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*Short Communication*

# Developmental regulation of expression of *Ran/M1* and *Ran/M2* isoforms of *Ran-GTPase* in mouse testis

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**ABSTRACT Two isoforms of *Ran-GTPase* have been described: *Ran/M1* and *Ran/M2*. *Ran/M2* is testis specific, whereas the *Ran/M1* isoform is also expressed in somatic tissues. Here we show that both mRNAs, differing in 35 of the 648 nucleotides included in the ORFs, are developmentally regulated during spermatogenesis. Real-time RT-PCR experiments demonstrated that the expression of *Ran/M1* and *Ran/M2* increased in pachytene spermatocytes with progressive transcript accumulation until they reached the round spermatid stage, in the seminiferous epithelium of adults. In the testis, the expression of both isoforms was found to be restricted to germ cells. An expression window from early pachytene spermatocytes to late round spermatids was detected by *in situ* hybridization.**

KEY WORDS: *Ran-GTPase, Ran/M1, Ran/M2, pachytene spermatocyte, round spermatid*

The *Ran-GTPase* genes encode for a small nuclear Ran-GTPase (Ran stands for Ras-related nuclear protein). The GTP-bound state of Ran-GTPase is mainly localized in the nucleus of non-dividing eukaryotic cells (reviewed in Dasso, 2002) and in the condensed chromosomes of mitotic cells (reviewed in Kahana and Cleveland, 1999). Ran-GTPase and its specific binding partners have been implicated in processes that are essential for cell viability, i.e., cell cycle control, nucleo-cytoplasmic transport, mitotic spindle formation and post-mitotic nuclear assembly (reviewed in Dasso, 2002). Participation of the Ran-GTPase pathway in the meiotic drive system of *Drosophila*, by segregation distorter, has been also suggested (Kusano *et al.*, 2001).

*Ran-GTPase* genes isolated from many species show a high level of conservation: yeast (Belhumeur *et al.*, 1993), plants (Haizel *et al.*, 1997), *Drosophila* (Koizumi *et al.*, 2001), fishes (Lundin *et al.*, 2000), *Xenopus* (Onuma *et al.*, 2000), chick (Trueb and Trueb, 1992) and mammals (Coutavas *et al.*, 1994). In mouse, two *Ran-GTPase* genes, *Ran/M1* and *Ran/M2*, have been described (Coutavas *et al.*, 1994) whose transcription is differentially expressed in adult tissues, being *Ran/M2* testis specific. Up to now, the presence of a homologous to mouse *Ran/M2* has been described only in the rat (Kierszenbaum *et al.*, 2002).

Recently, Zou *et al.* (2002) showed that the Ran-GTPase protein is localized in the nuclei of rat spermatocytes and spermatids and proposed that Ran-GTPase could be implicated in the organization of the manchette. In addition, Kierszenbaum (2002) and Kierszenbaum *et al.* (2002) presented a more detailed expression pattern and localization of the Ran-GTPase protein in rat spermatids. They proposed that Ran-GTPase might be involved in nucleo-cytoplasmic transport and microtubule assembly during spermiogenesis. On the other hand, Ran-GTPase protein from starfish oocytes is associated with meiotic chromosomes (Hinkle *et al.,* 2000). These data suggest an important role of this protein in gametogenesis.

Here we showed that *Ran/M1* and *Ran/M2* expression is developmentally regulated during spermatogenesis and that the *Ran-GTPase* mRNA presence in seminiferous epithelium of mouse adult testis is confined to the development of germ cells from pachytene spermatocytes to round spermatids.

### Ran-GTPase *Expression is regulated during Testis Development*

Northern-blot experiments in samples from different adult mouse tissues showed that the highest expression of *Ran-GTPase* was detected in testis (Fig. 1), confirming previous data (Coutavas *et al.*, 1994). We previously demonstrated a remarkable expression of *Ran-GTPase* in embryonic erythropoietic tissues (Lopez-Casas *et al.*, 2002) and in erythroid differentiation (Vanegas *et al.*, 2003). The expression detected in bone marrow (Fig. 1), as an adult erythropoietic tissue, is in consonance with these previous observations.

In testis, we analyzed the *Ran-GTPase* pattern of expression and regulation during development and differentiation of seminiferous epithelium cells. The results of Northern-blot experiments along the

*Abbreviations used in this paper:* ORF, open reading frame; Ran, Ras-related nuclear protein.

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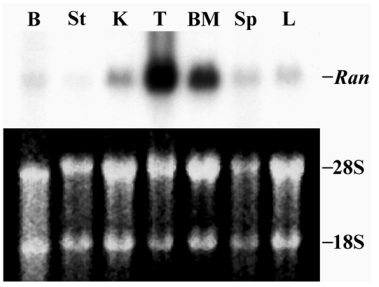


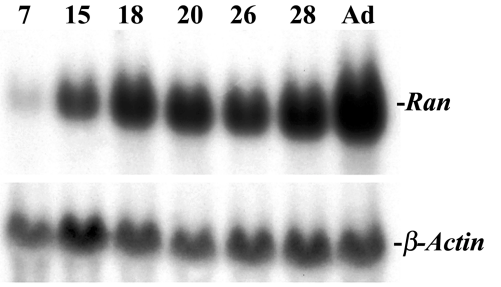


**Fig. 1. (Left) Expression of *Ran-GTPase* mRNA in different tissues.** *20 μg of total RNA isolated from adult brain (B), stomach (St), kidney (K), testis (T), bone marrow (BM), spleen (Sp) and liver (L), were hybridized in Northern-blot with a cDNA probe to the* M1 Ran-GTPase *isoform. The ethidium bromide staining as a control of loading is shown at the bottom.*

**Fig. 2. (Right) *Ran-GTPase* expression during development of the testis.** *20 μg of total RNA isolated from prepuberal (7, 15, 18, 20, 26 and 28 dpn) and adult (Ad) mouse testis were analyzed by Northern-blot using a radioactive labeled* Ran-GTPase *cDNA probe. The filter was stripped and rehybridized with a* β-Actin *probe as a loading control.*

postnatal development showed that *Ran-GTPase* transcripts increased from prepuber to adult stages. As shown in Fig. 2, a weak signal was detected at day 7 of postnatal life, when spermatogonias are not yet differentiated to the meiotic stage (Bellve *et al.*, 1977). *Ran-GTPase* transcript accumulation was observed at 15 days postnatal (dpn), when pachytene spermatocytes represent about 20% of the cells that make-up the seminiferous epithelium (Bellve *et al.*, 1977). The most conspicuous signal was detected at 18 dpn coinciding with the appearance of the first round spermatids (Bellve *et al.*, 1977). The mRNA level was sustained during the next days with an increase that was detected in adults, where the proportion of cells from pachytene to spermatids is greater than 75 %, with respect to the rest of the cell types (Bellve *et al.*, 1977).

### Ran-GTPase *Expression is restricted to Spermatocytes and Round Spermatids*

In mammals, the adult seminiferous epithelium presents different cell associations or defined grouping of germ cells along the seminiferous tubules (Oakberg, 1956). This allows identification of the precise cell type expressing the gene of interest.

*In situ* hybridization of *Ran-GTPase* on cryo-sections from adult mouse testis showed a restricted expression pattern. We identified several stages of the cycle of seminiferous epithelium from I to XII (Fig. 3) where the hybridization signal was restricted to certain layers (corresponding to specific cell types) from pachytene spermatocytes to round spermatids. The highest intensity of hybridization was observed from late pachytene cells to early round spermatids. No signal was detected in elongating spermatids. The diagram in Fig. 4 represents the *Ran-GTPase* signal (blue shading) throughout the cycle map of mouse spermatogenesis (Oakberg, 1956). This expression pattern is consistent with that observed in Northern-blot experiments, where we found an increasing signal coincident with the first differentiation of germ cells to spermatocytes and round spermatids.

The abundance of *Ran-GTPase* transcripts and its regulated expression during spermatogenesis suggest that Ran-GTPase plays an important role along this process, probably associated to nucleocytoplasmic transport and microtubule dynamics, as already suggested by Zou *et al.* (2002) and Kierszenbaum *et al.* (2002).

### *Expression Pattern of* Ran/M1 *and* Ran/M2 *Isoforms*

The nucleotide sequences similarity between both isoforms of *Ran-GTPase* did not allow generation of a specific probe for Northern-blots (see experimental procedures). However, oligonucleotides able to discriminate between these two isoforms were designed for real-time RT-PCR experiments. With such tools we analyzed the expression pattern of both somatic and testis specific mouse *Ran-GTPase* isoforms throughout testis development and monitored their relative levels of expression. Results showed in Fig. 5A demonstrated that only *Ran/M1* was present from 7 to 14 dpn. Between 14 and 18 dpn, *Ran/M1* mRNA increased and *Ran/M2* started to be present. At adult stages, the accumulation of *Ran/M1* and *Ran/M2* transcripts was greatly increased respect to the level observed at 14

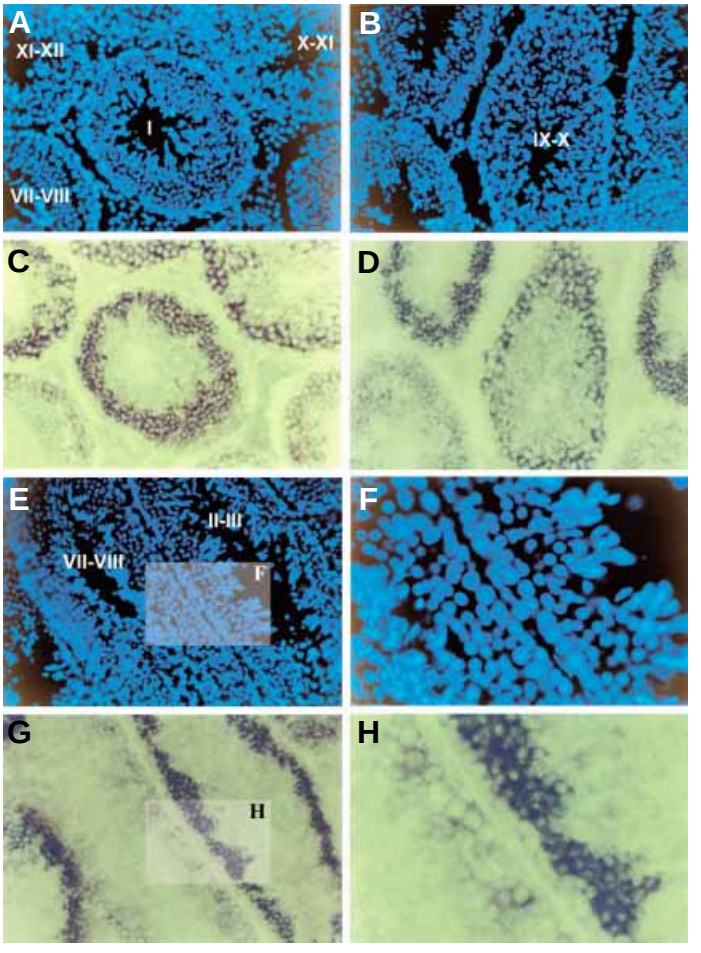


**Fig. 3. *In situ* hybridization of cryo-sections of mouse adult testis.** *Signal was developed using alkaline phosphatase with BCIP/NBT (Roche Applied Science).* **(A,B,E)** *Hoechst staining of the same testis sections hybridized in* **(C,D,G)***, respectively.* **(F,H)** *Enlarged images of the whitish rectangle marked in (E,G), respectively. Relevant stages of the cycle (following Oakberg pattern) are marked with roman numerals inside the cross-sectioned tubules of (A), (B) and (E).*

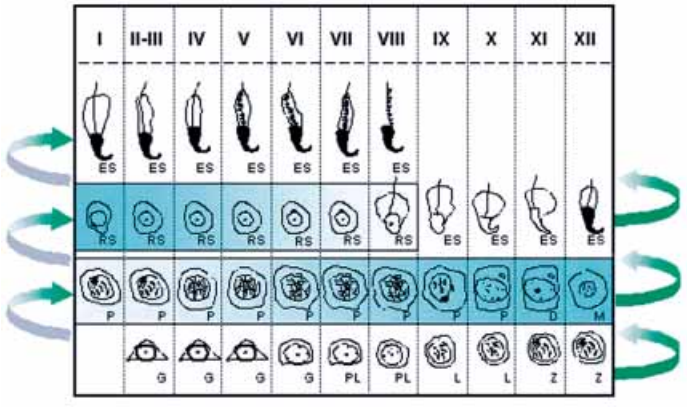


**Fig. 4. Cycle map of adult mouse seminiferous epithelium.** *The developmental progression of a cell is followed horizontally until the right hand border of the cycle map is reached; cell progression continues at the left of the map one row up (green/grey curved lines marked). Vertical columns, designated by roman numerals (from I to XII), depict cell associations. The cell types of cell developmental progression which were found to express* Ran-GTPase *by* in situ *hybridization, i.e., from pachytene spermatocytes to round spermatids, are marked in blue. D, diplotene; ES, elongate spermatid; G, spermatogonia; L, leptotene; M, secondary spermatocyte; P, pachytene; PL, preleptotene; RS, round spermatid; Z, zygotene. (Based on the model by Oakberg, 1956).*

days. These data confirmed the general results obtained by Northern-blot analysis and defined the contribution of both genes to the bulk of *Ran-GTPase* mRNAs detected in testis. The major contribution corresponded to the *Ran/M1* isoform. In addition, we analyzed the presence of *Ran-GTPase* isoforms in other adult tissues (Fig. 5B). We found that *Ran/M2* was detected at very low levels in all the tissues analyzed (Fig. 5B). While *Ran/M2* isoform was not identified in other tissues, we found a *Ran/M2* cDNA cloned from mouse hypothalamus reported in the databases (Acc. No. AI854010).

The presence of both *Ran-GTPase* isoforms in rodent genomes could be the consequence of an active genomic evolution of *Ran/M1* isoform. From preliminary loci assignment in the Mouse Genome Project Database, we can speculate that the locus for *Ran/M2* isoform is different of the *Ran/M1* locus. Multiple presence of *Ran-GTPase* loci have also been found in *Arabidopsis thaliana* (Haizel *et al.*, 1997) and *Salmo salar* (Lundin *et al.*, 2000), with at least three and two active genes, respectively. Comparing the mRNA sequences of mouse and rat *Ran/M2* and those from *S. salar* and *A. thaliana*, we did not observe a reasonable evolutionary relationship between them. However, in rodents, an evolution by duplication of the *Ran/M1* gene including some regulatory elements could had driven to the *Ran/M2* gene, maintaining the regulatory domains for expression in the testis, but lacking of some expression modifiers that are active in somatic tissues. In this way, *Ran/M2* gene would be evolving in rodents allowing changes in the nucleotide sequence as observed when both cDNA sequences were compared (Coutavas *et al.*, 1994).

Since *in situ* hybridization results showed that *Ran-GTPase* transcripts were detected from pachytene to round spermatid (Fig. 4), we measured, by real-time RT-PCR, *Ran/M1* and *Ran/M2* in these cell types, obtained from adult testis by the STA-PUT system (see experimental procedures). Both *Ran-GTPase* isoforms were expressed in both cell types (Fig. 5C).

In rat, Zou *et al.* (2002) and Kierszenbaum *et al.* (2002) detected Ran-GTPase protein in the differentiation from pachytene spermatocyte to elongating spermatids. However, transcript localization was not evaluated. Our results in mouse showed lack of transcript accumulation in elongating spermatids, despite this is the more abundant cell type in the seminiferous epithelium of adult testis. As a high level of both types of *Ran-GTPase* mRNAs was detected in adult testis (Fig. 5A), we suggest that meiotic cells and round spermatids continuously accumulate *Ran-GTPase* transcripts during development, which are translated before the differentiation of elongating spermatids.

## Experimental Procedures

### *Northern Blot*

Total RNA was extracted by the acid guanidinium thiocyanate method (Chomczynski and Sacchi, 1987) from mouse (Swiss) testis at different stages of development or from 100 mg of different somatic tissues. RNA

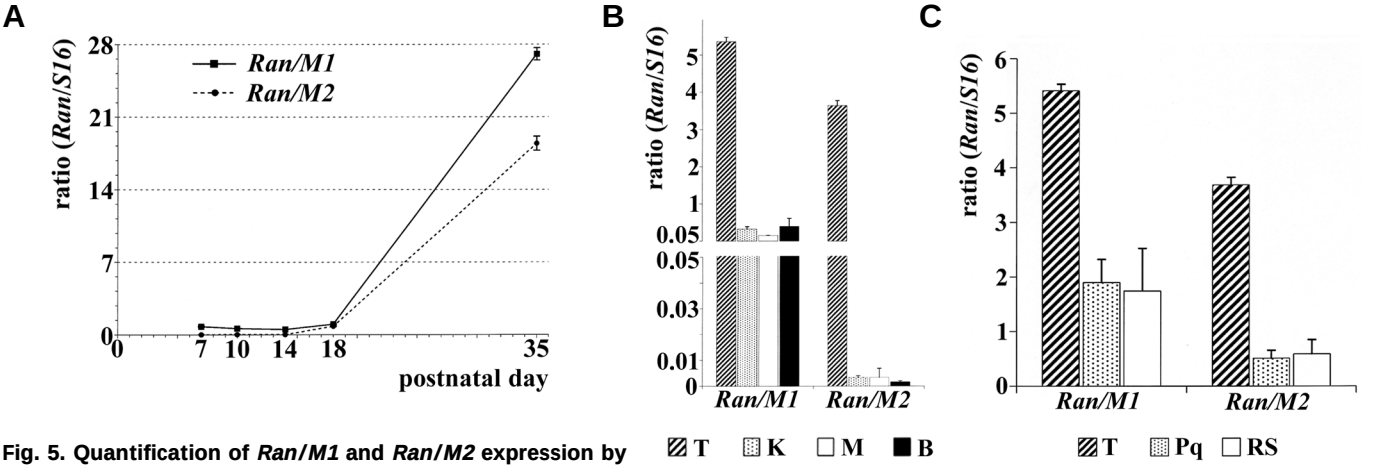


**Fig. 5. Quantification of *Ran/M1* and *Ran/M2* expression by real-time RT-PCR.** *The SYBR-Green dye was used to perform the fluorescence measurements. Triplicates were measured for each sample.* **(A)** Ran-GTPase *isoform* / S16 ribosomal protein *ratio, in arbitrary units, versus time of testis development (dpn).* **(B)** *Quantification in two scales of* Ran/M1 *and* Ran/M2 (Ran-GTPase *isoform* / S16 ribosomal protein *ratio) in adult testis, T, kidney, K, muscle, M, and brain, B.* **(C)** *Diagram like in (B) with the quantification of* Ran/M1 *and* Ran/M2 *(*Ran-GTPase *isoform* / S16 ribosomal protein *ratio) in adult testis, T, pachytene spermatocytes, Pq, and round spermatids, RS. Means ±SD are shown in all graphs.*

samples (20 μg) were electrophoretically separated under denaturing conditions (1% agarose / 8% formaldehyde) and transferred to nylon. Prehybridization and hybridization with $\alpha^{32}$P-dCTP labeled *Ran-GTPase* cDNA were carried in Church solution (Church and Gilbert, 1984) for 1h at 65°C and overnight at 68°C, respectively. Membrane was washed and X-ray film exposed during 60 h at –80°C. The same procedure was used with the stripped membrane and $\alpha^{32}$P-dCTP labeled *ß-Actin* cDNA, used as a probe for control of loading.

*Ran-GTPase* probe corresponded to a *Bam*HI/*Bam*II fragment (676 bp) that comprises the predicted ORF of *Ran/M1* cDNA (Coutavas *et al.*, 1994). *Ran/M1* cDNA was isolated from a MEL (Murine ErythroLeukemia) cell cDNA library (Vanegas *et al.*, 2003).

**In Situ *Hybridization***

Testis from adult mice (Swiss) were dissected and fixed in 4% paraformaldehyde/PBS at 4°C. They were processed for cryo-section *in situ* hybridization (Strahle *et al.*, 1994) with digoxigenin-labeled sense (control) and antisense riboprobes (Roche Applied Science) from the same *Ran-GTPase* cDNA used in RNA analysis. Hybridized samples were counterstained with Hoechst 33258 and analyzed under bright field and fluorescence microscopy. Sense control showed no hybridization (data not shown).

***Real-Time RT-PCR***

Pachytene and round spermatid cells were purified by sedimentation in a continuous BSA gradient (2-4%) with an STA-PUT system from adult mice testes (Bellve *et al.*, 1977). Total RNA was extracted from cells or tissues using the same method that described above.

Residual genomic DNA from total RNA was removed by DNase treatment (RQ1 RNase-free DNase, Promega). RNA was reverse transcribed using a 17-mer oligo-dT and Moloney murine leukemia virus Reverse Transcriptase (SuperScript II, Life Technologies), according to the manufacturer instructions.

Primer combinations for specific amplification of each *Ran-GTPase* isoform at region of maximal sequence divergence and for *S16 ribosomal protein*, which was selected as a normalization gene (Wagner and Perry, 1985), were designed (Primer Express, Applied Biosystems). The oligonucleotide sequences:

| | |
|---|---|
| *S16*-F | TTCTGGGCAAGGAGCGATT |
| *S16*-R | GATGGACTGTCGGATGGCA |
| *Ran/M1*-F | TGATTTAGAGGTTGCTCAGACGAC |
| *Ran/M1*-R | CACACGCGCTGCACCGCTGA |
| *Ran/M2*-F | TGATTTAGAGGTTGCTCAGACGAC and |
| *Ran/M2*-R | ACACTGCACCGCTGGCTGGC. |

Real-time PCRs were carried out in a 25 μl volume using 96-well plate format. Each PCR well contained 1 × SYBR Green PCR Master Mix (Applied Biosystems), 200 nM each primer and 0,25 U/μl AmpErase (uracil N-glycosylase from Applied Biosystems). Reactions were carried out and SYBR-Green fluorescence was detected on an ABI Prism 7700 Sequence Detection system (Applied Biosystems). PCR conditions were 5 min at 50ºC, 10 min at 95ºC and 40 cycles of 15 sec at 95ºC and 1 min at 60ºC. For each sample a replicate was run omitting the reverse transcription step and a template-negative control was run for each primer combination. Arbitrary standards were generated by serial dilutions of cDNA from adult testis, which were used to construct standard curves for each gene measurement. Each experiment was at least replicated three times.

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