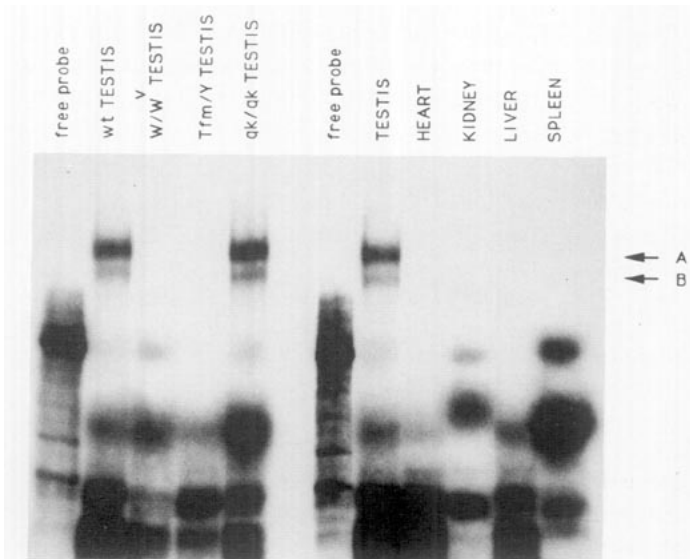


FIG. 4. RNA band shift analysis of *Prm-1* 3' UTR binding activity in testis extracts prepared from various mouse mutants and in adult tissues. In each lane the probe used was the full-length 156-nt *Prm-1* 3' UTR. Samples were treated as described in Fig. 2. The arrows labeled A and B refer to the *Prm-1*-specific bands described in Fig. 2.

(Bennett *et al.*, 1971). These animals normally transcribe and translate *Prm-1* (Hecht *et al.*, 1985). As expected, both binding activities are present in extracts prepared from *qk/qk* homozygotes.

Preparation of extracts from purified populations of spermatocytes and spermatids demonstrated directly that the factors are present in germ cells. RNA band shift assays performed with extracts prepared from three different germ cell types show that the factors are present in the cytoplasm of round spermatids and pachytene spermatocytes (Fig. 5). They are, however, absent from nuclear extracts of these cells and are greatly diminished in cytoplasmic extracts prepared from elongating spermatids. To eliminate the possibility that the binding activity detected in pachytene spermatocytes is due to contamination of the purified pachytene spermatocytes with round spermatids, we also prepared extracts from testes dissected from 18-day-old prepubertal animals, which do not contain postmeiotic cells. Both factors were detected, confirming that the binding activity is present in pachytene spermatocytes (data not shown).

Deletion Mapping and RNase T1 Footprint Analysis

To further map the binding sites in *Prm-1* for the activities referred to as A and B in Fig. 2, additional band shift analyses were performed with deletion variants of the *Prm-1* 3' UTR. A summary of these data is shown in Fig. 6. Formation of both complexes was observed with

an RNA containing as little as the first 37 nt of the *Prm-1* 3' UTR and with an RNA extending from nucleotides 16 through 81. Loss of both complexes was observed with an RNA terminating at nucleotide 23, or beginning at nucleotide 35. This analysis suggests that the binding site for both complexes is located within the 22-nt region of the *Prm-1* 3' UTR between nucleotides 16 and 37. To determine if the 22-nt region defined by the deletion endpoints is sufficient for complex formation, we used oligonucleotide-directed *in vitro* transcription (Milligan *et al.*, 1987) to synthesize just the 22-nt RNA. Binding of both complexes was detected with the 22-nt RNA (data not shown), confirming that sequences to either side of the deletion endpoints are not required for complex formation.

To determine the size and identity of the RNA fragment present in the *Prm-1* binding complex, RNase T1 footprint analysis was performed. To isolate the RNase T1 fragment present in the complex, the protein-bound fragment was excised and eluted from a preparative band shift gel. Due to the difficulty of separating the upper and lower bands on a preparative gel, the entire dou-

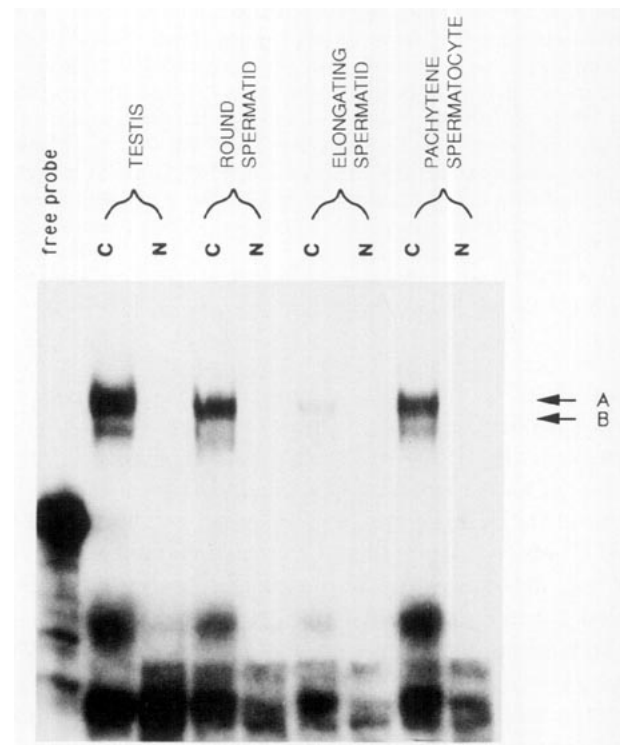


FIG. 5. RNA band shift analysis of *Prm-1* 3' UTR binding activity in extracts prepared from purified germ cells. Cytoplasmic (C) and nuclear (N) extracts were prepared from whole testis and purified round spermatids, elongating spermatids, and pachytene spermatocytes as described under Materials and Methods. In each lane the probe used was the full-length 156-nt *Prm-1* 3' UTR. Samples were treated as described in Fig. 2. The arrows labeled A and B refer to the *Prm-1*-specific bands described in Fig. 2.

linker sequence	<i>Prm-1</i> nucleotides		binding activity
22	156	<i>Prm-1</i> 3'-UTR AAUAAA →	+
38	81	→ 81	+
18	72	→ 72	+
18	51	→ 51	+
18	37	→ 37	+
18	23	→ 23	-
50	66	16 → 81	+
50	47	35 → 81	-
0	22	16 → 37	+

FIG. 6. Summary of deletion analysis of the *Prm-1* 3' UTR band shift complex. RNA probes were prepared that contain portions of the *Prm-1* 3' UTR RNA and polylinker sequence from the plasmid vector as indicated. The number of nucleotides in the RNA that was contributed by the linker sequence of the multiple cloning site in the vector is indicated. Band shift assays were performed with testis cytoplasmic extracts and each RNA. The full-length *Prm-1* RNA is 156 nt in length. Drawn adjacent to each RNA is the first and/or last nucleotide of each transcript.

blet was excised, eluted overnight, and extracted with phenol and chloroform. A single RNA species was found. It comigrated with the unique 16-nt RNase T1 product released from the original 37-nt RNA probe (Fig. 7, lanes 2 and 4). Treatment of the eluted fragment with RNase T1 did not alter its mobility, demonstrating that it did not have an internal RNase T1 site and suggesting that it was the same 16-nt fragment as was generated from the starting RNA probe (lane 5). Treatment of the eluted RNA with RNase A cleaved the RNA fragment into a number of smaller fragments, demonstrating that there was not a general inhibitor of RNase in the sample (Fig. 7, lane 6). Likewise, addition of the eluted fragment back to the starting RNA did not inhibit cleavage of the starting RNA with RNase T1 (Fig. 7, lanes 7 and 8). Last, hybridization of the eluted RNA fragment to a 12-nt oligonucleotide that is complementary to a region of the predicted 16-nt RNase T1 product of the starting RNA rendered it sensitive to cleavage by RNase H (lane 9), whereas hybridization of a random 21-nt oligonucleotide did not (lane 10). As summarized in Fig. 8, these results show that both the upper and lower bands visualized in the band shift assay contain a 16-nt RNase T1 fragment that maps between nucleotides 21 and 36 in the 5'-most region of the *Prm-1* 3' UTR.

To map the site responsible for formation of complex A and B in *Prm-2*, deletion variants of the *Prm-2* 3' UTR were prepared and used in RNA band shift experiments.

Figure 9 contains a summary of these experiments. Binding of both complexes was observed with RNAs extending from nt 1 through 104 and from 85 through 172, but not with an RNA terminating at nt 93 or with an RNA that initiates at nt 101. These data suggest that the binding site for both *Prm-2* complexes is located within the 20-nt region between nucleotides 85 and 104. Comparison of the sequences within the binding sites for *Prm-1* and *Prm-2* reveals a region of 7- to 9-nt sequence identity (Fig. 8), suggesting that sequences contained within this site may be important for protein binding.

uv Cross-linking

Ultraviolet light was used to covalently cross-link radiolabeled *Prm-1* and *Prm-2* 3' UTR RNA to proteins present in testis cytoplasmic extracts. RNA fragments and testis protein extracts were combined as for the

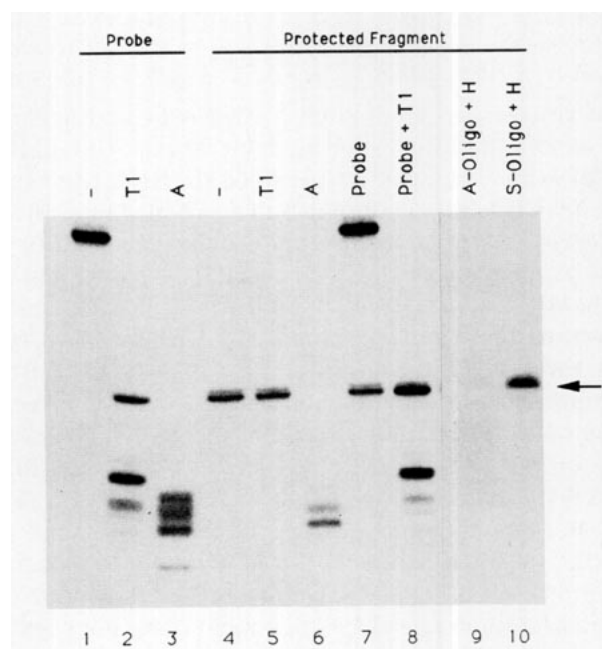


FIG. 7. RNase T1 footprint analysis of the *Prm-1* band shift complex. A [32 P]UTP-radiolabeled RNA probe containing the first 37-nt of the *Prm-1* 3' UTR and 18-nt of polylinker sequence was synthesized *in vitro* and purified on a 20% polyacrylamide-7 M urea gel (lane 1) as described under Materials and Methods. Treatment of the free RNA with RNase T1 (lane 2) and RNase A (lane 3) reduced it to a series of smaller RNAs. To determine the size of the RNA in the band shift complex, a preparative band shift was performed with testis cytoplasmic extracts and the RNA portion of the complex eluted from the gel as described under Materials and Methods (lane 4). The eluted RNA was treated with RNase T1 (lane 5), RNase A (lane 6), or combined with the starting 37-nt probe (lane 7) and treated with RNase T1 (lane 8). The eluted RNA was also hybridized to a complementary antisense oligonucleotide of 12-nt (lane 9) or a 21-nt random oligonucleotide (lane 10) and treated with RNase H. The arrow indicates the position of a 16-nt RNase T1 oligoribonucleotide product generated from the starting 37-nt *Prm-1* probe.

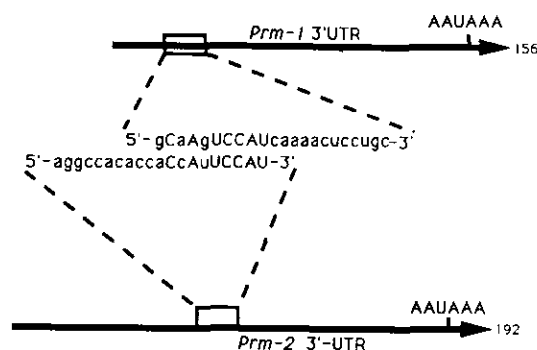


FIG. 8. Summary of deletion mapping analysis of *Prm-1* and *Prm-2* 3' UTR binding activity. Drawn as thick arrows are a representation of the complete 156-nt *Prm-1* and 192-nt *Prm-2* 3' UTRs. Below the *Prm-1* line is a 22-nt sequence defined by RNA band shift analyses of *Prm-1* 3' UTR deletion variants. Within the 22-nt sequence is a 16-nt sequence, 5'-UCCAUCAAAACUCCUG-3', that was identified by RNase T1 footprint analysis. Above *Prm-2* is a 20-nt sequence defined by RNA band shift analyses of *Prm-2* 3' UTR deletion variants. Comparison of the *Prm-1* and *Prm-2* sequences is a 9-nt stretch that shares identity over 7 nt and is capitalized.

band shift assay, treated with RNase T1 and heparin, and exposed to ultraviolet light for 30 min. One-half of the irradiated sample was retained for RNA band shift analysis while the remaining half was boiled in Laemmli buffer and analyzed by SDS-PAGE (Laemmli, 1970). uv irradiation of either the 37-nt sense *Prm-1* 3' UTR probe or the 88-nt sense *Prm-2* 3' UTR probe (Figs. 6 and 9) generated two bands of 53 and 55 kDa on SDS-PAGE (Fig. 10A), as did the minimal 22-nt *Prm-1* 3' UTR RNA binding region (data not shown). As controls, uv treatment of an RNA that contained an 88-nt anti-sense *Prm-2* probe or a 144-nt RNA containing the entire hGH 3' UTR did not generate bands of 53 and 55 kDa (Fig. 10A), although on longer exposures, a faint band of approximately 54 kDa was detected with the *Prm-2* anti-sense probe. No bands were detected with any of the RNAs in the absence of UV treatment (data not shown).

Three results suggest that the 53- and 55-kDa uv-generated bands are related to the RNA band shift complexes. First, uv irradiation of a preparative gel containing the RNA band shift complex, followed by extraction of the complex from the gel and reelectrophoresis of the sample in SDS-PAGE, generated a broad band of approximately 55 kDa (data not shown). Second, comparison of the intensity of the uv-generated complexes (Fig. 10A) with the RNA band shift complexes from the remaining half of the sample (Fig. 10B) shows that the relative intensity of the two bands within each doublet, as well as the relative intensities of the doublets between *Prm-1* and *Prm-2*, is the same. Third, competition experiments with an unlabeled 81-nt *Prm-1* sense 3' UTR RNA inhibited to the same extent the formation of the

band shift doublet and the uv cross-linking doublet of *Prm-1* (data not shown).

DISCUSSION

To determine if *Prm-1* mRNA isolated from round spermatids is translationally competent, we compared the translational efficiency of deproteinized *Prm-1* mRNA isolated from purified populations of round and elongating spermatids in an *in vitro* translation system. We found that *Prm-1* mRNAs isolated from round and elongating spermatids were translated with similar efficiencies in wheat germ extracts, suggesting that the *Prm-1* message present in round spermatids is competent for translation and that it is not translationally repressed by a covalent mechanism, unless wheat germ extracts contain a modification activity that renders the mRNA translationally active.

Previous studies in transgenic mice support the model that *Prm-1* is under negative regulation and that the *cis*-acting sequences required for its translational repression are contained within its 3' UTR (Braun *et al.*, 1989; Braun, 1990). Reasoning that any potential translational regulatory factors might act through the *Prm-1* 3' UTR, we attempted to identify such factors with an RNA band shift assay. Using this assay we identified a

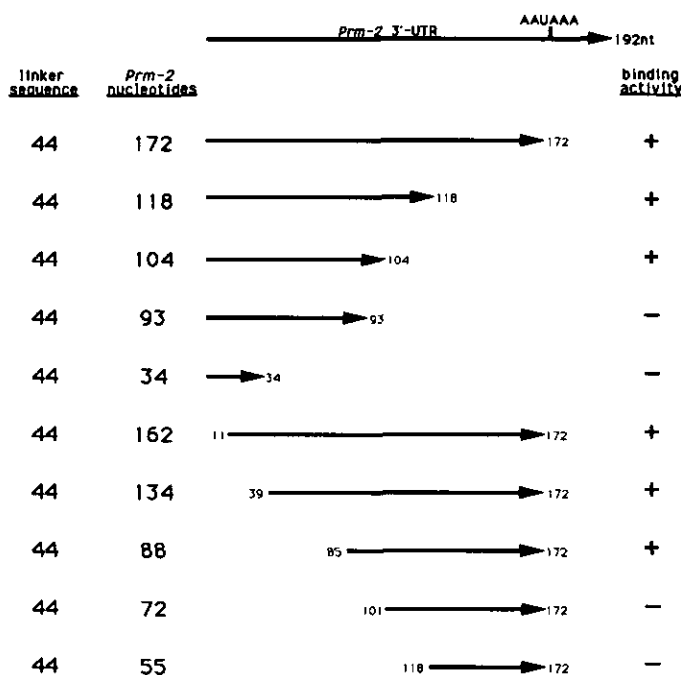


FIG. 9. Summary of deletion analysis of the *Prm-2* 3' UTR band shift complex. RNA probes were prepared that contain portions of the *Prm-2* 3' UTR RNA and polylinker sequence as indicated. Gel mobility shift assays were performed with testis cytoplasmic extracts and each RNA. The full-length *Prm-2* RNA is 172-nt in length. Drawn adjacent to each RNA is the first and/or last nucleotide of each transcript.

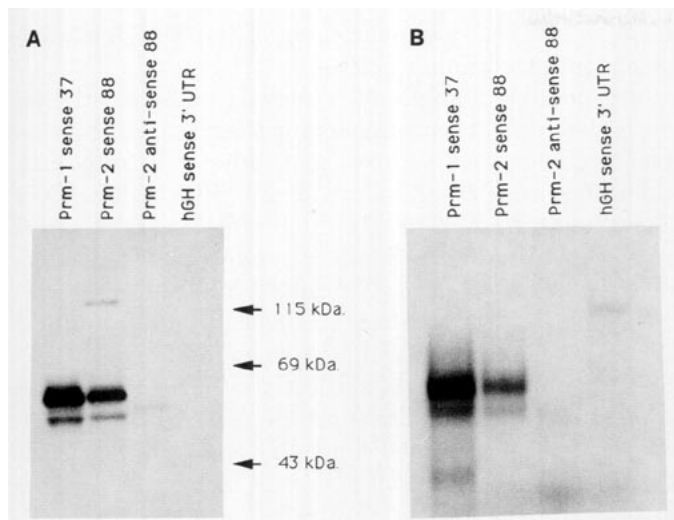


FIG. 10. *Prm-1* and *Prm-2* uv-cross-linked complexes contain the same proteins as the band shift complexes. (A) uv cross-linking. Radiolabeled RNA probes containing either *Prm-1* or *Prm-2* 3' UTR sense RNAs, antisense *Prm-2* 3' UTR or the hGH 3' UTR, were treated with testis cytoplasmic protein extracts, RNase T1, and heparin as described for the band shift analysis. Half of the sample was irradiated for 30 min with uv light and analyzed by SDS-PAGE. Molecular weight standards are indicated by arrows. (B) RNA band shift analysis. The remaining half of the samples described in A were analyzed in a standard band shift assay.

sequence-specific RNA binding activity, detected as a closely spaced doublet, that is present in the cytoplasmic fraction of testis protein extracts. RNA band shift assays performed with extracts prepared from a variety of adult tissues, the testes of mouse mutants that are arrested at different stages of fetal and testicular germ cell development, and from purified populations of germ cells suggest that the factors are testis- and germ cell-specific. Using a combination of deletion mapping and RNase T1 footprint analysis we were able to map the binding site to a region of the *Prm-1* 3' UTR between nucleotides 16 and 37 (Fig. 8). To determine if a similar binding site existed in *Prm-2*, we also performed band shift assays with RNA containing its 3' UTR. A band shift, migrating as a closely spaced doublet and with the same mobility as that detected with *Prm-1*, was detected. Using deletion variants of the *Prm-2* 3' UTR we mapped the region required for binding to sequences between nucleotides 85 and 104. Competition experiments between the *Prm-1* and *Prm-2* 3' UTRs strongly support the conclusion that the same protein(s) are binding to both *Prm-1* and *Prm-2* RNA. Neither binding site in *Prm-1* or *Prm-2* contain regions of obvious secondary structure. However, comparison of their primary sequence does reveal a region of homology, 5' CNANUC-CAU 3' (7/9 identity), that may constitute sequences important for protein binding (Fig. 8). In band shift assays performed with a mutant version of the *Prm-1* 22-nt

RNA that has all seven of the conserved sites altered, we failed to detect either complex (unpublished data).

The RNA band shift activity that we detected migrates as a closely spaced doublet. We also detected a binding activity for *Prm-1* and *Prm-2* by uv cross-linking that migrates on SDS-PAGE as a closely spaced doublet of approximately 53/55 kDa (Fig. 10A). Direct uv irradiation of the band shift doublet, extraction of the complex from the gel, and further analysis by SDS-PAGE strongly suggest that the band shift and uv cross-linking activities are the same (data not shown). Estimating that the RNA component of the uv-cross-linked complex is approximately 5 kDa leads us to predict that the protein moieties are approximately 48 and 50 kDa.

Kwon and Hecht (1991 and 1993) have described two regions of the *Prm-1* and *Prm-2* 3' UTRs, the "Y" and "H" boxes, that interact with proteins present in testis extracts. Using our numbering system, the "Y" box maps to nt 116–129 in the *Prm-2* 3' UTR and to nt 56–65 in the *Prm-1* 3' UTR, while the "H" box maps to nt 130–153 in *Prm-2* and to nt 70–87 in *Prm-1*. Kwon and Hecht (1991) have shown that the "Y" box in *Prm-2* can be uv cross-linked to a protein of 18 kDa. As described in this paper, we are able to detect three gel mobility shift complexes with the *Prm-2* 3' UTR. Two of these activities migrate as a doublet with the same mobility as the *Prm-1* doublet in the band shift assay and as a 53- to 55-kDa doublet in the UV cross-linking assay. Localization of the binding site for the 53- to 55-kDa complex by deletion mapping and RNase protection unequivocally demonstrated that neither complex maps to the "H" or "Y" boxes of *Prm-1* or *Prm-2* and that they are, therefore, distinct from the binding activities previously described.

Cytoplasmic extracts prepared from *Xenopus laevis* oocytes contain proteins of 54 and 56 kDa that are believed to constitute protein components of stored maternal mRNPs (Richter and Evers, 1984; Tafuri and Wolffe, 1990, 1993; Murray *et al.*, 1991, 1992). The genes that encode p54 and p56 have been cloned and shown to be highly similar but different genes, and p56 is the same as the germ cell-specific transcription factor FRG Y2 (Tafuri and Wolffe, 1990; Murray *et al.*, 1992). These proteins are believed to be sequence-specific DNA binding proteins and nonspecific RNA binding proteins (Tafuri and Wolffe, 1993). Homologues of the *Xenopus* p54/p56 proteins have recently been described in mouse testicular extracts and shown to migrate at 48/52 kDa on SDS-PAGE (Kwon *et al.*, 1993), and the gene for the 52-kDa protein has been cloned and is referred to as Mouse Y Box Protein MSY1 (Tafuri *et al.*, 1993). The mouse p48 and p52 homologues are highly enriched in the testis and have been shown to bind to RNA *in vitro*. We do not believe that the 53- and 55-kDa complexes that we have

described contain p48 and p52 for the following reasons. The factors that we have described here clearly bind to specific regions in the *Prm-1* and *Prm-2* 3' UTRs and not to the hGH 3' UTR or to numerous other RNAs tested (unpublished), whereas both the p48 and p52 proteins have been shown to bind RNA nonspecifically, including hGH. In addition, unlike p48 and p52, the RNA binding proteins that we have described are resistant to heparin up to 55 $\mu\text{g}/\text{ml}$ final concentration tested (unpublished data). Last, using an anti-frog-p54/p56 antibody that cross-reacts with the mouse p48 and p52 proteins (kindly supplied by M. Murray, Wayne State University), we have been unable to detect, by Western blotting or by immunoprecipitation, the p48 or p52 proteins in the uv cross-linked 53- to 55-kDa complex (unpublished data).

Initial mapping of the *cis*-acting sequences required for *Prm-1* translational control in transgenic mice showed that the full-length 156-nt *Prm-1* 3' UTR was sufficient to confer *Prm-1*-like translational control on a heterologous mRNA (Braun *et al.*, 1989). Later studies indicated that a *Prm*-hGH-*Prm* transgene that contains as little as 62 nt of the 3'-most region of the *Prm-1* 3' UTR was also sufficient for translational repression in round spermatids, whereas 23 nt was not (Braun, 1990). Surprisingly, the transgene containing 62 nt of *Prm-1* 3' UTR does not include the binding site for the germ cell-specific band shift activity described here, suggesting that it is not absolutely essential for translational repression *in vivo*. In addition, the 62-nt 3' UTR transgene also lacked the *Prm-1* versions of the "H" and "Y" boxes described by Kwon and Hecht (1993), suggesting that these sequences are also not essential for translational repression *in vivo*. The "H" and "Y" boxes of *Prm-2* have, however, been reported to confer translational repression on a heterologous mRNA in a rabbit reticulocyte extract supplemented with partially fractionated testis protein extracts (Kwon and Hecht, 1993). Therefore, if either of these factors are engaged in the translational repression of *Prm-1* and *Prm-2* *in vivo*, there must be redundancy in the *cis*-acting sequences which confer the translational repression. Redundancy is not uncommon in biological regulatory mechanisms, especially in instances where failure to regulate may have dramatic consequences. In the case of the importance of translational control of *Prm-1* for normal spermiogenesis, we (K.L. and R.E.B., manuscript in preparation) have recently shown that replacement of the *Prm-1* 3' UTR with the 3' UTR of hGH, resulted in premature translation of a *Prm-1*-hGH mRNA in round spermatids of transgenic mice and caused dominant sterility in males. Thus, the failure to inhibit translation of *Prm-1* mRNA can have dire consequences. Clearly, additional transgenic analysis is required to test the functional significance of these protein binding sites *in vivo* and to de-

termine if more than one region of the 3' UTR is sufficient for translational control.

It is possible that the 48- to 50-kDa binding activities described here are not important for translational control at all, but instead serve some other role in spermatid differentiation. Rastinejad and Blau (1993) have shown that the 3' UTRs of certain muscle-specific structural genes may act as *trans*-acting feedback regulators of muscle differentiation. If the 3' UTRs of other structural genes in other differentiated cell types also function in a feedback control loop, one might expect there to be factors present in those cells that interact with them. It is conceivable that the 48- and 50-kDa proteins that we have described here are such differentiation factors.

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