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Spermatogenetic Expression of RNA-Binding Motif Protein 7, a Protein That Interacts With Splicing Factors

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ABSTRACT: We have previously shown that a ubiquitously expressed RNA splicing factor, RNA-binding motif 7 (RBM7), cloned from a testis complementary DNA library, enhances messenger RNA (mRNA) splicing in vitro and is expressed in a cell-restricted fashion. Herein, we detail its mRNA and protein expression in the rodent testis. RNA in situ hybridization shows that *Rbm7* expression in rat germ cells closely parallels the entry and progression of meiosis. The expression commences in type B spermatogonia, it rises during the preleptotene stage, peaks in leptotene spermatocytes, and declines afterward, but increases again in stage-associated pachytene spermatocytes. An affinity-purified polyclonal antibody raised against a peptide corresponding to amino acids 202–224 of the mouse RBM7 recognized the predicted 35 kd protein both in testicular lysates and in in vitro translation reactions. Consistent with the in situ hybridization results, RBM7 immunoreactivity was also detected in type B spermatogonia, spanned the entire period of spermatocyte development, and extended to round and early elongated spermatids. Moreover, RBM7 appeared nuclear up to the mid pachytene stage and became cytoplasmic thereafter. Consistent with its role in RNA splicing, yeast 2-hybrid and glutathione *S*-transferase pull-down assays show that RBM7 interacts with splicing factor 3b subunit 2 (SAP145), and with the splicing regulator, SRp20. These interactions and the nuclear localization of RBM7 provide insights into its function in pre-mRNA processing in developing spermatocytes during entry into meiosis and progression through the meiotic prophase.

Key words: Testis, spermatogenesis, meiosis, RNA processing, RNA-binding protein.

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RNA-binding proteins are a conserved and diverse group that play critical structural and catalytic roles in many aspects of RNA functions, including transcription, splicing, transport, and translation. Several RNA-binding proteins play critical roles in meiosis and spermatogenesis. In fission yeast, the RNA-binding protein Mei2 regulates the cell cycle switch from mitosis to meiosis (Watanabe et al, 1997). Similarly, in metazoans, several testis-specific RNA-binding proteins play critical roles in the control of various steps of the mitotic and meiotic cell cycles (Cooke et al, 1996; Eberhart et al, 1996; Reijo et al, 1996; Yen et al, 1996; Delbridge et al, 1997; Habermann et al, 1998; Houston et al, 1998). *DAZ* (the *d*eleted in *az*oospermia gene) and *RBMY* (the Y RNA-binding motif gene) are both human Y chromosome gene families implicated in male infertility (Reijo et al, 1995; Ferlin et al, 1999). The mouse DAZ-like protein DAZL (Schrans-Stassen et al, 2001), and a related protein in fruit fly, Boule (Eberhart et al, 1996), are essential for successful spermatogonial mitotic expansion and meiotic completion, respectively. Whereas DAZ and DAZL probably play a role in the regulation of translation (Maines and Wasserman, 1999; Tsui et al, 2000b), RNA-binding motif (RBM), also known as y-chromosome ribonucleic acid recognition motif (YRRM) and encoded by *RBMY*, may participate in pre-mRNA processing during meiosis (Elliott et al, 1998). This protein localizes to the nucleus of germ cells and may have a role in pre-mRNA splicing (Elliott et al, 1998). This has been implied from the ability of RBM to interact with a number of splicing factors and serine-arginine (SR) proteins that play a role in both constitutive and alternative splicing (Elliott et al, 2000) and from biochemical evidence that it interferes with SR-dependent splicing reactions (Elliott et al, 2000). A close homologue of RBM, hnRNP G (heterogenous nuclear ribonucleoprotein G) encoded by *RBMX*, is ubiquitously expressed and is also believed to play a role in RNA splicing in the testes (Venables et al, 2000). A number of *RBMX*-like sequences (termed *RBMXL*s), many of them

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nonfunctional, are scattered throughout the human genome (Lingenfelter et al, 2001).

We have previously cloned a putative pre-mRNA splicing factor designated *RBM7* (RNA-binding motif protein 7; Guo et al, in preparation). It is highly conserved, and its homologs are found in many species. Its RNA-binding domain is highly similar to that of splicing factors such as spliceosome-associated protein 49 (SAP49), a component of splicing factor 3b (SF3b). RBM7 bares only a weak homology within its RNA-binding domain to RBMY, but it has stronger homology in this domain to other GenBank sequences (RBM11 and RBM4) grouped in the RBM family, but with a yet-unidentified function. Aside from mapping human *RBM7* to chromosome 11, we have also shown its RNA to be expressed in all tissues tested, with a unique shorter transcript expressed only in the testes. Despite its expression in a wide variety of tissues, in the rat, *Rbm7* was preferentially expressed in discrete cell populations within the brain. This raised the possibility that whereas RBM7 may not be important for constitutive mRNA splicing, it may enhance the splicing of a group of mRNAs in a cell-specific manner or in a certain developmental process, such as spermatogenesis. In this paper, we detail the testicular expression of RBM7 at both the mRNA and protein levels and demonstrate that RBM7 interacts with 2 essential splicing factors, SAP145 and SRp20.

Materials and Methods

Plasmids

Vectors pAS2/RBM7 and pBSmRBM7 containing the human and mouse RBM7 open reading frame (ORF) complementary DNAs (cDNAs), respectively, have been described previously (Guo et al, in preparation; GenBank accession numbers AF156098 and AF458961). A 0.73-kb *Sac*I-*Sal*I segment from pAS2/RBM7 was subcloned into pBluescript II KS(−) vector (Stratagene, La Jolla, Calif) for the generation of riboprobes. The *Bam*HI insert of pAS2/RBM7 containing the ORF was subcloned in-frame into pGEX-5X-1 (Amersham Pharmacia, Piscataway, NJ) to produce the glutathione *S*-transferase (GST)-RBM7 fusion protein. In parallel, pETGEXCT (Worthington, Lakewood, NJ) was used for control GST protein (~26 kd) production. For in vitro translation (IVT), the *Not*I insert of a full-length *SAP145* clone isolated from the 2-hybrid screen was subcloned into pcDNA3 (Invitrogen, Carlsbad, Calif). To subclone *SRp20* ORF, we used reverse transcriptase–polymerase chain reaction to amplify a fragment from mouse testis cDNA using primers 5′-TGAGCTTGGGCCTTTTGAAC-3′ (nucleotides 30–49) and 5′-CAATTTCCGCCAGTTGCTTT-3′ (nucleotides 909–890, GenBank accession number X53824), and cloned it into pCR3.1 (Invitrogen).

Tissue Preparation

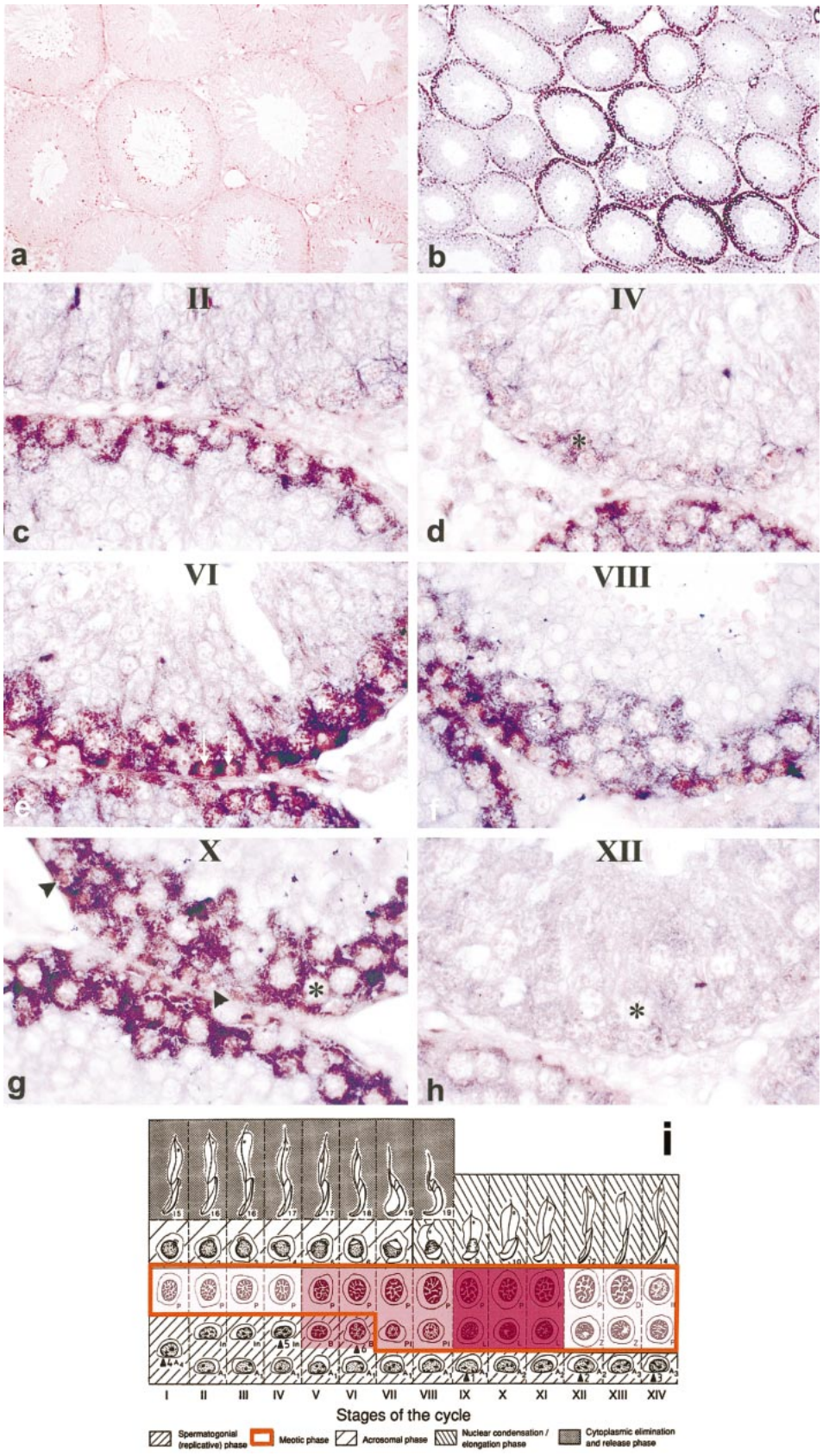
Animal handling protocols were approved by the institutional animal care and review committee. Adult Sprague-Dawley rats were obtained from B&K Universal (Fremont, Calif). C57/Bl6 mice were bred in-house. After the animals were killed, testes were either fixed in Bouins solution via intracardiac whole-body perfusion, or snap-frozen for protein extraction. Testicular lysates were prepared according to the *Research Applications* manual from Santa Cruz Technology (Santa Cruz, Calif). Proteins were solubilized in RIPA buffer (1× phosphate-buffered saline [PBS] supplemented with 1% NP-40, 0.5% sodium deoxycholate, 0.1% sodium dodecyl sulphate [SDS], 1× complete protease inhibitors [Roche Biochemicals, Indianapolis, Ind], 10 mg/mL sodium orthovanadate, and fresh 10 mg/mL phenylmethylsulphonyl fluoride).

RNA In Situ Hybridization

Sense and antisense riboprobes were generated by incubation of *Sac*I- or *Sal*I-linearized pBluescript templates with digoxigenin (DIG)-labeled *uridine* 5′-triphosphate in the presence of T7 (sense) or T3 (antisense) RNA polymerases according to the manufacturer's recommendations (Roche Biochemicals). RNA in situ hybridization was carried out on paraffin-embedded, 5-μm-thick testis transverse sections according to an established protocol (Millar et al, 1993), except for the omission of dithiothreitol (DTT) in the prehybridization buffer and RNase A in the posthybridization washes; in addition, the hybridization temperature was 46.5°C. The sections were counterstained with nuclear fast red for 10 minutes and photomicrographed with a camera mounted on an Olympus BH2 light microscope (Olympus Optical, Tokyo, Japan).

Antibodies, Immunoprecipitation, and Immunoblots

An antigenic peptide, which was not similar to other proteins in the databases, was synthesized. Its sequence was [Cys^{202}]-SHPYLADRHYSREQRYSDHGSD and corresponds to mouse RBM7 amino acids 203–224. Affinity-purified anti-RBM7 peptide rabbit polyclonal type G immunoglobulins (IgGs) were then produced (Bethyl Labs, Montgomery, Tex). Antibody specificity was determined by immunoprecipitation. [^{35}S]-labeled RBM7 was in vitro–translated in a T7 quick-coupled transcription/translation system (Promega, Madison, Wis) using pBSmRBM7 as the template. A 5-μL aliquot of the mixture was incubated on ice for 1 hour with 5 μL of either the antibody or preimmune serum and 90 μL of 0.02 M Tris-buffered saline with 0.1% Tween-20. An equal volume of 10% protein-A-agarose (Bio-Rad, Hercules, Calif) was added and incubated at 4°C for 1 hour with end-to-end rocking. The immunocomplex was analyzed by SDS-polyacrylamide gel electrophoresis (PAGE). The signals were detected by phosphor imaging (Molecular Imager FX; Bio-Rad). Tissue blotting was performed as previously described (Yamamoto et al, 2000). Primary antibodies were diluted 1:6000. For preabsorption, the same amount of primary antibody was preincubated for 30 minutes with 5× molar excess of the neutralizing peptide to probe a duplicate blot.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I II III IV V VI VII VIII IX X XI XII XIII XIV
Stages of the cycle
Spermatogonial (replicative) phase
Meiotic phase
Acrosomal phase
Nuclear condensation / elongation phase
Cytoplasmic elimination and release phase

Protein Isoform Detection by Electrospray Ion Trap Mass Spectrometry

Mouse testicular lysate was immunoprecipitated by the anti-RBM7 antibody overnight and the immunocomplex was resolved by SDS-PAGE and subjected to zinc staining (Bio-Rad). Protein bands (~35 kd and ~45 kd) were excised and electroeluted, and the residual SDS was removed by organic extraction (Hwang et al, 1996). Samples were reconstituted in 50% methanol:50% water with 1% glacial acetic acid and analyzed in the positive, full scan data acquisition mode using a Finnegan LCQ Deca ms_n (Palo Alto, Calif) mass spectrometer. The instrument is equipped with an electrospray ionization source tandem to the ion focusing (quadrupole)–ion trap–ion detector electron multiplier axis. During continuous sample injection, the instrument acquired data in the mass range of 500.00–2000.00 (*m/z*) in full scan mode. The acquired spectra were analyzed using Xcalibur BIOMASS Deconvolution settings in the automatic mass-tracking mode in order to determine the mass unit of proteins using their acquired mass/charge ratios.

Immunohistochemistry

We followed the protocol of the Unitect rabbit immunohistochemistry system (Oncogene Research Products, Cambridge, Mass). Primary antibody dilution was 1:400 (4 μg/mL in PBS supplemented with 1% bovine serum albumin). Equivalent concentrations of normal rabbit IgG were used as a negative control. A second negative control consisted of prior incubation of the primary antibody with an excess of neutralizing peptide.

Yeast Two-Hybrid Protein Interaction Screen

The bait vector pAS2/RBM7 that expressed the Gal4 DNA binding domain (GBD)-RBM7 fusion protein was cotransformed with the human MATCHMAKER testis cDNA library (10^7 transformants) into yeast strain y190. Transformants were screened according to the instructions provided by the manufacturer (Clontech). Putative positive clones were identified, isolated, and reintroduced into the Y190 host strain with pAS2/RBM7 to test for reporter activation. To eliminate potential false positives, each was transformed into yeast either without cotransformation with pAS2/RBM7, or cotransformed with a nonrelated vector, pAS2/mGSK3β.

GST Fusion Protein Production and GST Pull-Down Assay

pETGEXCT and pGEX-5X-1/RBM7 were transformed into *Escherichia coli* BL21(DE3) strain and induced for GST and GST-RBM7 fusion protein expression, respectively. Although the recombinant GST was partially soluble in 1% Triton X-100 sonicated bacterial lysate, GST-RBM7 was solubilized only by the sarkosyl method (Frangioni and Neel, 1993). Triton X-100 was added to this sonicated lysate prior to overnight incubation with glutathione Sepharose 4B beads (Amersham Pharmacia) with end-to-end rotation at 4°C. Beads were washed 6 times with ice-cold PBS and resuspended in storage buffer (50 mM Hepes pH 7.4, 0.15 M NaCl, 5 mM DTT, 10% v/v glycerol) at −20°C. The purity of the GST-fusion proteins was examined with Coomassie blue staining of a 12% gel as well as by immunoblotting a duplicate gel using the anti-RBM7 peptide antibody. For the GST pull-down assay, aliquots of RNase A–treated IVT products of [^{35}S]-labeled luciferase (2 μL), RBM7 (2 μL), SAP145 (15 μL), and SRp20 (10 μL) were mixed with 12.5 μL of 5% suspension of GST or GST-RBM7 immobilized on glutathione-Sepharose beads. The binding buffer contained 50 mM Tris-HCl pH 7.5, 0.5 M NaCl, 2 mM ethylenediamine tetraacetic acid (EDTA), 0.1% NP-40, and the complete protease inhibitor cocktail (Tsui et al, 2000). To examine weak protein-protein interaction, a low salt concentration (0.15 M) was substituted. After overnight incubation at 4°C, beads were pelleted and washed thrice with the binding buffer. Bound proteins were dissociated by boiling in reducing Laemmli buffer and analyzed by SDS-PAGE followed by phosphor imaging.

Results

RNA In Situ Hybridization

Although *Rbm7* is ubiquitously expressed, there is evidence of cell-specific expression in both the brain and the testis. We carried out in situ hybridization experiments to determine whether *Rbm7* expression is correlated with germ cell development. Cytoplasmic signals were visible in type B spermatogonia, and in preleptotene, leptotene, and pachytene spermatocytes at stages I through XII (Figure 1). The most intense staining was seen in preleptotene, leptotene, and pachytene spermatocytes at stages VI–XI (Figure 1, e–g). Some Sertoli cell staining could also be observed. There was no staining in the haploid cell populations or in other cell types in any of the other stages. Except in certain vessel walls, there was no staining in the interstitial compartment. Probing with a sense probe generated no signals (Figure 1a). A schema shown in Figure 1i illustrates the restricted, biphasic *Rbm7* germ cell expression: first in type B spermatogonia, prelepto-

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Figure 1. RNA in situ hybridization of *Rbm7* expression in the rat testis. Blue/purple staining represents positive signals. All sections were counterstained with nuclear fast red (red color). **(a)** Overview (200×) of a testis section probed with *Rbm7* sense cRNA. Note the absence of positive staining and the tissue integrity after fixation. **(b)** Overview (100×) of a testis section probed with *Rbm7* antisense cRNA. Note the differential positive staining in various stages of the seminiferous epithelium and the absence of interstitial staining. **(c–h)** High magnification (1000×) photomicrographs showing stage-specific patterns of *Rbm7* expression. Roman numerals indicate the stages of the seminiferous epithelial cycle, determined based on morphological criteria (Russell et al, 1990). Asterisks mark pachytene **(d–g)** or diplotene **(h)** cells. Arrows point to type B spermatogonia **(e)**, preleptotene cells **(f)**, and leptotene cells **(g)**. **(i)** Schematic summary of *Rbm7* expression in the germ cells, which was adapted from *The Physiology of Reproduction,* Raven Press, New York, 2nd edition, 1994, with permission. The cell types that express *Rbm7* are shaded in pink. The densities of the shades reflect levels of expression. *Rbm7* is expressed throughout the meiotic phase (framed in red) of rat spermatogenesis as well as in type B spermatogonia.

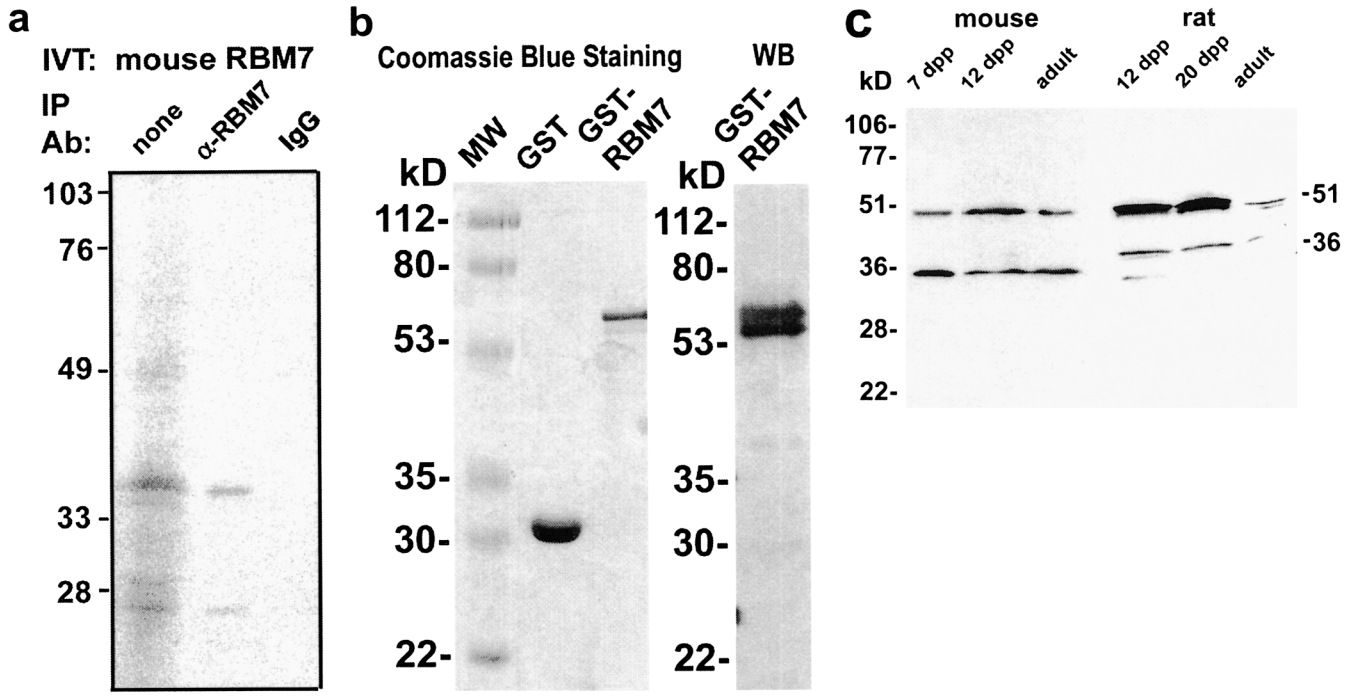


Figure 2. Expression of RBM7 protein. **(a)** IVT and characterization of anti-RBM7 antibody. IVT of mouse RBM7 protein is seen mainly at 35 kd and can be immunoprecipitated by anti-RBM7 peptide antibody (α-RBM7 Ab) but not by nonimmune rabbit IgG. **(b)** Expression and purification of recombinant GST and GST-RBM7 fusion proteins in *E. coli.* Purified proteins were separated on SDS-PAGE and stained with Coomassie blue (left panel). In parallel, α-RBM7 Ab specifically and strongly reacted with GST-RBM7 fusion protein, as a doublet in Western blot (right panel). **(c)** RBM7 expression in juvenile and adult mouse and rat testes. Western blot of proteins from mouse and rat testes obtained at the times indicated and probed with α-RBM7 Ab. Days postpartum are indicated (dpp).

tene, and leptotene cells; spanning the period when cells enter the meiotic cell cycle; and then in mid to late pachytene spermatocytes. The expression in type B spermatogonia and steady increase in preleptotene and leptotene cells suggests that it may play a role during the meiotic G1 to S transition. Its decline in zygotene and early pachytene cells and resurgence in mid to late pachytenes is evidence of coordinated developmental regulation during spermatocyte maturation.

RBM7 Protein Expression—Western Blotting

We generated and affinity-purified a rabbit polyclonal antibody against amino acid residues 203–224 of the mouse RBM7. This antibody specifically immunoprecipitated in vitro translated RBM7 proteins, shown in Figure 2a as a 35-kd band and a minor 27-kd band. Because the antibody was directed against a region near the C-terminus the latter band probably resulted from internal initiation of translation instead of premature termination. This antibody also strongly reacted with a GST-RBM7 fusion protein, which appeared as a doublet at ~60 kd (Figure 2b).

We used the antibody to study the ontogeny of RBM7 expression during rodent spermatogenesis, with an emphasis on meiosis. We chose 2 developmental stages in particular: immediately before meiosis I initiation, and early to mid pachytene stage. Thus, based on previous studies (Bellve et al, 1977; Yang et al, 1990; Malkov et al, 1998), we used mice at day 7 and day 12 postpartum (dpp) and rats at 12 dpp and 20 dpp. Western blotting (Figure 2c) of whole testis lysates detected at all time points revealed 2 bands: ~50 kd and ~35 kd in mice and ~50 kd and ~40 kd in rats. The rat ~50 kd form was consistently the more abundant form. Because all signals were abolished on a duplicate blot probed with the antibody preincubated with an excess of the antigenic peptide (data not shown), these bands appeared to be specific.

Some nuclear proteins, heavily charged RNA-binding proteins in particular, have a high degree of altered mobility on SDS-PAGE, often as a consequence of posttranslational modification (Query et al, 1989; Gozani et al, 1996; Klenova et al, 1997). Because the predicted size of RBM7 is ~30 kd, we explored the possibility that the 50-kd band is a posttranslationally modified isoform of RBM7 that exhibits aberrant mobility. Electrospray ion mass spectrometry (ESI/MS) has been used to resolve molecular weight differences attributed to aberrant gel migration (Iakoucheva et al, 2001). This has been attributed to its ability to reduce many tertiary protein conformational modifications, except those of phosphorylation (Roepstorff, 2000). To confirm that these 2 immunoreactive bands represented RBM7, RBM7 immunopre-

cipitates from mouse testis extracts were resolved by SDS-PAGE. Plasmid 35 (p35) and p50 were then eluted and subjected to electrospray ion trap mass specrometry. Results indicated that both bands had 28 213 mass units, and exhibited identical fragmentation patterns (Figure 3a and b) upon source fragmentation, wideband activation, or collision-induced fragmentation. These results show that the 50-kd band detected by the RBM7 antibody is an isoform with aberrant gel migration probably due to one or more forms of posttranslational modification.

RBM7 Protein Expression—Immunostaining

The anti-RBM7 antibody was used in standard avidin/biotin–mediated immunoperoxidase detection of RBM7 on rat testicular sections. Neither nonimmune IgG nor the antibody after immunoneutralization with the oligopeptide antigen yielded any signals (Figure 4a and b), attesting to the specificity of this antibody. RBM7 immunoreactivity was nuclear in type B spermatogonia (Figure 4d); and in preleptotene (Figure 4e and f), leptotene (Figure 4g and h), zygotene (Figure 4i), and early (up to stage VI) pachytene (Figure 4c, d, and i) spermatocytes. The staining intensity appeared strongest in active preleptotene, leptotene, and zygotene nuclei (stages VIII–XIII). RBM7 immunoreactivity then became cytoplasmic in mid and late pachytene (Figure 4e through h), diplotene, and dividing spermatocytes (Figure 4i), as well as in spermatids at steps 1 through 14. Step 11 spermatids had rather strong staining near the nuclear/acrosomal region (Figure 4h). Occasional type A spermatogonial staining could also be observed. Leydig cells and Sertoli cells were not stained. Virtually identical expression results were obtained from mouse testes (data not shown).

These results, summarized in Figure 4j, suggest that, consistent with the pattern of mRNA expression (Figure 1i), RBM7 expression is spatiotemporally regulated during rat spermatogenesis. The expression commences at the time of meiosis initiation at type B spermatogonia and preleptotene spermatocytes, and the protein is predominantly nuclear. As the mRNA levels peak at the leptotene stage, RBM7 protein continues to accumulate in the nucleus. During early pachytene development, when the message level becomes nearly undetectable, RBM7 immunoreactivity decreases progressively. At mid pachytene, a second and coincidental up-regulation of *Rbm7* mRNA and protein occurs, but interestingly, the protein is no longer nuclear. RBM7 immunoreactivity persists in the cytoplasm of late and dividing spermatocytes as well as postmeiotic spermatids prior to their cytoplasmic elimination, consistent with the decrease in mRNA levels in late pachytene cells. Interestingly, dividing spermatocytes show strong staining around the metaphase plates.

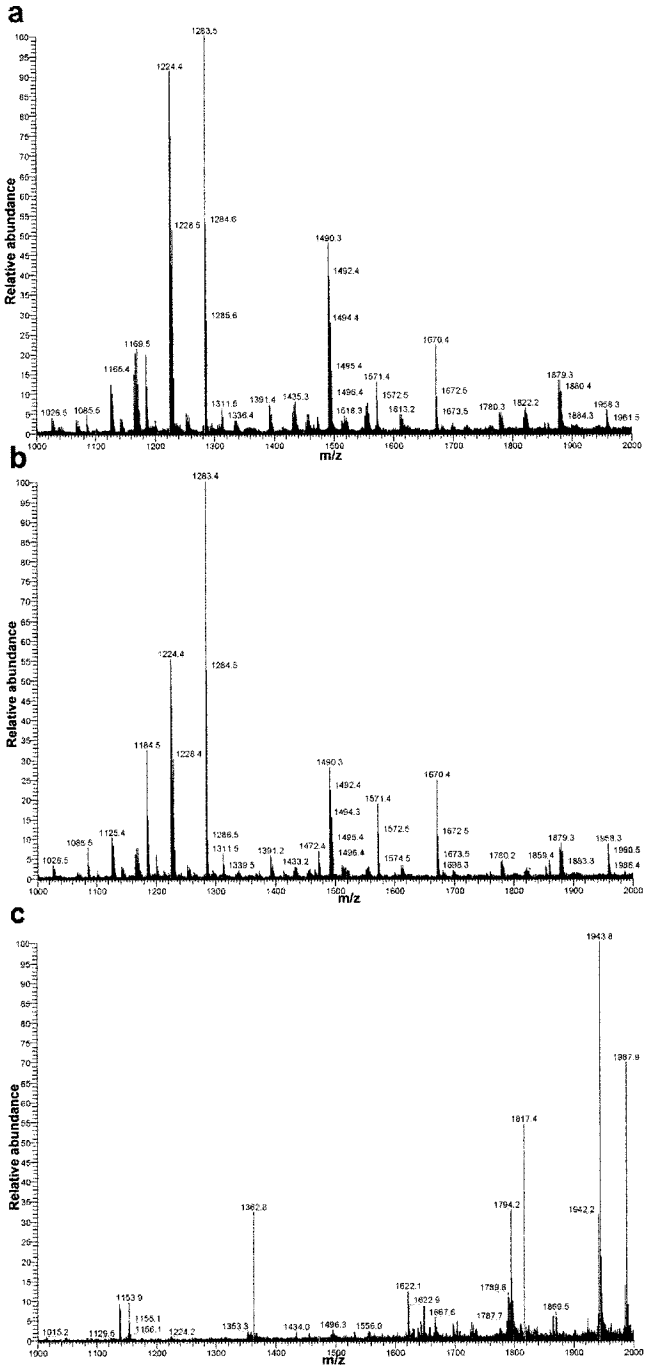
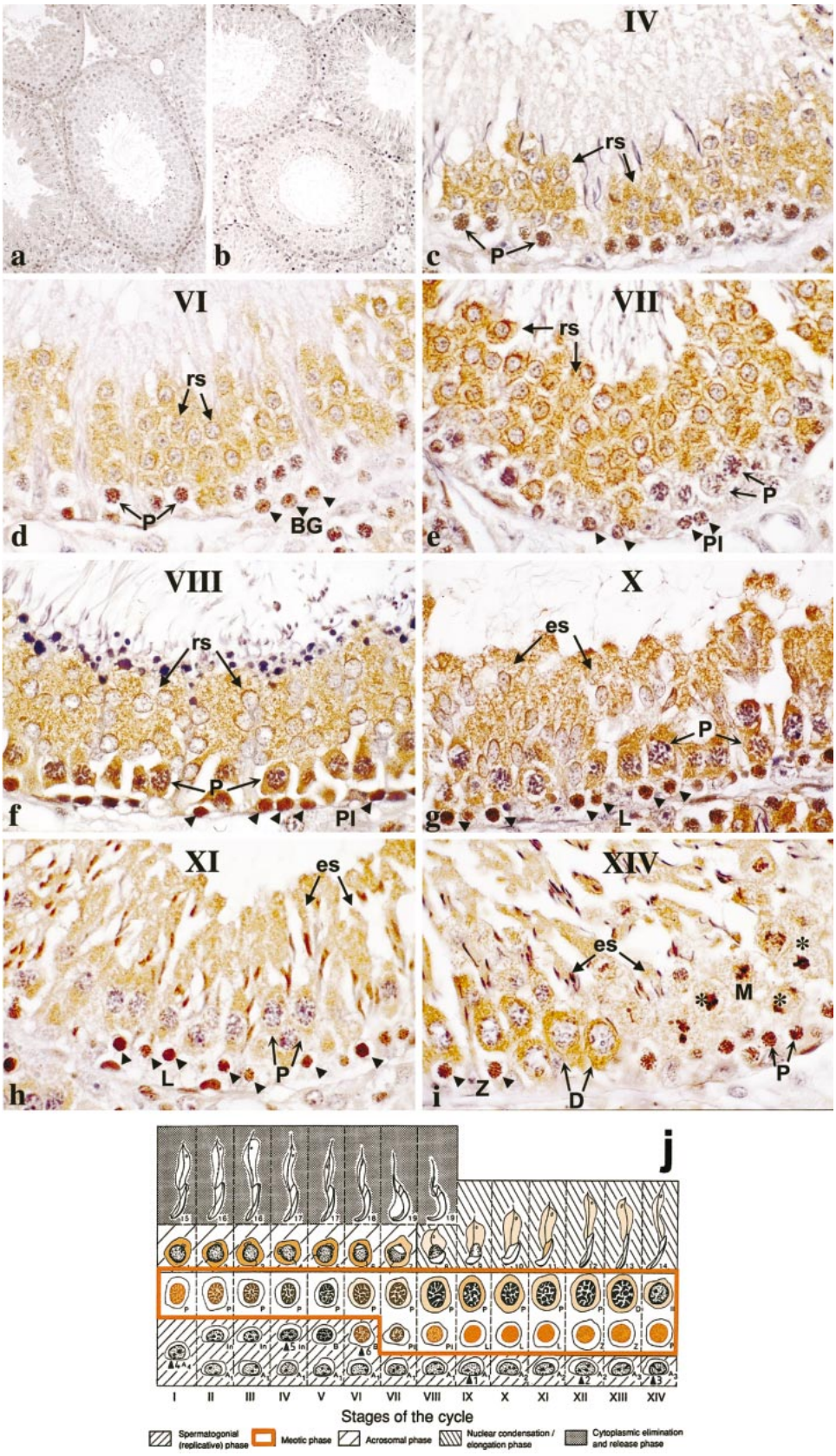


Figure 3. Mass spectral analyses of p35 and p45. The prominent p35 and p45 bands were immunoprecipitated by α-RBM7 antibody from mouse testis lysates, recovered from gel, cleaned up, and subjected to ESI/MS. The spectra shown here range from 1000 to 2000 *m/z*. Note the identical patterns in **(a)** p35 and **(b)** p45, but a completely different spectrum for an unrelated protein, trypsin, in **(c)**.

RBM7 Interacts With Splicing Factors SAP145 and SRp20

To understand the cellular function of RBM7, we used a yeast 2-hybrid protein assay to identify proteins that could interact with RBM7. Screening yielded 16 different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Stages of the cycle
Spermatogonial (replicative) phase
Meotic phase
Acrosomal phase
Nuclear condensation / elongation phase
Cytoplasmic elimination and release phase

clones, 12 of which carried varying lengths of the same gene, the essential spliceosomal SF3b subunit, *SAP145* (GenBank accession number NM_006842), with a majority of them aligned to the carboxy half of the protein. Closer examination identified a common region (amino acids 579–750) that could contain the minimal interaction domain. Another clone was the full-length *SRp20* (GenBank accession number NM_003017).

We confirmed the interactions in vitro by binding ^{35}S-labeled IVT proteins (SAP145 and SRp20) to recombinant GST-RBM7 immobilized on glutathione-Sepharose beads (Figure 5). As shown, only GST-RBM7 was able to pull down SAP145 and SRp20, whereas neither GST nor GST-RBM7 captured the luciferase control. The sizes and patterns of these IVT products were in good agreement with published reports (Wang et al, 1998; Elliott et al, 2000; Bryant et al, 2001). Of note, the SRp20 experiment was performed at physiologic salt concentration (0.15 M NaCl). In a high salt condition (0.5 M NaCl), essentially no SRp20 binding was detected. To examine the possibility of RNA-mediated protein-protein interaction, aliquots of IVT products were treated with RNase before the binding assay. There was no change in the amount of SAP145 recovered after RNase treatment, suggesting that RBM7 and SAP145 directly associate. However, compared with the input, much less SRp20 was retained by RBM7 and this interaction was sensitive to RNase treatment. Thus, SRp20 specifically but weakly associates with RBM7, and this interaction is enhanced by the presence of RNA.

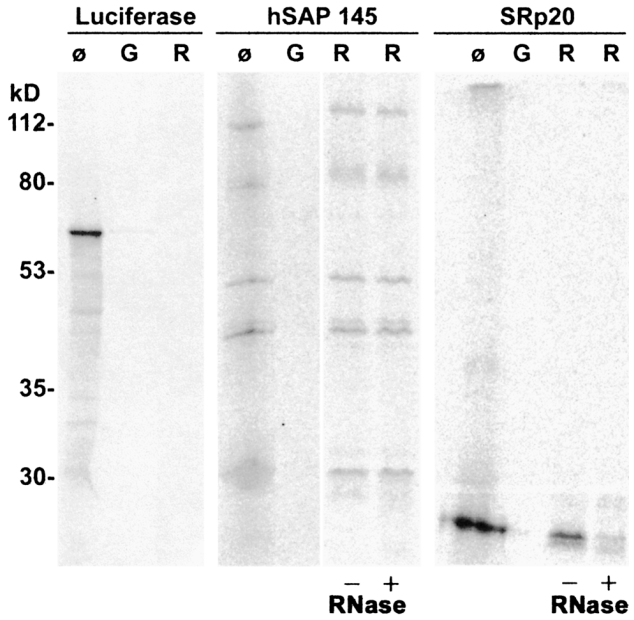

Figure 5. In vitro interactions of RBM7 with SAP145 and SRp20. ^{35}S-labeled IVT proteins (as indicated in the heading) were treated with RNase and either loaded directly as input marked by "ø" or after incubation with glutathione-Sepharose beads coated with GST alone (G) or GST-RBM7 (R). Samples with or without RNase treatment were so marked for comparison (−RNase vs +RNase). Luciferase served as positive control for IVT and negative control for the pull-down. Assay was performed at 0.15 M NaCl for SRp20 samples, whereas the rest were performed at 0.5 M NaCl.

Discussion

Diversity of gene function in the testis is achieved either through generation of testicular specific transcripts for many ubiquitously expressed genes or by generating multiple transcripts for testis specific genes (Wolgemuth and Watrin, 1991). This is believed to be achieved through the use of alternative promoters, alternative polyadenylation, and most commonly, alternative splicing (Braun, 1998; Eddy, 1998). Regulated splicing (ie, the ability to selectively and specifically include or exclude certain exons thereby creating multiple transcripts) affords the cell this functional diversity in response to constant environmental and genetic changes during differentiation and development. Herein, we have detailed the testicular expression of a putative splicing factor, RBM7, and provided evidence that it associates with SAP145 and SRp20. Together with our previous finding that overexpression of RBM7 increases intron-containing gene expression, we propose that RBM7 participates in pre-mRNA processing during germ cell development.

The idea that RBM7 may be a splicing factor was initially based on its homology with other splicing factors, especially SAP49. Also known as splicing factor 3b subunit 4, SAP49 is essential in the *Caenorhabditis elegans* worm (Fujita et al, 1998) and yeast (*Saccharomyces cerevisiae*) (Igel et al, 1998). It directly and stably interacts with SF3b subunit 2 (SAP145) and functions to tether U2 small nuclear ribonucleoprotein particle (snRNP) with pre-mRNA at the branch site during spliceosome assem-

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Figure 4. RBM7 immunoreactivity in adult rat testis sections. Probed with α-RBM7 antibody and detected with avidin-biotin-horseradish peroxidase complex and diaminobenzidine, which gave brown staining. Lightly counterstained with hematoxylin. **(a)** Negative control section incubated with immunoneutralized primary antibody (magnification 400×). **(b)** Negative control section incubated with equivalent nonimmune rabbit IgG (400×). **(c–i)** Representative stage-wise description of RBM7 immunostaining (1000×). Stages of the tubules are labeled with Roman numerals atop the panel. Type B spermatogonia (BG), preleptotene (Pl), leptotene (L), and zygotene (Z) spermatocytes are marked with solid triangles. Dividing spermatocytes (M) are marked with asterisks. Pachytene (P), diplotene (D), and spermatocytes are pointed out with sharp arrows, and round spermatids (rs) and elongated spermatids (es) are indicated by solid arrows. Note prominent nuclear staining in BG, Pl, L, Z, and early P cells; as well as strong cytoplasmic staining in late P, D, and spermatids. **(j)** Schematic summary of the differential staining according to stages and subcellular location.

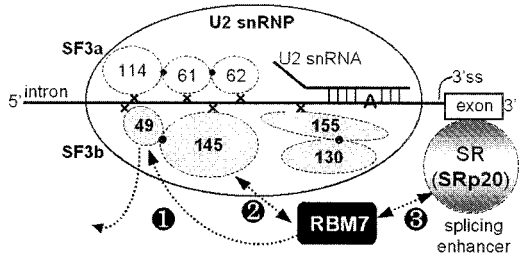

Figure 6. Possible routes of regulation by RBM7 in promoting splicing. Depicted is the 3′ exon of the pre-mRNA selected for splicing and portion of its 5′ intron with adjacent sequence bound by U2 snRNA at the branch point sequence during spliceosome assembly. The pre-mRNA is linearized and SF3a within U2 snRNP is simplified for illustration purposes. The nomenclature of SAPs is followed. RBM7 may either 1) substitute for SAP49 function in cells lacking SAP49, 2) modulate SAP49 and SAP145 interaction via its own interaction with SAP145, or 3) recruit SAP145 to near the branch site via SRp20 bound to an exon splicing enhancer (ESE) in ESE-dependent splicing. Note that the sphere of influence of SR extends throughout constitutive splicing and may recruit RBM7 via SRp20. 3′ss indicates the 3′ splice site; A, branch point adenosine; x, protein-RNA interaction; and ●, protein-protein interaction. Based on Will and Luhrmann (1997).

bly (Champion-Arnaud and Reed, 1994). This interaction between SAPs 49 and 145 is conserved for their respective essential yeast homologues, HSH49 and CUS1 (Wells et al, 1996). The spliceosome, the complex where the splicing reaction takes place, contains the pre-mRNA, several snRNPs, and a number of other splicing factors that enable splice-site selection and catalysis of the splicing reaction (Adams et al, 1996; Chabot, 1996; Will and Luhrmann, 1997). Among the non-snRNP splicing factors are a group of SR proteins that act as splicing activators through their facilitation of the recognition, selection, and pairing of splice sites (Will and Luhrmann, 1997; Tacke and Manley, 1999).

Our earlier results showed that RBM7 could promote pre-mRNA splicing (Guo et al, in preparation). However, the luciferase reporter assay therein does not yield clues as to how RBM7 promotes the reporter expression. It is conceivable that RBM7 may increase splicing efficiency via direct interaction with other splicing factors. Alternatively, it is also possible that RBM7 may enhance the nuclear export and stability of spliced transcripts, which have been shown to be coupled with splicing (Kim and Dreyfus, 2001). To explore these possibilities, we used yeast 2-hybrid screen and GST pull-down assay and established that RBM7 directly and stably associates with SAP145, and that it also interacts with SRp20. Both SAP145 and SRp20 are important splicing factors, thus this finding supports the theory that RBM7 participates in pre-mRNA splicing. There are several possible routes by which RBM7 may fulfill this role (Figure 6).

Route 1—Because RBM7 and SAP49 are homologous in their RNA-binding domain and both interact with SAP145, RBM7 may substitute for SAP49 in situations in which SAP49 is down-regulated during differentiation and development (Ruiz-Lozano et al, 1997). It will be interesting to determine the testicular expression of SAP49 and compare it with that of RBM7. Bouck et al (1998) have suggested that splicing efficiency could be decreased through increased binding of constitutive splicing factors such as SAP49.

Route 2—In the context of route 1, an alternative function for RBM7 would be to modulate the interaction between SAP145 and SAP49 through its binding to SAP145, theoretically increasing the splicing efficiency.

Route 3—SR proteins generally enhance splicing by facilitating recognition and pairing of 5′ and 3′ splice sites (Will and Luhrmann, 1997; Tacke and Manley, 1999). If the interaction of SRp20 and SAP145 is concomitant, RBM7 may act to recruit SF3b to tether U2 snRNP to the branch site near the 3′ splice site selected by SR proteins. Because some SR proteins, including SRp20, can facilitate selection of an otherwise unrecognized, weak 3′ splice site, this model may also be relevant in alternative splicing.

SAP145, like many splicing proteins, exhibits punctate nuclear localization (Bryant et al, 2001). It remains to be determined whether RBM7 also localizes within these speckles, the preferential sites of splicing components. On the other hand, SRp20 shuttles between the nucleus and the cytoplasm and can promote mRNA export (Huang and Steitz, 2001). Because SRp20 is abundant in the testis (Ayane et al, 1991), studying the extent and site of SRp20 and RBM7 colocalization could shed light on the functional nature of their interaction. Therefore, a detailed analysis of its expression vis-à-vis RBM7 in the germ cells is warranted.

RBM is a testis-specific spermatogenesis factor coded by *RBMY* genes that may be deleted in some infertile men. It is localized in human testis in punctate regions of the nucleus of spermatogonia and spermatocytes and may have a role in pre-mRNA splicing (Elliott et al, 1998). It interacts with several splicing factors. These include testis-signal transduction and activation of RNA (T-STAR; Venables et al, 1999), which is a homolog of Src-associated mitotic cell protein of 68 kd (SAM68), which is implicated in RNA splicing and is negatively regulated by signal transduction during the cell cycle. RBM also interacts along with hnRNP G proteins and Tra2β, which is the human homologue of Tra, a splicing factor that regulates sexual differentiation, spermatogenesis, and in fly courtship behavior (Venables et al, 2000). Finally, it interacts with a number of the SR family of splicing factors, chief among them, SRp20 (Elliott et al, 2000). The interaction between RBM and SRp20 occurs through the RRM and hinge regions of RBM and is not dependent on the RS domain, which is frequently used by SR proteins for interactions among each other or with other proteins (Elliott et al, 2000). We have not mapped the area of

interaction between RBM7 and SRp20, but because the RS domain in SRp20 is free while it binds RBM, a concomitant interaction of all 3 factors cannot be ruled out. It is thus conceivable that these proteins act in a common pathway to regulate testicular meiotic splicing events.

The testicular expression of RBM7 at both the mRNA and protein levels appears to be spatiotemporally correlated. Our results establish that 1) *Rbm7* is expressed in germ cells at the onset of meiosis initiation and throughout meiosis; and 2) RBM7 protein level follows that of its mRNA, but the protein itself relocates from the nucleus to the cytoplasm during the second round of mRNA up-regulation at mid pachytene phase. This relocation of RBM7 can be a means of separating this protein from other components of the splicing reaction. Alternatively, its translocation may suggest an additional function such as RNA transport. There is evidence that the localization, assembly, or substrate specificity of RNA-binding proteins can be altered by phosphorylation (Watanabe et al, 1997; Gu et al, 1998; Morales et al, 1998). SRp20 function is regulated through serine phosphorylation in its SR domain, which may affect its binding specificity to splicing enhancer sequences (Prasad et al, 1999) or its nuclear compartmentalization (Caceres et al, 1998). Although we have yet to determine the phosphorylation status of RBM7, the presence of several phosphorylation consensus sites near its C-terminus raises the possibility that similar mechanisms are operative in regulating its subcellular localization, its substrate specificity, and protein-protein interaction.

In summary, we propose that RBM7 is a cell-specific regulator of pre-mRNA splicing through its interaction with SAP145 and SRp20. The restricted mRNA expression and the differential protein subcellular localization in rodent germ cells indicate that RBM7 has a more specific role in meiosis entry and progression. It is conceivable that RBM7 and additional testis-specific RNA-binding proteins collaborate to regulate the splicing of specific pre-mRNA species that are important in the meiotic cell cycle. Elucidation of the mechanism of action of RBM7 and identification of its mRNA targets will remain the focus of future research.

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