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Molecular Cloning of Rat Macrophage-Stimulating Protein and Its Involvement in the Male Reproductive System

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Macrophage-stimulating protein (MSP), a member of the hepatocyte growth factor family, is a ligand for receptor tyrosine kinase STK/RON. Here we isolated a full-length cDNA of rat MSP and a partial cDNA of rat STK/RON, then characterized their expression in the male reproductive system. *In situ* hybridization revealed that MSP mRNA was localized to spermatogonia and spermatocytes in the testis and the epithelium lining the lumen of the epididymis. On the other hand, RT-PCR analysis showed that STK/RON mRNA was expressed in sperm collected from both testis and epididymis. These findings suggest that locally produced MSP may play a vital role in germ cell–germ cell interaction during spermatogenesis as well as in the acquisition of sperm motility and/or fertilizing capacity in the epididymis. Our findings reveal new possible roles of the MSP-STK/RON signaling pathway. © 1996 Academic Press, Inc.

MSP is a serum protein with homology to hepatocyte growth factor (HGF) (1,2). MSP and HGF belong to the same protein family characterized by highly conserved triple disulfide loop structures, kringle domains (2). STK/RON is a receptor tyrosine kinase that belongs to the HGF receptor family (3,4). Recently, STK/RON was identified as the receptor for MSP (5, 6). MSP has various biological effects on murine resident peritoneal macrophages including the induction of chemotaxis, phagocytosis and shape change, and the inhibition of NO production (7-9). MSP also stimulates bone resorption by osteoclasts (10). However, the functions of MSP other than those on mononuclear phagocytes have not been clarified.

To clarify the novel biological functions of MSP, we isolated cDNAs of rat MSP and STK/RON and characterized their expression in the male reproductive system. Here we show the possible involvement of the MSP-STK/RON signaling pathway in spermatogenesis and the regulation of sperm function in the epididymis.

MATERIALS AND METHODS

Screening of cDNA libraries. Rat liver and lung cDNA libraries (in λgt11, Clontech, Palo Alto, CA) were screened with [^{32}P]-labeled full-length mouse MSP cDNA and a partial mouse STK/RON cDNA, respectively.

Expression of rat MSP protein. A full-length cDNA of rat MSP tagged with a FLAG octapeptide (DYKDDDDK) was inserted into pMKITNeo, then transfected into COS7 cells using diethyl aminoethyl-dextran. To obtain an active form of rat MSP, the serum-free conditioned media of transfected COS7 cells were treated with 36.2nM human plasma kallikrein (Sigma Chemical Co., St. Louis, MO) at 37°C for 2 hr. Proteins in conditioned media were resolved by SDS-PAGE under reducing conditions and transferred to PVDF membranes, which were then

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The nucleotide sequence data reported in this paper will appear in the EMBL, Genbank, and DDBJ Nucleotide Sequence Databases under Accession No. X95096.

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(A)

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CAGCTTAGGAGAATGGGGTGGCTCCCACTACTGCTGCTTCTGGCACAGTGTTCAAGGGCTCTTGGGCAGCGCTCACCGCTGAATGACTTCCAGCTGCTTCGGGGCACAGAGTTAAGGAAC   120
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                                                                                                    K1
AGCTACCGGGGCACTGTGGCCAGGACAGCTGATGGCTTGCCCTGCCAAGCCTGGAGCCGCAGGTTCCCCAATGACCACAAGTACACGCCCACACCGAAGAATGGCCTGGAAGAGAACTTC   480
S  Y  R  G  T  V  A  R  T  A  D  G  L  P  C  Q  A  W  S  R  R  F  P  N  D  H  K  Y  T  P  T  P  K  N  G  L  E  E  N  F     156

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C  R  N  P  D  G  D  P  R  G  P  W  C  Y  T  T  N  R  S  V  R  F  Q  S  C  G  I  K  S  C  R  E  A  V  C  V  W  C  N  G     196
                                                                                                    K2
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E  D  Y  R  G  E  V  D  V  T  E  S  G  R  E  C  Q  R  W  D  L  Q  H  P  H  S  H  P  F  H  P  E  K  F  P  D  K  A  L  K     236

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AAAGGATCCAAGTCACAACAGCGCAACAAGGTCAAGGCTTCGAACTGCTTCCGCGGAAAAGGTGAAGACTATCGAGGCACAACCAATACCACCTCTGCGGGTGTGCCCTGCCAGCGCTGG   960
K  G  S  K  S  Q  Q  R  N  K  V  K  A  S  N  C  F  R  G  K  G  E  D  Y  R  G  T  T  N  T  T  S  A  G  V  P  C  Q  R  W     316
                                              K3
GATGCGCAGAATCCGCACCAGCACCGCTTTGTGCCGGAGAAATATGCTTGCAAGGACCTTCGTGAGAATTTCTGCCGGAATCCTGATGGCTCCGAGGCGCCTTGGTGCTTCACATCTCGA  1080
D  A  Q  N  P  H  Q  H  R  F  V  P  E  K  Y  A  C  K  D  L  R  E  N  F  C  R  N  P  D  G  S  E  A  P  W  C  F  T  S  R     356

CCTGGTTTGCGTGTGGCCTTTTGCTACCAGATCCCACGCTGCACAGAAGAAGTGGTGCCAGAGGGCTGCTACCATGGCTCAGGTGAACAGTATCGTGGCTCAGTCAGCAAGACACGCAAG  1200
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                                                                    K4
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TGTGGCAAGAGAGTTGACCAGAGTAATAGACTTCGTGTGGTGGGGGGTCATCCTGGGAACTCACCGTGGACAGTCAGCTTGCGGAATCGACAGGGCCAGCATTTCTGTGGGGGTTCCCTA  1560
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TGCTGGGTCCTACAGGGACTTATCATCCCGAACAGAGTGTGTGCACGGCCTCGCTGGCCAGCTATCTTCACACGTGTGTCTGTGTTTGTGGACTGGATTAACAAGGTCGTGCAGCTGGAG  2160
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TAGGCCTGCTTTCGATCTCTTAGAGATGACAAGGCTGCTTATCAAACTAAAGCAAACTTTTC                                                          2222
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(B)

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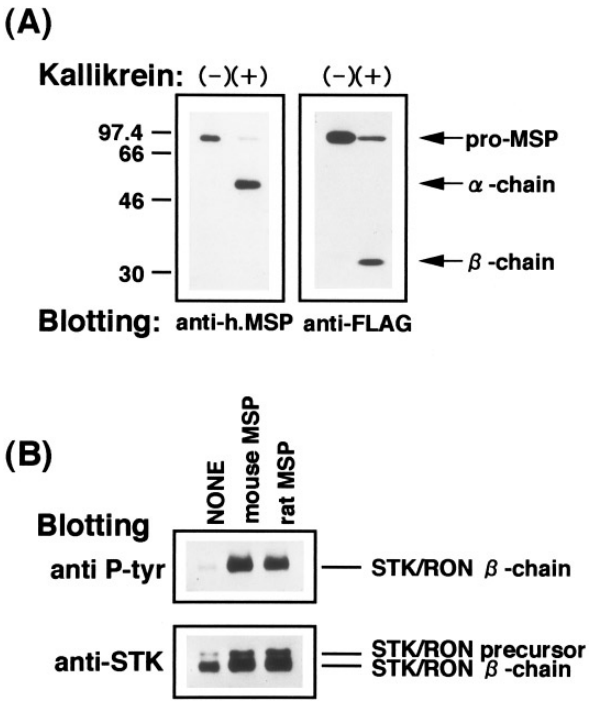

FIG. 2. Immunoblotting and *in vivo* tyrosine phosphorylation assay. (A) Detection of rat MSP tagged with FLAG octapeptide in conditioned media of transfected COS7 cells with and without treatment of kallikrein. Proteins were separated by SDS−PAGE under reducing conditions, then transferred to PVDF membranes and probed with anti-human MSP, which reacts with pro-MSP and α-chain but not with β-chain (lanes 1,2) and with anti-FLAG, which reacts with pro-MSP and β-chain (lanes 3,4). (B) Tyrosine phosphorylation of mouse STK/RON β-chain in BaF3/STK cells. BaF3/STK cells were stimulated with medium alone (lane 1), kallikrein-treated mouse MSP (lane 2) or kallikrein-treated rat MSP (lane 3). STK/RON protein in cell lysates was immunoprecipitated and Western-blotted with anti-phosphotyrosine (upper panel) and with anti-STK/RON (lower panel).

probed with either the anti-FLAG M2 (Eastman-Kodak, New Haven, CT) or rabbit anti-human MSP antibodies (11). Specific bindings were detected using the enhanced chemiluminescence detection system (ECL; Amersham International plc, Bucks., UK).

In vivo tyrosine phosphorylation assay. Ba/F3 cells transfected with mouse STK/RON (BaF3/STK) were stimulated with 10 % conditioned media of COS7 cells transfected with rat or mouse MSP at 37°C for 10 min. Cells were solubilized with lysis buffer containing 2 mM sodium orthovanadate and STK/RON was immunoprecipitated with anti-mouse STK/RON C-terminus (8). Tyrosine phosphorylation of mouse STK/RON was evaluated by Western blotting with anti-phosphotyrosine antibody (Upstate Biotechnology Inc, Lake Placid, NY).

Northern blotting. A Rat Multiple Tissue Northern Blot filter (Clontech) was blotted with [^{32}P]-labeled full-length rat MSP or a partial rat STK/RON cDNA.

In situ hybridization and immunohistochemistry. In situ hybridization (ISH) was performed on 6 μm frozen sections of the Sprague-Dawley rat tissues (8-12 week-old) as described previously (12). The digoxigenin (DIG)-labeled cRNA riboprobes were generated from the 332 bp cDNA fragment of rat MSP (nucleotides; 1-332 in Fig.1A) using a RNA Transcription kit (Stratagene, La Jolla, CA). MSP-specific hybrids were detected with anti-DIG antibody conjugated with alkaline phosphatase (Boehringer Mannheim, Mannheim, Germany), or with the gold-conjugated anti-DIG anti-

FIG. 1. The nucleotide and deduced amino acid sequences of the full-length rat MSP cDNA (A) and the partial rat STK/RON cDNA (B). The predicted cleavage sites for the signal peptide and for α- and β-chains are indicated by arrowhead and arrow, respectively. K1−K4 indicate each kringle domain. Sense and antisense primer sequences for RT-PCR analysis of STK/RON expression are underlined.

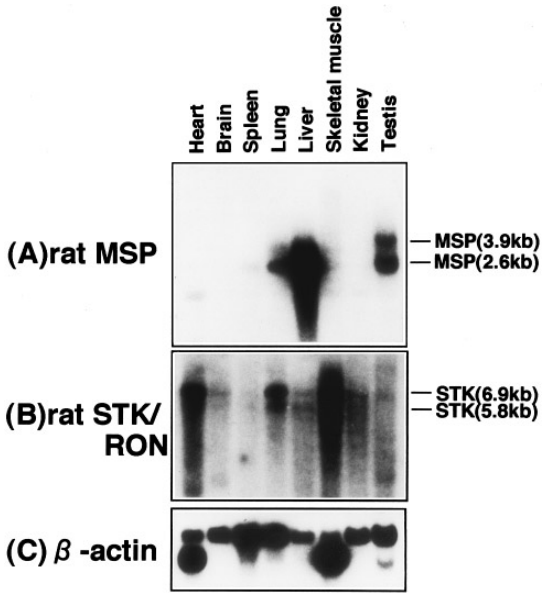

FIG. 3. Northern blot analysis of rat MSP (A) and STK/RON (B) in adult rat tissues. The filter was hybridized with ^{32}P-labeled full length MSP cDNA, or with a rat STK/RON cDNA (nucleotides: 289–688 in Fig. 1B). The blots of MSP and STK/RON were exposed for 1 day and 2 days, respectively. A β-actin probe was hybridized as a reference gene (C).

body using a silver enhancing kit (British BioCell International, Cardiff, UK). Immunostaining of rat testis with anti-PCNA (proliferating cell nuclear antigen) Ab was performed as previously reported (13).

RT-PCR. Epididymis and testis in Ham's F10 medium (Life Technologies, Inc., Grand Island, NY) were sliced with scissors. Sperm was separated from tissue fragments by repeated centrifugation (14). Total RNA was obtained from sperm by acid guanidinium thiocyanate-phenol-chloroform extraction, then treated with 5 μg/ml RNAase-free DNAase I at 37°C for 30 min. The first-strand cDNA was synthesized from all of the total RNA using Superscript II reverse transcriptase and random hexamer oligonucleotides (Life Technologies, Inc.). Amplification was performed using 10 % of the cDNA as a template. The cycling parameters were 1 min at 94°C, 2 min at 62°C, and 3 min at 72°C for 40 cycles. The primer sequences of STK/RON are indicated in Fig. 1B. Rat β-actin was used as a reference gene. The primer sequences were as follows: sense, 5-TAAGGCCAACCGTGAAAAGA-3′; and antisense, 5′-CAG-TAACAGTCCGCCTAGAA-3′.

RESULTS AND DISCUSSION

Isolation of rat MSP and STK/RON cDNAs. A full-length cDNA of rat MSP and a partial cDNA of rat STK/RON were isolated and their sequences determined (Fig. 1A and B). The predicted amino acid sequence of rat MSP showed 92.7 % and 79.4 % identity to mouse and human MSP, respectively (Fig. 2), and 42 % identity to rat HGF. On the other hand, the predicted amino acid sequence of rat STK/RON showed 94.2 % and 83.8 % identity to mouse and human STK/RON, respectively. Similar to mouse and human MSP, rat MSP has an arginine-valine bond (amino acid residues 488 and 489), a putative proteolytic cleavage site (Fig. 1A). As expected, we found that rat MSP was synthesized as a 85 kDa of single-chain precursor (pro-MSP), then cleaved into a heterodimer consisting of a 53 kDa α-chain and a 32 kDa β-chain by treatment with kallikrein (Fig. 2A). Kallilrein-treated rat MSP, as well as mouse MSP, induced the phosphorylation of STK/RON β-chain (144 kDa) in BaF3/STK cells (Fig. 2B), whereas rat MSP without kallikrein-treatment barely induced (data not shown), indicating that rat MSP exerts biological activities as a heterodimeric protein.

The expression of rat MSP and STK/RON. Northern blot analyses showed that MSP mRNAs

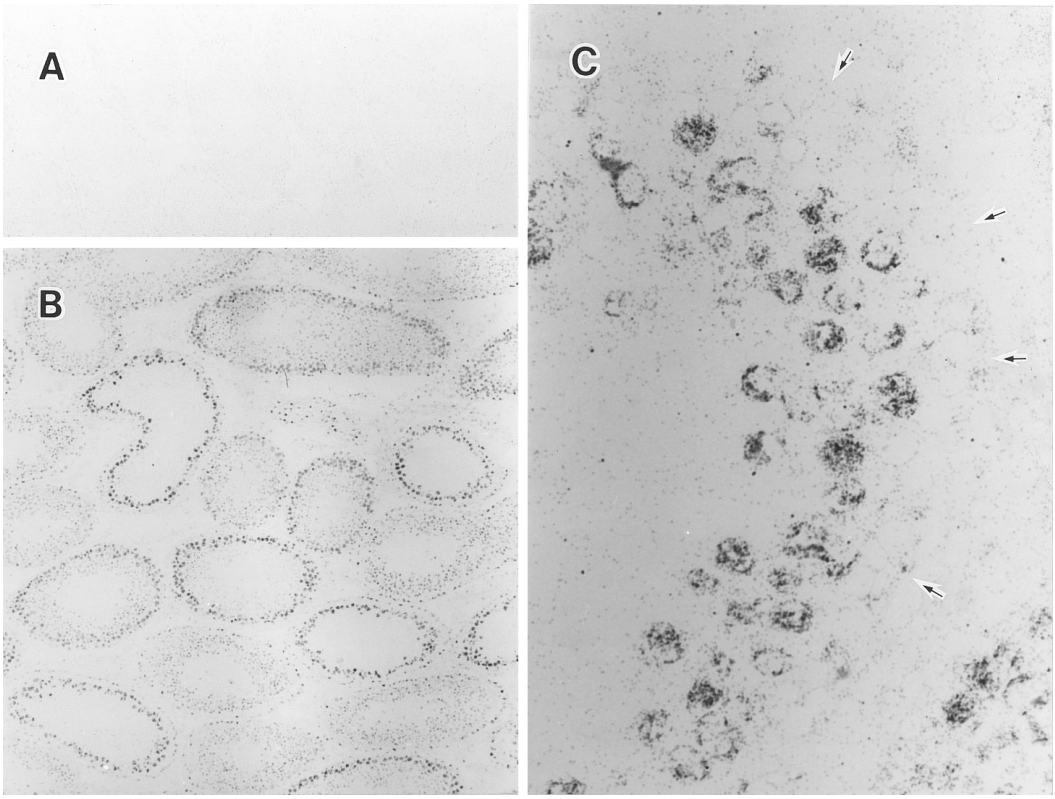

FIG. 4. *In situ* localization of MSP mRNA in the testis. Frozen sections were hybridized with digoxigenin-labeled sense (A) and antisense (B, C) RNA probes. Specific hybrids were detected by immunogold method. Note uneven intensity and distribution of positive cells among seminiferous tubules shown in (B). A higher magnification (C) shows a cluster of large positive cells localizing at inner part of one tubule, being one or two cells from its boundary layer (arrow) (A, B ×48; C ×480).

of 2.6 and 3.9 kb were strongly expressed in the liver and testis, and weakly in the lung (Fig. 3). Human and mouse MSP are expressed predominantly in the liver (1,2). The high level of expression in the testis is characteristic of rat MSP. On the other hand, rat STK/RON mRNAs of 5.8 and 6.9 kb were strongly expressed in the heart, lung and kidney, moderately in the liver and brain, and weakly in the spleen and testis (Fig. 3).

In situ hybridization and immunohistochemistry. To further characterize MSP and STK/RON expression, we identified the cell types that express their mRNAs in rat testis and epididymis by RNA ISH. A sense probe gave no specific signal above the background (Figs. 4A and 6A). Strong signals were detected in a layer of germ cells including spermatocytes (Figs. 4C and 5A) and spermatogonia (Fig. 5A). Anti-PCNA MoAb also reacted with the nuclei of similar types of cells, suggesting that MSP and PCNA may be coexpressed in these cells (Fig. 5). PCNA labeling is confined to the nuclei of all differentiating germ cells from spermatogonia, except for resting A-dark spermatogonia, up to pachytene spermatocytes (15). Since Sertoli cells scarcely proliferate, $PCNA^+$ cells would mostly correspond to germ cells. These findings confirmed that MSP mRNA in the seminiferous tubules are mostly expressed in germ cells including spermatogonia and spermatocytes, but not in germinal epithelia (Sertoli cells). The signal intensity of MSP in these cells varied in each seminiferous tubule (Fig. 4B), indicating that MSP expression is precisely regulated during differentiation of seminiferous

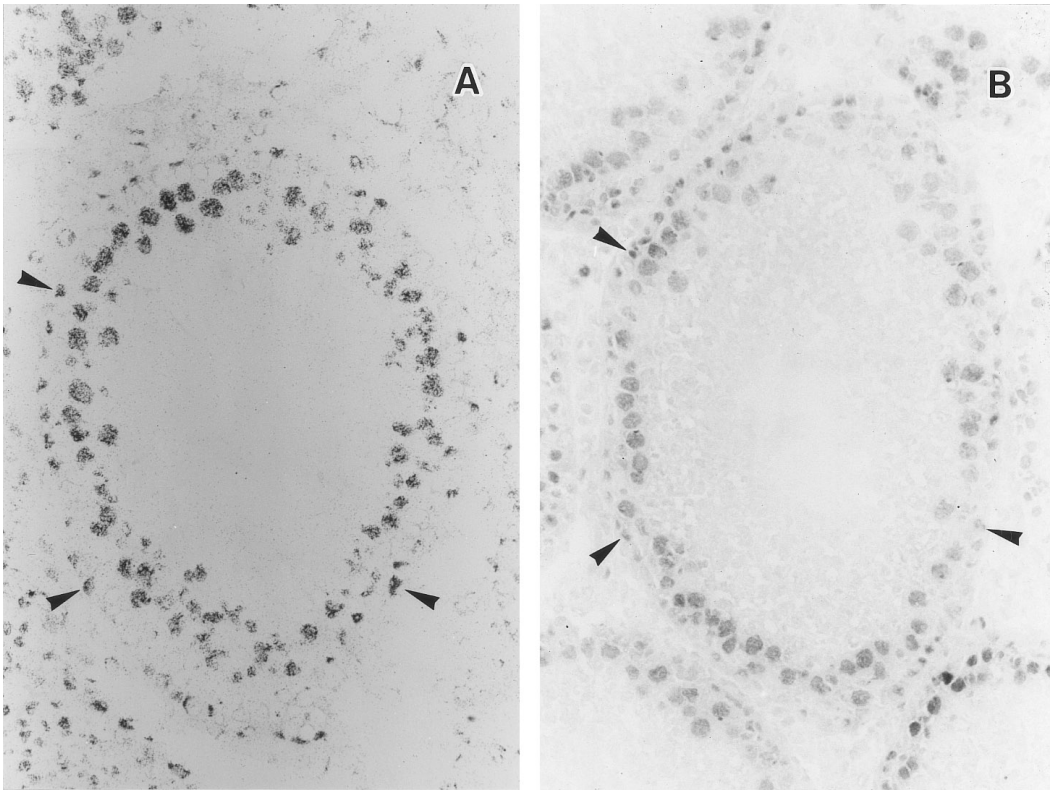

FIG. 5. *In situ* localization of MSP mRNA (A) and immunolocalization of the PCNA antigen (B) in the testis. Note positive cells are localized in a similar pattern in both cases. A cluster of large positive cells apart from the boundary layer may be the primary spermatocyte. Scattered small positive cells (arrowheads) close to the boundary layer may correspond to the spermatogonia. (A, B ×240)

epithelium. In the epithelium lining the lumen of epididymis, on the other hand, the even stronger signals of MSP were detected (Fig. 6).

Detection of STK/RON mRNA in sperm. RNA probes of STK/RON did not give significant signals in ISH (data not shown). However, RT-PCR analysis showed that STK/RON mRNA is expressed in sperm from both testis and epididymis (Fig.7).

The germinal epithelium in the adult testis is subjected to constitutive proliferation and differentiation. An array of locally produced growth factors are assumed to be involved in the regulation of spermatogenesis (16). Some of them are produced by Sertoli cells and regulate the mitotic and/or meiotic division of germ cells, while others are produced by germ cells and act on Sertoli cells. Spermatogenesis is regulated by these interactions between germ and Sertoli cells (16). On the other hand, basic fibroblast growth factor is expressed in pachytene spermatocytes and type A spermatogonia, and its receptor in round and elongated spermatids, suggesting germ cell-germ cell interaction during spermatogenesis (17). Although further analysis on the expression of STK/RON in the testis is needed, our findings suggest the involvement of MSP and its receptor in germ cell-germ cell interaction during spermatogenesis.

Sperm acquires its motility and fertilizing capacity in the distal parts of the epididymis. The underlying cellular mechanisms and molecules for this process remain to be determined. Among growth factors, HGF is expressed in the epithelium lining the lumen of mouse epididymis and induces sperm motility *in vitro* (18). It is of interest that MSP, a HGF-related protein, is also

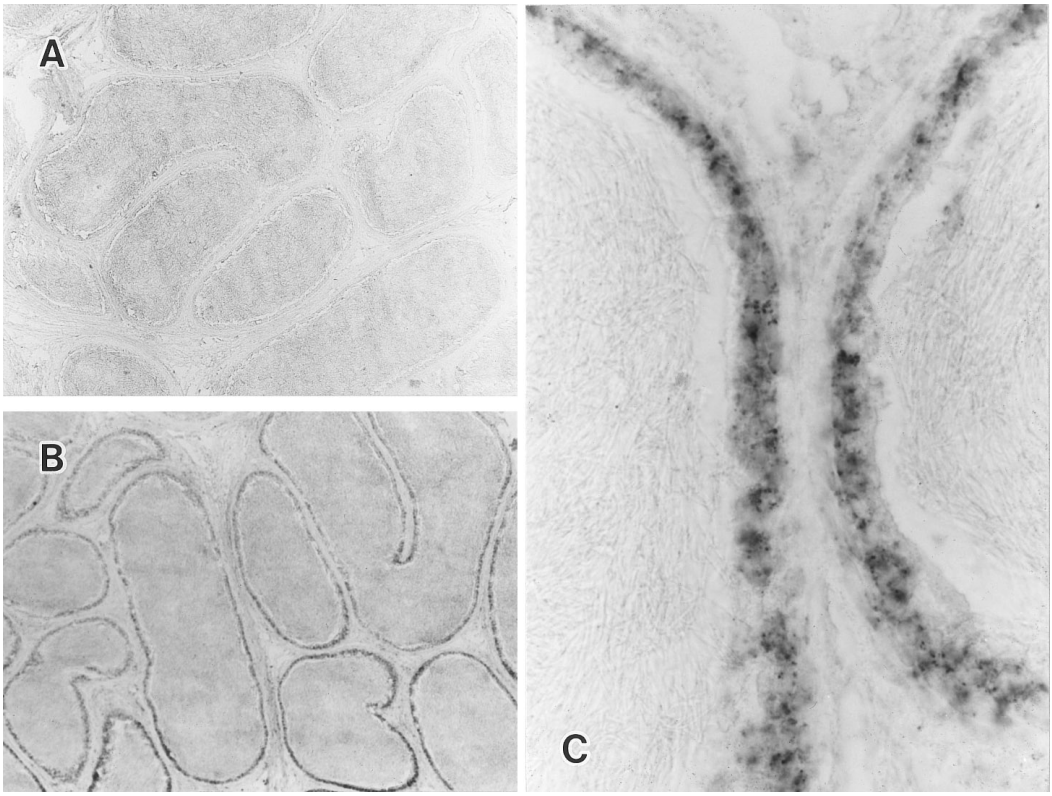

FIG. 6. *In situ* localization of MSP mRNA in the cauda epididymis. Frozen sections were hybridized with sense (A) and antisense (B, C) RNA probes, then detected by immunoalkaline phosphatase staining. Intense signals were evident in pseudostratified columnar-cuboidal epithelial cells lining the lumen of epididymis (A, B ×48; C ×480).

expressed in the epithelium of epididymis and that sperm express its receptor, STK/RON. Recently, we found that human STK/RON is expressed on the ciliated epithelium of mucociliary transport apparatus and that MSP stimulates ciliary motility (Sakamoto et al., in revise). Flagellum, an important component of sperm motility, has a structure closely related to that of cilia. These findings suggest that MSP plays an important role in the regulation of sperm function, especially in the acquisition of motility in the epididymis.

In summary, we showed that locally produced MSP may be involved in germ cell-germ

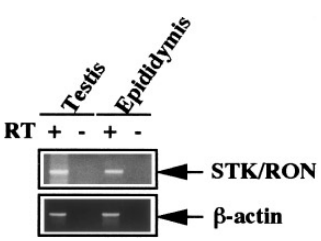

FIG. 7. RT-PCR analysis of STK/RON expression in sperm from testis and epididymis. The cDNA was amplified by PCR using STK/RON oligonucleotide primers (indicated in Fig. 1B). PCR products were resolved by electrophoresis on a 2% agarose gel and stained with ethidium bromide. The β-actin was used as a reference gene.

cell interaction during spermatogenesis and the regulation of sperm function in the epididymis. Further studies would give a novel insight into the field of male reproductive system.

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