MOLECULAR AND CELLULAR BIOLOGY, July 1993, p. 4146–4156 Vol. 13, No. 7
0270-7306/93/074146-11$02.00/0
Copyright © 1993, American Society for Microbiology

Testis-Specific Mak Protein Kinase Is Expressed Specifically in the Meiotic Phase in Spermatogenesis and Is Associated with a 210-Kilodalton Cellular Phosphoprotein

ATSUSHI JINNO,[1] KEIJI TANAKA,[1] HITOSHI MATSUSHIME,[1] TATSUJI HANEJI,[2] AND MASABUMI SHIBUYA[1]*

Department of Genetics, Institute of Medical Science, University of Tokyo, Minato-ku, Tokyo 108,[1] and Department of Anatomy, School of Medicine, Chiba University, Chuo-ku, Chiba 260,[2] Japan

Received 28 December 1992/Returned for modification 9 February 1993/Accepted 9 April 1993

The *mak* gene encodes a new protein kinase distantly related to cdc2 kinase, and its transcripts are expressed exclusively in testicular germ cells at and after meiosis (H. Matsushime, A. Jinno, N. Takagi, and M. Shibuya, Mol. Cell. Biol. 10:2261–2268, 1990). In this study, we prepared a series of antibodies against synthetic peptides and fusion products of the *mak* gene and characterized the subcellular localization, protein kinase activity, and association with other cellular proteins of Mak. Mak products were identified as 66- and 60-kDa proteins that specifically appeared in rat testes after puberty. Testicular germ cell fractionation revealed that Mak products were most abundant in the fraction of the late pachytene stage and that their levels were dramatically decreased in postmeiotic haploid cells. Mak products were localized mostly in the cytoplasm as a soluble form. [^{35}S]methionine labelling demonstrated that Mak products were associated with a 210-kDa cellular protein; in an in vitro kinase assay with immunoprecipitates of Mak products, the 210-kDa cellular protein was efficiently phosphorylated on serine and threonine residues. Furthermore, in a testicular cell culture system with $^{32}P_i$, the 210-kDa molecule associated with Mak was phosphorylated in vivo on serine and threonine residues. These results strongly suggest that the Mak complex may play a role in meiosis during spermatogenesis and that a phosphorylated 210-kDa protein is one of the physiological substrates for this protein kinase.

Spermatogenesis is known to be separated into three major stages, i.e., the mitotic phase of spermatogonia (stem cells), the meiotic phase of spermatocytes, and the maturation stage after meiosis to sperm. Spermatogenesis is one of the most interesting biological systems, in which both normal cell division and a unique cell cycle, meiosis, can be investigated. Recent studies revealed that two genes, c-*kit* and the stem cell factor gene, are directly involved in the mitotic cell cycle of spermatogonia: a tyrosine kinase receptor encoded by the c-*kit* gene in germ cells is activated by stem cell factor expressed in Sertoli cells in a paracrine manner (43, 48, 52). Since self renewal of the germinal stem cells involves the general cell cycle, cell cycle regulatory proteins, such as cyclins and cdc2-related kinases (11, 31, 37), are also suggested to be involved in germinal stem cell growth.

Although the mitotic stage of spermatogenesis has come to be more clearly understood at the molecular level, regulatory mechanisms of the meiotic process are still poorly characterized. Recently, several genes, such as *Hox-1.4* (53), members of the *HSP70* gene family (2, 3, 55), and *ferT* (13, 25), were reported to be expressed specifically near the time of meiosis. However, the physiological roles of their gene products in the meiotic phase are still not known. Other housekeeping genes, such as *PGK-2* (phosphoglycerate kinase 2), are known to be expressed in the testes as an isoform specific to meiosis and maturation (14, 15, 49). The switching of these genes from the somatic form (*PGK-1*) to the meiosis-specific form is thought to be due to the rapid inactivation of the *PGK-1* gene through chromosomal condensation or modification in the early phase of meiosis (15). It is not clear whether these testis-specific housekeeping genes play a regulatory role in the meiotic process.

Protein kinases have been shown to be directly involved in the initiation of meiosis in two different manners in yeast cells. Ran1$^+$/Pat1$^+$ kinase in *Schizosaccharomyces pombe* has a suppressive effect on meiosis, and inactivation of this kinase is essential for the commitment to meiosis (23, 34, 35). On the other hand, IME2/SME1 kinase in *Saccharomyces cerevisiae* is rapidly induced under conditions of low nitrogen in diploid cells, suggesting that this kinase is important for the initiation of meiosis (47, 54).

We have isolated a novel protein kinase gene, *mak* (male germ cell-associated kinase), the expression of which is strictly regulated at and after meiosis. *mak* mRNAs of 3.8 and 2.6 kb appeared in the testes after puberty, but no transcripts were observed at detectable levels in other normal tissues (33). The predicted product of *mak* consists of three regions, a kinase domain, a proline-glutamine-rich region, and a carboxy-terminal region. The kinase domain of Mak shows significant homology to that of the cdc2/CDC28 kinase and the IME2/SME1 kinase (38 to 43% identity). Since a highly conserved region, PSTAIRE, in the cdc2/CDC28 kinase family is divergent in the predicted Mak, a cellular protein(s) different from those that bind to the cdc2/CDC28 kinase could associate with Mak. In this study, using antisera specific for Mak, we characterized the localization, kinase activity, and association with other molecules of Mak. We found that Mak exists as a unique protein complex.

* Corresponding author.

MATERIALS AND METHODS

Preparation of antipeptide antibodies. Three synthetic peptides corresponding to the predicted rat Mak protein (see Fig. 1) [i.e., amino acid residues 388 to 406, (Cys)-Gly-His-Lys-Gly-Ala-Arg-Arg-Arg-Trp-Gly-Gln-Thr-Val-Phe-Lys-Ser-Gly-Asp-Ser (I); amino acid residues 546 to 566, (Cys)-Val-Pro-Ser-Phe-Leu-Lys-Glu-Val-Gly-Ser-Ala-Gly-Gln-Arg-Ile-His-Leu-Ala-Pro-Leu (II); and amino acid residues 603 to 622, (Cys)-Gln-Pro-Val-Pro-Ser-Val-His-Gly-Arg-Thr-Asp-Trp-Val-Ala-Lys-Tyr-Gly-Gly-His-Arg (III)] were coupled to keyhole limpet hemocyanin at the amino-terminal cysteine residue. The Cys residue in each peptide was added artificially for peptide-keyhole limpet hemocyanin coupling. About 200 μg of each coupled peptide was injected into rabbits five times, and the antisera were affinity purified by use of peptide-coupled epoxy-activated Sepharose 6B (Pharmacia) (32).

Preparation of antibodies against a TrpE-Mak fusion protein. The *SacI-EcoRI* fragment of *mak* cDNA containing the entire coding region (33) (the *EcoRI* site was digested with mung bean nuclease to form a blunt end) was ligated to the *SacI-SmaI* sites of pATH11, a *trpE* vector plasmid (26). The predicted 105-kDa TrpE-Mak protein also includes the 11 amino acid residues derived from the 5′-noncoding region of Mak. Recombinant pATH11 containing the *mak* insert was transformed into *Escherichia coli* HB101 and induced, and the insoluble fraction was prepared as described previously (26). Approximately 10 to 50 μg of the fusion protein band was cut from acrylamide gels and used for immunization. Affinity-purified anti-Mak antibodies were prepared from the crude anti-TrpE-Mak antiserum by use of affinity columns (45).

Western blotting (immunoblotting). Testicular lysates of rats (F344; SLC, Shizuoka, Japan) were prepared by use of modified RIPA buffer, containing 50 mM Tris-HCl (pH 7.4), 0.15 M NaCl, 1% Triton X-100, 1% sodium deoxycholate, 0.1% sodium dodecyl sulfate (SDS), 20 mM EDTA, 200 U of aprotinin per ml, and 1 mM phenylmethylsulfonyl fluoride (PMSF). Cell lysates were separated by SDS-polyacrylamide gel electrophoresis (PAGE) (28) and transferred to Immobilon polyvinylidene difluoride transfer membranes (Millipore). The membranes were blocked with 3.75% nonfat dry milk–phosphate-buffered saline (PBS) containing 0.1% Tween 20 for 2 h at 37°C, incubated for 10 to 12 h at 4°C with antibody, washed, probed with ^{125}I-labeled protein A solution (Amersham), washed again three times, dried, and exposed to Dupont X-ray film at −70°C. The protein concentration in the samples was determined by use of the Bio-Rad Laboratories protein assay (6).

Germ cell fractionation. Germ cell suspensions were prepared from 8-week-old rats by enzymatic dissociation of the testes with collagenase (1 mg/ml) and then digestion with trypsin (0.125 mg/ml) and then were fractionated by sedimentation on bovine serum albumin (BSA) gradients at one unit of gravity as previously described (42). The purity of the fractions was examined under a microscope and determined on the basis of the morphological features of the cells. Fractions that were more than 80% pure were used for protein analyses.

Subcellular fractionation of rat testes. Testes from 8-week-old rats were decapsulated and homogenized in 10 volumes of an ice-cold solution containing 0.25 M sucrose, 50 mM *N*-2-hydroxyethylpiperazine-*N*′-2-ethanesulfonic acid (HEPES)–NaOH (pH 7.5), 10 mM NaCl, 10 mM EDTA, 1 mM dithiothreitol (DTT), 200 U of aprotinin per ml, and 1 mM PMSF by use of a Potter-Elvehjem tissue homogenizer. The homogenate was centrifuged at 1,000 × *g* for 10 min at 4°C to yield the crude nuclear fraction. The supernatant was centrifuged at 100,000 × *g* for 1 h at 4°C in an SW41 rotor (Beckman Instruments) to obtain a pellet containing the membrane fraction (P100) and a supernatant containing the cytosolic fraction (S100).

The nuclei were separated from the crude nuclear fraction by centrifugation in the presence of 2.2 M sucrose (8). This separation accomplished by adding 12 volumes of a solution containing 2.4 M sucrose, 50 mM HEPES–NaOH (pH 7.5), 3 mM $CaCl_2$, 1 mM DTT, 200 U of aprotinin per ml, and 1 mM PMSF to the crude nuclear pellet. This mixture was shaken gently and centrifuged at 40,000 × *g* for 1 h at 4°C in an SW28 rotor (Beckman). The pellet obtained contained the nuclear fraction. Each fraction was analyzed by Western blotting.

Metabolic labelling of male germ cells, preparation of cell lysates, and immunoprecipitation. Germ cell suspensions prepared from 8-week-old rat testes were used for in vitro culturing. For labelling of proteins with [^{35}S]methionine, 1.0 × 10^7 germ cells were maintained for 6 h at 32°C with 5% CO_2 in a 60-mm petri dish containing 2.0 ml of Eagle's minimal essential medium (Nissui Seiyaku Corp.) supplemented with 10% (dialyzed) fetal bovine serum–6 mM sodium DL-lactate (Nakarai Chemicals)–1 mM sodium pyruvate (GIBCO)–10 μM methionine–200 μCi of Trans-^{35}S-label (>1,000 Ci/mmol; ICN) (38). For labelling of cells with $^{32}P_i$, 1.0 × 10^7 germ cells were cultured for 6 h at 32°C with 5% CO_2 in a 60-mm petri dish containing 1.5 ml of RPMI 1640 (GIBCO) supplemented with 10% (dialyzed) fetal bovine serum–6 mM sodium DL-lactate–1 mM sodium pyruvate–1 μg of kanamycin per ml–10 mM HEPES (pH 7.5)–1.5 mCi of $^{32}P_i$ (Amersham).

Labelled cells were washed twice with PBS and rapidly frozen in liquid nitrogen. [^{35}S]methionine-labelled cells were lysed in Nonidet P-40 (NP-40) lysis buffer, containing 50 mM HEPES–NaOH (pH 7.5), 150 mM NaCl, 1% NP-40, 10% glycerol, 5 mM EDTA, 1 mM DTT, 200 U of aprotinin per ml, and 1 mM PMSF. $^{32}P_i$-labelled cells were lysed in NP-40 lysis buffer containing 50 mM NaF, 10 mM sodium PP_i, 5 mM nitrophenylphosphate, and 1 mM sodium orthovanadate. The lysates were centrifuged, and the supernatants were precleared with protein A-Sepharose (Pharmacia). The supernatants were incubated with affinity-purified antibodies for 1 h at 4°C. For peptide competition experiments, immunoprecipitation experiments were performed in the presence of 100 to 200 μM peptide. The immune complexes were collected in protein A-Sepharose after incubation for 1 h at 4°C and washed three times with NP-40 lysis buffer and twice with HTG buffer, containing 20 mM HEPES–NaOH (pH 7.5), 0.1% Triton X-100, and 10% glycerol. The immune complexes were analyzed by SDS-PAGE.

In vitro transcription-translation system. Both the 622- and the 580-amino-acid forms of *mak* cDNA were digested with *SacI-EcoRI* and then inserted into the *SacI-EcoRI* sites of plasmid pSP73. The T7 or SP6 RNA polymerase in vitro transcription system and the rabbit reticulocyte cell-free translation system were purchased from Promega. The in vitro reactions were carried out as recommended by Promega. The translation products were labelled with [^{35}S]methionine (>1,000 Ci/mmol; ICN), diluted with NP-40 lysis buffer, and immunoprecipitated as described above. Immune complexes were subjected to SDS-PAGE and autoradiography.

In vitro kinase assays. For the protein kinase assay, the cytosolic fraction (S100) from testes and other tissues was

prepared by use of a buffer containing Tris-HCl (pH 7.4), 10 mM NaCl, 0.25 M sucrose, 5 mM EDTA, 1 mM DTT, 200 U of aprotinin per ml, and 1 mM PMSF. Immunoprecipitation with anti-Mak antibodies was carried out as described above. Immune complexes were washed three times with NP-40 lysis buffer and then twice with HTG buffer. The Mak product–protein A-Sepharose beads were then resuspended in 20 μl of assay buffer, containing 20 mM HEPES–NaOH (pH 7.5), 0.1% Triton X-100, 10% glycerol, 10 mM $MnCl_2$, 0.25 mM β-glycerophosphate, 1 μM ATP, and 10 μCi of [γ-^{32}P]ATP (3,000 Ci/mmol; Amersham) and incubated for 10 min at 22°C. The samples were mixed with a sample buffer for SDS-PAGE, boiled for 3 min, and analyzed by SDS-PAGE.

For the reimmunoprecipitation of Mak proteins from phosphorylated immune complexes, after the kinase assay, the complexes were washed twice with HTG buffer, 20 μl of denaturation buffer, containing 50 mM HEPES–NaOH (pH 7.5), 1% SDS, and 10 mM DTT, was added, and the mixture was boiled for 3 min. The supernatant was diluted 12-fold with NP-40 lysis buffer containing 0.1 mM sodium orthovanadate, 50 mM β-glycerophosphate, and 1 mg of BSA per ml. Immunoprecipitation reactions and the detection of phosphoproteins were performed as described above.

Labelled proteins were cut out of the dried gel, extracted, and hydrolyzed. The phosphoamino acids were separated by thin-layer chromatography as described previously (10).

Kinase assays for exogenous substrates were performed with assay buffer containing 0.5 mg of histone H1 (Boehringer) per ml or 0.25 mg of myelin basic protein (MBP) (Sigma) per ml.

Detection of kinase activity in an SDS-polyacrylamide gel containing MBP. Immunoprecipitates of testicular extracts, obtained with an anti-Mak antibody, were electrophoresed on an SDS-polyacrylamide gel containing 0.5 mg of MBP per ml (17, 24). SDS was removed, and the enzyme was denatured and then renatured as described previously (24). After renaturation, the gel was preincubated at 25°C for 30 min with 10 ml of 40 mM HEPES–NaOH (pH 7.5) containing 2 mM DTT, 0.1 mM ethylene glycol-bis(β-aminoethyl ether)-*N,N,N′,N′*-tetraacetic acid (EGTA), and 10 mM $MnCl_2$. Phosphorylation of MBP was carried out by incubating the gel at 25°C for 1 h with 10 ml of 40 mM HEPES–NaOH (pH 7.5) containing 0.5 mM EGTA, 10 mM $MnCl_2$, 10 μM ATP, and 100 μCi of [γ-^{32}P]ATP. After incubation, the gel was washed with a 5% (wt/vol) trichloroacetic acid solution containing 1% sodium PP_i until the radioactivity of the solution became negligible. The washed gel was dried and then subjected to autoradiography.

Gel filtration chromatography. The 8-week-old rat testis cytosolic fraction (S100) was prepared in the same buffer that was used for the kinase assay of immune complexes. S100 (500 μl) (7.5 mg of protein per ml) was fractionated by gel filtration on a Superose 12 column (Pharmacia). Proteins were eluted in a buffer containing 50 mM Tris-HCl (pH 7.4), 150 mM NaCl, 1 mM EDTA, 1 mM DTT, 5% glycerol, 200 U of aprotinin, per ml, and 1 mM PMSF. Aliquots (50 and 100 μl) of every 0.5-ml eluted fraction were used for Western blotting and the immune complex kinase assay, respectively.

RESULTS

The major products of the *mak* gene are 66- and 60-kDa proteins that appear specifically in the testes after puberty. To examine the *mak* gene products and their stage-specific expression in the testes, we prepared a variety of polyclonal antibodies against three synthetic peptides corresponding to different regions of the predicted Mak protein (I, II, and III) and against a TrpE-Mak peptide (IV) fusion protein (Fig. 1). Western blot analyses with antibodies against Mak peptide I (Fig. 2A, lane 2) and against Mak peptide III (Fig. 2A, lane 3) revealed that these antibodies recognized both the 66- and the 60-kDa proteins in testicular lysates from an 8-week-old rat. No bands were detected by preimmune sera (Fig. 2A, lane 1).

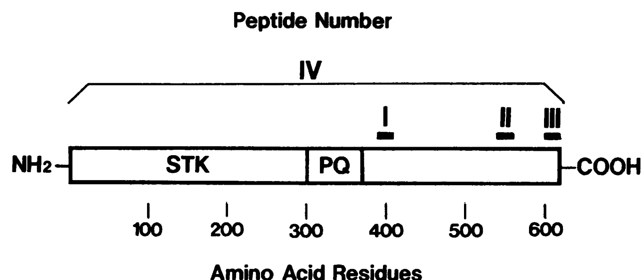

FIG. 1. Antigens used for the preparation of anti-Mak antibodies. The predicted Mak protein consists of 622 amino acids. STK and PQ represent the kinase domain and the proline-glutamine-rich domain in the Mak protein, respectively. Antisera were raised against three synthetic peptides, I, II, and III, and against the entire coding region of the Mak protein (IV) expressed in bacteria.

Although other faint bands of smaller sizes were detected by anti-Mak peptide III antibody (Fig. 2A, lane 3), these bands were not consistently detected by other antibodies produced against the same antigen by different rabbits (data not shown), suggesting that these smaller bands were due to nonspecific cross-reactions. Since the authentic *mak* mRNA encodes a 622-amino-acid protein, proteins of about 66 and 60 kDa are consistent with *mak* gene products. For confirmation that the two different antibodies recognized the same 66- and 60-kDa molecules, testicular lysates were immunoprecipitated with antibody against Mak peptide I, run on an SDS-polyacrylamide gel, and then analyzed by Western blotting with anti-Mak peptide III antibody. The results clearly showed that these 66- and 60 kDa proteins were specifically recognized by antibodies against different epitopes of the predicted Mak protein (lane 8). Thus, we concluded that the *mak* gene products are 66- and 60-kDa proteins ($p66^{Mak}$ and $p60^{Mak}$, or simply p66 and p60) under such denaturing conditions.

Previous analyses by Northern (RNA) blotting showed that *mak* mRNAs of 2.6 and 3.8 kb were specifically expressed in meiotic spermatocytes and early-stage spermatids but not in mitotic spermatogonia, Sertoli cells, or other interstitial cells (33). We examined the developmental expression of Mak products in rat testes by Western blotting with antibody against Mak peptide III (Fig. 2B). Mak products were detectable at 16 days after birth in the testes, and the levels of both p66 and p60 products increased gradually, reaching a plateau about 4 weeks after birth. The pattern of expression of Mak products coincided with the appearance of meiotic cells in the testes.

The two Mak proteins may correspond to heterogeneous *mak* mRNAs. The two different sizes of Mak products could be due to different coding sequences in *mak* mRNAs or to posttranslational modifications. Among the *mak* cDNAs isolated from the rat testis cDNA library, we found an

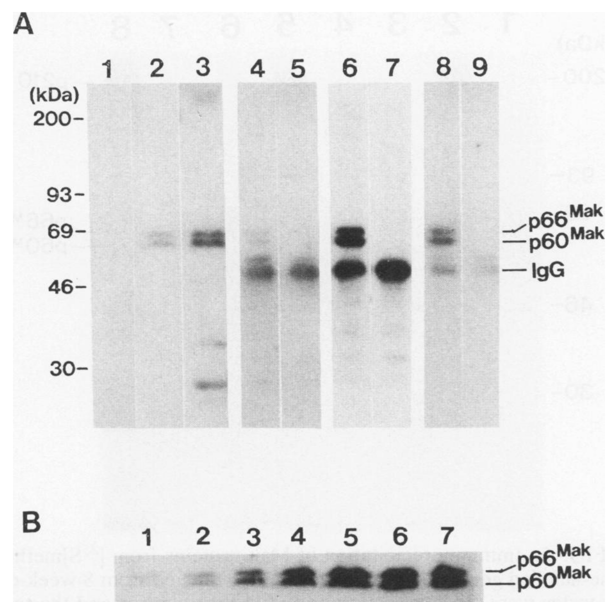

FIG. 2. Identification of *mak* gene products and developmental expression of Mak proteins in the testes. (A) Total soluble proteins (100 μg) in modified RIPA buffer prepared from the testes of an 8-week-old rat were separated by SDS-PAGE, transferred to a membrane, and reacted with antibody against Mak peptide I (lane 2), antibody against Mak peptide III (lane 3), and preimmune sera (lane 1). After a wash, the bands were detected with ^{125}I-labelled protein A. In lanes 4 to 9, after immunoprecipitation of Mak proteins with antibodies against Mak peptide I (lanes 4 and 8) and against Mak peptide III (lane 6), immune complexes were separated on an SDS-polyacrylamide gel and then analyzed by Western blotting with antibodies against Mak peptide I (lanes 4 and 5) and against Mak peptide III (lanes 6 to 9). For blocking experiments, these antibodies were used in competition with peptide I (lanes 5 and 9) and with peptide III (lane 7), respectively, in immunoprecipitation reactions. Molecular masses (in thousands) of marker proteins are indicated at the left. IgG, immunoglobulin G. (B) Total soluble extracts (100 μg) in the same lysis buffer as that mentioned above and prepared from the testes of 7 (lane 1)-, 16 (lane 2)-, 18 (lane 3)-, 20 (lane 4)-, 22 (lane 5)-, and 24 (lane 6)-day-old and 8 (lane 7)-week-old rats were analyzed by Western blotting with anti-Mak peptide III antibody.

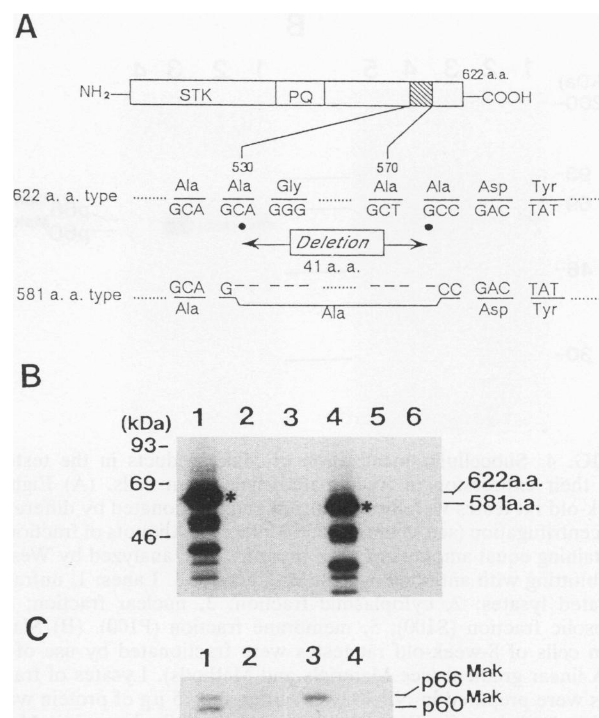

FIG. 3. The major difference in size between p66 and p60 Mak proteins could be due to different coding sequences in the carboxyl region. (A) The predicted *mak* gene product consists of 622 amino acids (622 a. a. type). Mak with a deletion (581 a. a. type) is expected to lose 41 amino acid residues, from Ala-530 to Ala-570, within the carboxyl region of 622 a. a. type. (B) Two types of *mak* cDNAs (622 and 581 amino acids long in the coding region) were transcribed and translated in vitro as described in Materials and Methods. RNAs for the sense strand of 622 a. a. type (lanes 1 and 3), the antisense strand of 622 a. a. type (lane 2), the sense strand of 581 a. a. type (lanes 4 and 6), and the antisense strand of 581 a. a. type (lane 5) were translated in the presence of [^{35}S]methionine, and the products were immunoprecipitated with antibody against Mak peptide III. In lanes 3 and 6, for the blocking experiment, this antibody competed with peptide III. Samples were separated by SDS-PAGE, and labelled proteins were detected by autoradiography. Asterisks indicate the major translational products of 622 a. a. type and 581 a. a. type. (C) Immunoprecipitates obtained with antibody against Mak peptide III of testicular lysates prepared in modified RIPA buffer from the testes of an 8-week-old rat were analyzed by Western blotting with antibodies against Mak peptide III (lanes 1 and 2) and against Mak peptide II (lanes 3 and 4). In lanes 2 and 4, antibody competed with peptide III in immunoprecipitation reactions.

aberrant *mak* coding sequence in a cDNA carrying a 123-bp in-frame deletion mutation (nucleotide residues 1828 [C] to 1950 [G]) within the carboxy-terminal region of the Mak protein (Fig. 3A). The predicted Mak product bearing this deletion mutation is expected to lose 41 amino acids, from Ala-530 to Ala-570, resulting in a product 581 amino acids long. cDNA polymerase chain reaction analysis of testicular RNA with primers directed towards the upstream and downstream sequences of the deletion region demonstrated that both the 622- and the 581-amino-acid-type *mak* mRNAs were expressed from 16-day-old to 10-week-old rat testes in similar ratios (49a; data not shown).

For examination of whether these 622- and 581-amino acid *mak* coding sequences correspond to the 66- and 60-kDa proteins in testicular lysates, both the *mak* cDNAs were ligated to an expression vector and the RNAs synthesized in the sense or antisense orientation were translated in vitro in the presence of [^{35}S]methionine. As shown in Fig. 3B, the major products immunoprecipitated with antibody against Mak peptide III (carboxy terminus) from the 622-amino-acid Mak mRNA (lane 1) and the 581-amino-acid Mak mRNA (lane 4) were approximately 66 and 60 kDa in size, respectively. No labelled proteins were precipitated with the same antibody either from antisense (lanes 2 and 5) or from sense (lanes 3 and 6) translational products in competition with peptide III. The smaller sizes of the radiolabelled bands might be due to protein degradation.

For further confirmation that the p66 and p60 Mak proteins differ because of to the different coding sequences in the carboxyl region, anti-Mak peptide II antibody specific for the deletion region (amino acid residues 546 to 566) was prepared (Fig. 1). As shown in Fig. 3C (lane 3), this antibody recognized only the 66-kDa protein among the Mak proteins. These results strongly suggest that the two types of Mak products in the testes are derived from two different transcripts.

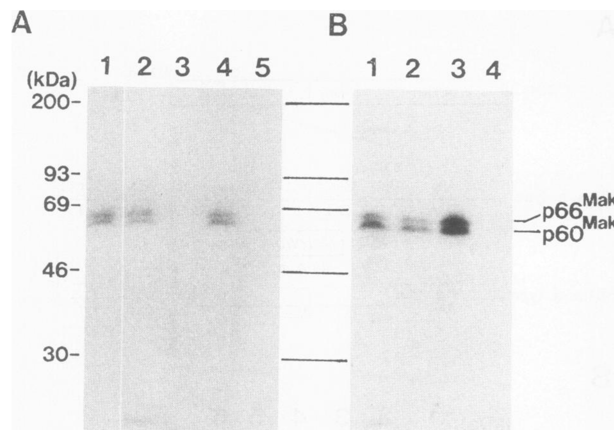

FIG. 4. Subcellular localization of Mak products in the testes and their expression in fractionated male germ cells. (A) Eight-week-old rat testes were homogenized and fractionated by differential centrifugation (see Materials and Methods). Aliquots of fractions containing equal amounts of Mak proteins were analyzed by Western blotting with antibody against Mak peptide I. Lanes: 1, unfractionated lysates; 2, cytoplasmic fraction; 3, nuclear fraction; 4, cytosolic fraction (S100); 5, membrane fraction (P100). (B) Male germ cells of 8-week-old rat testes were fractionated by use of a BSA linear gradient (see Materials and Methods). Lysates of fractions were prepared in NP-40 lysis buffer and 75 μg of protein was used in each lane for Western blotting with antibody against Mak peptide I. Lanes: 1, dissociated spermatogenic cells before fractionation; 2, fractionated spermatogenic cells containing spermatogonia, primary spermatocytes at the leptotene, zygotene, and early pachytene stages, and secondary spermatocytes; 3, primary spermatocytes at the late pachytene stage; 4, round spermatids.

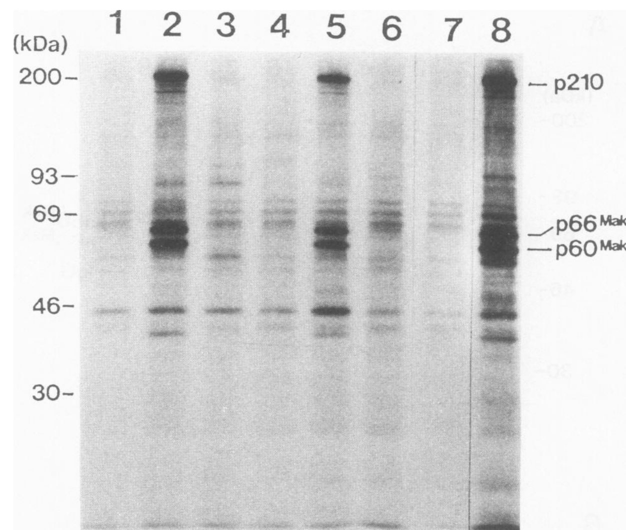

FIG. 5. Immunoprecipitation of Mak proteins from [^{35}S]methionine-labelled germ cells. Male germ cells obtained from 8-week-old rat testes were labelled in vitro with [^{35}S]methionine, and the total detergent (1% NP-40)-soluble cell lysates were immunoprecipitated with antibodies against Mak peptide I (lane 2), against Mak peptide III (lane 5), and against Mak peptide IV (lane 8) and with preimmune sera (lanes 1, 4, and 7). In a peptide competition analysis, antibodies against Mak peptide I (lane 3) and against Mak peptide III (lane 6) competed with the corresponding peptides. Samples were analyzed by SDS-PAGE, and labelled proteins were detected by autoradiography.

Mak products are mostly localized in the cytoplasm in a soluble form and are abundant in germ cells near the time of the late pachytene stage. Nonreceptor-type protein kinases involved in signal transduction or in the cell cycle are localized near the membrane, the cytoplasm, or nuclei, depending on their roles within the cell. For understanding the physiological function of Mak products, it appears important to clarify the subcellular localization and stage-specific expression of Mak proteins during spermatogenesis. A testicular homogenate prepared without detergent was fractionated by differential centrifugation (see Materials and Methods), and the fractionated samples were analyzed by Western blotting. As shown in Fig. 4A, both p66 and p60 Mak proteins were abundant in the postnuclear supernatant fraction (lane 2) but were hardly detectable in the nuclear fraction (lane 3). The postnuclear supernatant was further centrifuged at 100,000 × g to obtain the pellet (P100; membrane fraction) and the supernatant (S100; cytosolic fraction). Most of the Mak proteins were localized in S100 (lane 4), and very few were localized in P100 (lane 5), indicating that Mak proteins are present in a soluble form in the cytoplasm.

mak mRNAs are expressed at and after meiosis, the level of the 2.6-kb mRNA being higher in the postmeiotic stage and round-spermatid stage (33). For determination of which types of cells in the testes the Mak products exist in, testicular germ cells were fractionated by use of a BSA linear gradient, and the amounts of the Mak proteins were examined (Fig. 4B). The levels of both p66 and p60 Mak proteins were highest in the late pachytene cell fraction (lane 3), lower in the early pachytene cell fraction, which is a mixture of other types of cells (lane 2), and extremely low in the fraction of round spermatids (lane 4). The localization of Mak products in the testes was also examined by the indirect immunofluorescence method (19). Mak products were identified in the primary spermatocytes of the pachytene and diplotene stages but were not detectable in the spermatogonia or in the round spermatids (data not shown). These results suggest that Mak proteins may have a physiological function during the pachytene stage of meiosis. Since the level of the 2.6-kb *mak* mRNA is still high in round spermatids, a very low level of Mak proteins in these cells suggests poor efficiency in the translation of the 2.6-kb mRNA or rapid degradation of Mak products after meiosis.

Mak proteins are tightly associated with the 210-kDa cellular protein in the testes. In general, it has not been easy to find the physiological regulators and cellular substrates of protein kinases. As an exception, a few protein kinases are reported to be tightly associated with cellular proteins: for example, p13 and cyclin B are bound to cdc2 kinase and regulate its catalytic activity (5, 7, 27). We made an attempt to identify such molecules associated with Mak proteins by labelling testicular germ cells in cultures with [^{35}S]methionine. After labelling was done, cell lysates were prepared with NP-40 lysis buffer, and Mak proteins were immunoprecipitated with various anti-Mak antibodies. In addition to the p66 and p60 Mak proteins, a 210-kDa protein (p210) and a 40-kDa protein (p40) were specifically detected (Fig. 5, lanes 2, 5, and 8). These proteins were not immunoprecipitated by preimmune sera (lanes 1, 4, and 7) or by anti-Mak antibodies in the presence of competing peptides (lanes 3 and 6). Reproducible detection of p210 and p40 in the Mak immune complex with various antibodies against three different

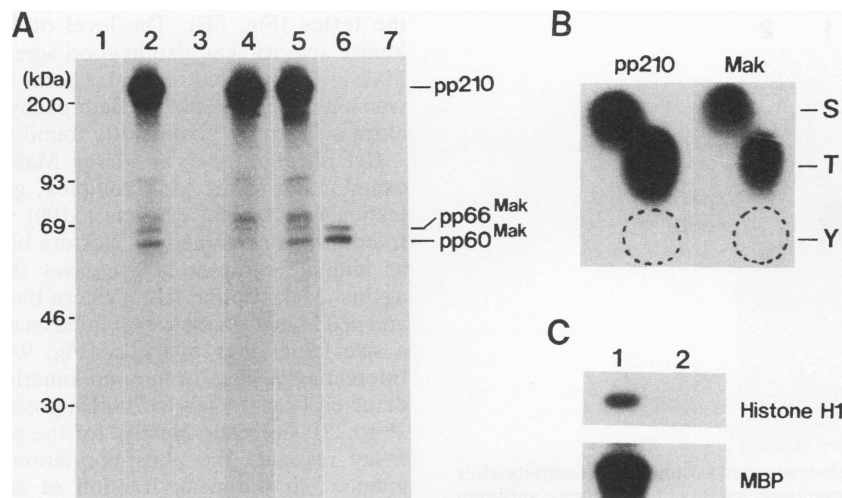

FIG. 6. In vitro kinase activity in Mak immunoprecipitates. (A) Testicular S100 fractions (each 200 μg) from 8-week-old rats were immunoprecipitated with antibodies against Mak peptide I (lane 2), against Mak peptide III (lane 4), and against Mak peptide IV (lane 5) and with preimmune serum (lane 1). In lane 3, antibody against Mak peptide I competed with peptide I. The in vitro kinase assay was performed with the Mak immune complex as described in Materials and Methods. Samples were analyzed by SDS-PAGE, and phosphoproteins were detected by autoradiography. In lanes 6 and 7, a phosphorylated immune complex obtained with antibody against Mak peptide IV was placed in 1% SDS–10 mM DTT and boiled for 3 min. Samples were diluted and reimmunoprecipitated with antibody against Mak peptide III (lane 6) or against Mak peptide III that had competed with peptide III (lane 7). Lanes 1 to 5 were exposed for 1 h and lanes 6 and 7 were exposed for 12 h with an intensifying screen at −80°C. (B) After the kinase assay, phosphorylated p210 (pp210) and Mak (both p66 and p60) were cut out of the dried gel and extracted, and a phosphoamino acid analysis was done. S, serine; T, threonine; Y, tyrosine. (C) Immunoprecipitates obtained with antibody against Mak peptide III were assayed for their ability to transfer ^{32}P from [γ-^{32}P]ATP to histone H1 and MBP (lane 1). In lane 2, immunoprecipitates were prepared in the presence of competing peptide III.

epitopes of the predicted *mak* gene product indicates that p210 and p40 are coprecipitated with Mak products, suggesting the formation of a Mak protein complex in association with p210 and p40. However, the possibility that p40 is a degradation product of p210 after immunoprecipitation cannot be completely ruled out.

The Mak immune complex is associated with serine/threonine-specific protein kinase activity and efficiently phosphorylates p210. For examination of whether protein kinase activity is associated with the Mak immune complex, an in vitro protein kinase assay was carried out. Immunoprecipitates formed with anti-Mak antibodies from S100 fractions of 8-week-old rat testes were incubated with [γ-^{32}P]ATP in the presence of 10 mM $MnCl_2$. As shown in Fig. 6A, protein kinase activity was specifically detected in the Mak immune complex (lanes 2, 4, and 5). The [^{35}S]methionine labelling experiments described above revealed that p210 was coprecipitated with Mak products (Fig. 5). In the in vitro kinase assay, p210 was highly phosphorylated (pp210) by the kinase activity associated with the Mak immune complex. Other, minor phosphorylated bands in the range of 60 to 93 kDa (pp60, pp66, pp70, and pp93) were also detected.

For examination of whether pp66 and pp60 are phosphorylated Mak products themselves, the Mak immune complex, after the in vitro kinase reaction, was denatured in the presence of SDS and DTT, diluted, and reimmunoprecipitated with anti-Mak antibody. As shown in Fig. 6B (lane 6), pp66 and pp60 were specifically recognized by the anti-Mak antibody. These results clearly indicate that both p66 and p60 Mak proteins are phosphorylated to some extent in vitro. Other phosphorylated bands, of p70 and p93, appear to be nonspecific, since these proteins also bound to the preimmune serum–protein A-Sepharose complex (Fig. 5).

Phosphoamino acid analysis of the in vitro-phosphorylated p210, p66, and p60 Mak proteins revealed both phosphoserine and phosphothreonine, indicating that a serine/threonine-specific kinase activity (rather than a tyrosine-specific kinase activity) is associated with the Mak immune complex (Fig. 6B).

We next analyzed the ability of the Mak immune complex to phosphorylate exogenous substrates. As shown in Fig. 6C, an immune complex containing Mak products phosphorylated efficiently both histone H1 and MBP. Casein used as an exogenous substrate was not phosphorylated by Mak-associated kinase activity (data not shown).

Kinase activities are associated with both p66 and p60 Mak proteins. Although the Mak protein complex clearly shows kinase activity and the Mak products contain a kinase domain, there is no direct evidence for the association of kinase activity with Mak proteins. A procedure for the identification of protein kinase activity after electrophoresis on SDS-polyacrylamide gels containing substrates, the in-gel kinase assay, was recently developed (24). To examine whether the Mak products really have protein kinase activity, we performed kinase reactions by use of a polyacrylamide gel with MBP as the substrate following denaturation and renaturation after SDS-PAGE of the Mak immune complex. As shown in Fig. 7, kinase activity was detected in both p66 and p60 Mak proteins (lane 1). No activity was detected in the control experiment carried out in the presence of a competing peptide (lane 2). These results clearly indicate that kinase activity is associated with Mak products. Furthermore, no kinase activity was detected in p210 under these conditions, suggesting that the kinase activity associated with the Mak immune complex is derived from the Mak products themselves.

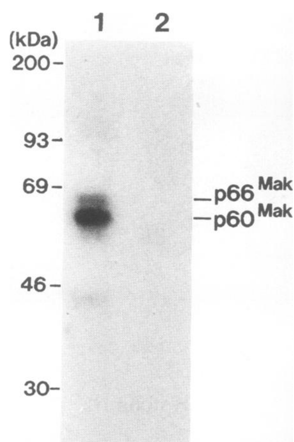

FIG. 7. Detection of Mak-associated protein kinase activity after SDS-PAGE with MBP. Immunoprecipitates obtained with antibody against Mak peptide III (lane 1) or against Mak peptide III that had competed with peptide III (lane 2) of the testicular cytosolic fraction (S100) from an 8-week-old rat were electrophoresed in an SDS-polyacrylamide gel containing MBP. After renaturation of proteins in the gel, kinase reactions and the detection of kinase activity were carried out as described in Materials and Methods.

Tissue- and stage-specific expression of Mak-associated protein kinase activity. For confirmation of the specificity of Mak-associated protein kinase activity, cell lysates of various tissues as well as testicular lysates at different stages were used for a kinase assay. The results (Fig. 8A) demonstrated that the kinase activity in the Mak immune complex was detectable only in the testes and that the level of activity increased in parallel with the increase in the level of Mak in the testes (Fig. 8B). The level of Mak-associated protein kinase activity was also in good agreement with the level of Mak in fractionated testicular germ cells (Fig. 8C); activity was highest in cells of the late pachytene stage (lane 3) but extremely low in postmeiotic round spermatids (lane 4).

Gel filtration analysis of the Mak complex. For further examination of the Mak complex, gel filtration analysis of testicular cytosolic extracts (S100) was carried out. Each fraction was examined by Western blotting (Fig. 9A) and by an immune complex kinase assay (Fig. 9B) with antibody against Mak peptide III. Western blotting revealed that p66 and p60 Mak proteins were eluted as a peak corresponding to a size larger than 500 kDa (Fig. 9A, fractions 19 to 23). Interestingly, few or no monomeric Mak products were detected near the 60- to 70-kDa position (Fig. 9A, fractions 26 to 27). Correspondingly, for the same region, the kinase assay revealed the phosphorylation of p210 in a similar manner, in the peak fraction of Mak products, as was observed with Western blotting (Fig. 9B). These results further support complex formation of Mak with p210.

p210 is phosphorylated in vivo. Although the p210 cellular protein is efficiently phosphorylated in the Mak immune complex in vitro, there is no direct evidence for the phosphorylation of p210 under physiological conditions. For examination of whether p210 is phosphorylated within cells, testicular germ cells were labelled with $^{32}P_i$ in cultures. After labelling was done, cell lysates prepared in NP-40 lysis buffer were immunoprecipitated with antibody against Mak peptide III. As shown in Fig. 10, the phosphorylated p210 band was clearly observed (lane 1). This phosphorylated band was specific, because no bands were detected when a competing peptide was added to the lysate prior to the immunoprecipitation reaction (lane 2). In correlation with the results of our in vitro kinase assay, we were able to

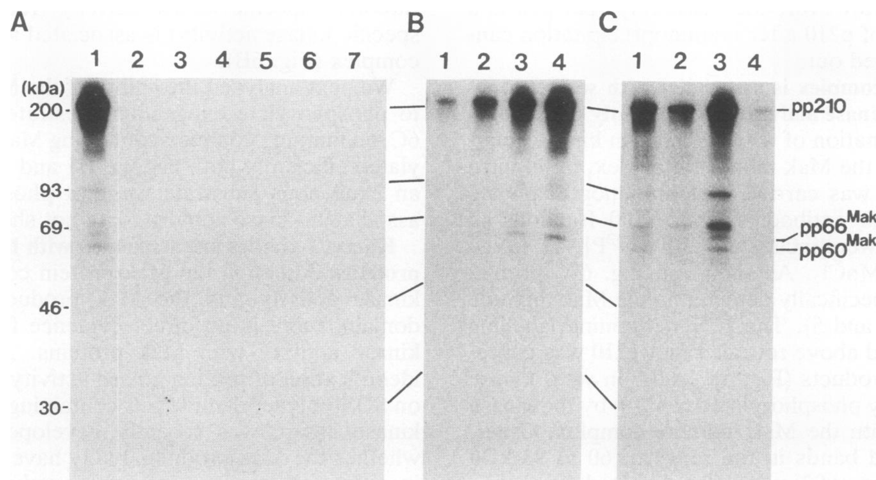

FIG. 8. Tissue- and stage-specific expression of Mak-associated protein kinase activity. (A) Cytosolic (S100) fractions (200 μg) prepared from various tissues of 8-week-old rats were immunoprecipitated with antibody against Mak peptide III, and an in vitro kinase assay was carried out. The protein samples were prepared from the testes (lane 1), brain (lane 2), thymus (lane 3), liver (lane 4), spleen (lane 5), and kidneys (lane 6). For lane 7, an S100 fraction of an ovary was prepared from a 15-week-old rat and used for the kinase assay. A similar analysis with NP-40 lysis buffer yielded essentially the same results (data not shown). (B) S100 fractions (200 μg) prepared from the testes of 16 (lane 1)-, 18 (lane 2)-, and 22 (lane 3)-day-old and 8 (lane 4)-week-old rats were immunoprecipitated with antibody against Mak peptide III, and the protein kinase activity was determined in vitro. (C) Male germ cells of 8-week-old rats were fractionated, and lysates were prepared by the same methods as those described in the legend to Fig. 4B. Lanes: 1, dissociated spermatogenic cells before fractionation; 2, fractionated spermatogenic containing spermatogonia, primary spermatocytes at the leptotene, zygotene, and early pachytene stages, and secondary spermatocytes; 3, primary spermatocytes at the late pachytene stage; 4, round spermatids. In each lane, 100 μg of protein was used for immunoprecipitation with antibody against Mak peptide III, and then an in vitro kinase assay was carried out. For unknown reasons, nonspecific pp93 and pp70 bands (see the text) seem to be enhanced (probably because of the use of a detergent-containing lysate).

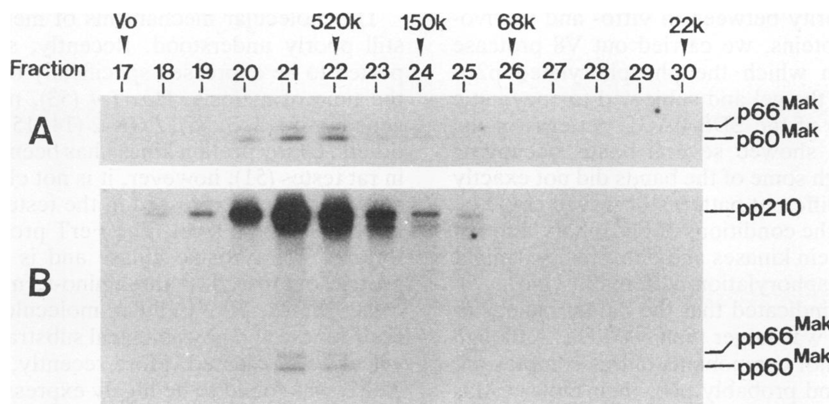

FIG. 9. Gel filtration analysis of S100 fractions from testes. (A) A Testicular cytosolic fraction (S100) obtained from an 8-week-old rat was fractionated by gel filtration on a Superose 12 column (Pharmacia) as described in Materials and Methods. Fifty microliters of each fraction was analyzed by Western blotting with antibody against Mak peptide III. (B) One hundred microliters of each fraction was immunoprecipitated with the same antibody as that used in panel A, and an in vitro protein kinase assay was carried out. Trypsin inhibitor (22 kDa), BSA (68 kDa), rabbit immunoglobulin G (150 kDa), and β-galactocidase (520 kDa) were used to calibrate the column for molecular mass. Vo, column void fraction.

observe the phosphorylation of p210 on serine and threonine residues only in vivo (data not shown). Since the Mak-p210 complex contains Mak products that have protein kinase activity (Fig. 7), it seems quite possible that at least some of the phosphorylation of p210 in vivo occurs through Mak.

Although the threonine residue for autophosphorylation in other protein kinases, such as cdc2 kinase (18) and mitogen-activated protein (MAP) kinase (40), is also present at Thr-156 of subdomain VIII (20), the phosphorylation of Mak products was not detectable under the culture conditions used in this study. It will be of interest to see what kind of mechanism or condition in vivo is involved in the regulation of kinase activity associated with the Mak products.

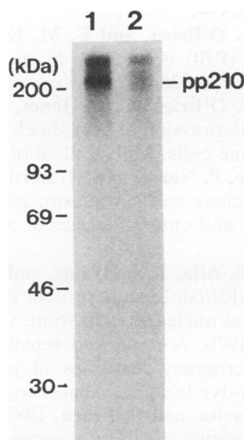

FIG. 10. Immunoprecipitation of Mak proteins from $^{32}P_i$-labelled germ cells. Male germ cells obtained from 8-week-old rat testes were labelled in vitro with $^{32}P_i$, and total detergent (1% NP-40)-soluble cell lysates were immunoprecipitated with antibody against Mak peptide III (lane 1) or against Mak peptide III that had competed with peptide III (lane 2). Samples were analyzed by SDS-PAGE and subjected to autoradiography.

DISCUSSION

In this study, we have shown that a novel protein kinase, Mak, is expressed as 66- and 60-kDa proteins, almost exclusively in the meiotic phase of testicular germ cells. Mak is associated with a p210 cellular protein that is efficiently phosphorylated in vitro in a Mak immune complex and also in vivo. These results strongly implicate that Mak plays an important role in the meiotic phase during spermatogenesis.

We detected two Mak coding sequences in rats, a 622-amino-acid coding sequence and a deletion-containing 581-amino-acid coding sequence, which correspond to the 66- and 60-kDa products. Our preliminary experiments suggest that mouse testes also contain the 66- and 60-kDa Mak proteins, detected by anti-rat Mak antisera. Thus, both types of Mak appear to be phylogenetically conserved in rodent species. Recently, we molecularly cloned human *mak* cDNAs from a testis cDNA library. In these cDNAs, the authentic structure of human Mak consists of a 623-amino-acid product that is highly homologous to the rat 622-amino-acid Mak product. However, we did not detect any deletion-carrying Mak similar to the 581-amino-acid Mak in rats. Instead, a few aberrant structures of human Mak, i.e., insertions in the carboxyl region, were detected (49b). Therefore, we suggest that the 622- or 623-amino-acid Mak is the fundamental Mak in mammalian species. Functional differences between the 622- and 581-amino-acid forms of Mak in rats are not clear yet.

Mak is associated with at least one species of cellular protein, p210. p210 is efficiently phosphorylated in the Mak immune complex in vitro and also in vivo under culture conditions. Although we cannot rule out the possibility that p210 is also another protein kinase, a simple explanation for the detection of kinase activity in Mak products by an in-gel kinase assay could be that Mak acts as protein kinase for p210 (Fig. 7). It will be interesting to examine whether p210 and p40 are also meiosis-specific proteins or are housekeeping proteins expressed in other tissues. Molecular cloning of these associated molecules may be important for a better understanding of the physiological function of Mak.

To examine the similarity between in vitro- and in vivo-phosphorylated p210 proteins, we carried out V8 protease cleavage experiments in which the phosphorylated p210 proteins were cut out of the gel and subjected to enzymatic digestion. A comparison of the SDS-PAGE patterns of the phosphorylated proteins showed several bands occupying similar positions, although some of the bands did not exactly match each other. The different patterns observed could be due to the differences in the conditions of phosphorylation of p210 proteins: other protein kinases and phosphatases might modify p210 protein phosphorylation patterns in vivo.

Gel filtration analysis indicated that the native molecular size of the Mak complex was larger than 500 kDa. Although we suggested that the major components of this complex are $p66^{Mak}$, $p60^{Mak}$, p210, and probably p40, their molar ratios and other possible minor components are still not yet clear. Since we detected a faint band of 180 kDa (p180) phosphorylated in a kinase assay after gel filtration (Fig. 9B, fractions 22 to 24) and since the p180-Mak complex could be separated from the p210-Mak complex by anion-exchange chromatography (data not shown), the Mak complex may not be present in a single form. Thus, it will be interesting to study the structural and functional relationships between p210 and other minor components, such as p180.

The primary structure of Mak shows significant homology to those of the cdc2 kinase and the cdk2 kinase (12, 36, 50), important in the cell cycle, the MAP kinase, involved in signal transduction (1, 16, 22, 44), and the IME2/SME1 meiosis-specific kinase in budding yeast cells (54). The functions of Mak may also be similar to those of these kinases. However, several differences between Mak and cdc2 kinase were also observed. The majority of Mak was localized in the cytoplasm, whereas cdc2 kinase appears to translocate to the nuclei (4, 5, 41). Furthermore, the molecular sizes of proteins associated with cdc2 kinase (p62, cyclin B, and p13) are different from those of proteins associated with Mak (7, 27). MAP kinase is also reported to be distributed in both the cytoplasm and nuclei (9). In addition, MAP kinase is present in a monomeric form within the cell (17).

Concerning the relationship between Mak and MAP kinase, several similarities can be pointed out. (i) The identity at the amino acid level in the kinase domain of these two protein kinases is about 35%, indicating significant structural similarity. (ii) Mak is able to phosphorylate in vitro MBP, which is considered to be one of the specific substrates for MAP kinase. (iii) Our preliminary data indicate that the Mak complex can elevate the level of phosphorylation of a p220 protein associated with microtubles in vitro, like MAP kinase can (46). This 220-kDa protein is different from the 210-kDa protein of the Mak complex, at least in size. On the basis of these observations, one interesting hypothetical model is that the Mak complex may act as a regulator of microtubles or related systems in testicular germ cells. This model needs to be further investigated by a more direct approach, such as a characterization of the similarity between p210 and MAP.

Although the structural similarity between Mak and the IME2/SME1 kinase in yeast cells is also significant, their functional relationship is still open to question: the IME2 kinase was reported to be able to initiate meiosis-specific DNA synthesis in IME2-overexpressing yeast cells, whereas the expression of Mak is high in the pachytene stage, in which DNA synthesis for meiosis is mostly terminated. Thus, there is so far no clear evidence supporting a functional similarity between Mak and the IME2 kinase.

The molecular mechanisms of meiosis in vertebrates are still poorly understood. Recently, several genes were reported to be expressed specifically or relatively highly near the time of meiosis: *Hox-1.4* (53), members of the *HSP70* gene family (2, 3, 55), *PGK-2* (14, 15, 49), *ferT* (13, 25), and others. c-*mos* protein kinase has been shown to be expressed in rat testes (51); however, it is not clear whether the human c-*mos* mRNAs expressed in the testes (39) are functional at the translational level. The FerT protein kinase is a variant form of Fer tyrosine kinase and is different from the Fer protein expressed at the amino-terminal region in somatic cells (13, 21, 30). Cellular molecules associated with the FerT kinase and physiological substrates for this kinase have yet to be elucidated. More recently, a new protein kinase, Nek1, was found to be highly expressed in the meiotic germ cells of mammals (29). Nek1 is distantly related to the NIMA gene product in *Aspergillus nidulans*, which has an important role in mitosis. Thus, it will be of interest to examine the functional relationship between Nek1 and NIMA and the biological role of Nek1 in spermatogenesis. Regarding Mak proteins, we have shown here the size, association with other proteins, and possible phosphorylation. Thus, the Mak system could present a useful tool for clarifying some aspects of the molecular mechanisms involved in the meiotic phase of spermatogenesis.

ACKNOWLEDGMENTS

We thank Fumiko Hirose and Akio Matsukage (Aichi Cancer Center) for fractionation of testicular germ cells. We are also grateful to Michiko Yamashita and Sachiko Yamaguchi for excellent technical assistance and Yasuhisa Fukui for helpful discussions.

This work was supported by a Grant-in-Aid for Special Project Research on Cancer-Bioscience (03258204) from the Ministry of Education, Science and Culture of Japan; a research grant from the Princess Takamatsu Cancer Research Fund; and a research grant from the Foundation for Promotion of Cancer Research in Japan.

REFERENCES

1. **Ahn, N. G., J. E. Weiel, C. P. Chan, and E. G. Krebs.** 1990. Identification of multiple epidermal growth factor-stimulated protein serine/threonine kinases from Swiss 3T3 cells. J. Biol. Chem. **265:**11487–11494.
2. **Allen, R. L., D. A. O'Brien, and E. M. Eddy.** 1988. A novel hsp70-like protein (P70) is present in mouse spermatogenic cells. Mol. Cell. Biol. **8:**828–832.
3. **Allen, R. L., D. A. O'Brien, C. C. Jones, D. L. Rockett, and E. M. Eddy.** 1988. Expression of heat shock proteins by isolated mouse spermatogenic cells. Mol. Cell. Biol. **8:**3260–3266.
4. **Bailly, E., M. Doree, P. Nurse, and M. Bornens.** 1989. $p34^{cdc2}$ is located in both nucleus and cytoplasm; part is centrosomally associated at G_2/M and enters vesicles at anaphase. EMBO J. **8:**3985–3995.
5. **Booher, R. N., C. E. Alfa, J. S. Hyams, and D. H. Beach.** 1989. The fission yeast cdc2/cdc13/suc1 protein kinase: regulation of catalytic activity and nuclear localization. Cell **58:**485–497.
6. **Bradford, M. M.** 1976. A rapid and sensitive method for the quantitation of microgram quantities of protein utilizing the principle of protein-dye binding. Anal. Biochem. **72:**248–254.
7. **Brizuela, L., G. Draetta, and D. Beach.** 1987. $p13^{suc1}$ acts in the fission yeast cell division cycle as a component of the $p34^{cdc2}$ protein kinase. EMBO J. **6:**3507–3514.
8. **Chauveau, J., Y. Moule, and C. H. Rouiller.** 1956. Isolation of pure and unaltered liver nuclei: morphology and biochemical composition. Exp. Cell Res. **11:**317–321.
9. **Chen, R.-H., C. Sarnecki, and J. Blenis.** 1992. Nuclear localization and regulation of *erk*- and *rsk*-encoded protein kinases. Mol. Cell. Biol. **12:**915–927.

10. **Cooper, J. A., B. M. Sefton, and T. Hunter.** 1983. Detection and quantification of phosphotyrosine in proteins. Methods Enzymol. **99:**387–402.
11. **Ducommun, B., P. Brambilla, M.-A. Felix, B. R. Franza, Jr., E. Karsenti, and G. Draetta.** 1991. cdc2 phosphorylation is required for its interaction with cyclin. EMBO J. **10:**3311–3319.
12. **Elledge, S. J., and M. R. Spottswood.** 1991. A new human p34 protein kinase, CDK2, identified by complementation of a *cdc28* mutation in *Saccharomyces cerevisiae*, is a homolog of *Xenopus* Egl. EMBO J. **10:**2653–2659.
13. **Fischman, K., J. C. Edman, G. M. Shackleford, J. A. Turner, W. J. Rutter, and U. Nir.** 1990. A murine *fer* testis-specific transcript (*ferT*) encodes a truncated Fer protein. Mol. Cell. Biol. **10:**146–153.
14. **Gold, B., H. Fujimoto, J. M. Kramer, R. P. Erickson, and N. B. Hecht.** 1983. Haploid accumulation and translational control of phosphoglycerate kinase-2 messenger RNA during mouse spermatogenesis. Dev. Biol. **98:**392–399.
15. **Goto, M., T. Koji, K. Mizuno, M. Tamaru, S. Koikeda, P. K. Nakane, N. Mori, Y. Masamune, and Y. Nakanishi.** 1990. Transcription switch of two phosphoglycerate kinase genes during spermatogenesis as determined with mouse testis sections *in situ*. Exp. Cell Res. **186:**273–278.
16. **Gotoh, Y., K. Moriyama, S. Matsuda, E. Okumura, T. Kishimoto, H. Kawasaki, K. Suzuki, I. Yahara, H. Sakai, and E. Nishida.** 1991. *Xenopus* M phase MAP kinase: isolation of its cDNA and activation by MPF. EMBO J. **10:**2661–2668.
17. **Gotoh, Y., E. Nishida, T. Yamashita, M. Hoshi, M. Kawakami, and H. Sakai.** 1990. Microtubule-associated-protein (MAP) kinase activated by nerve growth factor and epidermal growth factor in PC12 cells. Eur. J. Biochem. **193:**661–669.
18. **Gould, K. L., S. Moreno, D. J. Owen, S. Sazer, and P. Nurse.** 1991. Phosphorylation at Thr167 is required for *Schizosaccharomyces pombe* $p34^{cdc2}$ function. EMBO J. **10:**3297–3309.
19. **Haneji, T., and S. S. Koide.** 1987. Identification of antigen in rat spermatogenic cells interacting with an anti-human sperm monoclonal antibody. Biol. Reprod. **37:**467–477.
20. **Hanks, S. K., A. M. Quinn, and T. Hunter.** 1988. The protein kinase family: conserved features and deduced phylogeny of the catalytic domains. Science **241:**42–52.
21. **Hao, Q.-L., N. Heisterkamp, and J. Groffen.** 1989. Isolation and sequence analysis of a novel human tyrosine kinase gene. Mol. Cell. Biol. **9:**1587–1593.
22. **Hoshi, M., E. Nishida, and H. Sakai.** 1988. Activation of a Ca^{2+}-inhibitable protein kinase that phosphorylates microtubule-associated protein 2 *in vitro* by growth factors, phorbol esters, and serum in quiescent cultured human fibroblasts. J. Biol. Chem. **263:**5396–5401.
23. **Iino, Y., and M. Yamamoto.** 1985. Negative control for the initiation of meiosis in *Schizosaccharomyces pombe*. Proc. Natl. Acad. Sci. USA **82:**2447–2451.
24. **Kameshita, I., and H. Fujisawa.** 1989. A sensitive method for detection of calmodulin-dependent protein kinase II activity in sodium dodecyl sulfate-polyacrylamide gel. Anal. Biochem. **183:**139–143.
25. **Keshet, E., A. Itin, K. Fischman, and U. Nir.** 1990. The testis-specific transcript (*ferT*) of the tyrosine kinase *FER* is expressed during spermatogenesis in a stage-specific manner. Mol. Cell. Biol. **10:**5021–5025.
26. **Koerner, T. J., J. E. Hill, A. M. Myers, and A. Tzagoloff.** 1991. High-expression vectors with multiple cloning sites for construction of *trpE* fusion genes: pATH vectors. Methods Enzymol. **194:**477–490.
27. **Labbe, J.-C., J.-P. Capony, D. Caput, J.-C. Cavadore, J. Derancourt, M. Kaghad, J.-M. Lelias, A. Picard, and M. Doree.** 1989. MPF from starfish oocytes at first meiotic metaphase is a heterodimer containing one molecule of cdc2 and one molecule of cyclin B. EMBO J. **8:**3053–3058.
28. **Laemmli, U. K.** 1970. Cleavage of structural proteins during the assembly of the head of bacteriophage T4. Nature (London) **227:**680–685.
29. **Letwin, K., L. Mizzen, B. Motro, Y. Ben-David, A. Bernstein, and T. Pawson.** 1992. A mammalian dual specificity protein kinase, Nek1, is related to the NIMA cell cycle regulator and highly expressed in meiotic germ cells. EMBO J. **11:**3521–3531.
30. **Letwin, K., S.-P. Yee, and T. Pawson.** 1988. Novel protein-tyrosine kinase cDNAs related to *fps/fes* and *eph* cloned using antiphosphotyrosine antibody. Oncogene **3:**621–627.
31. **Lewin, B.** 1990. Driving the cell cycle: M phase kinase, its partners, and substrates. Cell **61:**743–752.
32. **Liu, F.-T., M. Zinnecker, T. Hamaoka, and D. H. Katz.** 1979. New procedures for preparation and isolation of conjugates of proteins and synthetic copolymer of D-amino acids and immunochemical characterization of such conjugates. Biochemistry **18:**690–697.
33. **Matsushime, H., A. Jinno, N. Takagi, and M. Shibuya.** 1990. A novel mammalian protein kinase gene (*mak*) is highly expressed in testicular germ cells at and after meiosis. Mol. Cell. Biol. **10:**2261–2268.
34. **McLeod, M., and D. Beach.** 1986. Homology between the $ran1^+$ gene of fission yeast and protein kinases. EMBO J. **5:**3665–3671.
35. **McLeod, M., and D. Beach.** 1988. A specific inhibitor of the $ran1^+$ protein kinase regulates entry into meiosis in *Schizosaccharomyces pombe*. Nature (London) **332:**509–514.
36. **Ninomiya-Tsuji, J., S. Nomoto, H. Yasuda, S. I. Reed, and K. Matsumoto.** 1991. Cloning of a human cDNA encoding a CDC2-related kinase by complementation of a budding yeast *cdc28* mutation. Proc. Natl. Acad. Sci. USA **88:**9006–9010.
37. **Nurse, P.** 1990. Universal control mechanism regulating onset of M-phase. Nature (London) **344:**503–508.
38. **O'Brien, D. A.** 1987. Stage-specific protein synthesis by isolated spermatogenic cells throughout meiosis and early spermiogenesis in the mouse. Biol. Reprod. **37:**147–157.
39. **Paules, R. S., F. Propst, K. J. Dunn, D. G. Blair, K. Kaul, A. E. Palmer, and G. F. Vande Woude.** 1988. Primate c-*mos* proto-oncogene structure and expression: transcription initiation both upstream and within the gene in a tissue-specific manner. Oncogene **3:**59–68.
40. **Payne, D. M., A. J. Rossomando, P. Martino, A. K. Erickson, J.-H. Her, J. Shabanowitz, D. F. Hunt, M. J. Weber, and T. W. Sturgill.** 1991. Identification of the regulatory phosphorylation sites in pp42/mitogen-activated protein kinase (MAP kinase). EMBO J. **10:**885–892.
41. **Pines, J., and T. Hunter.** 1991. Human cyclins A and B1 are differentially located in the cell and undergo cell cycle-dependent nuclear transport. J. Cell Biol. **115:**1–17.
42. **Romrell, L. J., A. R. Bellve, and D. W. Fawcett.** 1976. Separation of mouse spermatogenic cells by sedimentation velocity. Dev. Biol. **49:**119–131.
43. **Rossi, P., C. Albanesi, P. Grimaldi, and R. Geremia.** 1991. Expression of the mRNA for the ligand of c-kit in mouse Sertoli cells. Biochem. Biophys. Res. Commun. **176:**910–914.
44. **Rossomando, A. J., D. M. Payne, M. J. Weber, and T. W. Sturgill.** 1989. Evidence that pp42, a major tyrosine kinase target protein, is a mitogen-activated serine/threonine protein kinase. Proc. Natl. Acad. Sci. USA **86:**6940–6943.
45. **Segev, N., J. Mulholland, and D. Botstein.** 1988. The yeast GTP-binding YPT1 protein and a mammalian counterpart are associated with the secretion machinery. Cell **52:**915–924.
46. **Shiina, N., T. Moriguchi, K. Ohta, Y. Gotoh, and E. Nishida.** 1992. Regulation of a major microtubule-associated protein by MPF and MAP kinase. EMBO J. **11:**3977–3984.
47. **Smith, H. E., and A. P. Mitchell.** 1989. A transcriptional cascade governs entry into meiosis in *Saccharomyces cerevisiae*. Mol. Cell. Biol. **9:**2142–2152.
48. **Sorrentino, V., M. Giorgi, R. Geremia, P. Besmer, and P. Rossi.** 1991. Expression of the c-*kit* proto-oncogene in the murine male germ cells. Oncogene **6:**149–151.
49. **Tamaru, M., Y. Nagao, M. Taira, M. Tatibana, Y. Masamune, and Y. Nakanishi.** 1990. Selective activation of testis-specific genes in cultured rat spermatogenic cells. Biochim. Biophys. Acta **1049:**331–338.

49a. **Tanaka, K., A. Jinno, and M. Shibuya.** Unpublished results.

49b. **Tanaka, K., and M. Shibuya.** Unpublished results.

50. **Tsai, L.-H., E. Harlow, and M. Meyerson.** 1991. Isolation of the human *cdk2* gene that encodes the cyclin A- and adenovirus

E1A-associated p33 kinase. Nature (London) **353**:174–177.

51. **Van der Hoorn, F. A., J. E. Spiegel, M.-F. Maylie-Pfenniger, and S. K. Nordeen.** 1991. A 43 kD c-*mos* protein is only expressed before meiosis during rat spermatogenesis. Oncogene **6**:929–932.
52. **Witte, O. N.** 1990. Steel locus defines new multipotent growth factor. Cell **63**:5–6.
53. **Wolgemuth, D. J., C. M. Viviano, E. Gizang-Ginsberg, M. A. Frohman, A. L. Joyner, and G. R. Martin.** 1987. Differential expression of the mouse homeobox-containing gene *Hox-1.4* during male germ cell differentiation and embryonic development. Proc. Natl. Acad. Sci. USA **84**:5813–5817.
54. **Yoshida, M., H. Kawaguchi, Y. Sakata, K. Kominami, M. Hirano, H. Shima, R. Akada, and I. Yamashita.** 1990. Initiation of meiosis and sporulation in *Saccharomyces cerevisiae* requires a novel protein kinase homologue. Mol. Gen. Genet. **221**:176–186.
55. **Zakeri, Z. F., D. J. Wolgemuth, and C. R. Hunt.** 1988. Identification and sequence analysis of a new member of the mouse *HSP70* gene family and characterization of its unique cellular and developmental pattern of expression in the male germ line. Mol. Cell. Biol. **8**:2925–2932.