Spnr, a Murine RNA-binding Protein That Is Localized to Cytoplasmic Microtubules

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Abstract. Previous studies in transgenic mice have established the importance of the 3′ untranslated region (UTR) of the spermatid-specific protamine-1 (*Prm-1*) mRNA in its translational control during male germ cell development. To clone genes that mediate the translational repression or activation of the *Prm-1* mRNA, we screened cDNA expression libraries made with RNA from pachytene spermatocytes and round spermatids, with an RNA probe corresponding to the 3′ UTR of *Prm-1*. We obtained six independent clones that encode Spnr, a spermatid perinuclear RNA-binding protein. Spnr is a 71-kD protein that contains two previously described RNA binding domains. The *Spnr* mRNA is expressed at high levels in the testis, ovary, and brain, and is present in multiple forms in those tissues. Immunolocalization of the Spnr protein within the testis shows that it is expressed exclusively in postmeiotic germ cells and that it is localized to the manchette, a spermatid-specific microtubular array. Although the Spnr protein is expressed too late to be directly involved in the translational repression of *Prm-1* specifically, we suggest that the Spnr protein may be involved in other aspects of spermatid RNA metabolism, such as RNA transport or translational activation.

THERE are now numerous reports demonstrating the pivotal role of 3′ untranslated regions (UTR)[1] in mRNA stability, transport, localization, and translation (Jackson, 1993). Although antisense RNAs and proteins that bind to the 3′ UTRs of some mRNAs have been identified (Wightman et al., 1993; Lee et al., 1993; Wharton and Struhl, 1991; Harford et al., 1990), the molecular mechanisms by which 3′ UTRs control mRNA behavior remain largely unknown.

In addition to their 3′ UTR sequences, many localized messages also require microtubules and other cytoskeletal elements for their localization (Pokrywka and Stephenson, 1991; Yisraeli et al., 1990). Presumably, the *trans*-acting factors that bind to the 3′ UTRs of these mRNAs interact directly or indirectly with the cytoskeleton. Genetic studies in *Drosophila* have shown that the *exuperantia*, *swallow*, and *staufen* genes are required for the proper localization of the *bicoid* mRNA (Nusslein-Volhard et al., 1987; Stephenson et al., 1988; St Johnston et al., 1991), and the localization of the exuperantia and staufen proteins have been shown to be microtubule dependent (Wang and Hazelrigg, 1994; Ferrandon et al., 1994). Proteins that bind to the 3′ UTRs of actin, vimentin and tubulin mRNAs have been found in the cytoskeletal framework fraction of NIH3T3 cells (Sharpless et al., 1993) and may have a role in localizing these messages along the cytoskeleton within these cells.

Since 3′ UTR sequences are involved in many aspects of mRNA metabolism, including translational control, we were interested in finding proteins that could bind to sequences within the 3′ UTR of the spermatid-specific protamine 1 (*Prm-1*) mRNA. The mouse *Prm-1* gene encodes a male germ cell-specific mRNA that is stored as a cytoplasmic mRNP from the time it is first transcribed in postmeiotic round spermatids until its translation up to a week later in elongating spermatids (Balhorn et al., 1984; Kleene et al., 1984). *Cis*-acting elements necessary for the delay in translation have been mapped to the 3′ UTR through the use of reporter genes in transgenic mice (Braun et al., 1989; Braun, 1990). To clone genes that effect the translational repression or activation of the *Prm-1* message, we screened an expression library of cDNAs from pachytene spermatocytes and round spermatids with a *Prm-1* 3′ UTR RNA probe. The screen yielded five previously unidentified mouse genes. The protein product of one of these genes, spermatid perinuclear RNA-binding protein (Spnr), contains two RNA-binding domains similar to those found in the *Drosophila* staufen protein (St Johnston et al., 1992), and is localized to the manchette, a spermatid-specific microtubular array. Although the expression of the Spnr protein occurs too late for it to be involved in the translational repres-

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1. *Abbreviations used in this paper*: FISH, fluorescence in situ hybridization; *hGH*, human growth hormone; MBP, maltose binding protein; MBP-β-galactasidase; Spnr, spermatid perinuclear RNA-binding protein; UTR, untranslated region.

sion of *Prm-1*, its association with the manchette suggests that Spnr may link spermatid mRNAs with the cytoskeleton.

Materials and Methods

Mice

All mice were purchased from either the Jackson Laboratory (Bar Harbor, ME), or Simonsen Laboratories, Inc. (Gilroy, CA).

Expression Library Screen

Lambda gt11 expression libraries prepared with cDNAs from mouse round spermatid and pachytene spermatocyte mRNA were plated and expression was induced by overlaying the plaques with nitrocellulose filters soaked in 10 mM IPTG. The plates and filters were incubated at 37°C for 6 h. The filters were then blocked in Blotto (5% nonfat dry milk, 50 mM Tris HCl, pH 7.5, 50 mM NaCl, 1 mM EDTA, 1 mM DTT) for 1 h at 22°C. The filters were airdried at 22°C for 15 min and incubated at 4°C in Hepes-binding buffer (25 mM Hepes, pH 7.9, 25 mM NaCl, 5 mM $MgCl_2$, 0.5 mM DTT) with 6 M guanidine HCl two times for 10 min each. They were then incubated at 4°C in 2× serial dilutions of guanidine HCl (in Hepes-binding buffer) for 5 min each for a total of five dilutions (final dilution 0.188 M guanidine HCl). The filters were transferred to Hepes-binding buffer and washed two times for five minutes each, followed by incubation in Blotto for 30 min at 4°C. The filters were then soaked in Hepes-binding buffer with 0.25% dry milk for 1 min. The filters were hybridized with 100 ng/ml of a digoxigenin-labeled (Genius Kit, Boehringer Mannheim, Indianapolis, IN) sense-strand probe of the *Prm-1* 3′ UTR in binding buffer for 60 min at 22°C. The filters were washed four times in Hepes-binding buffer for a total wash time of 30 min. Positive plaques were detected with a Genius digoxigenin-labeled nucleic acid detection kit (Boehringer Mannheim, Indianapolis, IN).

DNA Sequencing

A series of nested deletions were produced from the *Spnr* cDNA clone (in a Bluescript KS(−) vector [Stratagene, La Jolla, CA]) as described by Sambrook et al. (1989). Both strands were completely sequenced by the dideoxy sequencing method using a Sequenase kit (USB, Cleveland, OH). DNA sequence analysis was performed with the Intelligenetics suite and Blast programs.

Fluorescence In Situ Hybridization

A cDNA probe containing a 3-kb insert corresponding to the *Spnr* gene was labeled with biotin-11-dUTP by nick translation (GIBCO BRL, Gaithersburg, MD). The size of the product was determined to be between 200 and 400 bp. Metaphase chromosome preparations from lymphocytes of a male C57BL/6J mouse and prometaphase chromosome preparations of lymphocyte cultures from a normal human male synchronized by methotrexate were obtained using 0.075 M KCl as a hypotonic buffer and methanol:acetic acid (3:1, vol/vol) as fixative. Slides were denatured in the presence of 70% formamide at 70°C. The hybridization to mouse and human metaphase chromosomes was carried out as previously described (Edelhoff et al., 1994). The hybridization signals were detected using a detection system from Vector Labs (Burlingame, CA). After incubation with goat anti-biotin antibody, slides were rinsed in posthybridization buffer (0.2 M NaH_2PO_4, 0.2 M Na_2HPO_4, 0.15 M NaCl, 0.1% Tween 20, and 0.15% BSA). A second incubation with fluorescein-labeled anti-goat IgG and a rinse in posthybridization buffer followed. The chromosomes were banded using HOECHST 33258–actinomycin D staining and counterstained with propidium iodide. The chromosomes and hybridization signals were visualized by fluorescence microscopy, using a dual band pass filter (Chroma or Omega, Brattleboro, VT).

Fusion Protein

A maltose-binding protein (MBP)-Spnr fusion protein was prepared by cloning a 1.5-kb HindIII fragment from the 3′ end of the *Spnr* cDNA into the HindIII site of the expression vector pMAL-c2 (NEB, Beverly, MA). The cloned fragment was placed in-frame with MBP by cutting in the polylinker with SalI, treating with mung-bean nuclease and religating the plasmid. Transformed DH5α *E. Coli* cells were grown at 30°C to an OD of 0.5. The cells were induced with IPTG, harvested, and lysed as described by NEB. The lysate was passed over an amylose resin column, washed, and the bound fusion protein eluted with excess maltose as described by NEB. Protein concentrations were determined by a Bradford Assay (Biorad, Hercules, CA). Protein containing fractions were pooled and concentrated in a Centricon-30 column (Amicon, Beverly, MA).

Northwestern Analysis

Purified fusion protein extracts were diluted in buffer and electrophoresed on 8% SDS-PAGE gels (Laemmli, 1970). The gels were blotted onto nitrocellulose using an electroblotter run with water cooling at 200 mA for 2 h in a Tris-glycine buffer (25 mM Tris, 192 mM glycine) at 4°C. The blots were denatured and renatured as described above for the expression library screen. RNA probes were transcribed from cloned DNA in Bluescript vectors (Stratagene) using SP6, T7, or T3 RNA polymerase (Promega, Madison, WI) with [α-^{32}P]UTP (Dupont, Boston, MA) as the labeled nucleotide. The full-length *Prm-1* 3′ UTR probe contained 156 nt of *Prm-1* sequence and 22 nt of sequence contributed from the polylinker in the vector (Fajardo et al., 1994). The *Prm-1* 3′ UTR 5′ probe contained the first 82 nt of the 3′ UTR and 38 nt of polylinker sequence. The *Prm-1* 3′ UTR 3′ probe contained 59 nt of the *Prm-1* 3′ UTR between 72–130 nt, and 22 nt of polylinker sequence. Blots were incubated for 1 h at 22°C in 2 ml of Hepes-binding buffer + 0.25% dry milk with ~45 pmol probe per blot. The blots were washed four times at room temperature for a total of 30 min in Hepes-binding buffer and exposed to X-ray film. The blots were subsequently washed on successive days in Hepes-binding buffer with increasing NaCl concentrations, up to 1.5 M NaCl. After each wash, the blots were exposed to X-ray film for ~14 h.

RNA Analysis

A 413-bp DNA fragment between the XbaI and SacI sites in the *Spnr* cDNA was cloned into Bluescript KS (Stratagene). The plasmid was linearized with SalI in the cloning site of the vector and transcribed with T7 RNA polymerase (Promega) using [α-^{32}P]UTP as the labeled nucleotide to make an antisense RNA probe. A control probe from the murine adult skeletal α-actin gene was prepared by cloning a 114-bp HindIII–BamHI DNA fragment into Bluescript KS (Stratagene). The plasmid was linearized with SalI and an anti-sense RNA probe was transcribed with T7 polymerase (Promega) using [α-^{32}P]UTP as the labeled nucleotide. Both probes were gel purified before use.

RNAs were extracted from several different adult mouse tissues by homogenization in guanidinium isothiocyanate, followed by precipitation with lithium chloride, as described by Cathala et al. (1983). RNAse protection experiments were carried out with RNAse I (Promega) as described by the manufacturer.

For Northern analysis 10 μg of each RNA was ethanol precipitated and pellets were resuspended in Formamide sample buffer (10 mM $NaPO_4$, 1 mM EDTA, 5 mM NaOAc, 50% formamide, 2.2 M formaldehyde). The samples were heated to 68°C for 5 min and electrophoresed on a 1.5% agarose gel immersed in Formaldehyde running buffer (10 mM $NaPO_4$, 1 mM EDTA, 5 mM NaOAC, 2.2 M formaldehyde). The gel was blotted to nitrocellulose in 20× SSC and the blot hybridized with an α-^{32}P-labeled probe corresponding to the entire 3.0-kb *Spnr* cDNA. The blot was washed in 2× SSC and 0.1% SDS at 22°C for 30 min and in 0.2× SSC and 0.1% SDS at 65°C for 30 min.

Antibody Production

Antibodies to the Spnr protein were made by injecting New Zealand white rabbits with 100 μg of the MBP-Spnr fusion protein emulsified in Freund's Complete Adjuvant (Sigma Chem. Co., St. Louis, MO) for the first injection and with Freund's Incomplete Adjuvant (Sigma) for subsequent injections. All rabbit work was performed by R&R Rabbitry (Stanwood, Washington). Antibodies to the Spnr fusion protein were purified by incubating the sera with a nitrocellulose strip that was blotted with the Spnr fusion protein (Sambrook et al., 1989). The antibodies were eluted in 0.2 M glycine, pH 2.8, 1 mM EGTA. The elute was neutralized with Tris base and concentrated in a Centricon-30 column (Amicon).

Western Analysis

Protein extracts were prepared from tissues dissected from sexually mature randomly bred Swiss Webster mice sacrificed by surgical dislocation. Each

of the tissues was suspended in RIPA buffer (150 mM NaCl, 50 mM Tris, 1% NP-40, 0.5% sodium deoxycholate, 0.1% SDS) and homogenized for 10 s with a tissue homogenizer. The extracts were cleared by spinning at high speed in a microfuge for 2 min at 4°C. Protein concentrations were measured with a Bradford assay (Biorad Labs). 100 μg of each extract were mixed with buffer (Laemmli, 1970), boiled for 5 min, and electrophoresed on an 8% SDS-PAGE gel. The gel was blotted to nitrocellulose using an electroblotter run with water cooling at 4°C at 200 mA for 2 h in a Tris-glycine buffer (25 mM Tris, 192 mM glycine, 20% vol/vol methanol, pH 8.3). The blot was dried and blocked in 5% dry milk for 30 min 22°C. Purified antibody was added at a dilution of 1:50 in 5% dry milk and incubated overnight at 22°C. The blot was then washed twice in 5% dry milk + 0.05% Tween-20 for 20 min each, and then in 5% dry milk for 20 min. The proteins were visualized with a horseradish peroxidase–conjugate substrate kit (Biorad Labs).

Immunofluorescence

Testis were removed from sexually mature mice, either randomly bred Swiss Webster mice or F1 or F2 hybrids from C57BL/6J X CBA/J or C57BL/6J X SJL/ J crosses. Testis were fixed overnight in Carnoy's fixative (60% Ethanol, 30% $CHCl_3$, 10% glacial acetic acid). The tissues were embedded in paraffin blocks and cut into 5-μm sections. The sections were deparaffinized with xylene and rehydrated using standard procedures. The sections were incubated with 1:100 to 1:10 dilutions of affinity-purified primary antibody in PBS for 2 h at 22°C. Sections were then treated with a fluorescently labeled secondary antibody (Zymed Labs, South San Francisco, CA and Vector Labs, Burlingame, CA). Confocal images were processed with the Adobe Photoshop program.

Results

Expression Library Screen for Prm-1 3′ UTR RNA-binding Proteins

To identify genes whose protein products bind to the 3′ UTR of the *Prm-1* mRNA, lambda-gt11 cDNA expression libraries prepared with RNA from pachytene spermatocytes and round spermatids were screened with a digoxigenin-labeled RNA probe corresponding to the 156-nt 3′ UTR of the mouse *Prm-1* mRNA. To increase the probability of finding *Prm-1*-specific RNA-binding proteins, positive plaques were re-screened with a negative control digoxigenin-labeled RNA probe corresponding to the 145 nt 3′ UTR of the human growth hormone (*hGH*) mRNA. The *hGH* 3′ UTR was used as a negative control because experiments in transgenic mice have shown that a chimeric message with the *hGH*-coding region and the 3′ UTR of *Prm-1* will be delayed in translation, whereas the 3′ UTR of *hGH* does not confer this delay (Braun et al., 1989). The screen yielded 19 positive plaques that bound the *Prm-1* 3′ UTR probe, but did not bind, or bound significantly less well, to the *hGH* 3′ UTR probe. Cross-hybridization with the cDNAs from these phage showed that they represent five different genes (data not shown).

The cDNA from one of these five genes, *Spnr*, was recovered from the screen six times; three times from the pachytene spermatocyte library and three times from the round spermatid library. The largest of the *Spnr* cDNAs is 3 kb in size, and was cloned into a Bluescript vector for further analysis. All the results discussed here are derived from this particular cDNA.

DNA Sequence of the Spnr cDNA

Both strands of the 3-kb *Spnr* cDNA were sequenced by making a series of nested deletions from each end of the cDNA and sequencing the appropriate deletions. The entire nucleic acid sequence and the deduced amino acid sequence are shown in Fig. 1 *a*. The *Spnr* cDNA codes for a 649–amino acid protein of 71 kD that has significant homology with a family of known RNA-binding proteins that includes *Xenopus* 4F-1 and 4F-2, *Drosophila* staufen, human Tar-binding protein and human PKR (Bass et al., 1994; St Johnston et al., 1992; Gatignol et al., 1993). The Spnr protein has two copies of the RNA-binding domain that defines this family of proteins. Both of these domains are located in the carboxy-terminal third of the Spnr protein, double-underlined in Fig. 1 *a*. Just upstream of both binding motifs are stretches of conserved amino acids, single-underlined in Fig. 1 *a*. This similarity is found upstream of the RNA-binding domains in many of the other family members. A comparison between the two domains found in the Spnr protein, the five domains found in staufen (St Johnston et al., 1992), and a consensus sequence of the motif (Gatignol et al., 1993), is shown in Fig. 1 *b*.

The Spnr protein also contains a putative leucine zipper domain between amino acids 381 and 402. Just upstream of this motif is a highly charged region with a short stretch of aspartic acid residues followed by a short basic region containing several lysine and arginine residues. Leucine zippers are often found preceded by highly charged regions (Landschulz et al., 1988; Busch and Sassone-Corsi, 1990).

The *Spnr* gene was mapped on mouse and human metaphase chromosomes by FISH using the entire 3-kb *Spnr* cDNA as a probe. In mouse, of 43 cells examined, 42 (97.6%) showed signals on both chromatids of one or both chromosomes 2 at region C1 (Fig. 2). In human, of 72 cells examined, 33 (45.8%) showed signals on both chromatids of one or both chromosomes 9 at band q34 (Fig. 2). There was no significant hybridization to other chromosomes. Southern blotting experiments using human, mouse, and rat genomic DNAs confirmed that the *Spnr* gene is conserved across these species (data not shown).

RNA-binding Properties of the Spnr Protein

To assess the binding capabilities of the RNA-binding domains found in the Spnr protein, a fusion protein between the MBP of *E. Coli*, and the most carboxy-terminal 223 amino acids, of the Spnr protein which includes 11 amino acids from the first RNA-binding domain the entire second RNA-binding domain, was expressed in *E. coli* and purified by affinity chromatography on amylose resin. Northwestern blots of this protein and a control protein (MBP-β-galactasidase [MBP-β-gal]) were probed with equal molar amounts of in vitro-transcribed α-^{32}P–labeled RNA probes including the entire *Prm-1* 3′ UTR, the 3′ end of the *Prm-1* 3′ UTR, the 5′ end of the *Prm-1* 3′ UTR, the *hGH* 3′ UTR, and adenovirus VA RNA (Green and Mathews, 1992). The blots were initially washed in 50 mM NaCl for 30 min and exposed to X-ray film. To determine the relative affinities of binding to each of the different probes at different salt concentrations, the blots were washed on successive days with increasing amounts of NaCl. Each wash was followed by an overnight exposure to X-ray film (Fig. 3). The control protein (MBP-β-gal) bound none of the RNAs whereas the Spnr fusion protein bound all of the RNA probes to varying extents. Binding was greatest for the 3′ portion of the *Prm-1* 3′ UTR and for the 5′ portion of the *Prm-1* 3′ UTR, both of

a

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1    CGGAGGCATA TGAGGAGGTA CTGTCACATA AAACAGTGTC TAGTGAAGAC GACAAGAAAG AGAGAAAAGG ATCGGAAAAA GAAGCTAAAA
91   TACCATAGGA AACC
105  ATG AGA TCT ATT AGA TCT TTT GCC AAT GAT GAT CGC CAT GTT ATG GTG AAA CAT TCA ACA ATC TAT CCA TCT CCA
       M  R  S  I  R  S  F  A  N  D  D  R  H  V  M  V  K  H  S  T  I  Y  P  S  P     25
180  GAG GAA CTT GAA GCT GTG CAA AAT ATG GTG TCT ACT GTT GAA TGT GCT CTT AAA CAT GTC TCA GAT TGG CTG GAC
       E  E  L  E  A  V  Q  N  M  V  S  T  V  E  C  A  L  K  H  V  S  D  W  L  D     50
255  GAA ACA AAC AAA GGC ACA AAA CCT GAG GGG GAA ACA GAA GTA AAG AAA GAT GAA GCT GTG GAA AAC TAT TCC AAG
       E  T  N  K  G  T  K  P  E  G  E  T  E  V  K  K  D  E  A  V  E  N  Y  S  K     75
330  GAT CAA GGT GGC CGG ACA TTG TGT GGT GTA ATG AGG ATT GGC TTG GTT GCA AAA GGC TTG CTG ATT AAA GAT GAT
       D  Q  G  G  R  T  L  C  G  V  M  R  I  G  L  V  A  K  G  L  L  I  K  D  D    100
405  ATG GAC CTG GAG CTA GTC TTA ATG TGC AAA GAC AAA CCC ACG GAA ACC CTG TTA AAT ACA GTT AAA GAT AAC CTT
       M  D  L  E  L  V  L  M  C  K  D  K  P  T  E  T  L  L  N  T  V  K  D  N  L    125
480  CCT ATT CAG ATT CAG AAA CTA ACA GAA GAG AAA TAC CAA GTG GAA CAA TGT ATA AAT GAA GCA TCC ATT ATA ATT
       P  I  Q  I  Q  K  L  T  E  E  K  Y  Q  V  E  Q  C  I  N  E  A  S  I  I  I    150
555  CGG AAC ACG AAA GAG CCA ACA CTG ACT TTG AAG GTG ATA CTT ACT TCC CCT CTA ATT AGG GAC GAA TTG GAG AAG
       R  N  T  K  E  P  T  L  T  L  K  V  I  L  T  S  P  L  I  R  D  E  L  E  K    175
630  AAG GAT GGA GAA AAT GTT ATG ATG AAA GAC CCT CCG GAC TTA TTG GAC AGG CAA AAA TGC CTG AAT GCC TTG GCT
       K  D  G  E  N  V  M  M  K  D  P  P  D  L  L  D  R  Q  K  C  L  N  A  L  A    200
705  TCT CTT CGA CAT GCC AAA TGG TTC CAG GCA AGG GCA AAT GGA TTA AAA TCA TGT GTA ATT GTC CTC CGC ATT CTG
       S  L  R  H  A  K  W  F  Q  A  R  A  N  G  L  K  S  C  V  I  V  L  R  I  L    225
780  CGT GAT TTG TGC AAC AGA GTA CCC ACA TGG GCA CCA TTG AAA GGA TGG CCA CTA GAG CTT ATA TGT GAA AAG TCT
       R  D  L  C  N  R  V  P  T  W  A  P  L  K  G  W  P  L  E  L  I  C  E  K  S    250
855  ATA GGT ACT TGT AAT AGA CCT TTG GGC GCT GGG GAG GCC TTG AGA CGA GTA ATG GAG TGT TTG GCA TCT GGA ATA
       I  G  T  C  N  R  P  L  G  A  G  E  A  L  R  R  V  M  E  C  L  A  S  G  I    275
930  CTA CTT CCT GGT GGC CCT GGT CTT CAT GAT CCT TGT GAG CGA GAC CCA ACA GAT GCT CTA AGC TAT ATG ACC ACT
       L  L  P  G  G  P  G  L  H  D  P  C  E  R  D  P  T  D  A  L  S  Y  M  T  T    300
1005 CAG CAA AAA GAA GAT ATT ACC CAT AGT GCA CAG CAT GCA CTT AGA CTA TCA GCC TTT GGC CAG ATT TAT AAA GTG
       Q  Q  K  E  D  I  T  H  S  A  Q  H  A  L  R  L  S  A  F  G  Q  I  Y  K  V    325
1080 CTG GAG ATG GAC CCT CTT CCG TCT AGT AAG CCT TTT CAG AAG TAT TCC TGG TCA GTT ACT GAT AAA GAA GGT GCT
       L  E  M  D  P  L  P  S  S  K  P  F  Q  K  Y  S  W  S  V  T  D  K  E  G  A    350
1155 GGG TCT TCA GCT CTA AAG AGG CCA TTT GAA GAT GGA TTG GGG GAT GAT AAA GAT CCC AAC AAG AAG ATG AAA CGA
       G  S  S  A  L  K  R  P  F  E  D  G  L  G  D  D  K  D  P  N  K  K  M  K  R    375
1230 AAC TTA AGG AAA ATT CTA GAT AGT AAA GCA ATA GAC CTT ATG AAT GCA CTA ATG AGA TTA AAT CAG ATC AGG CCC
       N  L  R  K  I  L  D  S  K  A  I  D  L  M  N  A  L  M  R  L  N  Q  I  R  P    400
1305 GGG CTT CAG TAT AAG CTT TTG TCA CAA TCT GGC CCG GTC CAT GCC CCA GTC TTC ACA ATG TCT GTA GAT GTG GAT
       G  L  Q  Y  K  L  L  S  Q  S  G  P  V  H  A  P  V  F  T  M  S  V  D  V  D    425
1380 GGC ACA ACA TAT GAA GCC TCA GGA CCA TCC AAG AAA ACA GCA AAG CTT CAT GTG GCA GTG AAG GTT TTA CAG GCA
       G  T  T  Y  E  A  S  G  P  S  K  K  T  A  K  L  H  V  A  V  K  V  L  Q  A    450
1455 ATG GGA TAC CCA ACA GGT TTT GAT GCA GAT ATT GAA TGT ATC AGT TCC GAT GAA AAA TCA GAT AAT GAA AGC AAA
       M  G  Y  P  T  G  F  D  A  D  I  E  C  I  S  S  D  E  K  S  D  N  E  S  K    475
1530 AAT GAT ACA GTA TCT TCA AAC TCA AGC AAT AAT ACT GGA AAT TCT ACA ACT GAG ACC TCC AGT ACC TTA GAG GTA
       N  D  T  V  S  S  N  S  S  N  N  T  G  N  S  T  T  E  T  S  S  T  L  E  V    500
1605 AGA ACT CAG GGT CCT ATC CTC ACA GCA AGT GGC AAA AAT CCT GTG ATG GAG CTC AAT GAA AAA AGA AGA GGT CTC
       R  T  Q  G  P  I  L  T  A  S  G  K  N  P  V  M  E  L  N  E  K  R  R  G  L    525
1680 AAA TAT GAA CTC ATC TCA GAA ACT GGT GGA AGC CAT GAC AAG CGC TTT GTA ATG GAG GTA GAA GTA GAT GGA CAG
       K  Y  E  L  I  S  E  T  G  G  S  H  D  K  R  F  V  M  E  V  E  V  D  G  Q    550
1755 AAA TTC AGA GGT GCA GGT CCA AAC AAG AAA GTG GCC AAG GCA AGT GCA GCA CTA GCT GCC CTG GAG AAG CTG TTT
       K  F  R  G  A  G  P  N  K  K  V  A  K  A  S  A  A  L  A  A  L  E  K  L  F    575
1830 TCT GGA CCC AAT GCG GCA AAT AAT AAG AAA AAG AAG ATC ATT CCT CAG GCA AAA GGT GTT GTG AAT ACA GCA GTA
       S  G  P  N  A  A  N  N  K  K  K  K  I  I  P  Q  A  K  G  V  V  N  T  A  V    600
1905 TCT GCG GCA GTC CAG GCA GTT CGG GGC AGA GGA AGA GGA ACT CTG ACA AGG GGA GCA TTC GTG GGG GCT ACA GCA
       S  A  A  V  Q  A  V  R  G  R  G  R  G  T  L  T  R  G  A  F  V  G  A  T  A    625
1980 GCT CCT GGA TAT ATA GCT CCA GGT TAC GGA ACC CCA TAT GGT TAC AGC ACA GCT GCC CCT GCA TAT GGT TAA
       A  P  G  Y  I  A  P  G  Y  G  T  P  Y  G  Y  S  T  A  A  P  A  Y  G  .      649
2052 TACTTTTAAG CAGTTTTCAG CACGTTTATC TCACTTTATT CTCGTTCTCC TCGGACCTCC GTAGCCATAT CACTTGTATA AAATAGGCTG
2142 AGATATAGTG ACTGTAAGCT GTGATCTCAG TATTCAAAGG GTCCTTCCTA TTATTCAAAG AAATATTTCA CTTAAAAAAC CAAACCAGCC
2232 AGGCATGGCA GCTCAGACCT ATACTATCAG CGGCTGGGAA GCTAAGACTG GAGGGCTACT GTGAATCTGT GGCCGGCTGG AGCTTCATTC
2322 TGAGACACAG AGAAGGTAAA GATGTCGCTG TCAGGAGTCT GTGGTGCAGC TGTGGCTGAG AGTGGGGTCT GCTTGGCAGA GCGGCGGAAG
2412 GCGGTGGCAA AACTGTAAAG TATCCGGCTC ACCTATAAGC AGAGATGTCA GGAAATTGGC TGTTATCAGT AAGACAGAAA CACTCTCCCT
2502 CCAGTGGCCA GTGACTCACA AAAGCATTTG CTGCTAGACT TCTTTTACAT GAAACAAGCA AAATCAACTT TTAAGGATAC AGTTCAGAAC
2592 AGCAGTTGTT TTTGTGTCTG GGTGTGTGTG TGTGTGTGTG TGTGTGTGTG TGTGTGTAAC AAAAGGGATC ACTCTTGAAT GCTAGACATG
2682 CTCTCTCTCT TAATCTGGGC AATGGTAGAA ACTCATGCAG GCTGCCACTG AGGAGGTCTG CACTTTATAC AGAGTCCTTT TCGGGTTCAA
2772 TGAGATAATG CAGCAAGTGA AGGCTTTCTG AGCAAGTCTG GTGACTAGAC CTGGATTCCC GAAACTCCAT AGGGTAGAAG GAGAGGACCA
2862 ATGCCAAGTT GTTAACTGAA TTGTATACCC CTACTCCCTG TATCATGTAC AAAAACATGC AATCATAATA AATATATAAA AAAAAAAAA
2952 AAAAAAAA
```

b

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Spnr I       NPVMELNEKAAGLKYELISETGGSHDKAFVMEVEVDGQKFAGAGPNKKVAKASAALAALEKL
Spnr II      NALMRLNQIRPGLQYKLLSQSGPVHAPVFTMSVDVDGTTYEASGPSKKTAKLHVAVKVLQAM
Staufen I    LVNELARVYNKITHQYRLTEERGPAHCKTFTVTLMLGDEEVSADGFKIKKAQHLAASKAIEET
Staufen II   SRFALPPPLGAHVHHGPNGPFPSVPTPPSKITLFVGKQKFVGIGRTLQQAKHDAAARALQVL
Staufen III  QVHEIGIKRNMTVHFKVLREEGPAHMKNFITACIVGSIVTEGEGNGKKVSKKRAAEKMLVEL
Staufen IV   QLQQTRKEKEPIFELIAKNGNETARRAEFVMEVSASGSTARGTGNSKKLAKRNAAQALFELL
Staufen V    QLLYLSKLLDFEVNFSDYPKGNHNEFLTIVTLSTHPPQICHGVGKSSEESQNDAASNALKIL
Consensus     PV..LNEY...P.Y.L...SGPAH.K.FTF.V.V.G....G.G.SKK.AK..AAE.AL..L
```

Figure 1. DNA and predicted amino acid sequence of the *Spnr* cDNA. (*a*) Nucleotides are numbered on the left, amino acids on the right. RNA-binding motifs are underlined, with the most highly conserved portion of the motif doubly underlined. Leucine residues within the putative leucine zipper motif are boxed. These sequence data are available from EMBL/Gen Bank/DDBJ under accession number X84692. (*b*) Spnr protein RNA-binding domain I and II sequences are compared to the five dsRNA-binding domains found in staufen (St Johnston et al., 1992), and the dsRNA-binding domain consensus (Gatignol et al., 1993). The peptide sequence used in in vitro RNA-binding assays by Gatignol et al. (1993) is underlined.

which bound better than the entire *Prm-1* 3′ UTR. Binding was the weakest for the 3′ UTR of the *hGH* RNA as expected from results of the expression library screen in which this probe was used as a negative selection. The fusion protein also bound the double-stranded VA RNA from adenovirus, a known inhibitor of PKR (Green and Mathews, 1992). Binding of all the RNA probes was greatly reduced with increasing concentrations of NaCl in the wash buffer. All binding of the *hGH* probe was removed by 200 mM NaCl, whereas the *Prm-1* 3′ UTR and the VA RNA probes remained bound until the 800-mM wash. Binding to the separate ends of the *Prm-1* 3′ UTR was detectable even after the 1.5 M wash, however binding of both probes was greatly reduced.

To test whether the fusion protein could bind to single-stranded or double-stranded DNA, the blots were probed with both denatured and renatured α-^{32}P-labeled λ phage

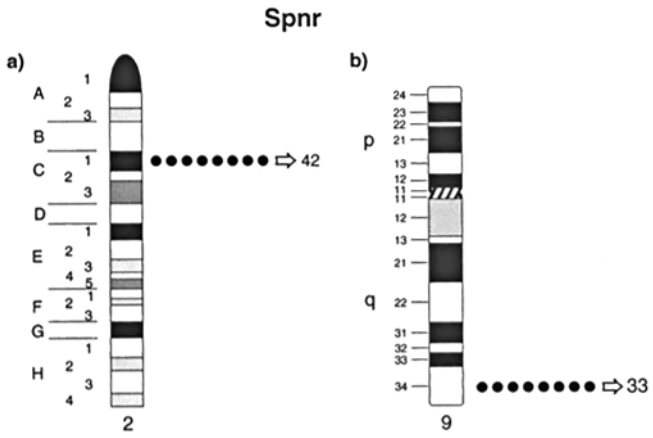


Figure 2. Assignment of *Spnr* to mouse chromosome 2 region C1 and human chromosome 9 band q34, as determined by FISH. (*a*) Distribution of 42 signals of hybridization on a diagram of mouse chromosome 2. (*b*) Distribution of 33 signals of hybridization on a diagram of human chromosome 9.

DNA. Neither the control MBP-β-gal nor the MBP-Spnr fusion protein bound to either probe at the lowest NaCl concentration wash of 50 mM (Fig. 3). Thus, the Spnr fusion protein does not appear to bind DNA in a nonspecific manner.

In summary, although the Spnr-MBP fusion protein bound to several RNAs with varying relative affinities, we conclude that the fusion protein binds RNA nonspecifically in vitro.

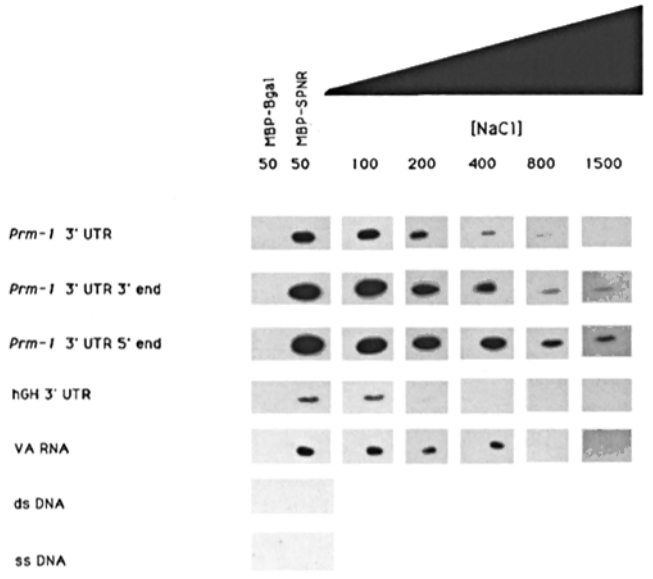


Figure 3. Northwestern analysis with MBP-Spnr fusion protein. Northwestern blots containing 10 μg of MBP-β-gal fusion protein and 750 ng of MBP-Spnr fusion protein were probed with equal molar amounts of sense-strand α-^{32}P-labeled RNA probes and were washed as described in Materials and Methods. The blots were stripped and reprobed with 100 ng of random primer-labeled lambda DNA that remained double stranded or that had been denatured by boiling.

Tissue Restriction of Spnr mRNA

An RNAse protection assay was performed to assess the level of *Spnr* mRNA expression in different mouse tissues. A 481-nt RNA probe from the middle of the *Spnr* cDNA was designed to protect a fragment of 413 nt. A 173-nt actin probe protecting a fragment of 114 nt was used as a control for RNA amounts. The highest level of mRNA was seen in the testis, with lesser amounts in ovary, thymus, and brain (Fig. 4 *a*). With much longer exposures, a protected fragment can also be seen in the liver, kidney, heart, and spleen. A second probe that protects a fragment of 326 nt from the 5′ end of the cDNA gave the same pattern of mRNA levels (data not shown).

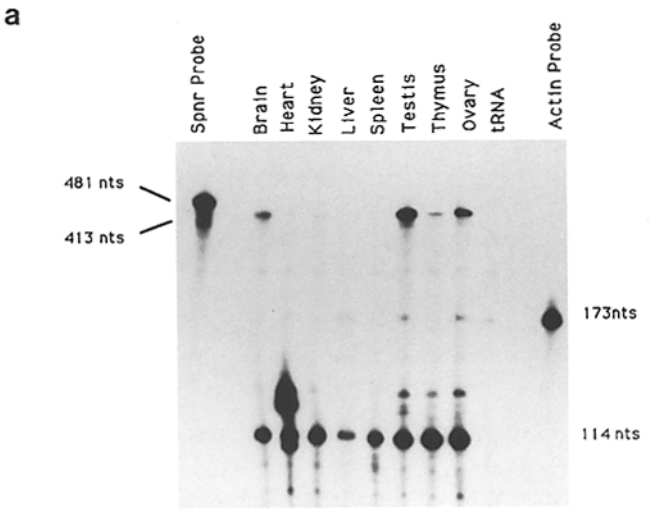
To confirm the RNAse protection results and to determine the size of the *Spnr* mRNA, a Northern blot was prepared with RNAs from various tissues and probed with the entire *Spnr* cDNA. Four different transcripts were detected in the testis; one of 3.8 kb, two of ~3.0 kb, and one of 2.0 kb (Fig. 4 *b*). The 2.0-kb transcript is the predominant message found in brain. Upon longer exposure, the 3.0-kb messages are seen at low levels in thymus, kidney, liver, and spleen. No transcripts were detected in RNA isolated from the heart or ovary. The Northern was stripped and reprobed with an actin probe to verify RNA amounts in each lane. Levels of actin mRNA in liver RNA preparations are consistently below the amount seen in other tissues when an equal number of μg of each tissue RNA are loaded.

To determine the level of Spnr protein in various tissues, Western blot analysis was performed using affinity-purified anti-Spnr polyclonal antibody raised against the MBP-Spnr fusion (Fig. 5). An abundant protein of ~71 kD was present in testis, but this protein was not detected in other tissues that express *Spnr* mRNA. Protein extracts from ovary were also blotted and probed with Spnr antibody, and again, no bands were detected (data not shown). We readily detected tubulin by Western blotting in all of the protein extracts, suggesting that our failure to detect the Spnr protein was not due to protein degradation during preparation of the extracts.

Localization of the Spnr Protein in the Testis

To determine the localization of the Spnr protein in the testis, immunofluorescence was performed with affinity-purified anti-Spnr polyclonal antibody on Carnoy's-fixed paraffin sections of adult testis. Incubation of testis sections with FITC-conjugated secondary antibody alone (Fig. 6 *A*), or with preimmune sera and FITC-conjugated secondary antibody (data not shown), did not generate an immunopositive signal. Treatment of testis sections with affinity-purified anti-Spnr antibody and FITC-conjugated secondary antibody revealed an immunopositive germ cell-specific staining that was restricted to elongating spermatids in a pattern dependent on the developmental stage of each tubule (Fig. 6 *B*).

Spermatogenesis in the mouse has been divided into XII developmental stages based on the presence of particular ages of developing germ cells within a given seminiferous tubule (Oakberg, 1956; Russell et al., 1990). Spermiogenesis, the haploid phase of spermatogenesis, has been divided into 16 different steps, depending on histological criteria that change as the haploid spermatids develop, including the size and position of the acrosome and nuclear shape (Oakberg, 1956). We determined the stages of each of the tubules in



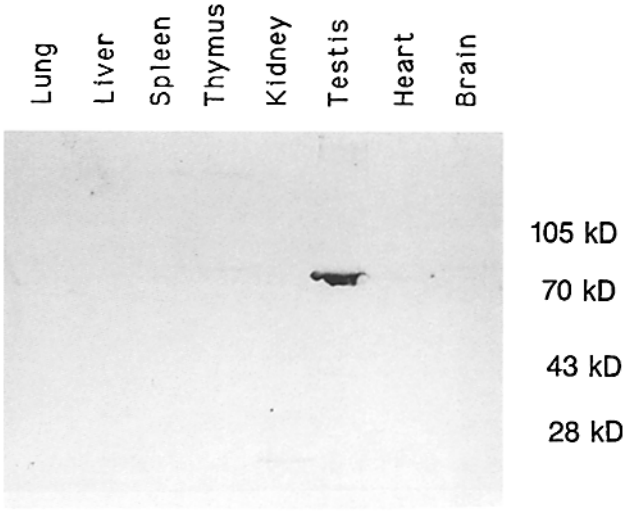


Figure 5. Western analysis showing the tissue distribution of the Spnr protein 100 μg of protein extract prepared from several different adult mouse tissues were separated on an 8% SDS-PAGE and blotted to nitrocellulose. The blot was incubated with affinity-purified Spnr antibody raised against the MBP-Spnr fusion protein. Bands were visualized with a horseradish peroxidase detection kit (Biorad Labs).

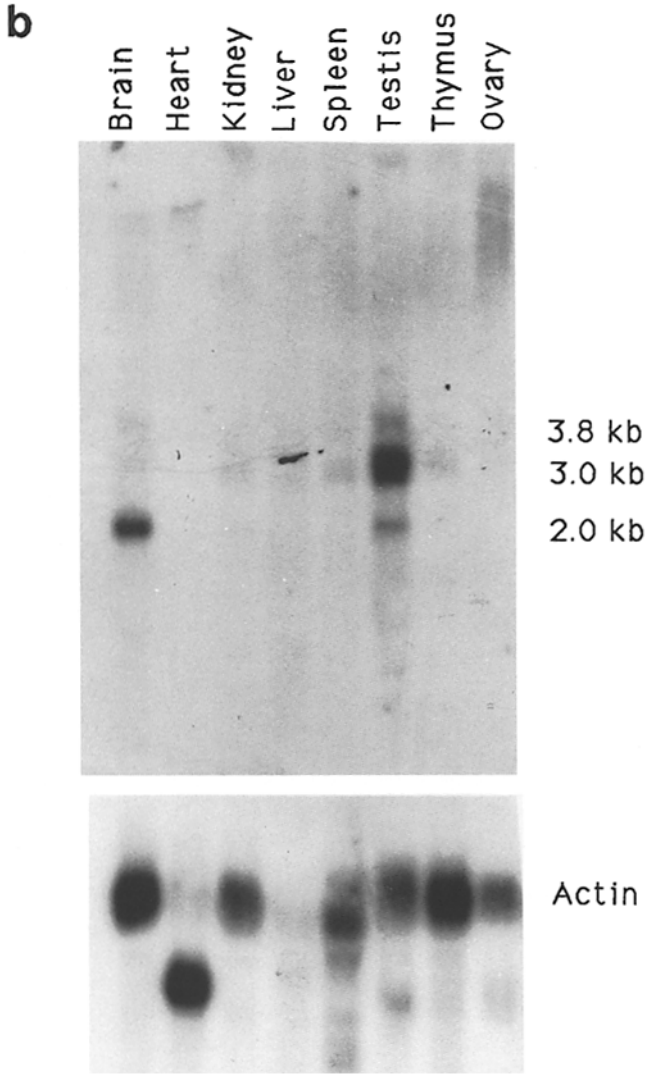


Figure 4. Tissue distribution of *Spnr* RNA. (*a*) RNAse protection analysis with a 481-nt anti-sense *Spnr* probe from the middle of the Spnr cDNA produced a protected RNA fragment of 413 nt. An actin probe of 173 nt that protected a fragment of 114 nt was used as a control for RNA amounts. (*b*) A Northern blot with 10 μg of total RNA from several different mouse tissues was hybridized with an α-^{32}P-labeled DNA probe corresponding to the entire *Spnr* cDNA. An actin probe was used as a control for RNA amounts.

which immunofluorescence of the Spnr protein was detected by staining serial testis sections with periodic acid schiff (an acrosomal marker) and hematoxylin (a nuclear stain) (data not shown). The Spnr protein is first detectable in step 9 spermatids as a perinuclear cap around the caudal end of the nucleus, while the acrosome is forming on the opposite, apical end of the nucleus (Fig. 6 *C*). We used an acrosomal specific antibody, 1D4 (O'Brien et al., 1988), in a double immunofluorescence to confirm this result (data not shown). The localization pattern of the Spnr protein is coincident with the formation of a testis-specific microtubular array, the manchette (Burgos and Fawcett, 1955). The manchette begins to form around the caudal end of the step 9 nucleus, and appears in cross-section as a cap-like structure. As the manchette develops, microtubules form a basket-like array surrounding the caudal end of the early-elongating nucleus; in cross-section this appears as a V of microtubules with the point at the very distal end of the nucleus. Confocal micrographs of Spnr localization at this stage (Fig. 6 *F, large arrow*) are indistinguishable from immunofluorescence micrographs with anti-tubulin antibodies that show an outline of the manchette (Aumuller and Seitz, 1988). As the spermatids elongate, the manchette extends caudally, at which point it becomes a hollow cylinder of microtubules that spreads into the caudal cytoplasm (Fig. 6 *D*). The confocal micrographs of the Spnr protein localization show circles of fluorescence; these are apparently cross-sections through the caudal-tube manchette (Fig. 6 *F, small arrow*). Staining with the Spnr antibody in late-elongated spermatids shows tube-like staining extending the length of the manchette and possibly beyond it onto the flagellar axoneme (Fig. 6 *E*). Immunostaining with the Spnr antibody decreases in step 14-15 spermatids, coincident with the disassembly of the manchette. There appears to be some residual staining in the lumen of early stage tubules (Fig. 6 *E*). This staining may

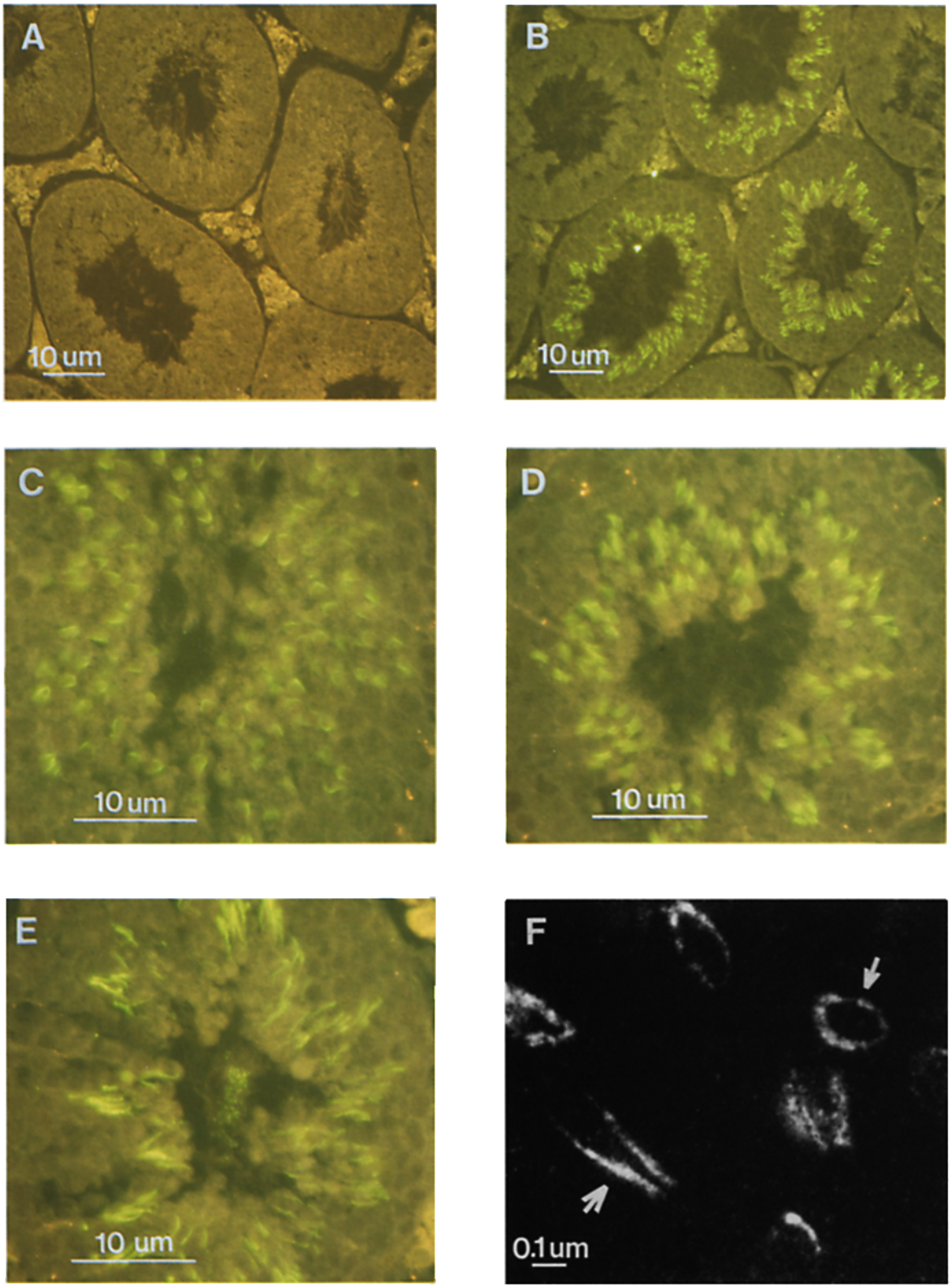


Figure 6. Localization of the Spnr protein in the testis. Testis sections were treated with rabbit polyclonal affinity-purified anti-Spnr antibody, washed, and treated with an FITC-labeled goat anti-rabbit secondary antibody (*B–F*), or treated with secondary antibody alone (*A*). (*A* and *B*) 300× magnification, (*C*) stage IX-X seminiferous tubule, (*D*) stage XII seminiferous tubule, (*E*) stage III-V seminiferous tubule, and (*F*) confocal image of stage XI seminiferous tubule (3 × zoom of 600× magnification). Bar, 10 μm.

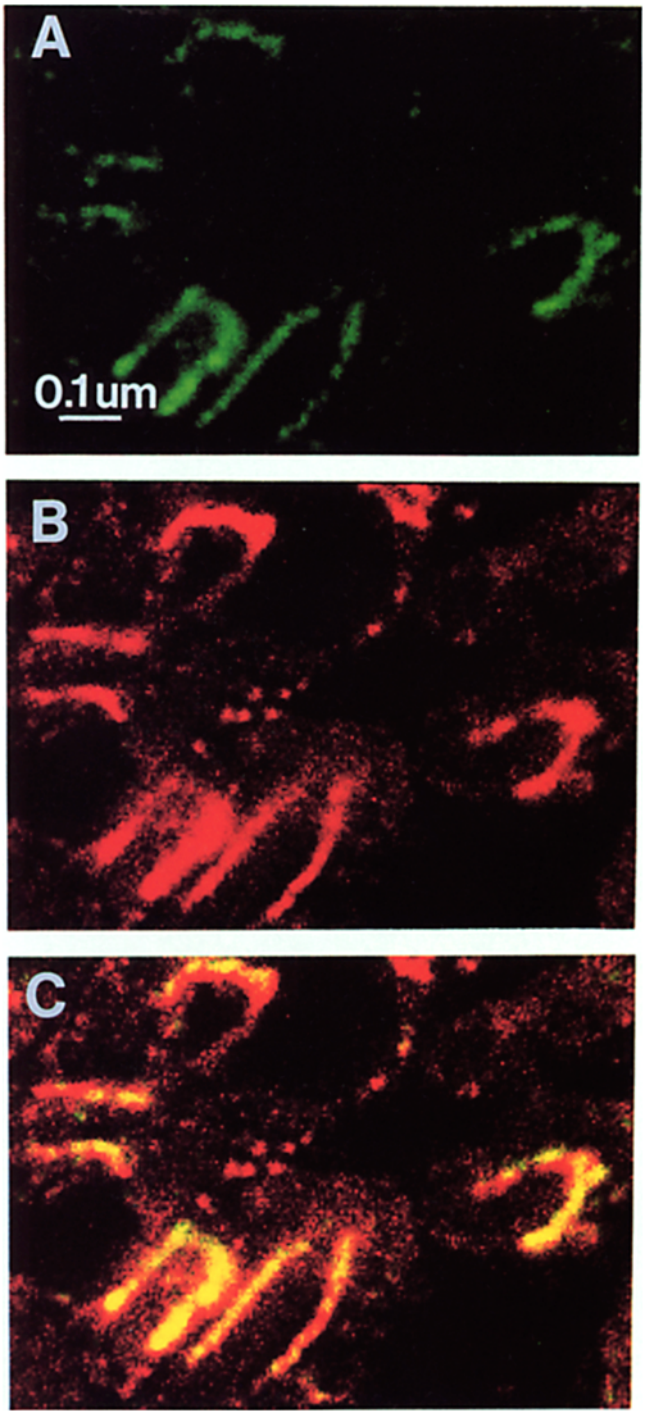


Figure 7. Double immunofluorescence of Spnr and Tubulin. Testis sections were treated with rabbit polyclonal affinity-purified anti-Spnr antibody and a mouse monoclonal anti-α-tubulin antibody (Amersham Corp.), washed, and treated with an FITC-labeled goat anti-rabbit secondary antibody and a rhodamine-labeled anti-mouse secondary antibody. (*A*) Confocal image of Spnr immunofluorescence alone. (*B*) Confocal image of tubulin immunofluorescence alone. (*C*) Merged confocal image of the Spnr and tubulin immunofluorescence (3 × zoom of 600× magnification). Bar, 0.1 μm.

correlate with the flagella of released spermatozoa after spermiation, or with their attached cytoplasmic droplets. The Spnr protein is not detected in mature, epididymal spermatozoa (data not shown).

To confirm that the Spnr protein is associated with the manchette microtubules, double immunofluorescence was performed with the Spnr antibody (Fig. 7 *A*) and an α-tubulin-specific antibody (Fig. 7 *B*). Spnr immunostaining is completely coincident with the anti-tubulin immunostaining of the manchette microtubules (Fig. 7 *C*).

Discussion

Spnr Protein Domains and In Vitro RNA-binding Properties

DNA sequencing of the *Spnr* cDNA revealed a predicted protein product of 71 kD that shows the greatest degree of similarity to the *Xenopus* 4F-1 and 4F-2 proteins (60% amino acid identity). Both of these proteins have been shown to bind RNA in vitro (Bass et al., 1994) and are members of a previously described family of RNA-binding proteins (St Johnston et al., 1992; Gatignol et al., 1993). The Spnr protein contains two copies of the RNA-binding domain that defines this family of proteins. Numerous studies addressing the in vitro binding properties of different members of this family of proteins suggest that they are sequence-independent RNA-binding proteins that prefer double-stranded RNAs and highly structured single-stranded RNAs that are rich in G-C base pairs (Green and Mathews, 1992; Gatignol et al., 1991; Gatignol et al., 1993; Manche et al., 1992). The most extensively studied member of this family of proteins, PKR, requires dsRNA substrates of at least 30 bp for minimal binding, and substrates of 85 bp or greater for maximum binding (Manche et al., 1992). However, despite the apparent nonspecific-binding properties of these proteins in vitro, genetic studies suggest that at least some of the members of this family, for example staufen (Ephrussi et al., 1991; Kim-Ha et al., 1991; St Johnston et al., 1991; Ferrandon et al., 1994), interact with selected mRNAs in vivo. The factors that confer message-specific binding to the staufen protein in vivo are unknown.

We have shown in a Northwestern assay that a fusion protein containing one of these domains from the Spnr protein is capable of binding the five RNAs that we tested in a salt-dependent manner and with varying relative affinities. These results demonstrate that the domain contained in the fusion protein can bind to different RNAs in a sequence-independent manner. However, given that the binding studies described here were performed with only one of the RNA-binding domains in the context of an MBP fusion, it is possible that the full-length protein may have binding characteristics and affinities that are different from those that are described here. At this time we have no evidence for a direct interaction between the Spnr protein and *Prm-1* mRNA in vivo. Clearly, the identification of Spnr's in vivo binding substrates will be informative as to the true function of the protein and its described RNA-binding domains.

The Spnr protein contains a putative leucine zipper that is preceded by a highly charged domain. Intriguingly, the leucine zipper in Spnr maps to a corresponding region in the *Xenopus* 4F-1 and 4F-2 proteins that contains a nuclear local-

ization signal (Bass et al., 1994), suggesting a modular evolution of these related RNA-binding proteins. Leucine zippers have been shown to be involved in protein–protein interactions between both homo- and hetero-dimers (Landschulz et al., 1988; Busch and Sassone-Corsi, 1990). It is very common for these domains to be associated with highly charged residues, especially in the case of leucine zipper containing transcription factors. The leucine zipper in the Spnr protein could be involved in dimerization, in docking the Spnr protein to a partner protein that is localized to the manchette, or it could act in localizing the Spnr protein to the manchette directly. Both tau and MAP2 are microtubule-associated proteins that have leucine zipper-like domains (Lewis et al., 1988), and tau has been localized to the manchette in bull spermatids (Ashman et al., 1992).

Chromosomal Localization of the Spnr Gene and Spnr mRNA Expression

By in situ hybridization, we found that the *Spnr* gene maps to mouse chromosome 2 region C1 and to human chromosome 9q34. This result confirms a region of conservation between human chromosome 9q and mouse chromosome 2 (Copeland et al., 1993). There are no known mouse mutants in this region with defects that could be easily attributed to a lack of the *Spnr* gene product. Since the *Spnr* gene appears to be evolutionarily conserved between humans and rodents, it will be of great interest to determine whether the Spnr protein is necessary for spermatogenesis and if so, whether mutations in the human *SPNR* gene are associated with male infertility in humans.

RNAse protection analysis revealed that the *Spnr* gene is expressed at a high level in testis, and that it is also expressed in the brain, ovary, and thymus. Northern blot analysis revealed several different Spnr transcripts in these tissues. The lack of detectable message in the ovary lane on the Northern blot is surprising given the amount of *Spnr* transcript that was detected in the RNAse protection experiments. It is possible that the transcript in the ovary is very large and is difficult to analyze by Northern blot analysis. The cloning of an ovary cDNA would clarify this issue.

Western blot analysis with the Spnr antibody shows a protein of the predicted molecular weight of 71 kD only in the testis. No proteins are detectable in extracts from other tissues. That the Spnr protein is not detected in the other tissues where the mRNA is expressed may be due to a variety of reasons. The protein products in these other tissues may be relatively unstable, the antibody may not be able to detect forms from other tissues (perhaps due to posttranslational modifications), or the mRNAs may not be translated at detectable levels in these tissues.

The Spnr Protein Is Associated with Microtubules

Immunolocalization of the Spnr protein within the testis shows that the protein is coincident both temporally and spatially with the formation of the manchette. The manchette is a spermatid-specific array of microtubules that begins to form around the caudal end of the early elongating spermatid nucleus (Burgos and Fawcett, 1955). The manchette forms in step 9 spermatids and persists until step 15 spermatids. The Spnr protein is only detected during this time. *Prm-1* is first transcribed in step 7 round spermatids (Mali et al., 1989) and is stored as a cytoplasmic mRNP until it is translated in step 12 elongating spermatids (Balhorn et al., 1984; Kleene et al., 1984). Because Spnr is expressed a few days after the onset of *Prm-1* transcription, it is highly unlikely that Spnr is involved in repressing translation of *Prm-1* mRNA. However, the appearance of Spnr just before and during the onset of *Prm-1* translation, suggest a possible role of Spnr in *Prm-1* translational activation (see below).

Two possible and not mutually exclusive functions for the manchette are in nuclear shaping and as a "track" for movement of organelles and vesicles (Russell et al., 1991; Fawcett et al., 1971). Electron micrographs have shown that the microtubules of the manchette are associated with vesicles and with the endoplasmic reticulum (Fawcett et al., 1971; MacKinnon and Abraham, 1972). It is known that microtubules and their associated proteins are involved in the movement of various cargos throughout the cytoplasm of somatic cells and germ cells. Given that the elongating spermatid is a highly polarized cell, there is an obvious need for transport of materials from the periphery of the nucleus down to the far reaches of the caudal cytoplasm where cellular products are needed for processes such as the construction of the axoneme. Since the manchette is a spermatid-specific microtubular array that forms in the elongating spermatid, and disappears when the spermatids are nearing completion, it is an obvious candidate as a mediator of such caudal movements.

Microtubules and other cytoskeletal elements clearly have a role in the localization of various RNAs in different cell types. We propose that the Spnr protein may bind to RNAs and transport them along the manchette microtubules. Spnr may interact with microtubule-associated proteins or it could bind to microtubules directly. Possible binding partners for Spnr include kinesin and dynein, both of which have been shown to be associated with the manchette in the rat (Hall et al., 1992). Determining how the Spnr protein is associated with microtubules and the identity of the proteins that assist in this interaction will be very informative as to the possible function of this protein. The expression of *Spnr* in the brain and ovary are consistent with Spnr having a role in RNA localization as well, since a number of examples of localized RNAs occur in neuronal cells and oocytes (Garner et al., 1988; Jeffery, 1989).

The need for the transport of RNAs within developing spermatids is potentially very great. Since transcription from the haploid genome ceases long before the cell has fully developed, many mRNAs, including the *Prm-1* mRNA, are stored as mRNPs until their protein products are needed later in spermiogenesis. The ultimate translation of RNAs near their sites of action has been well established for those mRNAs encoding cytoskeletal proteins (Fulton et al., 1980; Fulton and Wan, 1983), and is an obvious consequence of RNAs that are specifically localized. There is also a correlation between the association of mRNAs with the cytoskeleton and their translatability (Cervera et al., 1981). Since the spermatid is a highly polarized cell where many messages are under translational control, some of these RNAs may be localized to specific sites where they are stored until their ultimate translation and usage. The Spnr protein could be the link between such stored RNAs and microtubules that mediate their transport and ultimate translation.

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