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Dynamic changes in the subnuclear organisation of pre-mRNA splicing proteins and RBM during human germ cell development

David J. Elliott[1,*], Kathy Oghene[1], Genia Makarov[3], Olga Makarova[3], Timothy B. Hargreave[2], Ann C. Chandley[1], Ian C. Eperon[3] and Howard J. Cooke[1]

[1]Medical Research Council Human Genetics Unit and [2]Department of Urology, Western General Hospital, Crewe Road, Edinburgh, EH4 2XU, Scotland
[3]Department of Biochemistry, University of Leicester, Leicester, England
*Author for correspondence (e-mail: davide@hgu.mrc.ac.uk)

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SUMMARY

RBM is a germ-cell-specific RNA-binding protein encoded by the Y chromosome in all mammals, implying an important and evolutionarily conserved (but as yet unidentified) function during male germ cell development. In order to address this function, we have developed new antibody reagents to immunolocalise RBM in the different cell types in the human testis. We find that RBM has a different expression profile from its closest homologue hnRNPG. Despite its ubiquitous expression in all transcriptionally active germ cell types, RBM has a complex and dynamic cell biology in human germ cells. The ratio of RBM distributed between punctate nuclear structures and the remainder of the nucleoplasm is dynamically modulated over the course of germ cell development. Moreover, pre-mRNA splicing components are targeted to the same punctate nuclear regions as RBM during the early stages of germ cell development but late in meiosis this spatial association breaks down. After meiosis, pre-mRNA splicing components are differentially targeted to a specific region of the nucleus. While pre-mRNA splicing components undergo profound spatial reorganisations during spermatogenesis, neither heterogeneous ribonucleoproteins nor the transcription factor Sp1 show either developmental spatial reorganisations or any specific co-localisation with RBM. These results suggest dynamic and possibly multiple functions for RBM in germ cell development.

Key words: Pre-mRNA splicing, Germ cell, RBM, Human, Spermatogenesis

INTRODUCTION

RNA-binding proteins play a key role in eukaryotic gene expression and its regulation. This is illustrated by the drastic phenotypic effects that can be caused by their disruption. Mutations in *Drosophila* RNA-binding proteins affect neural development (Yao et al., 1993), spermatogenesis (Karschmizrachi and Haynes, 1993) and oogenesis (Matunis et al., 1994). In humans such defects might become manifested as genetic disease. For example, mutations in RNA-binding proteins are involved in the pathology of both fragile X mental retardation (Siomi et al., 1993, 1994) and Wilms tumour (Larsson et al., 1995; Charlieu et al., 1995). Recently deficiencies in two families of RNA-binding proteins have been implicated in problems in spermatogenesis, leading to either a huge reduction in spermatozoa (oligozoospermia) or their complete absence (azoospermia) (reviewed by Cooke and Elliott, 1997). One of these proteins, RBM (an acronym of RNA Binding Motif), is encoded by a multigene family, with genes on both the long and short arms of the human Y chromosome (Ma et al., 1993). The other, DAZ (an acronym of Deleted in Azoospermia), is encoded by a smaller gene family (Reijo et al., 1995). RBM is Y-encoded in mammals from humans to marsupials suggesting a conserved (and therefore important) function (Ma et al., 1993; Laval et al., 1995; Elliott et al., 1996; Delbridge et al., 1997), whilst DAZ is Y-linked only in old world primates (Cooke et al., 1996; Reijo et al., 1996; Cooke and Elliott, 1997).

All the major stages of spermatogenesis in the adult occur in the testis, which is anatomically divided up into seminiferous tubules. A cartoon summarising the major steps of spermatogenesis is shown in Fig. 1. Cells from the earliest stages of spermatogenesis are located at the periphery of the tubules, and these spermatogonia act both as stem cells to renew themselves and to provide precursors for the later stages of spermatogenesis, which are found more interior to the tubule. Cells sequentially undergo the first meiotic division (primary spermatocytes) and then the second division (secondary spermatocytes) to generate haploid round spermatids. These round spermatids then differentiate into elongating spermatids (a process called spermiogenesis) and ultimately into spermatozoa. Profound changes in gene expression occur during spermatogenic development, reflecting the different metabolic requirements of each stage. Recent evidence suggests that RNA-binding proteins are important regulators mediating these changes in genetic

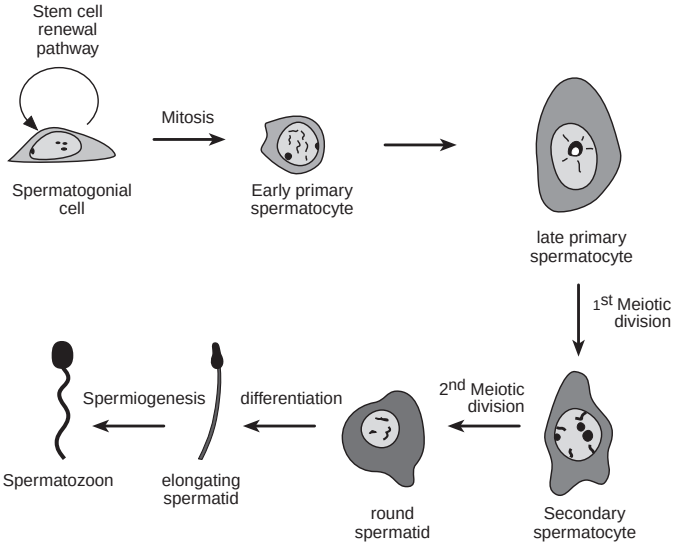

Fig. 1. Cartoon showing a time course of the major developmental stages of spermatogenesis in the adult testis.

activity. A number of transcripts encoding proteins critical for the completion of spermatogenesis are alternatively processed in the testis. An example of this is the CREM protein. A number of CREM isoforms are synthesised from alternatively spliced transcripts, of which some are activators and some are repressors of transcription. Prior to puberty, repressor isoforms of CREM are synthesised at low levels in germ cells. At puberty, Follicular Stimulating Hormone (FSH) secreted by the pituitary gland induces a change in polyadenylation site stabilising the mRNA encoding the transcriptional activator form CREMτ. This then stimulates the transcription of a number of structural genes in round spermatids, such as protamine genes, that have cyclic AMP responsive elements in their promoters (reviewed by Monaco et al., 1996).

Translational control is also found in the testis (Schafer et al., 1995). Delayed translation is necessitated by global changes in genome organisation over the course of spermatogenesis. Compaction of the genome in elongating spermatids as it is re-packaged, first with transition proteins and subsequently protamines, is incompatible with transcription and so any transcripts required at this stage are stored from earlier stages. Sequences in the 3′ untranslated regions of these stored transcripts are important for mediating translational control, and proteins that bind to these are being identified (Schumacker et al., 1995).

The amino acid sequence of RBM implies a function in RNA binding: RBM contains a single RNA recognition motif (RRM), which has been shown to mediate an interaction with RNA in several cases (Kenan et al., 1991; Ma et al., 1993). RBM is a nuclear protein (Elliott et al., 1997) that shows sequence homology (76% similarity and 60% identity at the amino acid level) to the heterogeneous ribonucleoprotein (hnRNP) G, a member of the ubiquitously expressed family of hnRNPs (Soulard et al., 1993; Ma et al., 1993). Both proteins have a single N-terminal RNA recognition motif (RRM) in addition to a number of sequence blocks common to other RNA-binding proteins (Ma et al., 1993). Sequence homology is found throughout the entire sequence, with the most obvious difference being a region in RBM that contains four tandem peptide repeats, called the SRGY box because of its high content of serine, arginine, glycine and tyrosine (Fig. 2A). While RBM expression is restricted to the testis, hnRNPG is thought to be one of the ubiquitously expressed hnRNP proteins (Dreyfuss et al., 1993).

The nuclear organisation of the molecules involved in gene expression has primarily been investigated in tissue culture cells, in which they appear to be spatially compartmentalised with regard to function, with molecules involved in similar metabolic processes being concentrated in the same nuclear regions (Larsson et al., 1995; Spector, 1993; Lamond and Carmo-Fonseca, 1993). Examples of this are proteins and snRNAs involved in pre-mRNA splicing, which are usually located throughout the nucleoplasm but are each also enriched in specific punctate sites. HnRNPs are located throughout the nucleoplasm and do not specifically co-localise with punctate regions enriched for pre-mRNA splicing components, although they are not excluded from these nuclear regions either. A number of RNA-binding proteins involved in rRNA maturation are located in the nucleolus.

The best place to study the function of a tissue-specific protein is in situ within the cognate tissue. With this in mind, we have examined the localisation of RBM and directly compared this with other components of nuclear RNA metabolism in human testes. In addition this study addresses for the first time the spatial organisation of factors involved in pre-mRNA splicing in a complex human tissue containing cells undergoing division and differentiation.

MATERIALS AND METHODS

Generation of antibodies against RBM

(1) Antibodies to whole RBM molecule. The entire RBM coding region was subcloned into pRSET (Invitrogen). The resulting plasmid pYRT3 was transformed into BL21 cells and a protein of around 70 kDa was induced by adding IPTG to 0.5 mM. The induced protein was purified by Ni^+ chromatography and injected into a rabbit. IgG was purified on protein A sepharose (Sigma).

(2) Antibodies to the SRGY region. The SRGY box and immediately upstream region of pMK5 (Ma et al., 1993) was PCR amplified using oligonucleotides 5′-AAAAAAA CTC GAG GCT GTG GCA AGA AGC AAT AGT and 5′-AAAAAAA GGT ACC ATG ATT TCT ATA TCC TCT AGA. The PCR product was cut with *Xho*I and *Kpn*I (restriction sites underlined) and ligated into PRSETA (Invitrogen). The resulting plasmid pH3 was transformed into *E. coli* BL21 cells and a polypeptide of around 28 kDa was induced by the addition of IPTG to 1 mM. This induced protein was purified as an inclusion body after cell lysis in 20 mM sodium phosphate/500 mM sodium chloride pH 7.8, then solubilised in 0.1 M sodium bicarbonate/0.05% SDS pH 9.6 and injected into a rabbit as an emulsion with Titre Max adjuvant (CytRx corporation, GA, USA). The rabbits were boosted after 9 weeks, and then bled 11 days and 3 weeks after this boost. IgG was purified using a protein A sepharose column (Sigma).

Detection of RBM in human testis extracts

Surgically removed human testis or prostate gland were Dounce homogenised, and then resuspended in an equal volume of 2× Laemmli buffer and DTT added to 0.1 M. Proteins were fractionated by SDS/PAGE on an 8% discontinuous gel and then transferred to an Immobilon filter using semidry western blotting (Millipore). RBM

was detected using either a 1:500 dilution of rabbit anti-RBM antiserum or a 1:133 dilution of anti-SRGY IgG, and a 1:2,500 dilution of HRP-conjugated anti-rabbit secondary (rabbit IgG-specific, clone RG96 from Sigma), in conjugation with chemiluminescence (Boehringer Mannheim). For the preabsorption experiment, 7.5 μl of anti-SRGY IgG in blocking solution (Boehringer Mannheim) was preincubated for 1 hour with either SRGY fusion protein or mGST1 protein (the RRM of mouse Rbm fused to GST) immobilised on an Immobilon filter. A further 800 μl of blocking solution was added to the mixtures and these were then used to probe immunoblots.

Immunohistochemistry

Surgically removed testes were fixed in either Bouin's solution or neutral buffered formalin and embedded in paraffin wax (Gurr, 1956). Bouin's solution contains a mixture of formaldehyde, picric acid and glacial acetic acid, and has been the most frequently used fixative for testis because of its high penetrative power and preservation of nuclear detail, while neutral buffered formalin has been used to fix tissue culture cells. Sections were processed for immunohistochemistry using standard techniques (Elliott et al., 1997). For DAB detection of antibody binding, a 1:400 dilution of biotin-conjugated swine anti-rabbit antibody (Dako) was used, followed by an avidin-biotin horseradish peroxidase system (Dako) and diaminobenzidene (DAB; Sigma). Sections were briefly counterstained in Harris hematoxylin and mounted in DPX mounting medium (BDH, Merck Ltd, UK). For fluorescent detection, sections were washed in TBS and then incubated for 30 minutes with FITC-conjugated donkey anti-rabbit IgG diluted 1:50 with 20% normal swine serum; and for double immunofluorescence also with Texas Red-conjugated donkey anti-mouse IgG at the same dilution (both minimal cross reaction: Jackson ImmunoResearch Laboratories Inc.). Sections were washed with TBS and then mounted in Vectastain (Vector Laboratories). In addition to the nuclear signal, weak fluorescent labelling of the tubules was apparent, particularly at their peripheries. This was not competed away by preincubation with the immunising polypeptide and so does not correspond to RBM staining. While some of this background may represent a non-specific cross-reaction of the antisera, much is due to autofluorescence of the tissue, since we observed it in the absence of secondary antibody. Considerably less autofluorescence was observed after fixation with neutral buffered formalin. Cell types were identified by their morphology and position in the tubule. Spermatogonia (abbreviated Spg) are present around the periphery of the tubule. Primary spermatocytes undergoing the first meiotic division are roughly divided into early cells (leptotene and early to mid pachytene; abbreviated early Spc) and late cells (late pachytene and diplotene; abbreviated late Spc) based on the stage in meiosis that they have reached. Late spermatocytes have visibly condensed chromosomes, larger nuclei and are deeper in the tubule. Round spermatids (abbreviated Rtd) have smaller round nuclei and are towards the center of the tubule, while elongating spermatids (abbreviated Spd) have much smaller, flattened nuclei. Sertoli cells (abbreviated SC) are usually just inset from spermatogonia and have a characteristic single central nucleolus. Myoid cells (abbreviated MC) are in the tubule wall.

Antibodies

Antibodies against the following antigens were used in this study.

RBM; anti-RBM (whole molecule), used at 1:2000 for immunofluorescence and western blotting.

SRGY; anti-SRGY, used at 1:50 for immunofluorescence and 1:500 for DAB detection.

Sp1; IC6, Santa Cruz Biotechnology, used at 1:10 for immunofluorescence.

CREM; Santa Cruz Biotechnology, used at 1:10 dilution for immunofluorescence.

SR proteins; monoclonal 16H3, provided by K. Neugerbauer and M. Roth, used at 1:50 for immunofluorescence.

U2B″ and SC35; provided by Y. Yannoni, used at 1:50 for immunofluorescence.

hnRNP A1; 9H10 provided by G. Dreyfuss and H. Kamma, used at 1:20 for immunofluorescence.

hnRNPs C1/C2; 4F4 provided by G. Dreyfuss, used at 1:20 for immunofluorescence.

hnRNPs A2, B1, B2; DP3, provided by H. Kamma, used at 1:20 for immunofluorescence.

hnRNP B1; 2B2, provided by H. Kamma, used at 1:20 for immunofluorescence.

hnRNPU; 3G6, provided by H. Kamma, used at 1:50 for immunofluorescence.

hnRNPG; dog autoimmune sera, provided by C. Larsen, used at 1:200 for immunofluorescence.

Fluorescence microscopy

Fluorescence microscopy was carried out on a Zeiss Axioplan microscope using a chromoseries 8100 multispectral filter. Images were captured on a CCD camera by Smartcapture (Digital Scientific, Cambridge, UK) and were analysed and manipulated using IP Lab Spectrum. Control experiments showed that there was no detectable cross reaction of secondary antibodies or bleedthrough between the FITC and Texas Red channels.

RESULTS

Biochemical analysis of RBM

We raised antisera independently to fusion proteins containing either the entire RBM coding sequence or the SRGY region alone (Fig. 2A), purified IgG from these antisera and used these to probe immunoblots of normal human testis. IgG prepared from both antisera recognised two major proteins of 55 and 50 kDa and a minor smear of protein of around 43 kDa (Fig. 2B, lanes 1 and 2). IgG raised against the full-length RBM additionally recognised a protein of around 40 kDa – this hence is likely to represent a cross-reacting protein (either specific or non-specific; Fig. 2B, lane 2). Amido black staining indicated equivalent amounts of protein in both lanes (Fig. 2B, lanes 3 and 4).

In order to determine if these proteins correspond to RBM, we carried out preabsorption experiments with the SRGY-containing fusion protein and the anti-SRGY antisera. Detection of p55, p50 and p43 was blocked by preincubation with recombinant SRGY fusion protein (Fig. 2C, lane 1, see Materials and Methods) but not by a fusion protein with an unrelated sequence (Fig. 2C, lane 2). As a further control in this experiment, a parallel untreated antiserum is shown (Fig. 2C, lane 3). The level of protein bound to filters was equal in each lane (determined by counterstaining with amido black; shown in lanes 4-6).

RBM is testis-specific in expression (Ma et al., 1993). We probed immunoblots containing both protein from testis from a man with a normal complement of germ cells and from prostate gland (which would be predicted to be RBM negative). These experiments showed that while p55, p50 and p43 are testis-specific, p40 is also detected in prostate extracts (Fig. 2D, lanes 1 and 2; parallel amido-black-stained filters are shown in lanes 3 and 4).

Based on the germ cell specificity, detection with two independent antisera and specific preabsorption, these experiments suggest two major and one minor isoform of the RBM protein in human testis (p53 and p50; and p43

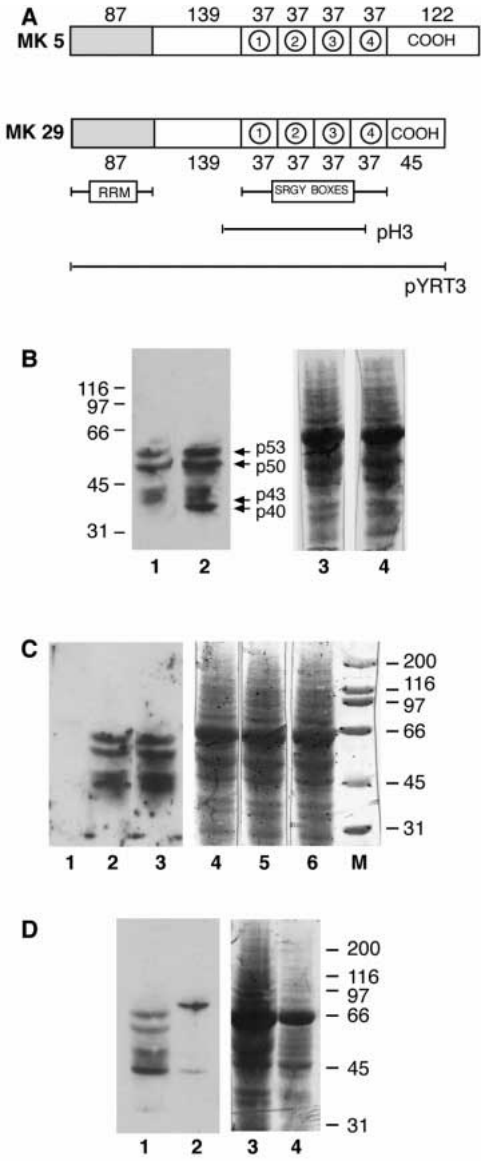

Fig. 2. Characterisation of antisera raised against RBM. (A) Cartoon of the domain structure of proteins conceptually translated from MK5 and MK29, which are the most abundant cDNAs encoding RBM. The RNA recognition motif (RRM) is shaded and the four SRGY boxes are numbered and shown as boxes. The sizes of each part of the protein is given in amino acids. Both MK5 and MK29 are identical apart from in the carboxy-terminal domain (COOH). The position of the bacterially expressed fusion peptides encoded by expression plasmids pH3 and pYRT3 used to raise the antisera is indicated underneath. PH3 encodes a fusion protein containing the SRGY box alone and pYRT3 a fusion protein containing the whole of RBM. (B) Western blot of protein from human testis with a normal complement of germ cells probed with antisera raised against either the SRGY region or the whole of RBM (lanes 1 and 2, respectively) or stained with amido black (lanes 3 and 4). (C) Western blot of protein from human testis with a normal complement of germ cells probed with either IgG raised against the SRGY polypeptide and then preabsorbed against the same polypeptide (lane 1), preabsorbed against a different bacterially expressed fusion protein not related in sequence (lane 2), or without preabsorption (lane 3). Corresponding filters stained with amido black are shown in lanes 4-6. (D) Western blot of normal human testis and prostate probed with IgG raised against the whole of RBM (lanes 1 and 2) or stained with amido black (lanes 3 and 4). The sizes of molecular weight markers run in parallel are indicated.

respectively). These isoforms might be generated either from different copies of the RBM gene, or alternative processing of the RBM transcript. cDNAs corresponding to two alternate versions of RBM differing in the length of their carboxy-termini have been identified (Ma et al., 1993; Fig. 2A), and there are a number of other cDNAs and genomic copies, some of which represent pseudogenes (Prosser et al., 1995). Alternatively, they might be generated by alternative modification of the RBM protein (e.g. another germ-cell-specific nuclear protein TSPY has two major isoforms of different apparent molecular weight differing in their phosphorylation status: Schneider et al., 1996). A further possibility since we are dealing with surgically obtained human tissue is that the smaller protein species might be degradation products of p55.

RBM has a distinct pattern of expression from hnRNPG

The closest homologue of RBM is hnRNPG, which is thought to be one of the ubiquitously expressed hnRNPs (see introduction). An important question concerning the relative functions of RBM and hnRNPG is whether they are expressed in the same spectrum of cells. To address this question, we directly identified cell types expressing RBM and hnRNPG by immunohistochemistry (Fig. 3). These experiments indicated that RBM is a nuclear protein (notice that the cytoplasm is unstained) expressed in spermatogonia, in both early and late spermatocytes and in round spermatids (which hence appear brown); but is not expressed in elongating spermatids or in any of the somatic cells in the testis (Sertoli cells, myoid cells or interstitial cells). These latter cells appear blue, which is the colour of the counterstain. Identical results were obtained with both IgG raised against the whole RBM and the SRGY region (Fig. 3A and B respectively). In contrast, hnRNPG (Fig. 3C) is strongly expressed in the nuclei of spermatogonia and (much more weakly) the nuclei of round spermatids (which hence appear a brownish blue). Unlike RBM, hnRNPG is also expressed in some Sertoli cells in the testis. Overall, the above results demonstrate the specificity of our antisera for RBM and indicate a different pattern of gene expression from hnRNPG.

RBM and pre-mRNA splicing components are spatially reorganised during spermatogenic development

While the use of a chromogenic reporter allows easy identification of cell types, it precludes subnuclear resolution, since the brown reaction product precipitates non-specifically on subnuclear structures (e.g. condensed chromosomes). In order to localise RBM at the subnuclear level, we probed sections of adult human testis by indirect immunofluorescence (Fig. 4). At this resolution, it is clear that, in addition to strong staining throughout germ cell nuclei, RBM is additionally enriched within discrete punctate sites in some cell types (spermatocytes; Fig. 4B). The localisation pattern of RBM was identical in tissues fixed with Bouin's solution and neutral buffered formalin (not shown and see also Fig. 5). Confirming the specificity of the antisera, nuclear staining was eliminated

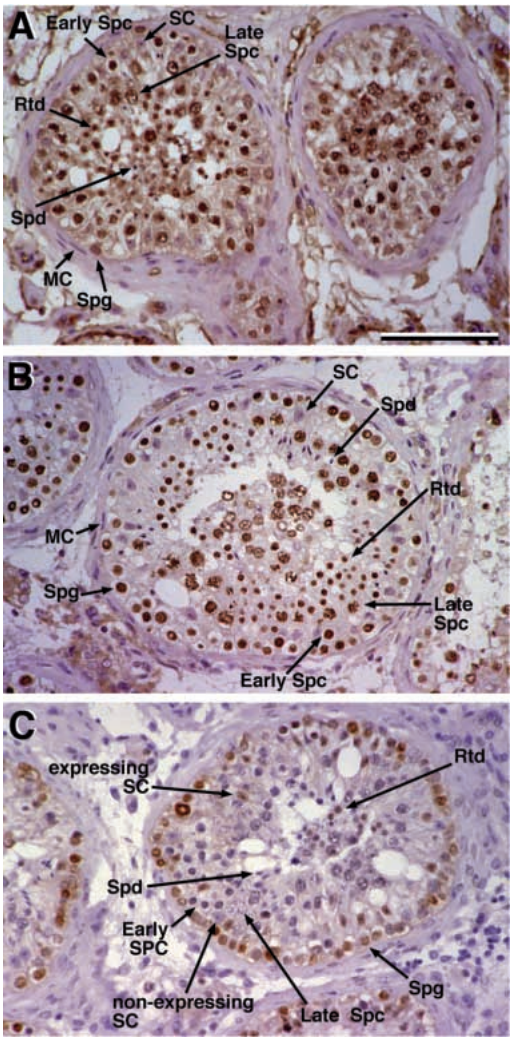

Fig. 3. RBM and hnRNPG have different expression patterns in the adult human testis. Sections of normal adult human testis were probed with either (A) IgG raised against the whole RBM molecule, (B) IgG raised against the SRGY region and (C) antibodies against hnRNPG. Antibody binding is indicated by a brown color. Sections were counterstained with haematoxylin which stains nuclei blue. The scale bar indicates 50 μm.

by preabsorption of the anti-SRGY antisera with the immunising SRGY protein (Fig. 4C) and identical results were obtained using either antisera raised against the SRGY box (Fig. 4B) or the whole RBM molecule (Fig. 4E).

An important indication of the function of RBM would be if it showed a similar pattern of nuclear localisation to any other proteins with known roles in gene expression. To address this, we examined the nuclear location of other molecules involved in gene expression during germ cell development. Direct comparison of testis sections stained for both RBM and SR proteins suggest that the punctate regions enriched for RBM in spermatocytes also contain high concentrations of SR proteins. SR proteins are an essential family of pre-mRNA splicing components that characteristically contain long stretches of arginine and serine residues (the so-called SR domains, from which the proteins get their name; Zahler et al., 1993). Monoclonal antibody 16H3 specifically recognises a subset of these that contain alternating arginine, serine and glutamine residues, including U170K and U2AF (but not SC35: Neugebauer et al., 1995). In tissue culture cell nuclei SR proteins are spatially localised in a number of punctate regions or speckles found throughout the nucleoplasm but are excluded from nucleoli. However, we find that the organisation of SR proteins during germ cell development is considerably more complex, with dramatic reorganisations taking place in the different cell types (Fig. 4A,D). SR proteins are distributed fairly evenly through the nuclei of spermatogonia, but become dramatically reorganised in the later stages of germ cell development. In spermatocytes, SR proteins become concentrated in a small number of intensely staining sites with little nucleoplasmic staining while, in round spermatids, the level of nucleoplasmic staining increases although high levels of SR proteins are still concentrated in one or two intensely labelled sites. Unlike RBM, SR proteins are expressed in somatic cells (Sertoli cells and myoid cells) as well as within germ cells.

The above experiments indicate that a population of SR proteins (those recognised by McAb 16H3) are reorganised during spermatogenesis. The splicing machinery has a large number of components including both snRNPs and non-snRNP proteins. To determine if all of these components undergo similar patterns of reorganisation, we carried out experiments to visualise both non-snRNP splicing proteins (SR proteins and SC35), an snRNP protein (U2B″) and snRNAs (visualising their characteristic m^3G cap structure). Although SC35 is an SR protein, it is not among those detected by McAb 16H3, and so was monitored independently in these experiments (Neugebauer et al., 1995; Fu and Maniatis, 1990). To additionally examine the extent of co-localisation between RBM and pre-mRNA splicing proteins, we independently localised RBM in these same cells and then superimposed the images (Fig. 5). In each case we got similar results. In spermatogonia, pre-mRNA splicing proteins (U2B″ in Fig. 5A-C) are distributed throughout the nucleoplasm and additionally concentrated in a number of punctate sites, which were of approximately equal staining intensity. This is similar to the localisation pattern that has been described in somatic cells grown in tissue culture (Neugerbauer et al., 1995). In these same cells, RBM is also both distributed throughout the nucleoplasm and concentrated in punctate sites, some of which (but not all) are coincident with those containing pre-mRNA splicing proteins.

Pre-mRNA splicing proteins become dramatically reorganised in spermatocytes, concentrating in a number of nuclear sites that also contain increased local concentrations of RBM (SR proteins recognised by McAb 16H3 and corresponding RBM images are shown in Fig. 5D-5F and 5H-5J). Despite this coincident punctate staining, it should be stressed that the level of nucleoplasmic staining is higher in the case of RBM: hence the staining patterns are not identical at this stage, only similar. These punctate structures are often internal to the nuclei in tissue fixed in neutral buffered formalin (Fig. 5D-F) while, in sections fixed in Bouin's solution, the punctate structures appear to be at the nuclear periphery (Fig. 5H-J). This difference is probably a result of tissue shrinkage caused by Bouin's solution, which causes some dehydration (Gurr, 1956). In late spermatocytes, the punctate regions

enriched for RBM and SR proteins physically disassociate (Fig. 5G shows a late spermatocyte nucleus labelled for both RBM and the SR protein SC35) with SR proteins being concentrated in one major intranuclear site while RBM is distributed throughout the nucleoplasm (any overlap would appear yellow).

In round spermatids SR proteins (in this case the protein SC35) are concentrated in (usually) a single major (arrowed) and a number of minor nuclear sites (arrowheads), as well as throughout the nucleoplasm. In contrast RBM is not found in any punctate structures and is distributed throughout the nucleoplasm (Fig. 5K-M). During spermiogenesis, the haploid genome of the round spermatid becomes condensed as it is re-packaged firstly with transition proteins, and then protamines as they differentiate into elongating spermatids. An elongating spermatid (Spd) and immediately adjacent round spermatid (Rtd) are shown in Fig. 4A,B, and an overlap of these images in Fig. 5N. Expression of both RBM and SR proteins is clearly reduced in elongating spermatids compared with round spermatids, with RBM expression decreasing more rapidly.

Hence both pre-mRNA splicing proteins and RBM have both punctate and more general nucleoplasmic populations, with the ratio of molecules in these two populations being dynamically modulated during germ cell development. Parallel phase and fluorescent images indicate that the fluorescent signal that we are detecting is nuclear (see also Fig. 3). A quantitative summary of the punctate structures recorded in these experiments is shown in Fig. 6, which shows the distribution of pre-mRNA splicing components in strongly staining sites at each stage of spermatogenesis (Fig. 6A), and the percentage of these sites which co-localised with RBM (Fig. 6B). For this analysis, we defined strongly staining sites as those containing quantitatively much higher levels of the splicing component relative to other nucleoplasmic sites (such sites are indicated by arrows in Fig. 5). Since in spermatogonia the punctate sites containing splicing factors were all somewhat similar in intensity, which was not much higher than that in the surrounding nucleoplasm, punctate structures are not recorded for this developmental stage. This quantitative analysis clearly indicates that each of the splicing factors that we examined re-localises to around two intensely staining punctate sites in spermatocytes, and that almost all of these punctate sites also contain high local concentrations of RBM. High concentrations of pre-mRNA splicing factors were still localised in a small number of intensely staining sites in late spermatocytes and round spermatids but, in this case, these did not overlap with regions enriched for RBM.

In summary, these experiments indicate that a number of different components of the pre-mRNA splicing machinery show developmental reorganisations in spermatogenesis and that punctate regions enriched for splicing factors show a temporally restricted pattern of co-localisation with RBM.

RBM and SR proteins behave differently in mitosis

Typically, proteins involved in gene expression exit the nucleus during mitosis. Spermatogonia comprise a population of stem cells, and also proliferating and differentiating cells which are mitotically more active. The mitotic behaviour of pre-mRNA splicing components has not been reported in the context of an intact tissue, whose cell division is likely to be controlled by complex paracrine and cell-cell interactions. By examining spermatogonia, we were able to determine the behaviour of both RBM and pre-mRNA splicing components in mitosis, during which both were cytoplasmic. A cell just completing mitosis is shown in Fig. 5Q. Interestingly, we found that SC35 was present in punctate structures in the cytoplasm of these mitotic cells (the red foci of staining arrowed in Fig. 5Q). Similar observations have been reported in tissue culture cells, where splicing components associated in punctate cytoplasmic structures during mitosis (Ferreira et al., 1994), which clustered on the nuclear membrane at anaphase (Spector et al., 1991), and indeed the regions enriched for SC35 appear to be

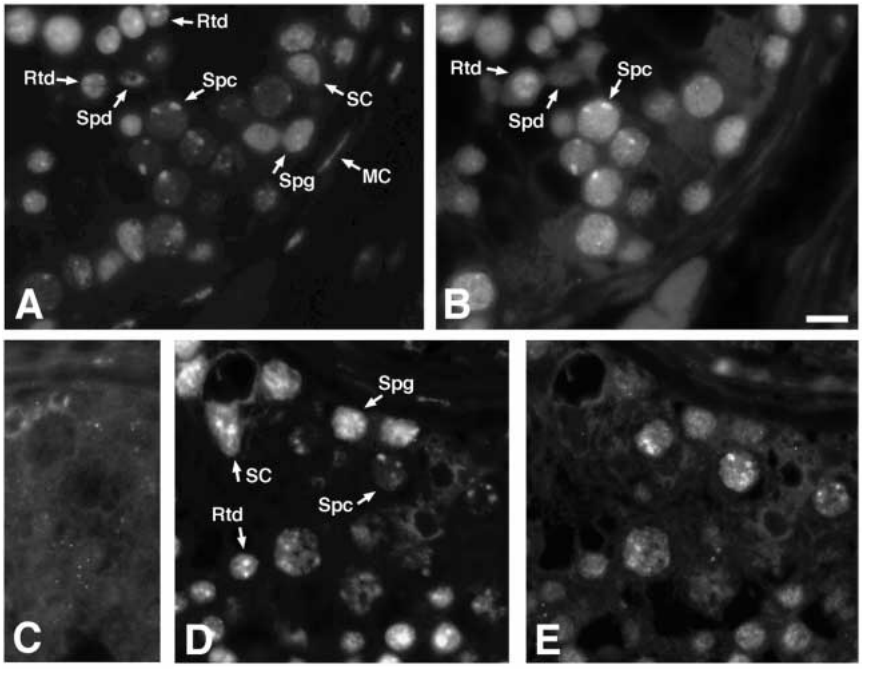

Fig. 4. RBM and a family of pre-mRNA splicing proteins are spatially reorganised during germ cell development. A section of normal human testis was stained for both (A) a subset of SR proteins (recognised by McAb 16H3, which contain alternating serine, arginine and glutamine residues, including U170K and U2AF but not SC35), and (B) RBM using the IgG raised against the SRGY region, and these were visualised independently at different wavelengths. (C) Staining of normal human testis with the anti-SRGY IgG after preincubation with the SRGY fusion polypeptide. (D) SR proteins visualised with McAb 16H3 and (E) immunolocalisation of RBM using IgG raised against the whole RBM molecule. Cell types were identified and labelled as in the materials and methods. Testis were fixed in neutral buffered formalin (A,B) or Bouin's solution (C-E) before embedding in parrafin wax. The scale bar is equal to 17.2 μm.

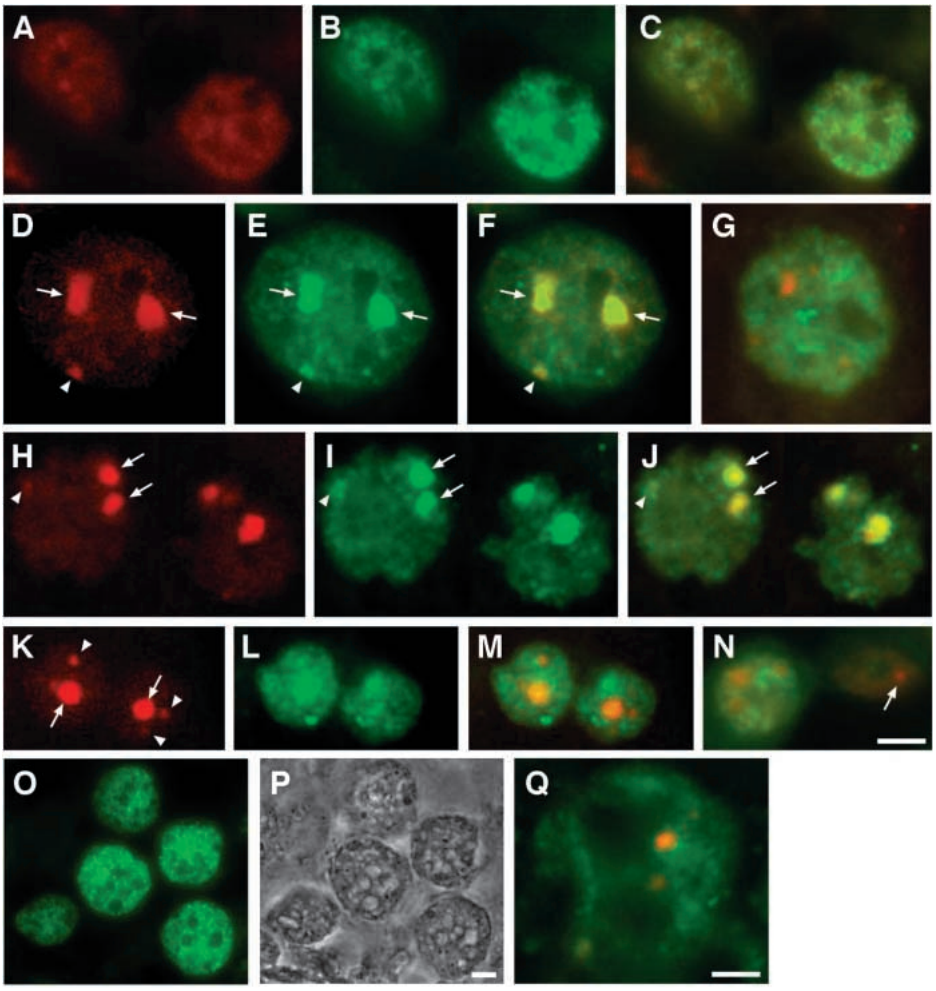

Fig. 5. RBM and pre-mRNA splicing factors show a dynamic pattern of co-localisation during germ cell development. RBM and splicing factors were visualised in germ cells at different stages of development by double immunofluorescence.
(A-C) Spermatogonia stained for both U2B″ (A) and RBM (B), and an overlap of these two images (C).
(D-F) Spermatocyte stained for both SR proteins using McAb 16H3 (D) and RBM (E) and an overlap of these images (F). This micrograph was taken from a section fixed in neutral buffered formalin.
(G) Late spermatocyte stained for both SC35 (red) and RBM (green). No colocalisation between the regions enriched for SC35 and RBM was observed, hence the region enriched for SC35 appears red rather than yellow.
(H-J) Spermatocytes stained for both SR proteins using McAb 16H3 (H), RBM (I) and an overlap of these images (N). In this case, the sections were fixed in Bouin's solution – notice more cell shrinkage than in spermatocytes fixed in neutral buffered formalin (above). (K-M) Round spermatids probed for SC35 (K), RBM (L), and an overlap of these images (M; SC35 in red and RBM in green).
(N) Overlapping image of round spermatid and elongating spermatid (arrowed) from Fig. 4A and 4B, with RBM pseudocoloured green and SR proteins pseudocoloured red. Notice the decrease in (particularly RBM) staining in the elongating spermatid. (O,P) Parallel fluorescent and phase micrographs of spermatocytes were taken to confirm the fluorescent signal was nuclear. (Q) Mitotic spermatogonium stained for both RBM (green) and SC35 (red). Punctate regions of SC35 staining are found outside the nuclear volume in these cells (arrowed), while RBM is distributed throughout the cytoplasm. In each panel, RBM is pseudocoloured green and pre-mRNA splicing components are pseudocoloured red. Any overlap between the red and the green images will appear yellowish. Subnuclear regions containing high concentrations of RBM and SR proteins are indicated by arrows, while regions containing slightly lower concentrations are indicated by arrowheads. The calibration bar corresponds to 10 μm (independent calibration bars are shown for A-N; O and P; and Q). RBM was visualised using the anti-SRGY IgG, and testis were fixed in Bouin's solution apart from D-F and N-P, which were fixed in neutral buffered formalin.

attached to the edge of the nuclear volumes in the cell shown in Fig. 5Q. Hence these focal concentrations are likely to be characteristic of at least some splicing components in vivo. In contrast, RBM (the green staining in Fig. 5Q which is absent from the nuclear volumes) was more uniformly distributed throughout the cytoplasm of mitotic cells. Hence these observations suggest distinct mechanisms control both the spatial localisation and nuclear re-entry after mitosis of SC35 and RBM. In contrast pre-mRNA splicing components are clearly nuclear throughout meiosis. Consistent with this, mRNA synthesis continues through meiosis but is halted in mitosis.

RBM has a different localisation pattern from hnRNPs or the transcription factor Sp1

The evidence described above suggests that both RBM and pre-mRNA splicing factors undergo stage-specific variations in nuclear organisation, and moreover specifically co-localise in the early stages of spermatogenesis (prior to and during the early stages of meiosis). Do all proteins involved in pre-mRNA metabolism undergo such reorganisations? In order to address this we examined the location of other molecules involved in gene expression in the testis.

Testis sections were probed with a panel of antibodies directed against RBM and a number of hnRNPs (see Materials and Methods). In each case, while hnRNPs were expressed in the nuclei of germ cells they did not concentrate in the subnuclear structures enriched for RBM. Typical results from these experiments are shown in Fig. 7, for hnRNPB1 in spermatogonia (Fig. 7A-C), hnRNPC in spermatocytes (Fig. 7D-F) and hnRNPG in round spermatids (Fig. 7G-I). Indeed, co-localisation indicates that hnRNPC is quantitatively reduced in the regions enriched for RBM (arrowed in 7D-F; in the overlap RBM is in green and hnRNPC in red).

To address whether transcription factors undergo developmental re-arrangements in nuclear localisation, we

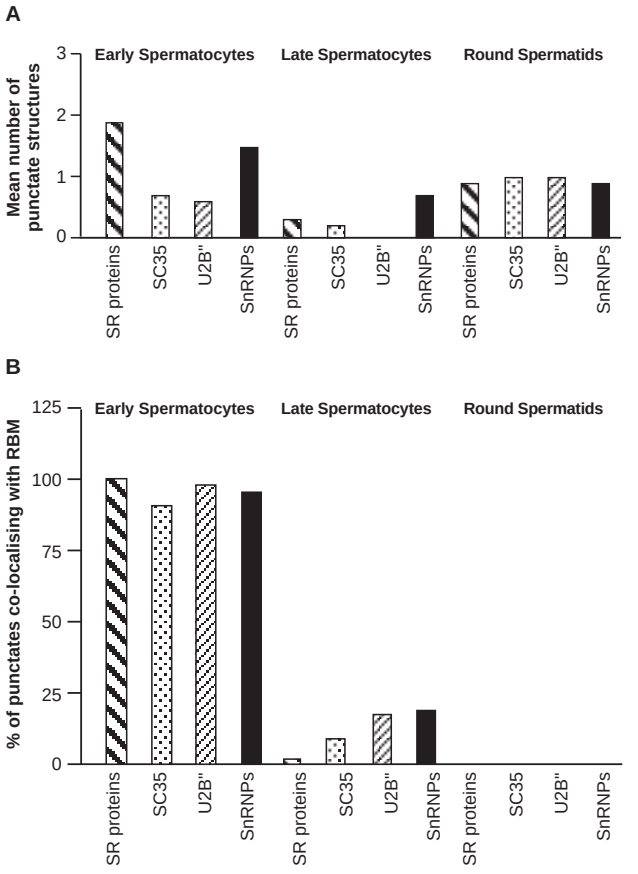

Fig. 6. Quantitative analysis of developmental reorganisations of pre-mRNA splicing components and RBM in germ cell nuclei. (A) The total number of punctate sites (defined as distinct structures containing an increased concentration of protein, for example, those indicated by arrows in Fig. 5D-K) enriched for each of the pre-mRNA splicing components at each developmental stage was determined by counting through serial sections of at least 10 nuclei. Each of the pre-mRNA splicing components is given the same shading on the chart for each developmental stage. In spermatogonia, all sites enriched for pre-mRNA splicing components are of approximately equal staining intensity. Since there were no large punctate structures (e.g. see Fig. 5B), this stage is not included on the bar chart. (B) The percentage of punctate sites enriched for both pre-mRNA splicing components which also contain RBM. Note that the number of co-localising sites is maximal in early spermatocytes and zero in round spermatids. The same shading for each splicing component is used as in A.

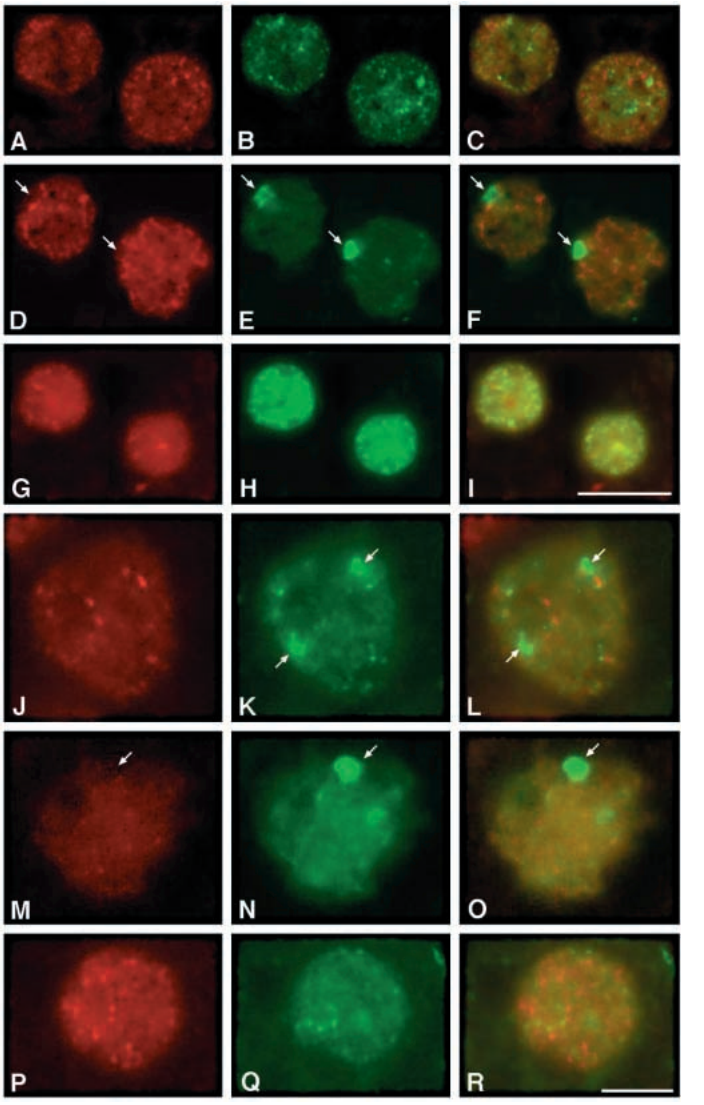

Fig. 7. Neither hnRNPs nor the transcription factor Sp1 undergo developmental reorganisations or specifically co-localise with RBM. (A-C) Micrographs of spermatogonia probed for hnRNPB1 (A) and RBM (B) and an overlap (C) of these images. Note that the regions enriched for RBM are not especially enriched for hnRNPB1. (D-F) Micrographs of spermatocytes probed for hnRNPC (D) and RBM (E), and an overlap (F) of these two images. (G-I) Micrograph of round spermatids probed for RBM (G) and hnRNPG (H), and an overlap of these images (I; in these three panels only, RBM in red and hnRNPG in green). (J-L) Micrographs of spermatogonium probed for Sp1 (J) and RBM (K), and an overlap of these images (L). (M-O) Micrographs of a spermatocyte probed for Sp1 (M) and RBM (N), and an overlap of these images (O). (P-R) Micrographs of a round spermatid nucleus probed for both Sp1 (P) and RBM (Q), and an overlap of these images (R). In each of the panels apart from G-I, RBM is pseudocoloured green and either hnRNPs or Sp1 are pseudocoloured red. Nuclear regions enriched for RBM are arrowed. The scale bar is equivalent to 12.8 μm (A-I) and 6.5 μm (J-R). RBM was visualised using the anti-SRGY antiserum.

carried out experiments to immunolocalise transcription factors during germ cell development. Data from the mouse have shown that a number of transcription factors, including CREM, are highly expressed in round spermatids, but are barely detectable earlier in spermatogenesis (Schmidt, 1996; Schmidt and Schibler, 1995). As such, they represent difficult subjects in which to examine developmental reorganisations in spatial localisation. In correspondence with this, we obtained a similar result for CREM in human testis (data not shown). Sp1 is another transcriptional activator which binds specifically to GC boxes in DNA, mediated by a zinc finger motif (Kadonaga et al., 1987). Indirect immunofluorescence using a monoclonal antibody directed against Sp1 showed that, unlike CREM, it is expressed throughout spermatogenesis from spermatogonia to round spermatids. Sp1 is found throughout

the nucleoplasm of spermatogonia and, although it is enriched in some regions (Fig. 7J), these do not correspond to the regions enriched for RBM (arrowed in Fig. 7K,L). The nuclear regions containing high concentrations of RBM in spermatocytes (Fig. 7N,O, arrowed) contain lower concentrations of Sp1 (arrowed in Fig. 7M-O). While both RBM and Sp1 are located throughout the nuclei of round spermatids, the distributions of the two proteins appear qualitatively different even at this stage (Fig. 7P-R).

Taken as a whole, these data indicate that the subnuclear localisation of RBM and pre-mRNA splicing components is different from both transcription factors and hnRNPs. Distinct subnuclear domains are specifically detected using antibodies directed against pre-mRNA splicing factors and RBM, and are not detected by antibodies against other classes of protein involved in different stages of nuclear gene expression. These results confirm that the structures that we are detecting are neither a non-specific aggregation of proteins, nor a particularly 'sticky' structure in the nucleus with a high affinity for non-specific antibody binding.

DISCUSSION

The conservation of RBM on the Y chromosome between man and marsupial suggest a critical role in mammalian germ cell development (Delbridge et al., 1997; Elliott and Cooke, 1997). The data described in this report show that this role is likely to be complex. While RBM is expressed in the nuclei of human germ cells between the spermatogonial and round spermatid stages, there are dynamic modulations in its spatial location between these different cell types. Specifically, the association of RBM with punctate regions of the nucleus enriched in splicing factors is transient and limited to the first two stages of spermatogenesis; it is found in spermatogonia and spermatocytes, but in round spermatids no co-localisation is observed. This observation suggests the intriguing possibilities either that the function of RBM is regulated during spermatogenesis, or that RBM has different functions at different stages of development. Neither the transcription factor Sp1 nor hnRNP proteins were targeted to splicing-factor-rich nuclear domains. Hence, despite its sequence similarity to hnRNPG, RBM is likely to have a different function.

The results described in this paper indicate that the nuclear organisation of the pre-mRNA processing machinery undergoes dynamic modifications in cell types undergoing division and differentiation in vivo, and so is more complex than in the more homogeneous populations of cells grown in tissue culture. We find that both snRNPs (snRNAs and associated proteins) and non-snRNP components of the spliceosome undergo spatial reorganisations during spermatogenesis, unlike either hnRNPs or the transcription factor Sp1. In tissue culture cells, splicing components are both concentrated in distinct areas called interchromatin granule clusters (ICGCs) and coiled bodies, and are also found throughout the nucleoplasm (reviewed by Spector, 1993; Dreyfuss et al., 1993; Lamond and Carmo-Fonseca, 1993; Mattaj, 1994; Fakan, 1996; Moen et al., 1995). This nucleoplasmic staining may correspond to structures identified under the electron microscope called perichromatin fibrils. It is possible that the punctate structures that we identify in the testis directly correspond to those described in tissue culture although, since they contain SC35, they are unlikely to be coiled bodies. Alternatively they may be represent more specialised structures unique to germ cells.

Quantitative relocalisations between subnuclear sites may be a feature of cell division and differentiation. Changes in splicing factor localisation also occur during differentiation of MEL cells to erythrocytes in tissue culture (Antoniou et al., 1993) and neuronal differentiation in the rat is also associated with stage-specific reorganisations of splicing components occurring concomitantly with changes in transcriptional activity (Santama et al., 1996). During spermatogenesis RNA synthesis peaks during the spermatocyte stage (Monesi, 1964; Erickson, 1990), is reduced in round spermatids and then ceases while haploid round spermatids differentiate into transcriptionally quiescent elongated spermatids. Splicing factors may concentrate into discrete regions of the round spermatid nucleus as a consequence of this global decrease in RNA synthesis compared with the previous spermatocyte stage. Other factors may affect the organisation of splicing factors into subnuclear domains in spermatocytes that are still transcriptionally active. Previous reports of the subnuclear localisation of splicing factors in the testis of rats and mice did not resolve these spatial reorganisations, either because of the experimental conditions (cytospins as compared with intact tissue) or choice of antibody probes (Richler et al., 1994; Moussa et al., 1994). The cellular location of molecules involved in controlling gene expression may be an important parameter controlling gene expression in other contexts. A differential association of the WT1 protein with either splicing factor-rich or transcription factor-rich regions of the nucleus has been reported and, in this case, is controlled by alternative splicing to generate two different isoforms of the protein (Larsson et al., 1995). It is tempting to speculate that the putative different isoforms of RBM that we detect on westerns may correspond to populations of RBM with different nuclear locations.

What implications does the subnuclear organisation of RBM have for its molecular function? Recent experiments have suggested the possibility that the nucleoplasmic population of splicing components is likely to be catalytically involved in pre-mRNA splicing, while the punctate structures are sites of storage and recruitment for nearby genes that become transcriptionally active (Zeng et al., 1997; Gama-Carvalho et al., 1997; Misteli et al., 1997). These punctate sites contain both pre-mRNA splicing factors and a proportion of the hyperphosphorylated form of RNA polymerase II, while the hypophosphorylated form, which can be recruited into transcriptional initiation complexes, is found throughout the nucleoplasm (Bregman et al., 1995). Hence RBM may be targeted to the punctate domains in spermatogonia and spermatocytes in association with either inactive pre-mRNA splicing components or RNA polymerase II. It is possible that RBM might be recruited from these punctate sites into the nucleoplasmic pool, which is functionally active. Nuclear RNA-binding proteins may be involved in many metabolic processes, such as binding to nascent RNA either as chaperones (Herschlag, 1995) or to display RNA to processing factors (Dreyfuss et al., 1993), in pre-mRNA splicing (either constitutive or alternative), in 3′ end formation and polyadenylation, and in nucleocytoplasmic RNA transport. It

is tempting to speculate that the targeting of RBM to splicing factor-rich domains is indicative of a direct function in pre-mRNA splicing, and we are currently testing this hypothesis.

The expression pattern of RBM also has important implications for any testicular pathologies that might result from either its deletion or inappropriate expression. Microdeletion of three regions (termed AZFa-c) of the human Y chromosome are associated with either oligo- or azoospermia (Vogt et al., 1996; Cooke and Elliott, 1997). RBM expression is reduced or eliminated in men with deletions of the AZFb region (Elliott et al., 1997). Germ cells in men with this deletion are arrested at the pachytene stage of spermatogenesis and do not proceed into the haploid stage of spermatogenesis (Vogt et al., 1996). This observation would imply that a gene present in the AZFb deletion interval is critical for the successful completion of meiosis. It is interesting to note that we observe co-localisation of RBM with pre-mRNA splicing components up to the end of meiosis.

Consistent with the notion that RNA-binding proteins control important steps in germ cell development, mutations in the *Drosophila* RNA-binding protein *boule* can arrest germ cell development in meiosis (Eberhardt et al., 1996). An *S. pombe* RNA-binding protein mei2 is required for transition into meiosis (Watanabe et al., 1997). Importantly for comparison with our study, the subnuclear localisation of mei2 is critically related to its function. In mitotically proliferating cells mei2 is cytoplasmic but, as cells enter meiosis, mei2 becomes concentrated in a single subnuclear region. This change in protein localisation is mediated by protein phosphorylation. Taken as a whole, these data indicate that both qualitative and quantitative aspects of the expression of RNA-binding proteins are likely to be important in germ cell development. In addition to any physiological role in modulating gene expression, differential expression of RNA-binding proteins between different germ cell types will also provide useful cell markers for assaying germ cell differentiation in vitro culture systems.

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