A mammalian germ cell-specific RNA-binding protein interacts with ubiquitously expressed proteins involved in splice site selection

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***RNA-binding motif* (*RBM*) genes are found on all mammalian Y chromosomes and are implicated in spermatogenesis. Within human germ cells, RBM protein shows a similar nuclear distribution to components of the pre-mRNA splicing machinery. To address the function of RBM, we have used protein–protein interaction assays to test for possible physical interactions between these proteins. We find that RBM protein directly interacts with members of the SR family of splicing factors and, in addition, strongly interacts with itself. We have mapped the protein domains responsible for mediating these interactions and expressed the mouse RBM interaction region as a bacterial fusion protein. This fusion protein can pull-down several functionally active SR protein species from cell extracts. Depletion and add-back experiments indicate that these SR proteins are the only splicing factors bound by RBM which are required for the splicing of a panel of pre-mRNAs. Our results suggest that RBM protein is an evolutionarily conserved mammalian splicing regulator which operates as a germ cell-specific cofactor for more ubiquitously expressed pre-mRNA splicing activators.**

The *azoospermia factor* was originally postulated as a gene on the long arm of the human Y chromosome deleted in some infertile men. Subsequently, candidate genes have been identified on the Y chromosome by positional cloning. One of these genes, *RBM* (an acronym for RNA-binding motif), encodes a germ cell-restricted nuclear protein (1, 2). Y chromosome *RBM* genes are found in all mammals and are related to the X chromosome gene which encodes hnRNP G (3, 4), a member of the hnRNP family of proteins (heterogeneous nuclear ribonucleoproteins, reviewed in ref. 5). The functions of RBM and hnRNP G are unknown.

Within germ cell nuclei, RNA-binding motif protein (RBMp) is found distributed throughout the nucleoplasm and within discrete punctate nuclear structures (6) which also contain pre-mRNA splicing factors. The latter include the SR proteins, which have been shown to play a crucial role in constitutive and alternative splicing (for reviews, see refs. 7 and 8). A strong prediction of these results is that RBMp might interact with components of the splicing machinery, and that these interactions may contribute to mediating the observed subnuclear distributions. Moreover, through such interactions RBMp might operationally affect pre-mRNA splice site choices in germ cells. To test these predictions, we carried out a series of protein–protein interaction and *in vitro* splicing assays. Our results show that RBMp interacts strongly with members of the SR family of pre-mRNA splicing factors and that this interaction can have functional consequences for splicing. These results suggest that RBM is a prototypical mammalian example of a cell-specific splicing factor that functions in conjunction with SR proteins to direct splicing in germ cell nuclei.

Materials and Methods

Plasmid Constructions. Constructs encoding amino acids 85–330 of mouse RBMp in pACTII and pAS1-CYH2 vectors (Clontech) were used to screen for interactions (Fig. 1). Deletion derivatives of this region were subcloned into pACTII. Human RBM nucleotides 145-1422 were subcloned into pAS1. SRp20 deletion plasmids were subcloned into pAS1. Mouse RBM protein (mRBMp)-glutathione *S*-transferase (GST) fusion (pmGST6) and mRBMp-thioredoxin fusion (pmHIS2) plasmids contain amino acids 113–232 subcloned into pGEX-5X and pET32a, respectively. SRp30c and 9G8 cloned in pACT were gifts of J. Venables and I. Eperon (University of Leicester, England).

Two-Hybrid Assays. Yeast transformations and assays were performed as described previously (9).

GST Pull-Down Assays. These were performed as described elsewhere (9). Bacterial proteins were made in *Escherichia coli* BL21 cells transformed with pGEX-5X1 and pMGST6. *In vitro* translated protein was prepared in a rabbit reticulocyte-coupled transcription and translation system (Promega) using either mouse RBM in pBluescript as a substrate and T3 RNA polymerase, or a PCR-derived template for SRp20 or the mRBM interaction region and T7 RNA polymerase. The primers used for PCR were 5′-TAATACGACTCACTATAGGGAGACCACATGGATGATGTATATAACTATCTATTC (T7 promoter for pACTII); and 5′-CATCATCATACCCATCTGTTGGCCACCTGTCATCGATGCCATCTCTATCACGTCCTCCAAG (mRBMp 3′ primer, extra methionine residues) and 5′-CATCATCATCATCATCATCATCATCATCATAGATCTGCGACGAGGTGGAG (SRp20 3′ primer, extra methionine residues) using pmRBMpACT and pSRp20pACT as templates. Luciferase was made using luciferase T7 control DNA (Promega) and T7 RNA polymerase. To measure the amount of *in vitro* translation product present before pull-down, 2.5 μl of a 100-μl volume was run in parallel with the protein pulled down (resuspended in a volume of 20 μl of 1× SDS running gel buffer). ^{35}S-labeled *in vitro* translated (IVT) proteins were separated by SDS-PAGE and visualized by fluorography.

Nuclear and S100 Extract Preparation and Treatment. HeLa cell nuclear extract (NE) and cytoplasmic S100 extract were prepared as described previously (10). One hundred microliters of NE was first incubated in 0.76 mM ATP, 24.8 mM creatin phosphate, and 3.2 mM $MgCl_2$. Either thioredoxin alone or

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Abbreviations: RBM, RNA-binding motif; GST, glutathione *S*-transferase; RRM, RNA recognition motif; mRBMp, mouse RBM protein; hRBMp, human RBM protein; mRBMIR, mouse RBM interaction region; NE, nuclear extract; IVT, *in vitro* translated; MN, micrococcal nuclease; ESE, exonic splicing enhancer; hnRNP, heterogeneous nuclear ribonucleoprotein.

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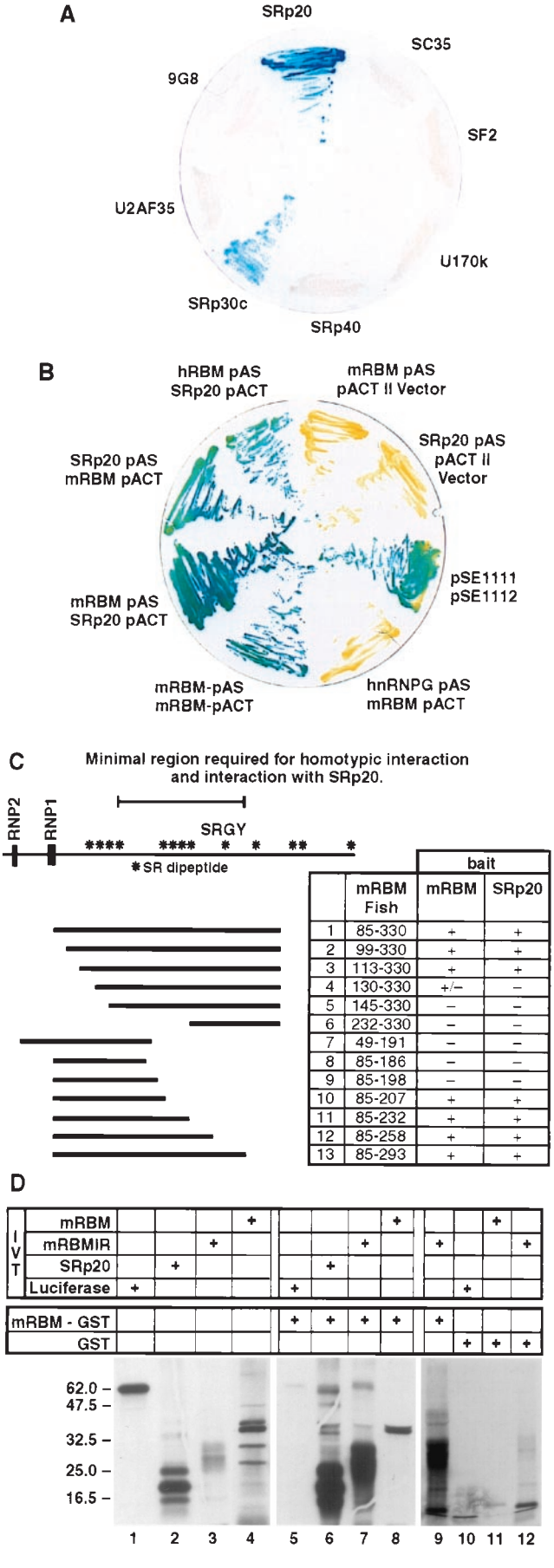


thioredoxin fused to the mRBMp interaction region was expressed in *E. coli* BL21 cells transformed with pET32 and pmHIS2 and bound to nickel agarose (Qiagen) according to the manufacturer's recommendations. Nickel agarose beads (20 μl) containing bound protein were saturated by preincubation with cytoplasmic S100 fraction for 20 min and then incubated with NE for 2 h with rolling agitation. The supernatant was recovered and used in splicing assays (see below).

Beads were washed twice in standard dialysis buffer (which contains 100 mM KCl), proteins bound to the beads were eluted by boiling in SDS sample buffer, and one-fifth of the elution was loaded on an SDS-polyacrylamide gel (corresponding to 20 μl of the initial NE). An aliquot, equivalent to 2 μl of control NE, was analyzed in parallel. After electrophoresis, proteins were transferred by Western blotting and probed with monoclonal antibodies specific for individual SR proteins 9G8, SC35, and SF2/ASF, or the SR family (mAb 10H3, as previously described in ref. 11), or U1-70K and TATA-binding protein (from Lazlo Tora, IGBMC, Illkirch, France).

***In Vitro* Splicing Assays.** These were performed as described previously (11) using nuclear or splicing competent S100 extracts, either with or without a prior depletion step (see above). Splicing competent extracts specific for individual SR species were reconstituted by mixing 9 μl of S100, 3–4 μl of 15–45% ammonium sulfate nuclear fraction, and one SR species (9G8 or ASF/SF2, 250 ng) or SRp20 (500 ng) as described previously (11). These competent extracts were depleted/mock depleted with beads as for the NEs (see above).

Add-back splicing assays were performed by addition of individual SR species to the extracts after depletion with mouse RBM interaction region (mRBMIR)-coated beads (see legend to Fig. 4).

Quantitations of splicing assays were made using a Bas-2000 BioImager (Fuji). The basal splicing efficiency was calculated as the ratio of mRNA:pre-mRNA (the exon 1 band is negligible and the intron is undetectable because it contains only three C residues). Three different efficiencies per construct were calculated (control, NE depleted by mRbm interaction region, and NE mock depleted by thioredoxin). The specific splicing inhibition by the mRBM interaction region was calculated as 100 − (ratio of percentage splicing efficiency in the depleted extract and in the mock depleted extract multiplied by 100).

Results

Mouse RBM Interacts both Homotypically and Heterotypically with Members of the SR Family of Pre-mRNA Splicing Proteins. Because human RBMp has a similar subnuclear distribution to members of the SR family of pre-mRNA splicing factors (6), we tested for direct physical interactions between these proteins using the yeast two-hybrid system (12). Initially, we used mRBMp as bait (cloned in pAS) and a panel of different SR proteins as fish

Fig. 1. RBMp interacts with both SR proteins and itself. (*A*) Interactions between mRBMp and a panel of SR proteins. (*B*) Heterotypic and homotypic interactions of RBM proteins. (*C*) Mapping of the minimum regions of mRBM required for homotypic and heterotypic protein interactions. mRBMp is shown as a cartoon with the positions of SR dipeptides shown as asterisks, and the position of the SRGY tetrapeptide (considerably expanded in hRBM) is indicated. The position of the RRM (RNP1 and RNP2 motifs) is indicated. The contents of each of the deletion constructs used to map the interaction region (cloned in pAS: mRBM fish) is shown as a solid line, alongside its amino acid content and whether it interacted (+) or not (−) with mRBM bait (amino acids 85–330 cloned in pACT) or SRp20 bait (full-length SRp20 cloned in pACT). Interactions were scored as positive if multiple (at least 10) individual colonies gave *LacZ* activity. (*D*) Agarose beads coated in the mRBMp interaction region (but not GST alone) pull-down IVT mRBM and SRP20 proteins.

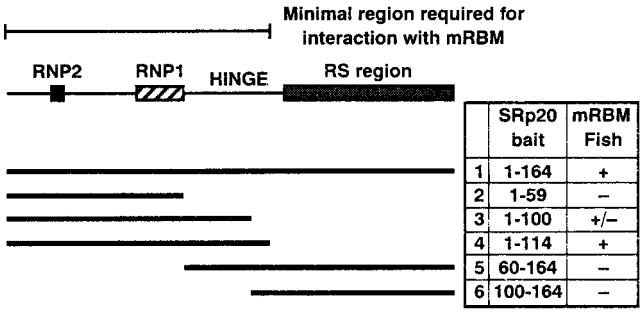
(cloned in pACT). Among these, SRp20 and SRp30c showed a clear strong positive interaction with mRBMp (Fig. 1*A*). The interaction between mouse RBMp and SRp20 occurs in both combinations of fish and bait (Fig. 1*B*). Since important protein–protein interactions are likely to be evolutionarily conserved, we tested whether there is any interaction between human RBMp (hRBMp) and SRp20. Although the amino acid sequence of SRp20 is identical between mouse and humans (13, 14), mRBMp and hRBMp are only 46% identical and 66% similar (15). In particular, the hRBMp has an extended repeat sequence called the SRGY box (rich in serine/arginine/glycine/tyrosine), which is reduced to a single SRGY tetrapeptide repeat in the mouse. Despite this relatively low level of sequence identity, hRBMp also interacts with SRp20 (Fig. 1*B*).

Since proteins are often involved in important homotypic interactions as well as heterotypic interactions, we next tested whether mRBMp is able to interact with itself using mRBM cloned as both bait and fish. These experiments indicated that mRBMp molecules do indeed show a strong homotypic interaction (Fig. 1*B*) which was comparable to that obtained with our positive control (after cotransformation with two plasmids pSE1111 and pSE1112 which encode known interacting proteins SNF4 and SNF1; Fig. 1*B*). We detected no interaction between mRBMp and the closely similar mouse hnRNPG protein and neither the pAS clones of mRBMp nor SRp20 gave any *LacZ* activity when cotransformed with the pACTII vector alone (Fig. 1*B*).

We mapped which parts of the mRBMp molecule were responsible for mediating the respective protein–protein interactions using a series of deletion constructs (Fig. 1*C*). Surprisingly, mRBMp sequences required for both the homotypic and the heterotypic interaction were almost identical. Both interactions required a sequence of 95 amino acids, although in the case of the homotypic interaction a weak interaction was observed without 17 N-terminal residues. Hence, mRBMp contains two largely overlapping (and possibly identical) protein interaction motifs capable of mediating interactions with at least two distinct primary amino acid sequences.

We next tested whether we could reproduce these interactions *in vitro* by assaying pull-down of [^{35}S]methionine-labeled IVT protein by agarose beads coated in the mRBMIR fused to GST (Fig. 1*D*). The amount of each of these IVT proteins originally present in the mixture before pull-down is shown in lanes 1–4. Although barely any luciferase protein was detected in the bound fraction (lane 5), both SRp20 (lane 6) and full-length mRBMp (lane 8) were quantitatively recovered. The mRBMIR was also quantitatively recovered: since this protein did not contain an RNA recognition motif (RRM) the observed interaction cannot be mediated by a linking RNA molecule. Beads coated in GST alone pulled down no IVT protein (Fig. 1*D*, compare lane 9 with lanes 10–12). We conclude that the interactions we observed in the yeast two-hybrid system are likely to represent *bona fide* molecular interactions.

SRp20 Interacts with mRBMp Independently of Its RS Domain. SR proteins frequently interact with each other and with other proteins by means of their RS domains. SRp20 has a C-terminal RS domain and an N-terminal RRM linked by an internal "hinge" region (Fig. 2). We mapped the interaction domain using deletion derivatives of SRp20 in a two-hybrid assay. Sequences from both the RRM and the hinge region of SRp20 are required to support an interaction with mRBMp, although neither of these two regions by themselves was able to support a detectable interaction. The RS domain alone was not able to mediate an interaction with mRBMp (interaction test 6 was negative), nor was it required for the interaction (interaction test 4 was positive, in which SRp20 lacked the RS region).



	SRp20 bait	mRBM Fish
1	1-164	+
2	1-59	–
3	1-100	+/–
4	1-114	+
5	60-164	–
6	100-164	–

Fig. 2. The SRp20 interaction region does not include the RS region. The SRp20 protein is shown as a cartoon with the relative positions of the RRM (RNP1 and RNP2 motif), hinge region, and RS region indicated. A series of deletion constructs were constructed in pACT (baits) and tested for interaction with mRBMp cloned in pAS (fish: amino acids 85–330) as for Fig. 1*C*.

The mRBM Interaction Region Interacts with a Functionally Active Population of SR Proteins from NEs. SR proteins are known to be activators of pre-mRNA splicing (8, 16–18). The protein interaction results described above suggest the hypothesis that RBMp has a role in controlling germ cell-specific pre-mRNA splicing, and that this role is mediated at least in part by an interaction with SR proteins such as SRp20/SRp30c. If RBM protein interactions are limited to SR proteins among the splicing machinery, mRBMIR should interact with SR proteins in NEs, and depletion of these extracts with an affinity matrix containing mRBMIR should inhibit the function of only SR proteins in pre-mRNA splicing. To test this, we coated agarose beads with equal amounts of either mouse mRBMIR fused to thioredoxin or with thioredoxin alone (Fig. 3*A*) and preincubated these with NEs competent for splicing. After removing the beads by centrifugation, bound protein was eluted. Samples of total NE (T, which shows the level of SR proteins in the untreated NE), supernatant (U, showing the proteins not bound by the beads), and bound protein (B, bound by the coated beads) were separated by SDS-PAGE and Western blotting. Filters were then probed for specific SR proteins (Fig. 3*B*). Probing with monoclonal antibody 10H3, which is reactive with members of the SR protein family, showed that SRp20, SRp30, SRp40, and (reproducibly less) SRp55 were consistently recovered by the beads coated in the mRBMIR but not SRp75 (lanes 2 and 3). None of these SR proteins were bound by the thioredoxin beads alone (lanes 4 and 5). For the individual SRp30 species, we observed that ASF/SF2, 9G8, and reproducibly in greater quantities SC35 were bound to the mRBMIR beads, but not to thioredoxin alone (lanes 6–20).

We calculated the amount of SR proteins pulled down by the mRBMIR-coated beads as only 10% of the SR proteins in the original NE. This calculation was done by comparing the level of SR proteins detected in the pulled down material with that in the total NE (compare lanes 1 and 3; lanes 6 and 8; lanes 11 and 13; and lanes 16 and 18), accounting for the relative amounts of protein loaded in each well of the gel (see *Materials and Methods*). This reproducible 10% depletion is reflected in the observation of only a very slight decrease in SR protein concentration seen in the unbound versus total SR protein.

Since some SR proteins did not interact with mRBM in the two-hybrid assay, we considered the possibility that mRBMIR binding may be due to either an RNA link or a protein link. To test this first possibility, we pretreated the NE with micrococcal nuclease (MN) before testing the interaction with mRBMIR (Fig. 3*C*). MN treatment did not modify the pattern of the bound SF2/ASF protein, nor of the other SR protein species detected with monoclonal antibody 10H3 (data not shown). We performed additional experiments to assess whether other nuclear

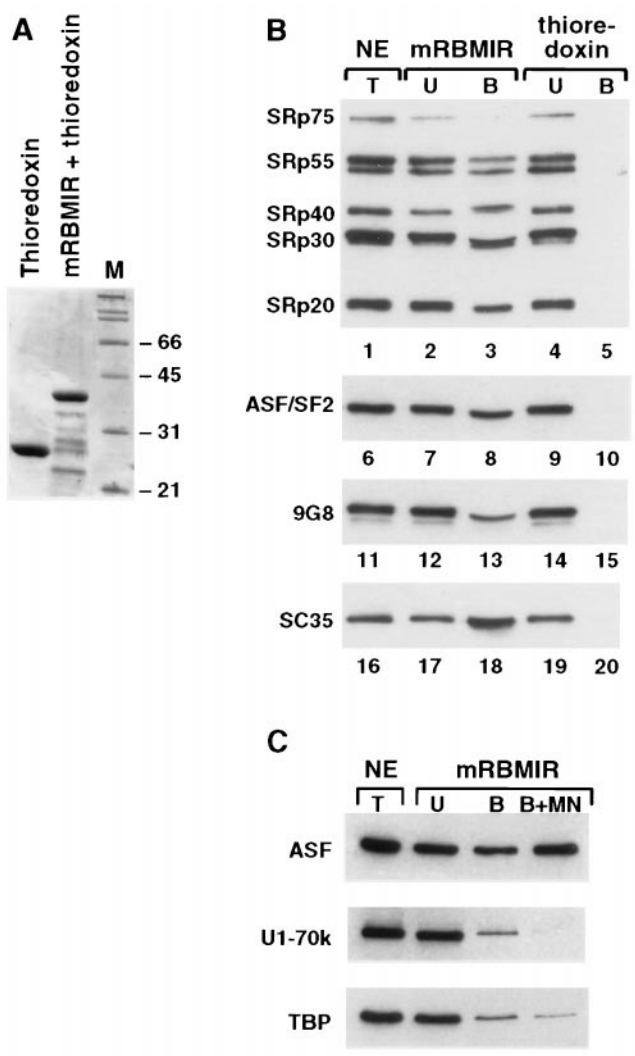


Fig. 3. RBM interacts with SR proteins in NEs. (*A*) Samples of thioredoxin and mRBMIR were attached to agarose beads. To show equal loading protein was eluted, separated by SDS-PAGE, and stained with Coomassie blue. (*B*) SR proteins pulled down from NEs by the respective beads were analyzed by SDS-PAGE followed by Western blotting, and probing with mAb 10H3 (lanes 1–5); or for single SR species ASF/SF2 (lanes 6–10), 9G8 (lanes 11–15), or SC35 (lanes 16–20). Note that the presence of high amounts of mRBMIR in lanes 3, 8, 13, and 18 alter the mobilities of the SRp40 and SRp30 protein. (*C*) Pull-down of ASF/SF2 is RNA independent, while U1-70K or TATA-binding protein are pulled down very weakly by linking RNAs.

proteins or spliceosomal components were bound by mRBMIR. All nuclear proteins are not pulled down as a complex with mRBMIR since the TATA-binding protein (TBP) was not bound efficiently. A similar result was obtained with U1 small nuclear RNP (detected by an antibody directed against U1-70K), and the very weak binding observed became undetectable after MN treatment. Although it is not an SR protein, U1-70K does contain an RS-rich domain (19).

The above results suggest that RBM specifically interacts with SR proteins, and that through this interaction may affect pre-mRNA splicing in germ cell nuclei. However, in our *in vitro* pull-down system, only a small fraction of the total SR proteins in the NE were bound. To test whether this small depletion could impact on pre-mRNA splicing, we utilized a panel of specific pre-mRNAs derived from the adenovirus E1A pre-mRNA. One of these pre-mRNAs contains endogenous E1A sequences (Sp1 wt) and is constitutively spliced in NEs, whereas each of the others contain an SR-specific exonic splicing enhancer (ESE) required for splicing which needs one specific SR protein for activity (11). We tested pre-mRNAs dependent on the SR proteins 9G8, SRp20, and ASF/SF2. The functional activity of different SR proteins in the depleted (ΔmRBMIR) or mock-depleted (Δthioredoxin) extracts was tested by assaying the splicing of these specific pre-mRNA substrates in an *in vitro* splicing assay.

The results of these experiments (Fig. 4*A*) show clearly that splicing of each of the pre-mRNAs was inhibited by the mRBMIR (as evidenced by an accumulation of pre-mRNA relative to mRNA in lanes 2, 5, 8, and 11). In each case, splicing was not inhibited by preincubation with the thioredoxin beads (compare control extracts from untreated NE for each pre-mRNA in lanes 1, 4, 7, and 10 with splicing in NE preincubated with thioredoxin-coated beads in lanes 3, 6, 9, and 12). Quantitation of the *in vitro* splicing data are shown in Fig. 4*B*. Within the range of experimental variation, the splicing of each of the SR protein-dependent pre-mRNAs was inhibited to the same level (by around 50%). In contrast, the splicing of the pre-mRNA containing the wild-type E1A sequence was inhibited to a much lower extent (25%). This suggests that splicing of the ESE-dependent pre-mRNAs was inhibited as a result of a 2-fold effect: both a depletion of SR proteins required for constitutive splicing and a depletion of SR proteins involved in ESE-dependent activated splicing.

We tested whether the readdition of individual SR proteins alone would be enough to restore splicing activity to a mRBMIR-depleted NE in an add-back experiment. Using both 9G8- and SRp20-dependent pre-mRNAs (Fig. 4*C*, *left* and *right panels,* respectively), the results clearly show that the splicing capacity of the mRBMIR-depleted extracts (lanes 3 and 9) is restored by the addition of the specific SR protein 9G8 or SRp20, respectively (lanes 4 and 11, respectively). Also as predicted, addition of SR proteins other than those that specifically bind to the ESE resulted in a much reduced activation of splicing (lanes 5–7, 10, 12 and 13). However, the SRp20-dependent ESE was almost completely rescued by the addition of the similar 9G8 protein which may recognize some SRp20-specific targets in NEs (11).

The depletion and add-back experiments indicated that mRBMIR binds a number of SR proteins in NEs and that as a result these become limiting for splicing. However, NEs contain each of the SR proteins and other splicing components, and therefore interactions with individual SR protein species may be either direct or indirect (i.e., protein bridged). Indeed, the two-hybrid data suggested that only a subset of SR proteins interact with RBM. To distinguish between these possibilities, we carried out similar depletion and add-back experiments in S100 cytoplasmic extracts, which do not support efficient splicing unless they are complemented by an individual SR protein (Fig. 4*D*, compare lanes 2, 6, and 10 with lanes 3, 7 and 11). The SR-complemented S100 extracts (which contain more limiting concentrations of all splicing components) were depleted using mRBMIR-agarose beads as for the NE and then assayed for splicing activity. As shown previously for the NE, we observed that the mRBMIR depletion results in a 2- to 2.5-fold reduction of splicing activity for the specific 9G8-, SRp20-, and ASF-dependent transcripts (compare lanes 4, 8, 12 to lanes 3, 7, and 11, respectively). Importantly, add-back complementation with each of the specific SR species resulted in a significant restoration of splicing activity (lanes 5, 9, and 13), indicating that the alteration of the reconstituted extracts is primarily targeted to each individual SR protein that complements the extract. Since these S100 extracts only contain single SR protein species, interactions with RBM must be direct and not bridged by other SR species. Hence, ASF/SF2 is likely to interact directly with

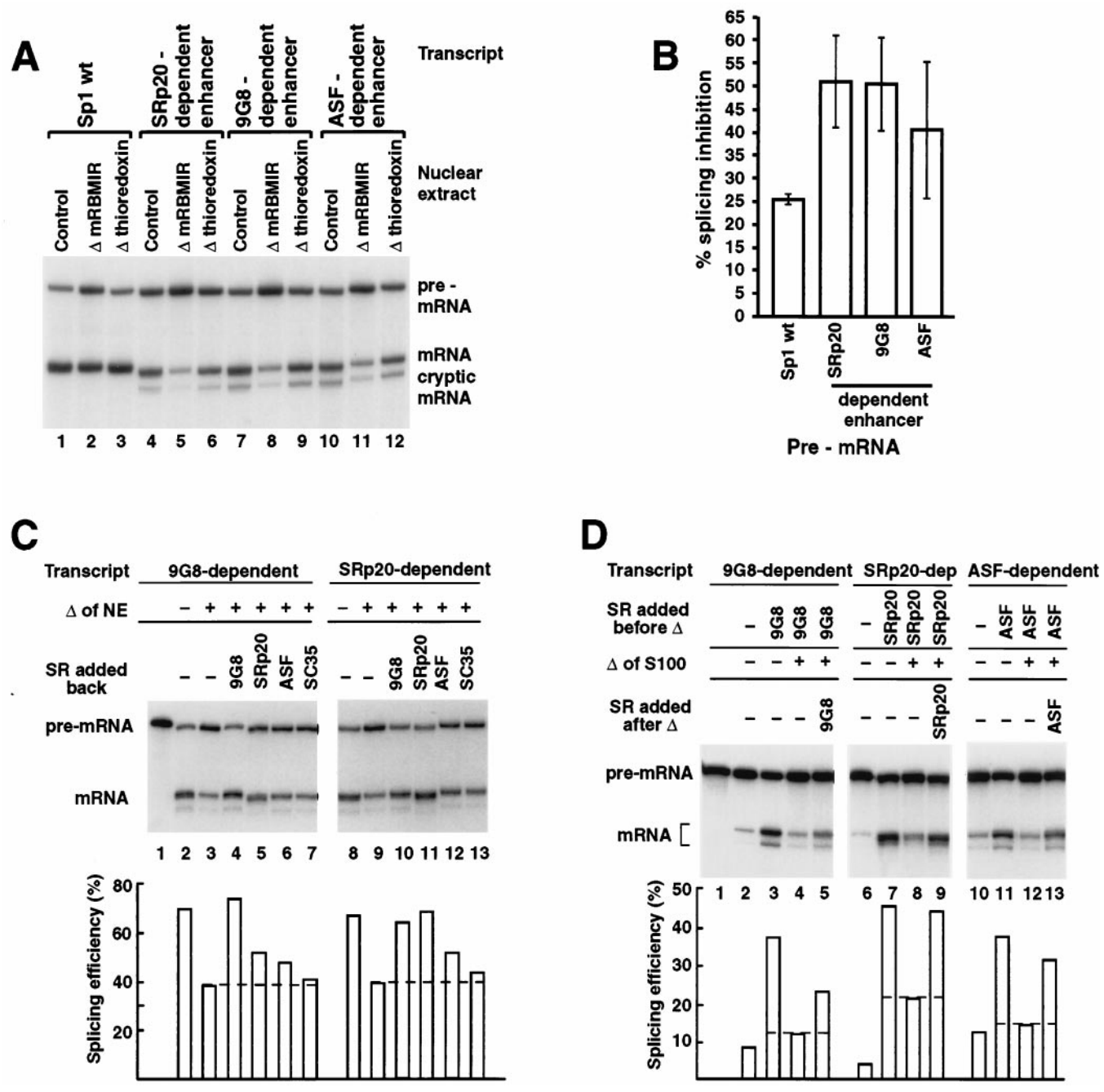


Fig. 4. mRBMIR interacts with a functionally active population of SR proteins *in vitro*. (*A*) Depletion of NEs with beads coated with mRBMIR but not thioredoxin alone inhibited the splicing of a panel of SR protein-dependent pre-mRNA substrates. (*B*) Quantitation of data from independent pre-mRNA splicing assays showing the percentage of splicing inhibition for each substrate. In one of the experiments, there was slight RNA degradation in the ASF/SF2 sample, resulting in the increased size of the error bar. (*C*) Add-back of individual SR proteins to mRBMIR-depleted extract can restore splicing of specific substrates. The individual SR species 9G8, ASF/SF2, or SC35 (150–200 μg) or SRp20 (400 μg) were added to the depleted extract. Pre-mRNA alone is shown in lane 1. The percentage of splicing efficiency of each of the pre-mRNA substrates is shown underneath. (*D*) 9G8, SRp20, and ASF/SF2 interact specifically with mRBMIR in an S100 extract. The same amount of each specific SR species that was added initially was readded after depletion in the add-back experiments. Pre-mRNA alone is shown in lane 1. The percentage of splicing efficiency in each lane is shown underneath.

mRBMIR in mammalian nuclei even though it does not detectably interact in the yeast two-hybrid system.

Discussion

In this article, we demonstrate molecular interactions between RBMp and members of the SR family of splicing proteins. At least two SR proteins directly interact with RBMp based on two-hybrid analysis (SRp20 and SRp30c) and GST pull-down assays (SRp20). We have mapped the mRBMIR with SRp20. In NEs, this protein interaction domain also interacts with 9G8, the other SRp30s (SC35, SF2/ASF), and SRp40, but has a much weaker association with SRp75. A comparison of the data of Figs. 3 and 4 indicates that a depletion of no more than 10% of SR proteins from the NE by mRBMIR results in a large reduction of splicing activation (from 25% for the constitutive to 50% for the enhancer-dependent splicing). An important impli-

cation of this result is that the SR proteins that are most accessible to mRBMIR in NEs are also those which are primarily involved in the *in vitro* splicing reaction.

In cell extracts, these protein interactions could be either direct or indirect (mediated by other proteins), but they are not bridged by RNA (they are MN insensitive). However, we favor the hypothesis that they are direct since depletion with mRBMIR prevents SF2/ASF-mediated splicing activation of an S100 extract, even though SF2/ASF does not interact with mRBMIR in the two-hybrid assay. Some protein interactions might not be reproduced efficiently in a yeast two-hybrid system because of differences in protein secondary structure/phosphorylation status in yeast compared with mammalian cell extracts. Alternatively the binding of given SR species in nuclear or S100 extracts could be facilitated by protein partners. For instance, it has been shown that SF2/ASF is bound to a protein called p32 (20), and this could dramatically influence interactions with RBMp. SR proteins are known to frequently interact with each other through their RS-rich regions (7, 21). Although RBMp does contain some RS dipeptides (Fig. 1*C*), it does not bind to SRp20 through interactions involving the SRp20 RS domain, indicating that SR proteins may be involved in a more complex than expected set of protein–protein interactions. Since RBMp interacts with SRp20 via the SRp20 RRM, it might override the SRp20's intrinsic RNA sequence specificity, thereby tethering SRp20 to pre-mRNAs that it would not normally have affinity for. Moreover, this would leave the RS region of SRp20 free to interact with other splicing factors.

Do other splicing factors such as SR-like or non-SR proteins also interact with RBM? The depletion/add-back experiments indicate that *in vitro* the only proteins RBM interacts with that are functionally required for splicing of our panel of pre-mRNAs are SR proteins, and RBM does not appreciably bind any constitutive splicing factors. Other potential interacting partners of hRBM are SAM68 and T-STAR (22) and SAM68 has been implicated in long-range branch point selection in the tropomyosin pre-mRNA (23). The SR proteins SRp30c and 9G8 were pulled out in a two-hybrid screen with hRBM, along with the SR-like protein Tra2β (30). Taken as a whole, these results suggest that RBMp complexes with a number of regulators of pre-mRNA splicing, with SR proteins as an important constituent (compare *Drosophila dsx* regulation described in refs. 24 and 25).

An important focus of current research concerns the specificity and function of SR proteins in splicing. SR proteins are ubiquitously expressed in many different cell types, whereas splicing patterns are frequently tissue and even cell-type specific (26). Hence, there is no obvious correlation between the expression of specific SR proteins and the execution of specific pre-mRNA processing pathways. Although SR proteins can be the only modulators involved in controlling single alternative splicing reactions, a possible way in which specificity could be obtained is if cell- or tissue-specific splicing cofactors operate with them. Although cofactors have been identified (20, 27, 28), how these might operate to control cell-specific splicing is largely unknown. Since RBMp is only expressed in germ cells, it is a clear (and among the first mammalian) candidate for a cell-type-specific regulator of alternative pre-mRNA splicing. Since RBMp has its own RRM, it is likely to bind directly to target RNAs and perhaps influence the splicing of adjacent exons. In principle, this could result in either exon inclusion as a result of splice site activation by recruited SR proteins (compare ref. 10) or exon exclusion by recruited splicing repressors such as RSF1 (29). Alternatively, RBMp may replace the function of a protein required for splicing but not expressed in germ cells. In either case, a prediction of this article is that important pre-mRNA splicing pathways will be disrupted in some infertile men.

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