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Tra2α and hnRNP K might be Functional Partners of Rbm for Regulation of RNA Processes during Spermatogenesis

Jungmin Lee, Euisu Kim, Sung Key Jang[1] and Kunsoo Rhee*

School of Biological Sciences, Seoul National University, Seoul 151-742, Korea;
[1]Department of Life Science, Pohang University of Science and Technology, Kyungbuk 790-784, Korea

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***Rbm* is a male infertility gene located in the *AZFb* region of the Y chromosome. Expression pattern of *Rbm* indicates that Rbm is critical for early phase of male germ cell development. It shares strong structural homology with *hnRNP G*, suggesting a function as an RNA processing factor. In order to gain a clue on the molecular mechanisms of Rbm on male germ cell development, we examined interactions of Rbm with selected proteins in yeast. The results revealed specific interactions between Rbm, hnRNP K and Tra2α. These results suggest that hnRNP K and Tra2α may be functional partners of Rbm in male germ cells. We propose a model in which hnRNP K may play a role as a platform for Rbm and Tra2α.**

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). *Rbm* is a male infertility gene located in the *AZFb* region of the Y chromosome (Ma et al., 1993). Cumulative reports revealed frequent observation of micro-deletions at *Rbm* loci among infertile men (Foresta et al., 2001). Furthermore, Y^{dl} mice which lacked most of *Rbm* copies revealed defects in spermatogenesis (Mahadevaiah et al., 1998). These observations indicate that Rbm is a male infertility gene critical for spermatogenesis. Recently, we reported that *Rbm* is expressed in early phase of spermatogenesis, but not in mid-pachytene spermatocytes or subsequent stages of differentiation, including haploid germ cells, indicating that Rbm is critical for early phase of male germ cell development (Lee et al., 2004). 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 may function as an RNA processing factor (Delbridge et al., 1999; Mazeyrat et al., 1999).

*To whom correspondence should be addressed.
Tel: 82-2-880-5751, Fax: 82-2-873-5751
E-mail: rheek@snu.ac.kr

The proteins named heterogenous nuclear ribonucleoproteins or hnRNPs are group of proteins that conduct posttranscriptional processing of primary transcripts. About twenty hnRNPs have been identified and form complexes for specific functions in RNA processing. hnRNP K is located in multiple subcellular compartments including the nucleus, cytoplasm and mitochondria, and is involved in a host of processes that comprise gene expression, such as chromatin remodeling (Denisenko et al., 2002), transcription (Michelotti et al., 1996; Du et al., 1998), pre-mRNA splicing (Expert-Bezancon et al., 2002), mRNA export (Michael et al., 1997) and translation (Ostareck et al., 1997). The involvement of hnRNP K in these various processes probably reflects interactions of its multiple domains with diverse molecular partners including DNA (Ito et al., 1994; Ostrowsky et al., 1994), RNA (Swanson et al., 1999), protein kinases (van Seuningen et al., 1995; Schullery et al., 1999; Wadd et al., 1999; Ostareck-Lederer et al., 2002), and a GTP/GDP exchange factor (Hobert et al., 1994; Bustelo et al., 1995). In fact, it was suggested that hnRNP K bridges signal transduction pathways for RNA processes (Ostrowski et al., 2000; 2001). It was also proposed that hnRNP K serves as a platform to dock otherwise separated molecules (Bomsztyk et al., 1997; Ostareck-Lederer et al., 1998).

Transformer-2 (Tra2) in *Drosophila* functions as a splicing activator for sex determination and sex-specific neural splicing and as a repressor of its own splicing in the male germ line (Mattox et al., 1990; 1991). Several mammalian *Tra2* homologues, including *Tra2α* and *Tra2β*, have been identified (Dauwalder et al., 1996; Matsuo et al., 1995; Segade et al., 1996; Beil et al.,

1997). A recent study showed that mammalian Tra2α can replace *Drosophila* Tra2 in activation of the *dsx* enhancer and bind preferentially to the purine-rich element within this enhancer, suggesting that its RNA binding specificity is similar to that of *Drosophila* (Lynch and Maniatis, 1995). Tacke et al. (1998) demonstrated that these two Tra2α proteins have indistinguishable RNA binding specificities. Neither functioned in constitutive splicing *in vitro*, but both activated enhancer-dependent splicing in a sequence-specific manner (Tacke et al., 1998). Rbm was shown to interact with Tra2β and to inhibit RNA splicing activities *in vitro* through sequestering splicing factors from nascent RNA (Venables et al., 2000; Elliott et al., 2000). These results suggested that Rbm may be involved in testis-specific splicing events during spermatogenesis (Venables et al., 2000).

In the current study, we examined specific interaction of Rbm with several candidate proteins. The results show that hnRNP K and Tra2α interact specifically with Rbm suggesting possible functional mechanisms of Rbm in RNA processing during spermatogenesis.

Materials and Methods

Construction of the fusion genes

The mouse *Rbm* cDNA was obtained by screening a testis cDNA library as described previously (Lee et al., 2004). *Tra2α* and the *hnRNP* constructs were generated as described previously (Kim et al., 2000; Seong et al., 2002). Wild type and mutant constructs of *Rbm*, *hnRNP K* and *Tra2α* were subcloned into *pGBT9* and *pGAD10* for yeast two-hybrid interaction assay.

Yeast two-hybrid interaction

Yeast two-hybrid interaction experiments were performed as described previously (Kim et al., 2000). In brief, constructs of the fusion proteins with the *GAL4* DNA-binding domain (BD) and the *GAL4* transcriptional activation domain (AD) were co-expressed in the yeast strain CG-1945 (*MATa, ura3-52, his3-200, lys2-801, ade2-101, trpl-901, leu2-3, 112, gal4-542, gal80-538, cyh^{r}2, LYS2::GAL1$_{uas}$ −GAL1$_{tata}$ −HIS3, URA3::GAL4$_{17mers(x3)}$ −CYC1$_{tata}$ −lacZ*). CG-1945 encodes *HIS3* and *LacZ* as reporters and has *trp1, leu2, and cyh^{r}2* as marker genes for transformant selection (Feilotter et al., 1994). Double transformants containing plasmids of the *pGAD10* and *pGBT9* were selected by growing yeast cells on the SD synthetic plates lacking leucine and tryptophan. For the β-galactosidase activity assay, yeast colonies were transferred to a filter paper after which liquid nitrogen was applied to the filter to break the cells open. The filter paper was then placed on top of a second filter paper presoaked with Z-buffer (60 mM Na_2HPO_4, 40 mM NaH_2PO_4, 10 mM KCl, 1 mM $MgSO_4$) containing 0.82 mM 5-bromo-4-chloro-3-indolyl-β-D-galactopyranoside (X-gal). The filters were kept at 30°C for several hours. A blue color change indicated β-galactosidase activity in the yeast cells. Alternatively, viability of the transformed yeast cells on the SD synthetic plates lacking leucine, tryptophan, and histidine was evaluated to determine protein-protein interaction.

Results

Interaction of Rbm with hnRNP K in yeast

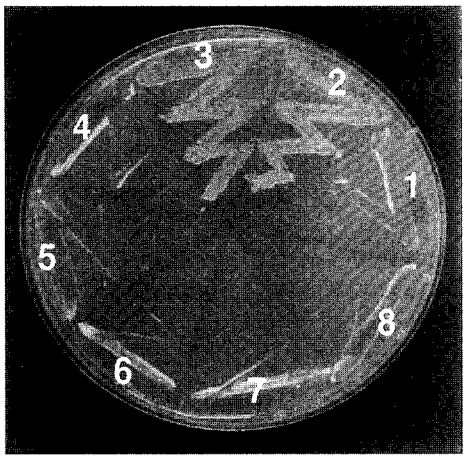
Yeast two-hybrid analysis was carried out to identify proteins that interact with Rbm. We initially tested a possible interaction of Rbm, a homologue of hnRNP G, with other kinds of hnRNP proteins. The fusion gene of Rbm linked to the *GAL4* AD was co-transformed with those of several hnRNPs linked to the *GAL4* BD. The results showed that, among the hnRNPs tested, only hnRNP K revealed a positive interaction with Rbm (Table 1). Specific interaction of Rbm with hnRNP K was confirmed with survival test in the SD plate deficient of leucine, tryptophan and histidine (Fig. 1). When hnRNP K or Rbm was transformed into yeasts separately, the cells did not survive (Fig. 1). However, when BD-hnRNP K and AD-Rbm were co-transformed into the yeasts, they survived, indicating a specific interaction between the two proteins (Fig. 1). Interestingly, we could not observe positive interaction between BD-Rbm and AD-hnRNP K, suggesting that orientation between the two proteins is critical for their interaction.

Rbm contains unique structural domains such as two RNPs (ribonucleic acid particle) for RNA binding and the SRGY domain for protein-protein interaction (Fig. 2). In order to define the site for interaction with hnRNP K, we generated truncated mutants of Rbm and examined their ability to interact with hnRNP K. The results showed that Rbm$^{1-245}$ interacted with hnRNP K with somewhat reduced binding activity, while no other mutants showed specific interaction (Fig. 2). These results suggest that the C-terminal end of Rbm is important for interaction with hnRNP K.

hnRNP K has several characteristic domains such as NLS (nuclear localization sequence), SH3 (src homology

Table 1. Yeast two-hybrid interactions of Rbm with selected hnRNP proteins

	hnRNP A1	hnRNP C1	hnRNP E2	hnRNP I	hnRNP K	hnRNP L
Rbm	−	−	−	−	+	−



	BD	AD	Interaction
1	-	-	-
2	hnRNP I	hnRNP I	++
3	hnRNP K	Rbm	++
4	Rbm	hnRNP K	-
5	-	Rbm	-
6	hnRNP K	-	-
7	-	hnRNP K	-
8	Rbm	-	-

Fig. 1. Specific interaction of Rbm with hnRNP K in yeast. Fusion proteins of Rbm and hnRNP K linked to the *GAL4* DNA-binding domain (BD) or *GAL4* transcription activation domain (AD) were transformed into yeast. The cells were streaked on SD plate deficient of leucine, tryptophan and histidine. hnRNP I was used as a positive control. Specific interactions between the two fusion proteins are indicated in an arbitrary unit.

domain 3), KH (K protein homology domain) and KNS (K protein nucleus shuttling domain) (Fig. 2). In order to define the site for interaction with Rbm, we generated truncated mutants of hnRNP K and examined their interactions with Rbm. The results showed that mutants with the C-terminal end interacted specifically with Rbm (Fig. 2). These results suggest that the C-terminal region of hnRNP K is important for interaction with Rbm.

Interaction of Tra2α with Rbm and hnRNP K

It was reported that the human RBM interacts with Tra2β *in vivo* (Venables et al., 2000). In the present study, we tested whether Rbm also interact with Tra2α, another

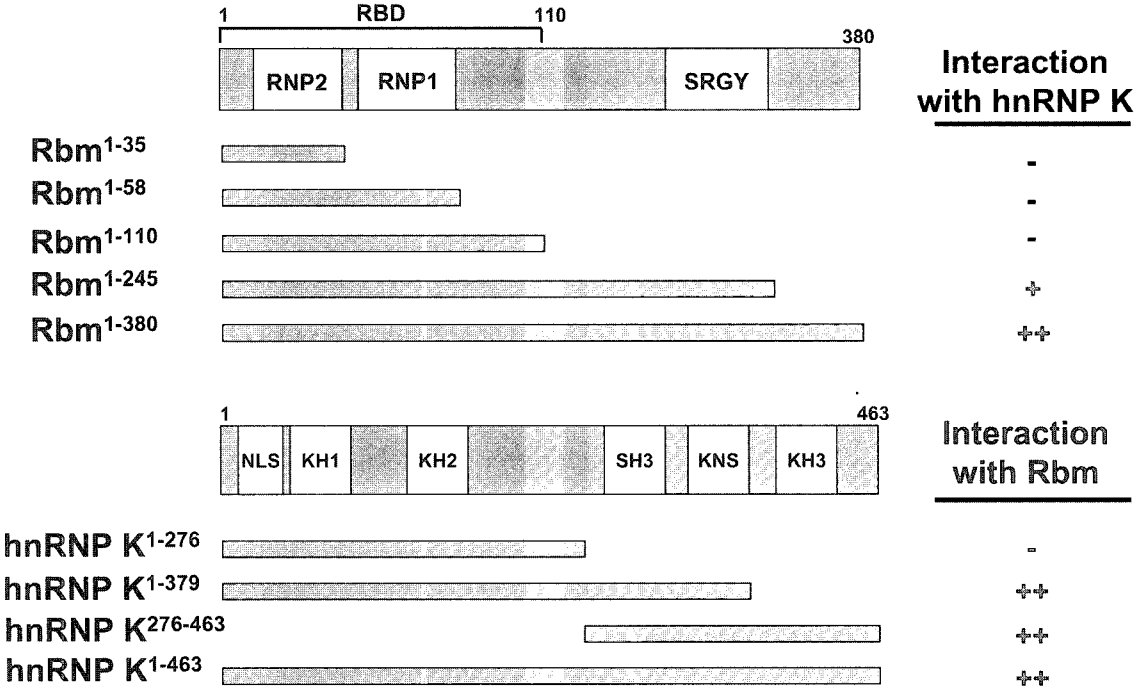


Fig. 2. Definition of interacting domains of Rbm and hnRNP K in yeast. Truncated mutants of Rbm and hnRNP K were generated and tested for their specific interactions. Intensities of yeast two-hybrid interactions are indicated on the right side in an arbitrary unit. Characteristic domains of Rbm and hnRNP K are indicated. RNP, Ribonucleic particle; RBD, RNA binding domain; SRGY, SRGY (serine, arginine, glycine, tyrosine) domain; NLS, nuclear localization sequence; KH, K protein homology domain; SH3, Src protein homology domain; KNS, K protein nucleus shuttling domain.

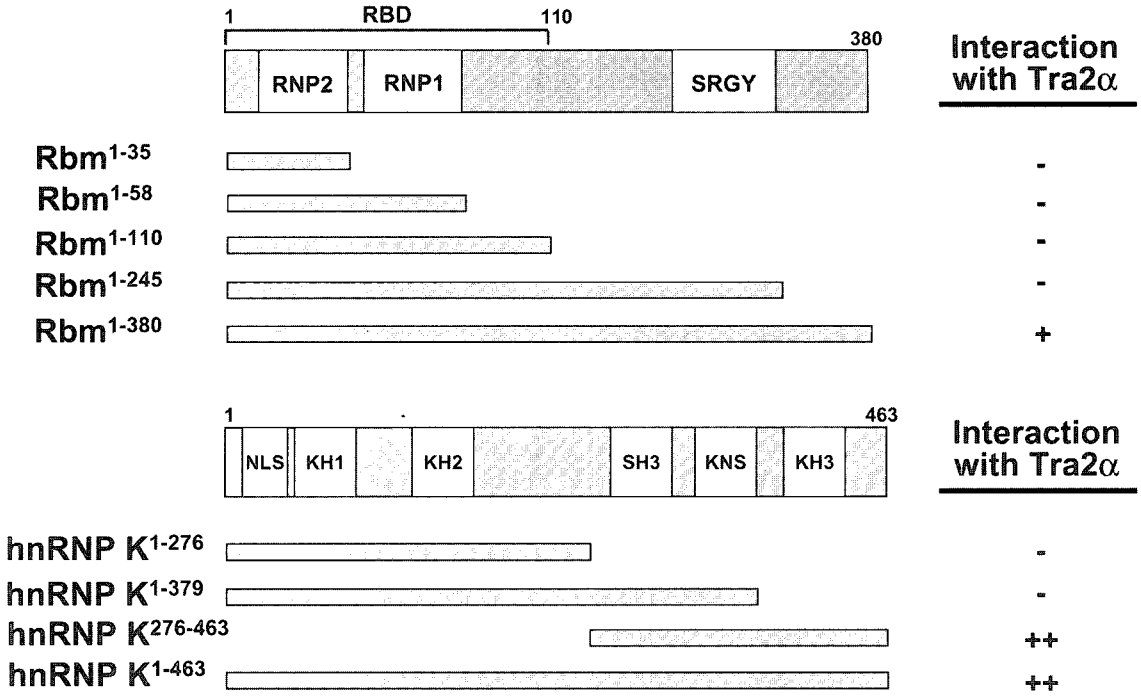


Fig. 3. Definition of interacting domains of Rbm and hnRNP K with Tra2α in yeast. Wild types and truncated mutants of Rbm and hnRNP K were tested for their specific interactions with Tra2α. Intensities of yeast two-hybrid interactions are indicated on the right side in an arbitrary unit. Characteristic domains of Rbm and hnRNP K are indicated in Fig. 2.

splicing factor homologous to Tra2β. We confirmed that both Rbm and hnRNP K interact with Tra2α in yeast (Fig. 3). In order to define binding sites for interaction with Tra2α, we used truncated mutants of Rbm for yeast two-hybrid analyses. The results showed that only intact Rbm protein but not C-terminal truncated mutants interacted with Tra2α, suggesting that the C-terminal end of Rbm is critical for interaction with Tra2α (Fig. 3). In order to define binding sites of hnRNP K for Tra2α, we used truncated mutants of hnRNP K for yeast two-hybrid analyses. The results showed that hnRNP $K^{276-463}$ interacted with Tra2α specifically (Fig. 3), suggesting that the C-terminal end of hnRNP K is critical for interaction with Tra2α.

Discussion

In this paper, we report specific interactions between Rbm, hnRNP K and Tra2α in yeast. These results suggest that hnRNP K and Tra2α may be functional partners of Rbm in male germ cells.

The results indicated that the C-terminal domain of Rbm is important for interaction with both hnRNP K and Tra2α. Since the SRGY domain is located at the C-terminal domain, it is possible that the SRGY domain functions as a binding site. In fact, it has been suggested that the SRGY domain interact with other proteins, such as Tra2β (Venables et al., 2000) and SRp30 (Elliott et al., 2000). In the case of hnRNP K, Rbm appeared to interact with the C-terminal end where SH3, KNS and KH3 domains are located. Tra2α also interacted with the C-terminal domain of hnRNP K containing minimal site of the KH3 domain. The same region was known to be important for association with Zik, a transcriptional repressor (Denisenko et al., 1996), c-Src and K protein kinase (van Seuningen et al., 1995). In fact, it was proposed that hnRNP K may serve as a docking platform that promotes molecular interactions and facilitates cross-talks among protein complexes (van Seuningen et al., 1995).

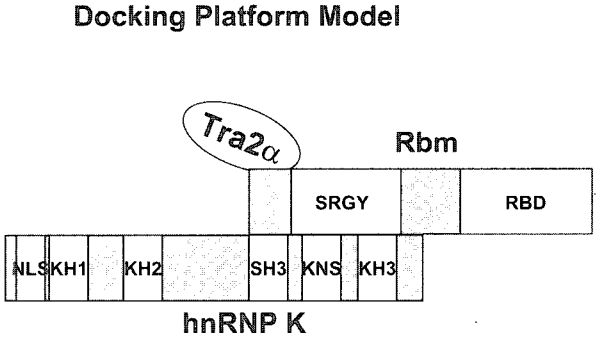


Fig. 4. A docking platform model for interactions between Rbm, hnRNP K and Tra2α. hnRNP K plays a role as a docking platform for recruitment of Rbm and Tra2α together.

Interaction of Rbm with hnRNP K and Tra2α allowed us to propose a model as in Fig. 4. It is possible that hnRNP K may play as a platform for Rbm and Tra2α. As a result, the Rbm-hnRNP K-Tra2α ternary complex is formed and controls pre-mRNA splicing. It was known that hnRNP K binds to pyrimidine-rich sequences (Matunis et al., 1992), whereas Tra2α interacts with purine-ruch sequences (Tacke et al., 1998). If the Rbm-hnRNP K-Tra2α ternary complex is formed, this complex could interact with both pyrimidine-rich and purine-rich sequences of the pre-mRNA. Obviously additional experiments should be carried out to examine this model.

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