The Expression and Cellular Localization of the Sperm Flagellar Protein MC31/CE9 in the Rat Testis: Possible Posttranscriptional Regulation during Rat Spermiogenesis*

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Summary. We isolated the MC31 cDNA clone coding the antigen specifically recognized by the monoclonal antibody mMC31, and found that MC31 was identical to rat CE9. Therefore, this molecule is called MC31/CE9. MC31/CE9, a member of the immunoglobulin superfamily molecules, was localized on the rat sperm flagellar plasma membrane. We analyzed the expression and cellular localization of MC31/CE9 mRNA and protein in the adult rat testis by use of Northern hybridization, *in situ* hybridization, and immunohistochemical analyses. In the course of spermatogenesis, MC31/CE9 mRNA first appeared in type B spermatogonia. The mRNA signal intensity increased progressively to pachytene spermatocytes and remained constantly at a considerable level throughout the subsequent phases of spermatocytes and round spermatids, and then decreased gradually from step-11 spermatids to disappear in step-15 spermatids. On the other hand, MC31/CE9 protein expression showed a bimodal pattern. Immunohistochemical analysis for the MC31/CE9 protein revealed its most intense immunoreactivity on the flagella of step-8 to step-19 elongated spermatids. The cytoplasmic immunoreactivity of the MC31/CE9 protein also appeared in preleptotene to early pachytene spermatocytes and elongated spermatids, with particularly intense immunoreactivity in the Golgi complexes of zygotene and early pachytene spermatocytes (stage XIII to III) as well as step-8 to step-13 spermatids. Between these two phases, the MC31/CE9 protein proved undetectable in the cytoplasm of any spermatogenic cells. Sertoli cells and Leydig cells were devoid of MC31/CE9 mRNA and its protein. Therefore, the production of MC31/CE9 is thought to be posttranscriptionally regulated during spermiogenesis.

The seminiferous tubule of the testis is the site of spermatogenesis, a developmental process by which spermatogenic cell progenitors become spermatozoa. Spermatogenesis undergoes three different phases: the mitotic phase of spermatogonia, the meiotic phase of spermatocytes, and the postmeiotic phase of spermatids to produce spermatozoa, i.e., spermiogenesis. Spermiogenesis is a complex developmental process by which postmeiotic cells differentiate into spermatozoa. This process involves major morphological and functional changes including formation of the flagellum and the acrosome and DNA condensation. Different genes are expressed in each phase of spermatogenesis to produce the relevant proteins with specific patterns of expression (EDDY et al., 1993). The rat CE9, which is a member of the immunoglobulin superfamily, was originally identified as a basolateral-specific integral plasma membrane glycoprotein of rat hepatocytes (HUBBARD et al., 1985; BARTLES et al., 1985a, b). CE9 has also been known as rat OX-47 (FOSSUM et al., 1991) and RET-PE2 antigen (FINNEMANN et al., 1997), mouse gp42 (ALTRUDA et al., 1989) and basigin (MIYAUCHI et al., 1990), avian neurothelin (SCHLOSSHAUER and HERZOG, 1990), HT7 (SEULBERGER et al., 1990) and antigen 5A11 (FADOOL and LINSER, 1993), rabbit 4D4 (SCHUSTER et al., 1996), bovine RPE7 (YOSHIMOTO et al., 1998) and human M6 (KASINERK et al., 1992), EMM-PRIN (BISWAS et al., 1995) and OK^a (SPRING et al., 1997). Later, immunocytochemical analysis revealed that CE9 was a domain-specific integral plasma

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membrane protein of the rat spermatozoon; on testicular spermatozoa, it was located within the plasma membrane of the principle piece, whereas during epididymal maturation it was redistributed and confined to the midpiece. This change in the localization of CE9 was observed to take place in a progressive fashion during the passage of the spermatozoa from the caput epididymis to cauda epididymis (PETRUSZAK et al., 1991; NEHME et al., 1993; CESARIO and BARTLES, 1994). Additionally, during rat spermatogenesis, the cellular localization of CE9 was observed in the cytoplasm of early primary spermatocytes and step-8 to step-12 spermatids, and in the flagellum of step-8 to step-19 spermatids (CESARIO et al., 1995).

MC31 was identified as a rat sperm flagellar surface antigen using the monoclonal antibody mMC31 against rat epididymal sperm (TOSHIMORI et al., 1992). This antigen is an acidic 26-35 kDa glycoprotein which is synthesized in the testis. It is first expressed in the cytoplasm of early primary spermatocytes and then gradually concentrated to the principal piece of the sperm flagellum during spermatogenesis. During epididymal maturation, the location of the MC31 antigen changes from the principal piece to the midpiece. Immunoelectron microscopy has also revealed that the MC31 antigen is present on the flagellar plasma membrane. Taking all this in consideration, we noticed that the MC31 molecule was very similar to CE9 in respect to their having almost same molecular weight and a pattern of temporo-spatial localization and redistribution in rat sperm.

In the present study, we isolated the MC31 cDNA clone coding the antigen specifically recognized by the monoclonal antibody mMC31, and we found that MC31 was identical to rat CE9. However, the expression and cellular localization of CE9 mRNA during rat spermatogenesis remained unknown. Therefore, we examined the cellular distribution of MC31 mRNA in the rat testis to compare the expression and cellular localization of the MC31 protein.

MATERIALS AND METHODS

Animals and tissue preparation

Male wistar rats at the age of 10 weeks were purchased from Nippon SLC, Inc. (Hamamatsu, Japan) and grown under an automatically controlled environment, 12 h light/12 h dark, 20°C, in the laboratory animal center of Miyazaki Medical College and Kyushu University. All procedures were performed

according to guidelines for the care and use of laboratory animals. Rats were anesthetized with diethyl ether, sacrificed by bleeding from the right atrium, and perfused transcardially *in vivo* with a cold culture medium. They were then perfused transaortally with Bouin fixative. The testes were dissected out, further immersed in the same fixative for 4 h, dehydrated in a graded alcohol series for 24 h and embedded in paraffin. They were then cut into 4 µm thick sections by routine procedure.

RNA preparation

Total RNA extraction from the rat testis was carried out according to CHOMCZYNSKI and SACCHI (1987). Subsequently, poly(A)⁺RNA was purified by two cycles of batch chromatography using Oligotex dT-30 (Rosch, Tokyo, Japan), according to the manufacturer's instructions.

cDNA cloning

An oligo(dT)-primed TripleEX cDNA library from rat testis poly(A)⁺ RNA was prepared in *E. coli* BM 25.8 and screened for production of MC31 with monoclonal antibody mMC31. Monoclonal antibody mMC31 was previously characterized (TOSHIMORI et al., 1992). A total of 2×10^5 independent plaques were screened and three positive plaques were isolated, representing the different clones in length. Positive plaques were purified three times to ensure homogeneity.

The phage DNA was prepared as described by SAMBROOK et al. (1989). It was then digested with EcoRI restriction enzyme to release the cloned insert cDNA, which was subcloned into plasmid vector pBluescript SK (+) (Stratagene, CA, USA) for restriction mapping and DNA sequencing.

The complete sequence was determined on both strands by the dideoxy termination method (SANGER et al., 1977), using the Big Dye Terminator Kit (PE Applied Biosystems, Chiba, Japan) according to the manufacturer's instructions. The sequences were analyzed and compared for homology using the DNASIS DNA sequence program (Hitachi, Tokyo, Japan).

Northern hybridization

The total RNA samples, each 20 µg in amount, were denatured by glyoxal and electrophoresed in 1% agarose gel in the presence of ethidium bromide (THOMAS, 1980). As a molecular size marker, an RNA ladder (Gibco BRL Life Technologies, Rockville, USA) was used. After being analyzed for ethidium bromide-stained bands, the RNA samples were blotted onto a High Bond-N⁺ nylon membrane (Ama-

sham Pharmacia Biotech, Tokyo, Japan) and cross-linked by UV irradiation (Stratagene). MC31 cDNA was labeled ^{32}P -dCTP (Amasham Pharmacia Biotech) with a Megaprime DNA labeling system (Amasham Pharmacia Biotech) and used as a probe. The membrane was pre-hybridized at 65°C for 2 h with 1 M NaCl, 50 mM Tris-HCl (pH 7.5), $10\times$ Denhardt's solution, 0.1% sodium N-lauroyl sarcosinate (SDS), 10 mM ethylenediaminetetraacetic acid (EDTA), and 250 $\mu\text{g}/\text{ml}$ denatured salmon sperm DNA. The conditions of hybridization were identical to those of the pre-hybridization except for the addition of ^{32}P -labeled probes. After incubation at 65°C for 24 h, the membrane was washed once in $2\times\text{SSC}$ at room temperature for 20 min, then twice in $2\times\text{SSC}$ with 0.1% SDS at 65°C for 20 min each, and exposed to Kodak BIOMAX MR film with intensifying screens at -80°C for 24 h.

In situ hybridization

In situ hybridization was carried out as described previously (MILLAR et al., 1993). To synthesize sense and antisense riboprobes, a PCR amplified fragment corresponding to the partial MC31 cDNA (45-228) was subcloned into pGEM3Zf(+) (Promega, WI, USA). It was linearized with restriction enzymes, and antisense and sense single-stranded RNA were synthesized with T7 and SP6 RNA polymerase respectively, in the presence of digoxigenin (DIG)-conjugated uridine triphosphate (Boehringer Mannheim, Mannheim, Germany). Hybridization of the labeled probe with the fixed tissue sections was performed overnight at 42°C using a hybridization buffer containing 50% deionized formamide, $4\times\text{SSC}$, 0.1 M phosphate buffer (pH 7.2), $1\times$ Denhardt's solution, 2% SDS, 250 $\mu\text{g}/\text{ml}$ yeast tRNA, 250 $\mu\text{g}/\text{ml}$ denatured salmon sperm DNA and 10% dextran sulfate. Excess and mismatched probes were removed by treating sections with 20 $\mu\text{g}/\text{ml}$ RNase A (Boehringer Mannheim) in NTE buffer (0.5 M sodium chloride, 10 mM Tris-HCl, pH 8.0 and 1 mM EDTA, pH 8.0) at 37°C for 30 min, followed by sequential 20 min washes in $2\times\text{SSC}$ and in $0.2\times\text{SSC}$ at 42°C . The hybridized probe was localized on tissue sections using an anti-DIG alkaline phosphatase conjugated antibody (diluted 1:750) (Boehringer Mannheim). Excess antibody was removed by washing in 100 mM TBS, pH 9.5 containing 50 mM MgCl_2 . The antibody bound DIG probe-mRNA complexes were visualized by an enzyme catalyzed color reaction using 175 mg/ml 5-bromo-4-chloro-3-indoxyl-phosphate (DAKO, Kyoto, Japan) and 337.5 mg/ml nitroblue tetrazolium chloride (DAKO) in 100 mM TBS (pH 9.5), 50 mM MgCl_2 containing 1 mM levamisole (DAKO) to reduce

endogenous alkaline phosphatase activity. According to criteria by HESS (1990), the stages of the cycle of rat seminiferous epithelium were identified by the help of another monoclonal antibody mMN7 against an intra-acrosomal antigen (TANII et al., 1994).

Immunohistochemistry

The tissue sections were treated with 0.3% H_2O_2 in methanol for 10 min for the inhibition of intrinsic peroxidase activity, and 5% normal goat serum for 30 min for the prevention of non-specific antibody binding. Subsequently, the sections were incubated overnight at room temperature with monoclonal antibody mMC31 (TOSHIMORI et al., 1992). After the sections were washed in PBS, the sites of immuno-

Fig. 1. Northern blotting analysis of the expression of MC31/CE9 mRNA in the rat testis. Autoradiogram showing total RNA sample (20 μg) from a 10-week-old rat testis hybridized with ^{32}P -labeled MC31/CE9 cDNA probe (lane 1). The size of the hybridization band (bp) is indicated. Fluorescence of the same total RNA samples stained with ethidium bromide showing intact 18S and 28S ribosomal RNA bands (lane 2).

reaction were visualized by incubating the sections successively with biotinylated goat anti-mouse IgG antibody for 1 h, horseradish peroxidase-conjugated

streptavidin for 1 h, and 3',3'-diaminobenzidine solution containing H_2O_2 for a few minutes, using a LSAB2 kit (DAKO).

Fig. 2. *In situ* hybridization of MC31/CE9 mRNA in the rat testis. Bouin fixed-paraffin sections of the rat testis were hybridized with antisense (**a**, **c**, **d**, **e** and **f**) and sense (**b**) digoxigenin-labeled MC31/CE9 riboprobe. **a.** Various levels of the signal are demonstrated in the seminiferous tubules. $\times 50$. **b.** No signal is recognized in the seminiferous tubules. $\times 50$. **c.** At stages V, middle pachytene spermatocytes and round spermatids have a very strong signal, whereas elongated spermatids have no signal. $\times 200$. The **inset** shows a moderate signal in a type B spermatogonium (*arrowhead*) and no signal for a Sertoli cell (*arrow*). $\times 450$. **d.** At stage IX, late pachytene spermatocytes have a very strong signal, whereas elongated spermatids have a weaker signal than late pachytene spermatocytes. $\times 200$. The **inset** shows a late pachytene spermatocyte (*arrowhead*) and elongated spermatid (*arrow*). $\times 450$. **e.** At stages XI-XII, late pachytene spermatocytes have same level of signal as those at stage IX, whereas elongated spermatids have a gradually declining signal. $\times 200$. The **inset** shows a late pachytene spermatocyte (*arrowhead*) and elongated spermatid (*arrow*). $\times 450$. **f.** At stage I, round spermatids have a very strong signal, whereas elongated spermatids have almost no signal. $\times 200$. The **inset** shows a round spermatid (*arrowhead*) and elongated spermatid (*arrow*). $\times 450$

RESULTS

Sequence analysis of MC31 cDNA

A representative clone out of the three MC31 cDNA clones was 1236 bp in length. Comparison of the sequence of this clone with the GeneBank database indicated that the clone had an identical sequence with the known rat gene CE9 (EMBL/GenBank/DBJ; accession number X67215) (NEHME et al., 1993). Therefore, this rat cDNA clone is called MC31/CE9 in the present study.

Expression of MC31/CE9 mRNA in the rat testis

On Northern blot analysis, the total RNA sample from the adult rat testis was hybridized with the labeled MC31/CE9 cDNA probe and formed a single band about 1.5 kilobases in length (Fig. 1). This was in agreement with the 1.5 kilobases of the reported size of rat CE9 mRNA (NEHME et al., 1993), indicating the expression of MC31/CE9 mRNA in the adult rat testis as well as the specificity of the present probe.

Localization of MC31/CE9 mRNA in the adult rat testis

In situ hybridization with DIG-labeled antisense RNA probe demonstrated the signal for MC31/CE9 mRNA in the seminiferous epithelium of rat testis (Fig. 2a). The signal intensity was dependent on the stage of the seminiferous tubules. In contrast, no signal was seen when the sense riboprobe was used (Fig. 2b), confirming the specificity of the signal obtained with the antisense probe. In the course of differentiation of the spermatogenic cell population, the signal was first detected in the type B sper-

matogonia which was spherical in shape and located adjacent to the basement membrane in the seminiferous tubules of stage V (Fig. 2c), whereas no signal was detectable in the type A and intermediate spermatogonia also located adjacent to the basement membrane in the seminiferous tubules until stage III. In the seminiferous tubules of stage V, type B spermatogonia appear first as more rounded cells than

Fig. 3. Immunohistochemical localization of MC31/CE9 protein in the rat testis. Bouin fixed-paraffin sections of the rat testis were reacted with the monoclonal antibody mMC31, and visualized with the streptavidin-biotin-peroxidase complex. **a.** Intense immunoreaction was recognized in the flagella at various stages. $\times 80$. **b.** At stage I, the Golgi complexes (arrowheads) are strongly immunostained, and the cytoplasm is moderately stained in the early pachytene spermatocytes. $\times 200$. The inset shows the Golgi complex (arrowhead) in the early pachytene spermatocyte. $\times 1,000$. **c.** At stage X, the Golgi complexes (arrowheads) and the cytoplasm are moderately immunostained in the elongated spermatids. $\times 200$. The inset shows the Golgi complex (arrowhead) in the elongated spermatid. $\times 1,000$

intermediate spermatogonia (HESS, 1990). The signal increased in intensity as the spermatogenic cells progressed through preleptotene/leptotene/zygotene spermatocytes (stage VI to XIII), and became most intense in pachytene spermatocytes (Fig. 2d, e). Thereafter the signal intensity was constant in round spermatids (Fig. 2c) and gradually declined in step-11 elongated spermatids located in the apical portion of the seminiferous tubule (Fig. 2e), then became undetectable in the step-15 elongated spermatid of stage I (Fig. 2f). These results are summarized in Table 1. No signal for MC31/CE9 mRNA was detectable in Sertoli cells or Leydig cells (Fig. 2e).

Localization of MC31/CE9 immunoreaction products in the adult rat testis

Immunohistochemistry demonstrated that the MC31/CE9-immunoreaction products were also localized on spermatogenic cells in a stage-specific pattern (Fig. 3a). The most intense immunoreactivity was localized to the flagella of step-8 to step-19 spermatids, which were located in the apical portion of seminiferous tubules (Fig. 3a). Relatively intense immunoreaction products were also found in the cytoplasm of either preleptotene (stage VIII) to early pachytene spermatocytes (stage III) (Fig. 3b) or step-7 to step-13 spermatids (Fig. 3c). In particular, an intense im-

munoreactivity was found in the Golgi complexes of both zygotene and early pachytene spermatocytes (stage XIII to III) (Fig. 3b) and step-8 to step-13 elongated spermatids (stage VII to XIII) (Fig. 3c). These results are summarized in Table 1. We confirmed by immunoelectron microscopy that these immunopositive products were actually deposited in the Golgi complexes (detailed data will be separately reported). A weaker immunoreactivity was recognized on the plasma membrane of pachytene spermatocytes to spermatids. In contrast, Sertoli cells in the seminiferous tubules and Leydig cells exhibited no immunoreactivity (Fig. 3a).

DISCUSSION

The present study, using *in situ* hybridization and immunohistochemistry, has clarified that MC31/CE9 is expressed only in the spermatogenic cells in the rat testis. Since the seminiferous epithelium is a complicated tissue composed of Sertoli cells and spermatogenic cells at different phases that are arranged in specific spatial patterns called "stages", it is sometimes difficult to distinguish the cell type with positive mRNA signal by *in situ* hybridization. We especially experienced such difficulty when we used radioisotope-labeled probes on frozen sections. In the

Table 1. Expression of MC31/CE9 mRNA and its protein in the Golgi complexes in rat spermatogenic cells.

Cell type	mRNA	Protein
Spermatogonia		
Type A	—	—
Intermediate	—	—
Type B	+	—
Primary spermatocytes		
Preleptotene	++	—
Leptotene	++	—
Zygotene	++	+++
Pachytene		
early	+++	+++
middle	+++	+/-
late	+++	—
Diplotene	+++	—
Metaphase I	+++	—
Secondary spermatocytes		
Metaphase II	+++	—
Spermatids		
Step 1-3 (Golgi phase)	+++	—
Step 4-7 (cap phase)	+++	+/-
Step 8-14 (acrosome phase)	++~+	++
Step 15-19 (maturation phase)	—	+~-

—: negative, +/-: weak, +: medium, ++: strong, +++: very strong

present study, using DIG-labeled riboprobe and Bouin-fixed, paraffin-embedded sections (MILLAR et al., 1993), the well-preserved cellular architecture in the seminiferous tubules made it possible to analyze the localization of the MC31/CE9 mRNA signal at the cellular level. Accordingly, we can unequivocally identify the positive cell types in combination with another appropriate antibody, such as the mMN7 used in this study.

MC31/CE9 mRNA expression is first recognized in type B spermatogonia, increases in intensity to early pachytene spermatocytes, persists to step-9 spermatids, and decreases gradually up to step-15 spermatids. It is generally accepted that the rate of RNA synthesis in the spermatogenic cell population is high in type A spermatogonia through mid-pachytene spermatocytes, but the rate decreases in the subsequent phases undergoing meiotic divisions (HECHT, 1990). For instance, some signal transduction related genes, such as *raf-1* (SORRENTINO et al., 1988; WOLFES et al., 1989; WADEWITZ et al., 1993), *H-ras* (WOLFES et al., 1989) and *K-ras* (SORRENTINO et al., 1988; WOLFES et al., 1989) have been reported. However, genes for some basic nucleoproteins, such as mouse protamines 1 and 2, are transcribed predominantly in the postmeiotic periods, and the resulting mRNA molecules are stored for about a week before the onset of protamine synthesis in the later spermatids (HECHT, 1990). The present results indicate that the expression of MC31/CE9 mRNA begins in the premeiotic period and persists during the postmeiotic periods. Certain genes, such as those of acrosin (BABA et al., 1994) and calmeglin (IKAWA et al., 1997), are known to have a similar spatial and temporal expression pattern as that of MC31/CE9 mRNA.

The present study has also revealed an apparent discrepancy between the cellular distributions of MC31/CE9 mRNA and the coding protein in the rat seminiferous tubules. The occurrence of the MC31/CE9 protein, as revealed by immunohistochemistry, is split between the meiotic and postmeiotic phases of spermatogenic cells. In the meiotic phase, the immunoreactivity is localized in Golgi complexes of early pachytene spermatocytes at stages XIII to III. In the postmeiotic phase, the immunoreactivity is localized to either the Golgi complexes of step-8 to step-13 elongated spermatids or the flagella of step-8 to step-19 elongated spermatids. Between these two phases, middle pachytene spermatocytes to step-7 spermatids, only a low level expression of the MC31/CE9 protein is detectable in the plasma membrane. CESARIO et al. (1995) reported the cellular localization of the CE9 protein by immunohistochemistry and

pointed out the immunoreaction in the cytoplasm and the Golgi complexes in early primary spermatocytes at stages VIII to IV, and in the Golgi complexes in step-8 to step-12 elongated spermatids. Furthermore, they found that the CE9 immunoreactivity on step-1 to step-7 round spermatids was weak. These results and ours indicate that the process of MC31/CE9 protein synthesis proceeds in bimodal phases in the rat testis. However, we could not recognize any positive reactivity on developing acrosome in spermatids as reported by CESARIO et al. (1995). Also different from our results was the finding that basigin, the mouse MC31/CE9 homolog, was expressed in step-2 to step-6 of spermatids, but the immunoreaction in the Golgi complexes was not observed in any types of spermatogenic cells (MAEKAWA et al., 1998). This discrepancy could be owing to the difference of either the species or antibodies used. Taking into account that MC31/CE9 mRNA is continuously expressed during this period, a mechanism for the posttranscriptional regulation of MC31/CE9 gene is highly probable. During step-1 to step-7 of spermiogenesis, the Golgi complex is known to contribute actively to the formation of the acrosome on the nuclear surface (CLERMONT and TANG, 1985). After step-8, the Golgi complex detaches from the acrosome, remains suspended in the elongated cytoplasm, and then degenerates during step-16 (CLERMONT and TANG, 1985). The absence of intense MC31/CE9 immunoreactivity on the Golgi complex of step-1 to step-7 spermatids as revealed in the present study is coincident with the period in which the Golgi complex engages in the acrosome formation. There may be a mechanism by which the translation of MC31/CE9 mRNA is inhibited during the acrosome formation and then reactivated in the later steps of spermiogenesis.

Recently, *basigin* knockout mice were generated and analyzed for spermatogenesis (IGAKURA et al., 1998). These mice showed that a majority of spermatocytes had degenerated at the metaphase of the first meiosis, and a few spermatocytes could differentiate into step-1 spermatids. However, basigin expresses itself during spermiogenesis after step-1 spermatids in the wild mice (MAEKAWA et al., 1998). Therefore, the biological role of the MC31/CE9 protein translated in the early pachytene spermatocytes may be related to the process of meiosis in the rat as well. On the other hand, MC31/CE9 proteins translated predominantly in the postmeiotic elongated spermatids may presumably have another significant role such as epididymal maturation and fertilization.

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