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Source: Zoological Science, 19(5) : 539-544

Published By: Zoological Society of Japan

URL: https://doi.org/10.2108/zsj.19.539

ZOOLOGICAL SCIENCE **19**: 539–544 (2002)

Analyses of mRNA Expression Patterns of Cohesin Subunits Rad21 and Rec8 in Mice: Germ Cell-Specific Expression of *rec8* mRNA in Both Male and Female Mice

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ABSTRACT—A multisubunit protein complex called cohesin is required for the cohesion between sister chromatids in both mitosis and meiosis in yeast. We investigate here the mRNA expression patterns of mouse homologues of the yeast mitotic cohesin *rad21* and the meiotic cohesin *rec8* in various organs, with special attention to their expression in gonads. Northern blot and reverse transcription-polymerase chain reaction (RT-PCR) analyses revealed that, in contrast to the ubiquitous expression of r*ad21* mRNA in all of the organs examined, *rec8* was expressed only in the gonads. We conducted *in situ* hybridization analysis to identify the cells that express *rad21* and *rec8* mRNAs in the gonads. In the testis, *rad21* mRNA was expressed in somatic cells and spermatogonia but not in spermatocytes, and conversely, *rec8* mRNA was expressed in spermatocytes but not in spermatogonia or somatic cells. Spermatids expressed *rad21* and *rec8* mRNAs simultaneously. In the ovary, *rad21* mRNA was detected in all of the ovarian cells including germ cells and somatic cells, whereas *rec8* mRNA was detected only in oocytes. Unlike the widespread expression of *rad21* gene, therefore, the gene expression of *rec8* is strictly confined to spermatocytes and spermatids in male mouse and oocytes in female mouse. The restricted expression pattern of rec8 mRNA implies its essential role in meiosis in both sexes of mammals, as has been reported in yeast. We also discuss the cooperative functions of Rad21 and Rec8 on the basis of the finding that their mRNAs are coexpressed in oocytes and spermatids.

Key words: *rec8*, *rad21*, testis, ovary, mRNA

INTRODUCTION

The establishment of sister chromatid cohesion during the S phase in the cell cycle and its dissolution at the metaphase-anaphase transition are essential for the faithful segregation of sister chromatids in mitosis. The establishment and maintenance of sister chromatid cohesion necessitate a multisubunit protein complex, cohesin. In the budding yeast *Saccharomyces cerevisiae*, cohesin contains at least four subunits: Scc1 (sister chromatid cohesion) [also called Mcd1 (mitotic chromosome determinant) or Rad21 (radiation-sensitive mutants) in the fission yeast *Schizosaccharomyces pombe*], Scc3, Smc1 (structural maintenance of chromosomes), and Smc3 (Michaelis *et al*., 1997; Tóth *et al*., 1999). The proteolysis of Scc1 by separin, a cysteine protease related to caspases, leads to sister chromatid separation (Uhlmann *et al*., 1999, 2000). *Xenopus* cohesin, which exists as 14S and 9S protein complexes in interphase nucleus, has also been proved to play an essential role in sister chromatid cohesion in this species (Losada *et al*., 1998, 2000).

Meiosis is a special type of the cell division, in which the number of chromosomes is halved through two successive rounds of chromosome segregation without an intervening chromosome duplication. During meiosis I, the paired homologous chromosomes are separated to each other but the duplicated sister chromatids remain attached at their centromeres. The sister chromatids are separated during meiosis II as in mitosis (reviewed in Miyazaki and Orr-Weaver, 1994). Therefore, the meiotically dividing cells must be equipped with special molecules that ensure the specificity of meiosis, to maintain cohesion between the sister chromatids in meiosis I and to destroy it in meiosis II. It has been known in yeast that a meiosis-specific cohesin subunit, Rec8, replaces a mitotic cohesin subunit, Scc1/Rad21, during meiosis and that this replacement is needed for prevent-

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ing sister chromatids from separating precociously in meiosis I (Michaelis *et al*., 1997; Watanabe and Nurse, 1999). Homologous chromosome separation in meiosis I is promoted by the proteolytic cleavage of Rec8 by separin (Buonomo *et al*., 2000). To date, putative homologs of *rad21* and *rec8* genes have been isolated in various species including mammals, and the functions of Rad21 in mitosis and Rec8 in meiosis are thought to be evolutionary conserved from yeast to humans (McKay *et al*., 1996; Parisi *et al*., 1999; Warren *et al*., 2000; Dong *et al*., 2001; Pasierbek *et al*., 2001).

In spite of growing evidence obtained from the studies in yeast and *Xenopus* for the conservative functions of Rad21 and Rec8, studies of cohesin in mammals are scant in both quantity and quality. For example, only a few studies have so far been made at the gene expressions of *rad21* (Mckay *et al*., 1996) and *rec8* (Parisi *et al*., 1999) in mammals. In these previous reports, various mouse and human organs (but excluding ovary) were examined only by Northern blot analysis, which manifested the gross outline of the gene expression in humans and mice but did not specify the cells expressing *rad21* and *rec8* mRNAs. Therefore, our knowledge of the gene expression of *rad21* and *rec8* during gametogenesis, especially information on histological and cytological aspects, is still limited. In the present study, we conduct *in situ* hybridization analysis, as well as Northern blot and reverse transcription-polymerase chain reaction (RT-PCR) analyses, of various mouse organs including testis and ovary, and provide new additional data for the better comprehensive understanding of the gene expression of *rad21* and *rec8* in male and female germ cells.

MATERIALS AND METHODS

Preparation of RNA probes

Total RNA was isolated from testes of adult C57BL/6 mice with ISOGEN RNA extraction reagent (Nippon Gene Inc., Tokyo, Japan). Single-stranded cDNAs were generated from the RNA with a first-strand cDNA synthesis kit (Gibco BRL LIFE Technology, Tokyo, Japan) according to manufacturer's instruction. The resulting cDNAs were used as templates for PCR to amplify cDNA fragments of *rec8* and *rad21*. According to the known sequences (DNA database of Japan accession number, *rec8*: AF262055, *rad21*: AF332086), we designed the following oligonucleotide primer sets for PCR: for *rec8;* 5'-GGAATTCCTGCTTCACTACCACTGGATG-3' (5' primer introducing *Eco*RI site, indicated by underline) and 5'-GTGCTCGAGGGGGAATTTGGGTCCAG-3' (3' primer introducing *Xho*I site, indicated by underline); for *rad21*, 5'-CGGGAAGCAGCTTATAATGC-3' (5' primer) and 5'-TGACAGTTATATCAATGGGC-3' (3' primer).

The resultant *rec8* PCR product (1034 bp) was digested with *Eco*RI and *Xho*I, and subcloned into the *Eco*RI/*Xho*I site of pBluescript II SK (–) plasmid (Stratagene, La Jolla, CA). The *rad21* PCR product was digested with *Eco*RI and *Hind*III, which produced a 573-bp fragment by cleaving the product at the recognition sites within it. The resulting fragment was then subcloned into the *Eco*RI/*Hind*III site of pBluescript II SK (–). Using a DIG RNA labeling kit (Roche Diagnostics, Mannheim, Germany), digoxigenin (DIG)-labeled sense or antisense RNA probes were synthesized from the plasmids containing *rec8* or *rad21* cDNA with T3 or T7 RNA polymerase, respectively, after linearization of the plasmids with restriction enzymes (*Xho*I for *rec8* sense prove, *Eco*RI for *rec8* antisense probe, *Hind*III for *rad21* sense probe and *Eco*RI for *rad21* antisense probe).

Northern blot analysis

FirstChoice™ Mouse Blot I (Ambion, Inc., Austin, TX) was used for Northern blot analysis. The Mouse Blot I contains 2 μg of poly(A) RNA from ten mouse tissues. The mice are mixed sex, 8–10 weeks old (except for embryo, which are 14 days). The membrane was prehybridized at 50°C for 2 hr in hybridization buffer (50% formamide, 2.38% DIG blocking reagent (Roche Diagnostics), 0.1% sodium N-lauroyl sarcosinate, 0.02% SDS, 5×SSC [1×SSC; 16.6 mM sodium citrate, 16.6 mM NaCl, pH 7.0]). The membrane was then hybridized with antisense or sense DIG-labeled RNA probe in the hybridization buffer at 50°C overnight. After sequential wash in 2×SSC containing 0.1% SDS at room temperature and 0.1×SSC containing 0.1% SDS at 68°C, the signal was detected with alkaline phosphatase-conjugated anti-DIG Fab-fragments and CSPD, according to the manufacturer's instruction (Roche Diagnostics). The signal was visualized by exposing the membrane to X-ray film (Fujifilm, Tokyo, Japan).

For reprobing the membrane, the hybridized probe was stripped off by incubating the membrane in a solution containing 50% formamide, 1% SDS and 50mM Tris-HCl (pH 8.0) at 68°C for 30 min twice, followed by washing in 2×SSC. The next Northern blot analysis was started from the prehybridization step.

RT-PCR

Total RNA was isolated from liver, ovary and testes of C57BL/6 mice with ISOGEN RNA extraction. Single-stranded cDNAs were generated from the RNA (1 μg) with a first-strand cDNA synthesis kit. The resulting cDNAs were subjected to RT-PCR analysis. To amplify cDNA fragment of *rec8*, the above-described primers were used. cDNA fragments of β-*actin* was amplified as a control using a primer set: 5'-TGGAATCCTGTGGCATCCATGAAAC-3' (5' primer) and 5'-TAAAACGCAGCTCAGTAACAGTCCG-3' (3' primer). After 30 cycles of amplification, the DNA products were separated on 1% agarose gels and visualized by staining the gels with ethidium bromide.

In situ hybridization

Testes and ovaries were isolated from adult male and female C57BL/6 mice and fixed immediately in Bouin's solution (saturated picric acid solution: 36% formaldehyde solution : acetic acid = 15 : 5 : 1) or 4% paraformaldehyde in PBS overnight at 4°C. The samples were dehydrated in ethanol, embedded in paraffin, cut into 3–5 μm sections, placed onto gelatinized slides, and subjected to hematoxylin-eosin staining or *in situ* hybridization analysis.

For *in situ* hybridization, the sections were deparaffinized, permeabilized, HCl-treated, proteinase K-digested, and incubated with prehybridization buffer containing 66% formamide and 2×SSC. They were then incubated with sense or antisense RNA probe in a buffer containing 0.83 mg/ml *E. coli* tRNA, 16.6 mM Tris-HCl (pH 8.0), 2 mM EDTA, 0.83×Denhardt's solution (0.02% BSA, 0.02% polyvinyl pyrrolidone, 0.02% Ficoll 400), 250 mM NaCl, 58% formamide, and 8.3% dextran sulfate. After sequential wash in 50% formamide-2×SSC and 2×SSC, the samples were treated with 20 μg/ml RNase A (Sigma, St. Louis, MO) and washed again in 0.2×SSC at 50°C. Following 1 hr-blocking in a blocking buffer (1.5% DIG blocking reagent, 150 mM NaCl, 100 mM Tris-HCl, pH 7.5), the samples were incubated with alkaline phosphatase-conjugated anti-DIG Fab-fragments in the blocking buffer. The signal was developed by incubating the sections in a color-substrate solution (0.2 mM each of nitroblue tetrazolium and 5-bromo-chloro-3-indolyl phosphate, 100 mM Tris-HCl (pH 9.5), 100 mM NaCl, 50 mM $MgCl_2$) in a dark room overnight at 4°C.

RESULTS

Northern blot analysis of the expression of *rad21* and *rec8* mRNAs in various mouse organs

To determine the organ specificity of mRNA expression of *rad21* and *rec8*, we examined the expression patterns in various mouse organs by Northern blot analysis using DIG-labeled antisense RNA probes specific to *rad21* and *rec8*. A 3.8 kb *rad21* mRNA was detected in all of the samples examined except for the testes, in which a 2.2 kb transcript was produced as a main form (Fig. 1a). In addition to the 2.2 kb transcript, three different forms of transcripts, including the 3.8 kb form found ubiquitously in the mouse organs with other 3.2 and 2.6 kb forms, were weakly detected in the testes.

In contrast to the commonly expressed *rad21* mRNA, evident expression of *rec8* mRNA (a 2.3 kb transcript) was found only in the testes (Fig. 1b). *rec8* mRNA was hardly detected in the ovary by a standard procedure but was faintly detected in an over-exposed film (2 hr-exposure instead of 5 min-exposure in the standard procedure, data not shown). When the blot was hybridized with DIG-labeled sense RNA probes, any signal was not observed (data not shown).

To confirm the expression of *rec8* mRNA in ovary, we analyzed the gene expression by RT-PCR using primers specific to *rec8* mRNA. The expression of *rec8* was observed in ovary as well as testis, but was not in liver, although the expression of β-*actin* as a control was observed in the three organs (Fig. 1c). Therefore, *rec8* mRNA is specifically expressed in both male and female gonads.

In situ hybridization analysis of the expression of *rec8* and *rad21* mRNAs in mouse testis and ovary

To determine which types of cells express *rad21* and *rec8* mRNAs in the testis and the ovary, we conducted *in situ* hybridization analysis using the DIG-labeled RNA probes. Sense RNA probes for *rad21* and *rec8* did not yield any signal in the sections of testis (data not shown) and ovary (Figs. 3b and 3d).

In the testis, *rad21* mRNA was expressed strongly in spermatids and weakly in spermatogonia and somatic cells (Sertoli cells and Leydig cells), but not in primary and secondary spermatocytes (Fig. 2a). We were aware that spermatogonia did not uniformly express *rad21* mRNA; obviously, the positive signal was absent in some cells. The absence of the mRNA was not dependent on the stages of spermatogonia (i.e., type A or B spermatogonia).

Roughly speaking, the expression patterns of *rec8* mRNA were reverse to those of *rad21* mRNA. A specific signal was not detected in spermatogonia but was detected in both primary and secondary spermatocytes, although the testis contained considerable numbers of primary spermatocytes expressing no *rec8* mRNA (Fig. 2d). As in the case of

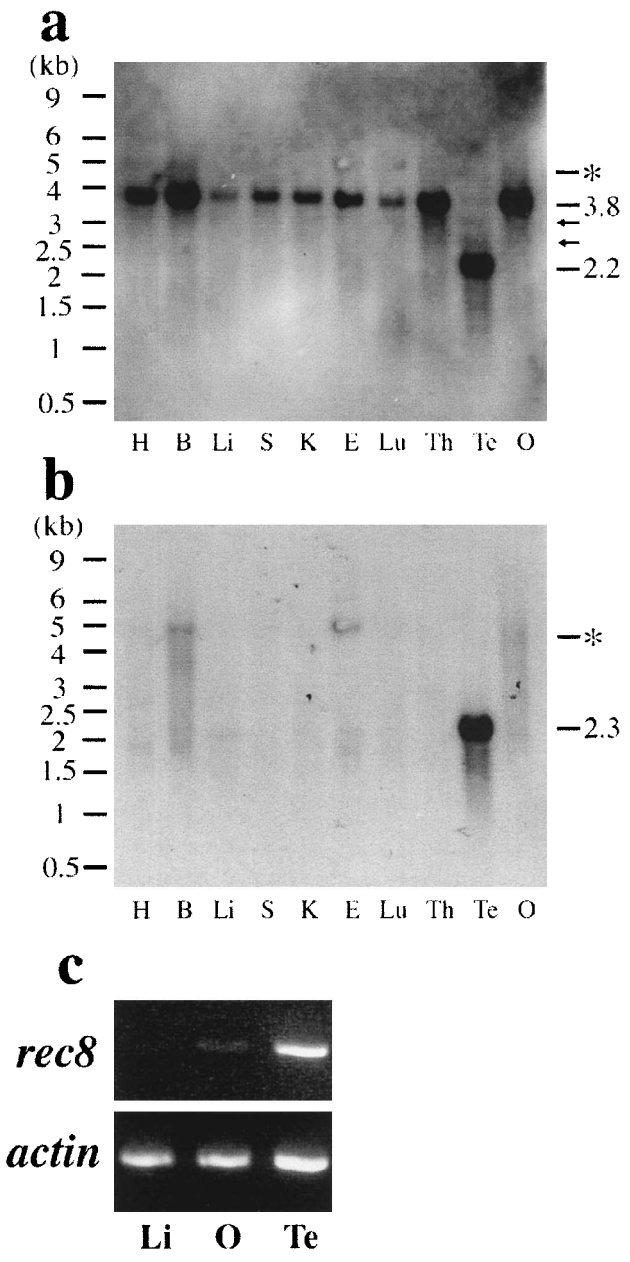

Fig. 1. Northern blot analysis of *rad21* (a) and *rec8* (b) mRNA and RT-PCR analysis of *rec8* mRNA (c) in various mouse tissues. (a and b)The membrane was first hybridized with a DIG-labeled *rec8* RNA probe, and after deprobing it was hybridized again with a *rad21* RNA probe. Each lane loads 2 μg of poly(A)$^+$ RNA extracted from the heart (H), brain (B), liver (Li), spleen (S), kidney, (K), embryo (E), lung (Lu), thymus (Th), testis (Te) and ovary (O). The *rad21* 3.8-kb and 2.2-kb transcripts and the *rec8* 2.3-kb transcript are indicated. Arrows indicate transcripts between the 3.8-kb and 2.2-kb transcripts in testis. Asterisks show an unidentified 5-kb transcript recognized by both *rad21* and *rec8* probes used in this study. (c) After reverse transcription of total RNAs from liver (Li), ovary (O) and testis (Te), cDNA fragments of *rec8* and β-*actin* were amplified 30 cycles using specific primer sets, run on 1% agarose gels, and visualized by staining with ethidium bromide.

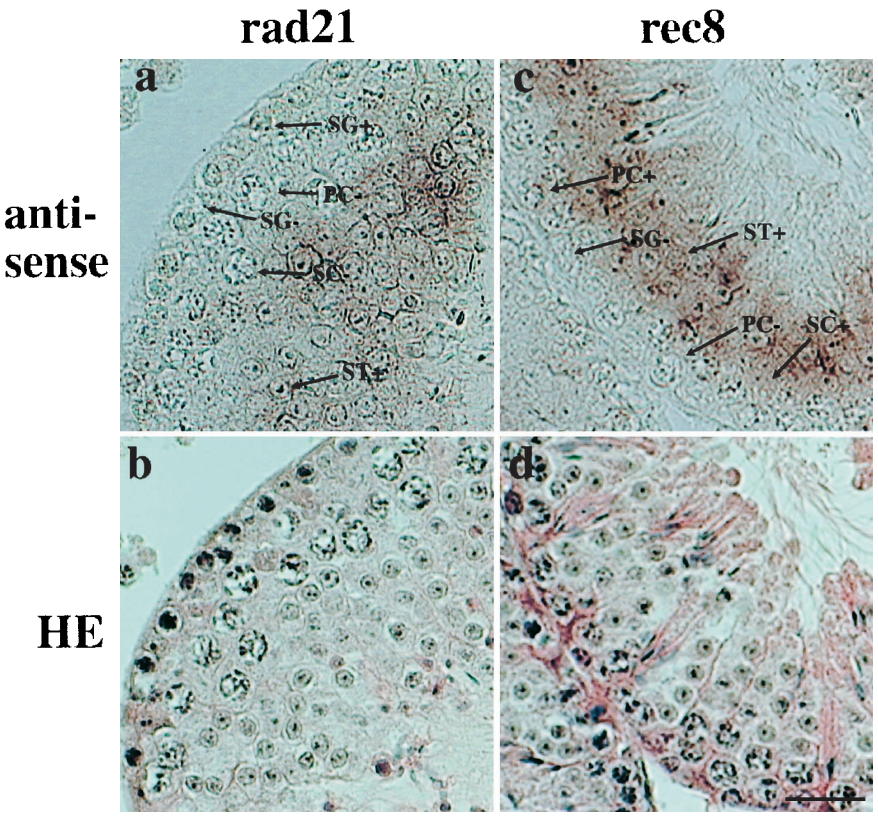

Fig. 2. Histological localization of *rad21* (a and b) and *rec8* (c and d) mRNA expression in the mouse testis. Testis was fixed in Bouin's solution. Serial sections (a-b and c-d) were hybridized with antisense RNA probes (a and c) or stained with hematoxylin-eosin (b and d). Positive cells are marked "+" and negative cells are marked "–". SG, spermatogonium; PC, primary spermatocyte; SC, secondary spermatocyte; ST, spermatid. Note two types of primary spermatocytes, those with (PC+) and without (PC-) *rec8* mRNA expression. Bar = 25 μm.

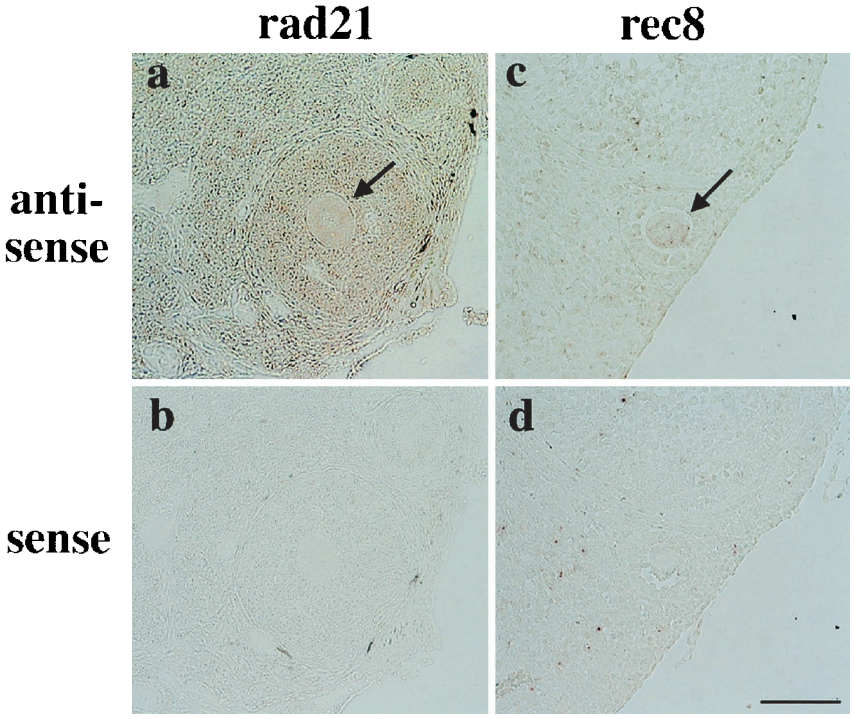

Fig. 3. Histological localization of *rad21* (a and b) and *rec8* (c and d) mRNA expression in the mouse ovary. Ovary was fixed in 4% paraformaldehyde in PBS. Serial sections (a-b and c-d) were hybridized with antisense (a and c) or sense (b and d) RNA probes. An arrow indicates an oocyte. Bar = 100 μm.

Table. 1. Expression of *rad21* and *rec8* mRNA in mouse testicular and ovarian cells

	Testis					Ovary		
	SG	PC	SC	ST	Somatic cells	Oogonium	Oocyte	Somatic cells
rad21	+	−	−	++	+	+	+	+
rec8	−	+/−	+	++	−	+/−	+	−

The intensity of *rad21* and *rec8* mRNA expression was evaluated as −, not detected; +/−, weak or not detected; +, weak; ++, strong. SG: spermatogonium; PC: primary spermatocyte; SC: secondary spermatocyte; ST: spermatid.

rad21 mRNA, *rec8* mRNA was expressed in spermatids at a high level (Fig. 2a).

All of the ovarian cells including oogonia, oocytes and somatic cells expressed *rad21* mRNA (Fig. 3a), whereas a clear positive signal for *rec8* mRNA was localized to the oocytes (Fig. 3b). It is not certain that oogonia did not express *rec8* mRNA at all, because its small size makes it difficult to detect a weak signal inside the cell.

Table 1 summarizes the expression patterns of *rad21* and *rec8* mRNAs revealed by *in situ* hybridization analysis of the testis and ovary. From the data obtained, we can draw the following conclusions: 1) The expression patterns of *rad21* mRNA differ in males and females in that meiotically dividing cells in the testis (spermatocytes) did not express the mRNA but those in the ovary (oocytes) did. This difference results in the exclusive existence of *rad21* and *rec8* mRNAs in spermatogonia and spermatocytes in contrast to their coexistence in oocytes. 2) The presence of *rec8* mRNA is confined to meiotic cells in both sexes, although postmeiotic cells (spermatids) in the testis also express the mRNA, resulting in the simultaneous strong expression of *rad21* and *rec8* mRNAs in spermatids.

DISCUSSION

The present Northern blot analysis has shown that *rad21* mRNA is expressed in all of the adult mouse organs examined in this study. The analysis also revealed that the major mRNA transcript in the testes was shorter (2.2 kb) than that in the other organs (3.8 kb). The expression of such a short *rad21* mRNA in postmeiotic cells (spermatids) has already been reported in the mouse (McKay *et al*., 1996). In the present study, however, we have demonstrated the expression of the standard size of *rad21* mRNA in the ovary, thereby indicating that the expression of the short transcript is specific to male gonads but not to female gonads. The short *rad21* transcript may be produced by a testis-specific alternative splicing or may be a testis-specific *rad21* homolog.

We have shown in this study that mouse *rec8* mRNA is expressed in both testis and ovary (though the content in the ovary seems to be extremely low), despite its absence in the other organs. This finding suggests the specific expression of *rec8* in mammalian gonads. Contrary to this, the expression of *rec8* mRNA in a nongonadal organ, the human thymus, has been reported using a human *rec8* cDNA probe (Parisi *et al*., 1999). This contradiction may reflect either the difference between the two species or the difference in stringency in Northern hybridization between the RNA probe used in this study and the cDNA probe used in the previous study.

To our knowledge, the present paper reports the first *in situ* hybridization analysis of *rad21* and *rec8* expression in mammals. The obtained results allow us to confirm or modify the previous conclusions mainly obtained by Northern blot analyses. Using a cell fractionation technique and Northern blot analysis, McKay *et al*. (1996) reported the existence of *rad21* mRNA in the fraction that consists mainly of spermatocytes. However, our *in situ* hybridization experiment showed that *rad21* mRNA was hardly detected in spermatocytes. This discrepancy might be explained either by the contamination of spermatogonia and other somatic cells to the spermatocyte fraction in the previous study or by the failure in detecting signals due to an extremely low expression of *rad21* mRNA in spermatocytes in our *in situ* hybridization study. Consistent with our observation, however, the protein content of Rad21 in rat spermatocytes is reported to be low, relative to those of the SMC proteins (Eijpe *et al*., 2000). It is therefore likely that *rad21* mRNA is expressed in spermatogonia but not in spermatocytes.

The present *in situ* hybridization analysis has demonstrated a weak expression of *rec8* mRNA in spermatocytes and a strong expression in spermatids. This finding is in good agreement with a previous study, in which the cells fractionated from mouse testes were probed with a human *rec8* cDNA; it reported that *rec8* mRNA was detected weakly in the fraction enriched with meiotic cells and strongly in the fraction enriched with postmeiotic cells (Parisi *et al*., 1999). We also demonstrated the exclusive expression of *rad21* and *rec8* mRNAs in mouse spermatogonia and spermatocytes, which is consistent with the replacement of Rad21 by Rec8 in yeast meiosis. However, the present *in situ* hybridization analysis presented a new finding that some primary spermatocytes do not express *rec8* mRNA, implying that *rec8* mRNA is expressed only in a certain phase (it might be premeiotic DNA synthesis) during meiosis I. In this context, it should be notable that spermatogonia did not constantly express *rad21* mRNA as well. Like that of *rec8* mRNA, the expression of *rad21* mRNA might be regulated in a cell cycle-dependent manner.

The expressions of *rad21* and *rec8* mRNA in mammalian ovary have never been investigated previously. In con-

tradiction to the assumption deduced from the studies in yeast that Rad21 is replaced by Rec8 in meiotically dividing cells, the present *in situ* hybridization study has revealed that the expression level of *rad21* mRNA in oocytes is similar to that in somatic cells. One possible explanation for the presence of *rad21* mRNA in oocytes is that the mRNA is stockpiled for mitotic divisions during the early stages of embryonic development, as many other stored mRNAs are (Gosden *et al.*, 1997). Further studies including detailed observations of the behaviors of *rad21* mRNA and Rad21 protein during fertilization are required for the biological significance of the *rad21* mRNA expression in oocytes and spermatids. Alternatively, *rad21* mRNA and its product Rad21 might be used during female meiosis complementary to a meiosis-specific cohesin subunit(s). If this is the case, the meiotic and mitotic cohesin subunits would not be exclusive to each other but cooperative for assuring the well-organized chromosome cohesion and separation during meiosis at least in female germ cells. Functional evaluation for Rad21 and Rec8 in mammalian germ cells is necessary to verify whether these proteins act under similar mechanisms to those in yeast and *Xenopus*.

In summary, rec8 mRNA was expressed specifically to the germ cells in both male and female gonads, whereas rad21 mRNA was expressed almost universally except for spermatocytes. The specific expression of rec8 mRNA in germ cells suggests that Rec8 protein plays a pivotal role during male and female meiosis in mammals, as has been proposed in yeast meiosis. In addition, coexpression of rad21 and rec8 mRNAs in oocytes implies their cooperative functions in the cells.

ACKNOWLEDGMENTS

The authors thank Dr. Takayuki Takahashi (Hokkaido University) for his generous gift of primers for β-*actin*. This work was supported in part by a Grant-in-Aid for Scientific Research from the Japan Society for the Promotion of Science to J.L.

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(Received February 13, 2002 / Accepeted March 18, 2002)