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Developmental stage-specific expression of *Rbm* suggests its involvement in early phases of spermatogenesis

Jungmin Lee[1], Jinpyo Hong[1], Euisu Kim[1], Kyungjin Kim[1], Soo Woong Kim[2], Hanumanthappa Krishnamurthy[3], Sanny S.W.Chung[3], Debra J.Wolgemuth[3] and Kunsoo Rhee[1,4]

[1]School of Biological Sciences, Seoul National University, Seoul 151-742,[2]Department of Urology, Seoul National University College of Medicine, Seoul 110-744, Korea and[3]Columbia University College of Physicians and Surgeons, Departments of Genetics and Development and Obstetrics and Gynecology and The Institute of Human Nutrition, New York, NY 10032, USA

[4]To whom correspondence should be addressed. E-mail: rheek@snu.ac.kr

***Rbm* is a male infertility gene located on the Y chromosome that is expressed in the testis. To investigate the specific events of spermatogenesis in which Rbm plays a role, the precise pattern of expression of *Rbm* in the mouse testis was determined. An antibody was generated against the Rbm protein and used to detect a single specific band of 43 kDa in size in mouse testicular lysates. *In situ* hybridization, immunoblot and immunohistochemistry analyses together indicated that *Rbm* was expressed in spermatogonia, preleptotene spermatocytes, late leptotene to early pachytene spermatocytes but not in mid-pachytene spermatocytes or subsequent stages of differentiation, including haploid germ cells. These observations suggest that Rbm functions in early but not later stages of male germ cell development.**

Key words: male infertility/Rbm/spermatogenesis

Introduction

One of the genetic bases for male infertility was established by a cytogenetic study in which deletions in the long arm of the Y chromosome were detected among infertile men with a high frequency (Tiepolo and Zuffardi, 1976). These frequently deleted loci in specific regions of the Y chromosome were designated as *azoospermia factor* (*AZF*) (Vergnaud *et al*., 1986; Andersson *et al*., 1988). It was proposed recently that homologous recombinations between Y-specific repeats called amplicons result in deletions in the *AZF* loci (Kuroda-Kawaguchi *et al*., 2001; Repping *et al*., 2003). Microdeletions in *AZF* were described in significant proportions of oligo- and azoospermic patients (Ma *et al*., 1992; Foresta *et al*., 2001). Taken together, it was logical to predict that genes in *AZF* loci play a critical role in spermatogenesis.

Rbm was the first of these candidate genes identified in *AZF* regions of the Y chromosome (Ma *et al*., 1993). *Rbm* is present in multiple copies distributed throughout the Y chromosome, with many of them being clustered at the *AZFb* region (Chai *et al*., 1997). The open reading frame of the *Rbm* cDNA contains an RNA-binding domain and the SRGY domain, a 37-residue repeat of a serine-arginine-glycine-tyrosine (SRGY) or similar tetrapeptide twice in each repeat (Ma *et al*., 1993). *Rbm* shares strong structural homology with *hnRNP G*, suggesting that Rbm functions as an RNA processing factor (Delbridge *et al*., 1999; Mazeyrat *et al*., 1999). Indeed, Rbm was shown to interact with Tra2β, an activator of pre-mRNA splicing, and to inhibit RNA-splicing activities *in vitro* through sequestering splicing factors from nascent RNA (Elliott *et al*., 2000; Venables *et al*., 2000). These results suggested that Rbm may be involved in testis-specific splicing events during spermatogenesis (Venables *et al*., 2000).

Despite strong genetic evidence suggesting involvement of Rbm in male germ cell development, the role that Rbm plays in spermatogenesis remains to be determined. Cumulative reports on Y chromosome microdeletions in infertile men reveal that phenotypes associated with *AZFb* deletions are variable, ranging from Sertoli cell-only syndrome to spermatogenic arrest (Foresta *et al*., 2001). One explanation for these observations could be that Rbm is involved in multiple functions during spermatogenesis. However, it is important to consider other mutually non-exclusive reasons for interpreting phenotypes of Rbm, such as (i) extension of deletion regions in the Y chromosome, (ii) genetic background of the patients, and (iii) progression of the spermatogenic failure (Foresta *et al*., 2001).

The current study was undertaken to explore the biological functions of Rbm during spermatogenesis by examining the precise expression pattern of *Rbm* in the mouse model. The results demonstrated that *Rbm* expression was limited to spermatogonia and preleptotene, late leptotene and early pachytene spermatocytes.

Materials and methods

Source of tissues, cell lines and DNA

Normal adult tissues were obtained from CD1 mice (Charles River, USA) and normal immature testes were obtained from mice on postnatal days 7, 9 and 17. Ethical approval for animal care was granted by the ethics committee of the Seoul National University. The mouse mutant strains *atricosis* (*at*; ATEB/Le *a/a d/d* + *at/eb* +) were obtained from the Jackson Laboratory (USA). Dissected tissue specimens were frozen in liquid nitrogen prior to RNA isolation. Tissues for *in situ* hybridization and immunohistochemical analyses were fixed in 4% paraformaldehyde in phosphate-buffered saline overnight at 4°C.

Radioactive nucleotides used in this study were obtained from New England Nuclear (USA) or from Amersham (USA). Riboprobes were prepared using T7

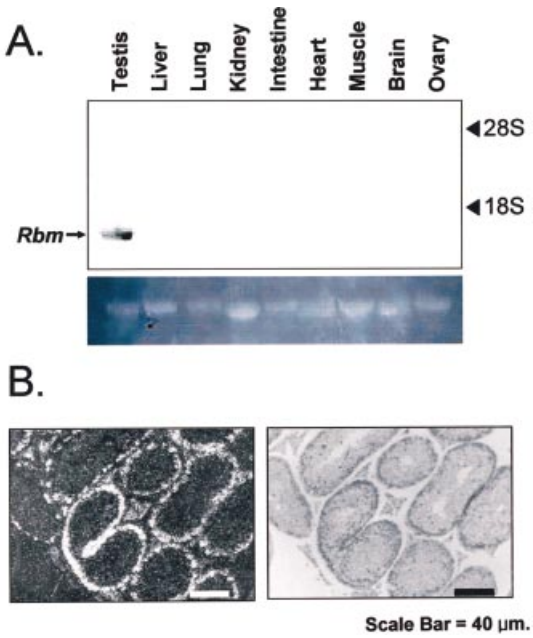

Figure 1. *Rbm* expression at the RNA levels. (**A**) Northern blot hybridization of *Rbm* in selected mouse tissues. The 28S and 18S ribosomal RNA bands are indicated on the right. The lower figure shows the 28S rRNA stained with ethidium bromide. Exposure time was 5 days. (**B**) *In situ* hybridization analysis of *Rbm* in the mouse testis. *Rbm*-specific signals and testicular morphology were observed in the dark and light field microscopes respectively. Exposure time was 2 weeks. Scale bar = 40 μm.

or T3 RNA polymerases in the presence of [^{32}P]UTP for northern blot hybridization analysis and [^{35}S]UTP for *in situ* hybridization analysis. For the northern blot and *in situ* hybridization analyses of *Rbm* expression, the coding sequence was used to generate both sense and antisense riboprobes.

The mouse *Rbm* cDNA was obtained by screening a testis cDNA library as described previously (Rhee and Wolgemuth, 1997). A probe for mouse *Rbm* was prepared initially with the reverse transcription/PCR method with primer sets of ACGCGTCGACCTCGAGAAGCAGAAACTGATCAGCCTGGGA and ACGCGTCGACCTCGAGTATATCTGCTTTCTCCACGACCTCCA. The mouse testis cDNA library was screened following the protocols outlined by Sambrook *et al*. (1989). Clones isolated from this screen were sequenced using an Applied Biosystems Model 373A DNA sequencer (Applied Biosystems, USA). The *Rbm* clones that we had isolated were 1.5 kb in size.

Northern blot hybridization analysis

Northern blot hybridization analysis was carried out as described previously (Rhee and Wolgemuth, 1997). Total RNA from various mouse tissues were prepared using the RNeasy Mini kit (Qiagen, USA). Total RNA from these tissues were run on a denaturing 1% agarose gel containing 2.5 mol/l formaldehyde, transferred to a nitrocellulose membrane, and baked for 2 h at 80°C in a vacuum oven. Ethidium bromide staining of the 28S RNA was used to determine equal loading for each sample. Prepared membranes were hybridized for 2–4 h at 65°C in prehybridization solution [60% formamide, 5× standard saline citrate (SSC), 20 mmol/l phosphate buffer pH 6.8, 1% sodium dodecyl sulphate (SDS), 5×Denhardt's solution]. After prehybridization, ^{32}P-labelled riboprobes corresponding to the full coding sequence of Rbm were added into hybridization solution (60% formamide, 5×SSC, 20 mmol/l phosphate buffer pH 6.8, 1% SDS, 5×Denhardt's solution, 0.1 mg/ml salmon sperm DNA, 0.1 mg/ml yeast tRNA, 10 μg/ml Poly A RNA, 7% dextran sulphate) and incubated at 65°C overnight with shaking. Hybridized membrane was washed twice with washing solution I (2×SSC, 1% SDS) at room temperature for 30 min and once with washing solution II (0.2×SSC, 1% SDS) at 80°C for 1 h. After washing, the membrane was exposed onto X-ray film at –70°C for 5 days.

In situ hybridization analysis

Paraffin-embedded adult mouse testis tissues were cut into 5 μm sections, and analysed by *in situ* hybridization as described previously (Rhee and Wolgemuth, 1995). In brief, sections were hybridized with a [^{35}S]UTP-labelled riboprobe that corresponded to the coding sequence of *Rbm*, covered with nuclear track emulsion (Kodak type NTB-2), and developed after a 21 day exposure. Background labelling was determined from sections hybridized with an identical quantity of the sense probe.

Generation of an antibody specific to Rbm

To generate an antibody specific to Rbm, the full coding sequence of the mouse *Rbm* was cloned into the *pGEX4T-1* bacterial expression vector (Amersham, USA). The resulting plasmid was transformed into BL21 and the 70 kDa GST–Rbm fusion protein was purified by immobilized GST protein column and injected into a rabbit. The antiserum was affinity-purified by incubation with a strip of nitrocellulose membrane blotted with the GST–Rbm fusion protein and eluted with 100 mmol/l glycine, pH 3.0.

Immunoblot analysis

Protein samples from mouse tissues were solubilized in the Laemmli sample buffer, resolved by 8% SDS–polyacrylamide gel electrophoresis, and blotted onto a nitrocellulose membrane. The membrane was blocked by soaking in Blotto (Tris-buffered saline with 0.3% Triton X-100 and 5% non-fat dried milk) for 1 h 30 min, and incubated overnight with the primary antibody in the blocking solution. The membrane was then washed three times with TBST (Tris-buffered saline with 0.3% Triton X-100), incubated with a secondary antibody conjugated with horseradish peroxidase for 45 min, and washed five times with TBST. The signal was detected with the ECL western blotting detection reagents (Amersham, USA) following the manufacturer's recommendations. The affinity-purified Rbm antibody was diluted 1:100, preimmune serum was diluted 1:100, and secondary antibody was diluted 1:5000.

Immunohistochemistry

Testis samples were fixed in 4% buffered paraformaldehyde, embedded in paraffin wax, and processed as described previously (Rhee and Wolgemuth, 1997). In brief, after deparaffinization, slides were boiled in 0.01 mol/l citrate buffer, pH 6.0 for 10 min and washed extensively with H_2O (Shi *et al*., 1991). The slides were then treated with 0.03% H_2O_2 in methanol for 20 min, washed with PBST (phosphate-buffered saline with 0.1% Triton X-100), preincubated with the blocking solution (PBST with 3% normal goat serum) for 1 h at room temperature and incubated with the primary antibody in a humidified 4°C chamber overnight. The slides were then washed three times with PBST, stained with the Vectastain ABC kit (Vector Laboratories, USA), and counterstained with haematoxylin. For controls, the slides were incubated with preimmune serum. The affinity-purified Rbm antibody was diluted 1:10, preimmune serum was diluted 1:100, and secondary antibody was diluted 1:200.

Flow cytometric analysis of testicular cells

The flow cytometric analysis of testicular cells was performed as described previously (Krishnamurthy *et al*., 2001). In brief, the methanol-fixed testicular cells were washed twice and resuspended in propidium iodide (PI) staining solution (25 μg/ml PI, 40 μg/ml RNase A in PBS) at room temperature for 20 min. The dual parameter flow cytometry was performed on a Becton Dickinson FACS Calibur flow cytometer equipped with 15 mW air-cooled argon ion laser. The green signals of GFP were collected on log scale using a 520-band pass filter (505–545 nm) and red signals of PI staining were collected on linear scale using a 620-band pass filter (605–635 nm). Based on DNA content, the PI-stained testicular cells can be discriminated as elongated and round haploid spermatids (HC and 1C), diploid spermatogonia and somatic cells (2C), spermatogonia synthesizing DNA (S-Ph) and primary spermatocytes and G2 spermatogonia (4C) (Krishnamurthy *et al*., 2001). Since the expression of *RARα-EGFP* is under control of the *protamine* promoter, one should expect the expression of GFP protein only in the haploid cells, i.e. elongated spermatids (S.S.W.Chung, H.Krishnamurthy, X.Wang and D.J.Wolgemuth, unpublished data).

Flow cytometric sorting of testicular cells

Testicular cells from the *Prm/RARα-EGFP* transgenic mouse strain were prepared according to the procedure described previously (Krishnamurthy *et al*., 2001). Briefly, the monocellular suspension of testicular cells was suspended in Dulbecco's modified Eagle's medium containing 1 g/ml DNase I at a concentration of 1×10^6 cells per ml. The cells were sorted based on their EGFP expression on a Becton Dickinson (USA) FACStarplus flow cytometer equipped with a 500 mW water-cooled laser. The cells were excited at an excitation wavelength of 488 nm. Forward-scatter was used as a triggering parameter and the green fluorescence of *EGFP*-negative and positive cells were collected on log scale using a 520-bandpass filter (505–545 nm). The sort window was set on the dot plot showing side-scatter on the *y*-axis and green signals of EGFP on the *x*-axis as the threshold between background signals and EGFP-positive staining. Testicular cells from the non-transgenic littermates were used as negative control. The lysates of the sorted cells were used for immunoblot analysis with the EGFP antibody of 1:200 dilution (Santa Cruz Biotech, USA).

Results

Rbm expression at the RNA level

Northern blot hybridization analysis was carried out to detect mouse *Rbm* transcripts. As expected, an *Rbm*-specific band of 1.7 kb in size was detected only in the testis among tissues tested (Figure 1A; Elliott *et al*., 1996). *In situ* hybridization analysis was then carried out in order to determine the testicular cell types in which *Rbm* was expressed. *Rbm*-specific signals were detected in the periphery of all seminiferous tubules of the mouse testis (Figure 1B), where spermatogonia and early spermatocytes reside. No specific signal was detected in more central regions of the tubules where late stage spermatocytes and haploid germ cells are found. However, the resolution of the radioactive signal *in situ* hybridization was insufficient to permit precise identification of the exact cell types.

Developmental stage-specificity in Rbm expression

We therefore wished to use immunohistochemistry to determine the localization pattern of Rbm protein in the testis. To this end, antisera were raised against the bacterially expressed Rbm fusion protein and were affinity-purified. Immunoblot analysis revealed a single band of 43 kDa in size that, among tissues tested, was specific to the testis (Figure 2A). The molecular weight of the Rbm-specific band concurred with the predicted size of the protein. Moreover, ectopic expression of *Rbm* in cultured cells also produced a single band of the identical size (data not shown). These results demonstrate that the antibody is specific to the mouse Rbm protein and that it does not cross-react with other structural homologues such as hnRNP G, which is known to be expressed ubiquitously (Bennett *et al*., 1992; Dreyfuss *et al*., 1993; Venables *et al*., 2000).

In the adult testis, nuclei of the cells in the periphery of seminiferous tubules that correspond to male germ cells in early developmental stages were stained (Figure 2Ba). No specific signals were detected in haploid germ cells that were located at the centre of the tubules. Spermatogenesis is initiated shortly after birth. Consequently, the 7 day old testis consists mostly of spermatogonia, while the 9 day old and 17 day old testes consist of germ cells of early developmental stages down to leptotene and pachytene spermatocytes respectively (Bellve *et al*., 1977). The presence of the Rbm protein in male germ cells of early developmental stages was confirmed with immunohistochemical analysis with immature testes. Spermatogonial expression of *Rbm* was confirmed by immunostaining of the 7 day old testis (Figure 2Bd). In the 9 day old and 17 day old testes, immunostaining was limited to the periphery of the tubules, indicating the *Rbm* expression in early developmental stages of male germ cells, earlier than leptotene and pachytene spermatocytes which were located in the middle of the tubule (Figure 2Bb, c). No specific staining was observed in germ cell-deficient testis, indicating male germ cell-specific expression of *Rbm* (Figure 2Be). The specificity of the staining pattern was also confirmed by successful competition of the Rbm antibody with the antigen, the GST–Rbm fusion protein, in the reaction mixture (Figure 2Bf).

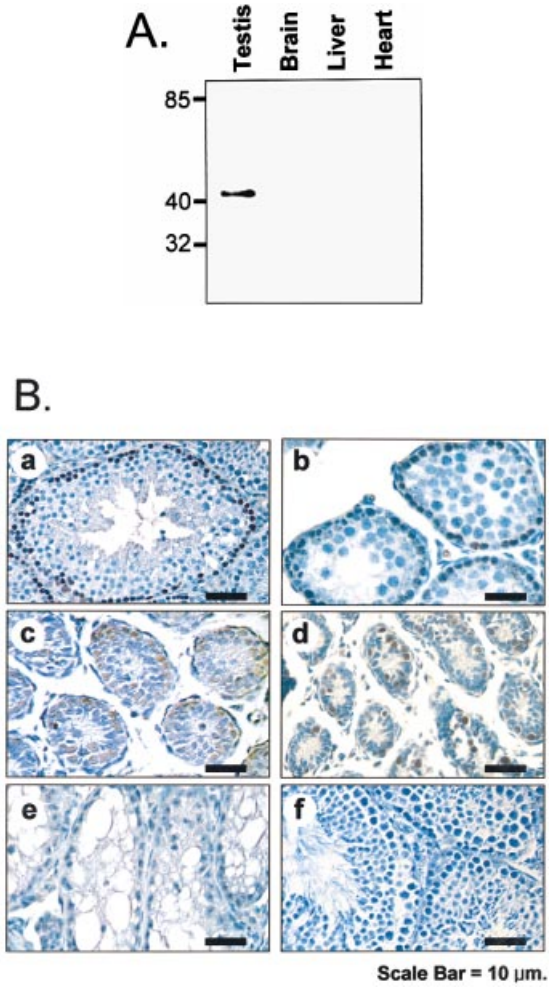

Figure 2. Rbm expression at the protein level. (**A**) Immunoblot analysis was carried out with four different mouse tissues, using the rabbit anti-mouse Rbm-specific polyclonal antibody generated. The sizes of the protein markers ($M_r\times10^{-3}$) are indicated on the left side of the figure. (**B**) Immunohistochemical analysis of Rbm was carried out with testicular sections from (**a**) adult, (**b**) 17 day old, (**c**) 9 day old, (**d**) 7 day old, and (**e**) *at/at* germ cell-deficient mice. In (**f**), the antigen (the GST–Rbm fusion protein, 1 μg protein per section) was added into the antibody reaction mixture. The DAB staining resulted in brownish Rbm-positive cells. Scale bar = 10 μm.

The distinct developmental programme of spermatogenesis in the adult mouse testis has been well characterized in histological sections. Since male germ cells develop in the temporally defined progression along the tubule, there exists a characteristic association of germ cells in particular stages of spermatogenesis. In the mouse, this progression of the cycle of the seminiferous epithelium can be divided into 12 stages, each with a characteristic set of spermatogenic cells in association with one another (Oakberg, 1956). The developmental stage-specificity of Rbm was therefore analysed by staging seminiferous tubules, following the criteria as described in Russell *et al*. (1990).

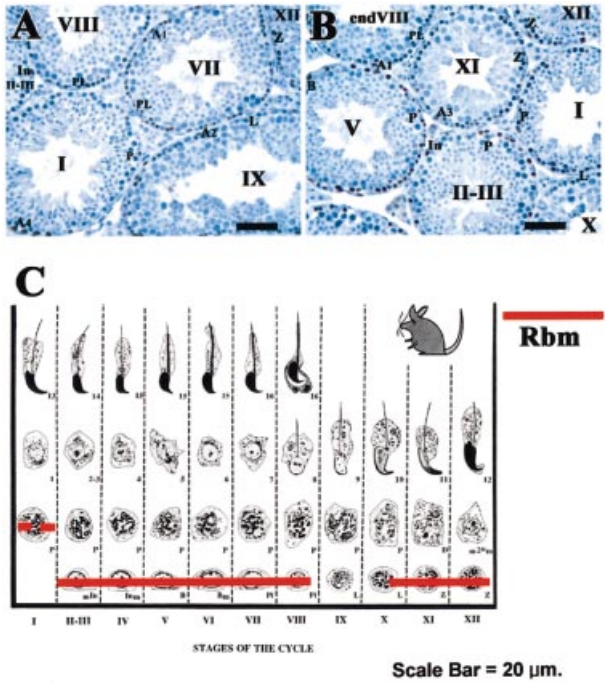

Figure 3. Determination of developmental stages at which Rbm was expressed during spermatogenesis. (**A** and **B**) Immunohistochemical analysis of Rbm protein localization was carried out on testis from adult mice and the tubules were assessed as to the stage of the cycle of the seminiferous epithelium (Russell *et al*., 1990). The Roman numerals indicate the stage of the seminiferous tubule. Scale bar = 20 µm. (**C**) Summary of developmental stage-specific expression of *Rbm* in the mouse testis. *Rbm* was expressed in early stages of spermatogenesis, starting in spermatogonia. The Rbm protein was detected consistently in spermatogonia and preleptotene spermatocytes, and intermittently in leptotene spermatocytes. The Rbm was detected again at zygotene to early pachytene spermatocytes. No Rbm-specific signal was detected in pachytene spermatocytes at stage II–III onwards.

(A) Flowcytometric Profile of the GFP-Positive Testicular Cells

Cell Type	% GFP Positive	
	Ptm/RARα-GFP	Littermate
Diploid	4	1
Haploid	43	2

(B) Immunoblot Analysis with Sorted Testicular Cells

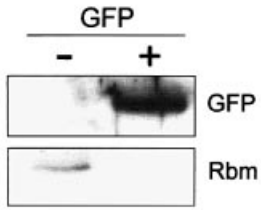

Figure 4. Confirmation of the absence of the Rbm protein in haploid germ cells. (**A**) Testicular cells from *Prm/RARα-EGFP* transgenic mice and from non-transgenic littermate mice controls were analysed with a FACS Calibur flow cytometer using cell size and granularity parameters. The EGFP expression was confirmed with the green fluorescent signal. (**B**) The testicular cells of *Prm/RARα-EGFP* transgenic mice were FACS-sorted into EGFP-negative or positive subsets. The cell lysates were subjected to immunoblot analysis with antibodies specific to EGFP and Rbm.

In the adult testis, Rbm was expressed in nuclei of A-spermatogonia in all stages of the cycle of the seminiferous epithelium (Figure 3A and B). Rbm continued to be expressed in intermediate spermatogonia at stage II–III, B-spermatogonia at stage V, and preleptotene spermatocytes at stage VII–VIII (Figure 3B). At the end of stage VIII (Figure 3B), there was an apparent drop in expression of Rbm in leptotene spermatocytes to almost undetectable level at stage IX (Figure 3A). Interestingly, Rbm was then detected again in late leptotene spermatocytes at stage X (Figure 3B), through zygotene spermatocytes at stage XI–XII (Figure 3A and B), up to early pachytene spermatocytes at stage I. The expression of Rbm in early spermatocytes appeared intermittent. It was detected in some tubules (Figure 3B), but in others was absent or at much-reduced levels if present (Figure 3A). However, there was no expression in pachytene spermatocytes at stage II–III and onwards in all tubules examined (Figure 3A and B). The developmental stage-specific expression of Rbm is summarized in Figure 3C.

Absence of the Rbm protein in haploid germ cells

The results from *in situ* hybridization and immunohistochemical analyses consistently ruled out the possibility of *Rbm* expression in haploid germ cells. However, there have been previous reports in which the Rbm protein was detected in haploid germ cells as well as in spermatogonia (Elliott *et al*., 1996, 1997, 1998; Mahadevaiah *et al*., 1998; Venables *et al*., 2000). To more rigorously assess the lack of expression of Rbm protein in haploid male germ cells, immunoblot analysis with testicular cell lysates from enriched populations of germ cells was performed.

To this end, a transgenic mouse in which the *RARα-EGFP* chimeric gene is under the control of the *protamine* promoter (*Prm/RARα-EGFP*) was used. The testicular morphology and fertility of this transgenic mouse appeared normal (S.S.W.Chung, H.Krishnamurthy, X.Wang and D.J.Wolgemuth, unpublished data). The transgene is expressed exclusively in haploid spermatids and thus cells can be sorted into enriched populations of haploid spermatids by virtue of their expression of EGFP. FACS analysis with testicular cells from the transgenic mice was performed to confirm the haploid-specific expression of EGFP (Janca *et al*., 1986; Krishnamurthy *et al*., 2001). The results showed that 43% of haploid germ cells (both round and elongated spermatids) of the transgenic mice were EGFP-positive, while only 4% of diploid cells exhibited the EGFP fluorescence. As a control, we observed that <2% of testicular cells from the non-transgenic littermates sorted as 'EGFP-positive'. These results indicate that most EGFP-positive testicular cells in the *Prm/RARα-EGFP* transgenic mice are haploid germ cells. Next, the testicular cells were sorted into EGFP-positive and EGFP-negative populations, and protein lysates were subjected to immunoblot analysis. The Rbm-specific band was detected only in the EGFP-negative population and none was detected in the EGFP-positive cellular lysate comprised primarily of spermatids (Figure 4B).

Discussion

In the present study, we have shown that the mouse *Rbm* gene was expressed in male germ cells in early developmental stages, beginning from spermatogonia and down to early pachytene spermatocytes. The *Rbm* expression disappeared in pachytene spermatocytes at stage II–

III and onwards. Consistent with our results, results from a recent microarray work investigating expression profiles of total mouse testis transcripts also revealed that Rbm transcript was ~10 times more abundant in immature testes than in the adult testes (Schultz *et al*., 2003).

The Rbm expression pattern reported here is somewhat contradictory to the previous report of Mahadevaiah *et al*. (1998) in which Rbm antibody reacted with spermatogonia, early spermatocytes and elongating spermatids in mouse testis sections. Our results at both the RNA and protein levels, using *in situ* hybridization, immunohistochemistry and immunoblot analyses, consistently showed that *Rbm* gene products were absent from haploid germ cells. Since Mahadevaiah *et al*. (1998) observed that Rbm immunostaining was detected in elongated spermatids in the near *Rbm*-deficient Y^{d1} mice, it is possible that the signals from elongated spermatids may have been an immunohistochemical artefact. In the human, it has been reported that the human Rbm protein was detected in spermatogonia, in both early and late spermatocytes and in round spermatids, but not in elongating spermatids (Elliott *et al*., 1998). Presently, it is not clear whether the differences seen in *Rbm* expression between human and mouse are attributable only to species specificity. To the best of our knowledge, no *in situ* hybridization analysis of human *Rbm* mRNA expression has been published.

Although the *Rbm* gene apparently exists as multiple copies on the Y chromosome, it is still not clear whether all *Rbm* copies produce functional gene products or are largely transcriptionally silent. Two functional types of *Rbm* cDNA, *RBM1* and *RBM2*, that share 80% identity have been identified in the human genome (Chai et al., 1997, 1998). An immunoblot study using the human Rbm antibody reported the presence of multiple bands; two major bands (53 and 50 kDa) and one minor (43 kDa) band (Elliott *et al*., 1998). However, it remains to be determined whether these bands represent different isoforms of the Rbm protein. In mouse, there is evidence to support the presence of a single Rbm protein. First, mouse *Rbm* cDNA clones from several laboratories, including our own, are identical except for a few minor base changes (Elliott *et al*., 1996; Mahadevaiah *et al*., 1998; Wang *et al*., 2001; data not shown). Second, proteins translated from 14 independent *Rbm* cDNA clones had identical sizes of expected products (Mahadevaiah *et al*., 1998). In accordance with these findings, we detected a single band of 43 kDa in size for the Rbm protein. This suggests that even if multiple copies of the mouse Rbm gene exist, a single Rbm protein is produced.

As mentioned previously, interactions of Rbm with splicing factors such as Tra2β and SR protein suggest that Rbm is involved in RNA splicing (Elliott *et al*., 2000). Therefore, to deduce at what stages of spermatogenesis Rbm functions, it is critical to identify which mRNA species are controlled by Rbm. Since Rbm is present in mouse spermatogonia and early spermatocytes, candidate Rbm-downstream mRNA producing testis-specific transcripts at very early phases of germ cell development would be logical starting-points for analysis.

Note added in proof: Another report of *Rbm* expression appeared while this paper was under review. Saunders *et al*. (2003) also observed the specific presence of the Rbm protein in male germ cells of early developmental stages.

Acknowledgements

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References

Andersson M, Page DC, Pettay D, Subrt I, Turleau C, de Grouchy J and de la Chapelle A (1988) Y;autosome translocations and mosaicism in the aetiology of 45,X maleness: assignment of fertility factor to distal Yq11. Hum Mol Genet 79,2–7.

Bellve AR, Cavicchia JC, Millette CF, O'Brien DA, Bhatnager YM and Dym M (1977) Spermatogenic cells of the prepubertal mouse: isolation and morphological characterization. J Cell Biol 74,68–85.

Bennett M, Pinol-Roma S, Staknis D, Dreyfuss G and Reed R (1992) Differential binding of heterogeneous nuclear ribonucleoproteins to mRNA precursors prior to spliceosome assembly in vitro. Mol Cell Biol 12,3165–3175.

Chai NN, Salido EC and Yen PH (1997) Multiple functional copies of the RBM gene family, a spermatogenesis candidate on the human Y chromosome. Genomics 45, 355–361.

Chai NN, Zhou H, Hernandez J, Najmabadi H, Bhasin S and Yen PH (1998) Structure and organization of the RBMY genes on the human Y chromosome: transposition and amplification of an ancestral autosomal hnRNPG gene. Genomics 49,283–289.

Delbridge ML, Lingenfelter PA, Disteche CM and Graves JA (1999) The candidate spermatogenesis gene RBMY has a homologue on the human X chromosome. Nature Genet 22,223–224.

Dreyfuss G, Matunis MJ, Pinol-Roma S and Burd CG (1993) hnRNP proteins and the biogenesis of mRNA. Annu Rev Biochem 62,289–321.

Elliott DJ, Ma K, Kerr SM, Thakrar R, Speed R, Chandley AC and Cooke H (1996) An RBM homologue maps to the mouse Y chromosome and is expressed in germ cells. Hum Mol Genet 5,869–874.

Elliott DJ *et al.* (1997) Expression of RBM in the nuclei of human germ cells is dependent on a critical region of the Y chromosome long arm. Proc Natl Acad Sci USA 94,3848–3853.

Elliott DJ, Oghene K, Makarov G, Makarova O, Hargreave TB, Chandley AC, Eperon IC and Cooke HJ (1998) Dynamic changes in the subnuclear organization of pre-mRNA splicing proteins and RBM during human germ cell development. J Cell Sci 111,1255–1265.

Elliott DJ, Bourgeois CF, Klink A, Ste'venin J and Cooke HJ (2000) Mammalian germ cell-specific RNA-binding protein interacts with ubiquitously expressed proteins involved in splice site selection. Proc Natl Acad Sci USA 97,5717–5722.

Foresta C, Moro E and Ferlin A (2001) Y chromosome microdeletions and alterations of spermatogenesis. Endocr Rev 22,226–239.

Janca FC, Jost LK and Evenson DP (1986) Mouse testicular and sperm cell development characterized from birth to adulthood by dual parameter flow cytometry. Biol Reprod 34,613–623.

Krishnamurthy H, Danilovich N, Morales CR and Sairam MR (2001) Qualitative and quantitative decline in spermatogenesis of the follicle-stimulating hormone receptor knockout male mouse. Biol Reprod 62,1146–1159.

Kuroda-Kawaguchi T, Skaletsky H, Brown LG, Minx PJ, Cordum HS, *et al.* (2001) The AZFc region of the Y chromosome features massive palindromes and uniform recurrent deletions in infertile men. Nature Genet 29,279–286.

Ma K, Sharkey A, Kirsch S, Vogt P, Keil R, Hargreave TB, McBeath S and Chandley AC (1992) Towards the molecular localisation of the AZF locus: mapping of microdeletions in azoospermic men within 14 subintervals of interval 6 of the human Y chromosome. Hum Mol Genet 1,29–33.

Ma K, Inglis JD, Sharkey A, Bickmore WA, Hill RE, Prosser EJ, Speed RM, Homson EJ, Jobling M and Taylor K (1993) A Y chromosome gene family with RNA-binding protein homology: candidates for the azoospermia factor AZF controlling human spermatogenesis. Cell 75,1287–1295.

Mahadevaiah SK *et al.* (1998) Mouse homologues of the human AZF candidate gene RBM are expressed in spermatogonia and spermatids, and map to a Y chromosome deletion interval associated with a high incidence of sperm abnormalities. Hum Mol Genet 7,715–727.

Mazeyrat S, Saut N, Mattei MG and Mitchell MJ (1999) RBMY evolved on the Y chromosome from a ubiquitously transcribed X-Y identical gene. Nature Genet 22,224–226.

Oakberg EF (1956) A description of spermiogenesis in the mouse and its use in analysis of the cycle of the seminiferous epithelium and germ cell renewal. Am J Anat 99,391–409.

Repping S *et al.* (2003) Polymorphism for a 1.6-Mb deletion of the human Y chromosome persists through balance between recurrent mutation and haploid selection. Nature Genet 35,247–251.

Rhee K and Wolgemuth DJ (1995) Cdk family genes are expressed not only in dividing but also terminally differentiated mouse germ cells, suggesting their possible function during both cell division and differentiation. Dev Dyn 204,406–420.

Rhee K and Wolgemuth DJ (1997) The NIMA-related kinase 2, Nek2, is expressed in specific stages of the meiotic cell cycle and associates with meiotic chromosomes. Development 124,2167–2177.

Russell LD, Ettlin RA, Sinha Hikim AP and Clegg ED (1990) Histological and Histopathological Evaluation of the Testis Cache River Press, Clearwater, FL.

Sambrook J, Fritsch EF and Maniatis T (1989) In Nolan C (ed) Molecular Cloning: A Laboratory Manual. Cold Spring Harbor Laboratory Press, Cold Spring Harbor, New York.

Saunders PT, Turner JM, Ruggiu M, Taggart M, Burgoyne PS, Elliott D and Cooke HJ (2003) Absence of mDazl produces a final block on germ cell development at meiosis. Reproduction 126,589–597.

Schultz N, Hamra FK and Garbers DL (2003) A multitude of genes expressed solely in meiotic or postmeiotic spermatogenic cells offers a myriad of contraceptive targets. Proc Natl Acad Sci USA 100,12201–12206.

Shi SR, Key ME and Kalra KL (1991) Antigen retrieval in formalin-fixed, paraffin-embedded tissues: an enhancement method for immuno-histochemical staining based on microwave oven heating of tissue sections. J Histochem Cytochem 39,741–748.

Tiepolo L and Zuffardi O (1976) Localization of factors controlling spermatogenesis in the nonfluorescent portion of the human Y chromosome long arm. Hum Mol Genet 34,119–124.

Venables JP, Elliott DJ, Makarova OV, Makarov EM, Cooke HJ and Eperon IC (2000) RBMY, a probable human spermatogenesis factor, and other hnRNP G proteins interact with Tra2β and affect splicing. Hum Mol Genet 9,685–694.

Vergnaud G, Page DC, Simmler MC, Brown L, Rouyer F, Noel B, Botstein D, de la Chapelle A and Weissenbach J (1986) A deletion map of the human Y chromosome based on DNA hybridization. Am J Hum Genet 38,109–124.

Wang PJ, McCarrey JR, Yang F and Page DC (2001) An abundance of X-linked genes expressed in spermatogonia. Nature Genet 27,422–426.

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Erratum

Developmental stage-specific expression of Rbm suggests its involvement in early phases of spermatogenesis

Jungmin Lee, Jinpyo Hong, Euisu Kim, Kyungjin Kim, Soo Woong Kim, Hanumanthappa Krishnamurthy, Sanny S.W.Chung, Debra J.Wolgemuth, Kunsoo Rhee,

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The publisher wishes to apologise for the incorrect reproduction of Figure 2 and 3 in this paper. The figure appears in its correct form below. The article appears in it's correct form online.

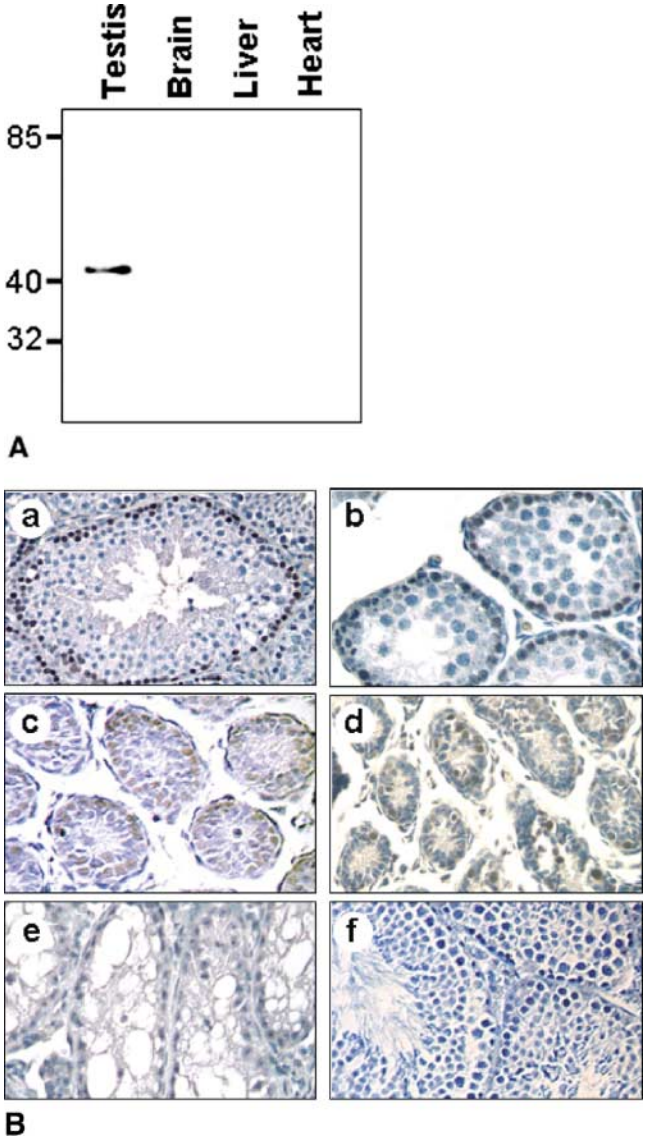

Figure 2. Rbm expression at the protein level. (**A**) Immunoblot analysis was carried out with four different mouse tissues, using the rabbit anti-mouse Rbm-specific polyclonal antibody generated. The sizes of the protein markers ($M_r \times 10^{-3}$) are indicated on the left side of the figure. (**B**) Immunohistochemical analysis of Rbm was carried out with testicular sections from (**a**) adult, (**b**) 17 day old, (**c**) 9 day old, (**d**) 7 day old, and (**e**) *at/at* germ cell-deficient mice. In (**f**), the antigen (the GST-Rbm fusion protein, 1 μg protein per section) was added into the antibody reaction mixture. The DAB staining resulted in brownish Rbm-positive cells.

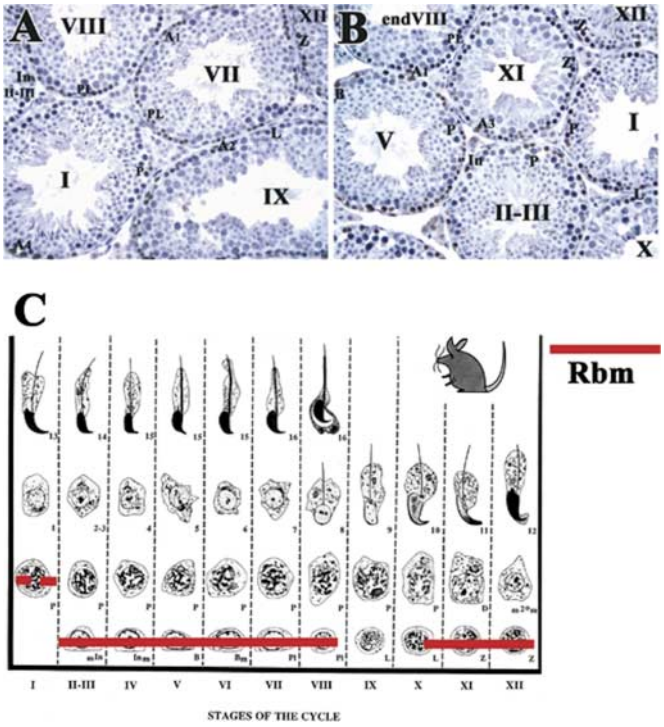

Figure 3. Determination of developmental stages at which Rbm was expressed during spermatogenesis. (**A** and **B**) Immunohistochemical analysis of Rbm protein localization was carried out on testis from adult mice and the tubules were assessed as to the stage of the cycle of the seminiferous epithelium (Russell *et al*., 1990). The Roman numerals indicate the stage of the seminiferous tubule. (**C**) Summary of developmental stage-specific expression of *Rbm* in the mouse testis. *Rbm* was expressed in early stages of spermatogenesis, starting in spermatogonia. The *Rbm* protein was detected consistently in spermatogonia and preleptotene spermatocytes, and intermittently in leptotene spermatocytes. The *Rbm* was detected again at zygotene to early pachytene spermatocytes. No *Rbm*-specific signal was detected in pachytene spermatocytes at stage II-III onwards.