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A Novel Mammalian Protein Kinase Gene (*mak*) Is Highly Expressed in Testicular Germ Cells at and after Meiosis

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We isolated a novel gene designated *mak* (male germ cell-associated kinase) by using weak cross-hybridization with a tyrosine kinase gene (v-*ros*). Sequence analysis of the cDNA corresponding to the 2.6-kilobase transcript revealed that the predicted product of rat *mak* consisted of 622 amino acids and contained protein kinase consensus motifs in its amino-terminal region. Comparison of the deduced amino acid sequence of *mak* in the kinase domain with those of other protein kinase genes demonstrated that *mak* was approximately 40% identical to the *cdc2-CDC28* gene family in *Schizosaccharomyces pombe*, *Saccharomyces cerevisiae*, and humans but less identical to most other protein kinase gene products. Expression of *mak* was highly tissue specific, and its transcripts were detected almost exclusively in testicular cells entering and after meiosis but hardly detectable in ovarian cells including oocytes, after the dictyotene stage. These results suggest that the *mak* gene plays an important role in spermatogenesis.

More than half of the proto-oncogenes and related genes known are classified in the protein kinase family and are considered to have important functions in cell proliferation or differentiation. However, the physiological roles of most of these genes are unknown (9).

Although meiosis is the essential step of sexual reproduction, the molecular mechanism of meiosis is not well understood. In the fission yeast *Schizosaccharomyces pombe*, inactivation of *ran1*$^+$(*pat1*$^+$) protein kinase activity induces the cells to enter meiotic differentiation through activation of the *mei2*$^+$ gene product (30, 37, 51). In *Xenopus laevis*, the c-*mos* product has an intimate relationship with oocyte maturation (46). Furthermore, some of the proto-oncogenes classified in the protein kinase family are expressed in gonadal tissues in mammals and may contribute to gametogenesis and/or embryogenesis (39, 42, 50).

Although many serine(threonine)- and tyrosine-specific protein kinase genes have been isolated, many unidentified kinase genes appear to be involved in a complex network system of signal transduction and in many important physiological processes in the cells. These protein kinase genes are structurally homologous to each other at the levels of the amino acid and the nucleotide sequences. Thus, cross-hybridization is a useful technique to isolate new genes belonging to a gene superfamily. Recently we obtained several DNA sequences derived from protein kinase gene family members by weak cross-hybridization with the v-*ros* tyrosine kinase gene (35, 36). In this study, we showed that a novel mammalian gene that cross-hybridized with v-*ros* was not a typical tyrosine kinase gene but was similar to the cell cycle control genes of *S. pombe* and *Saccharomyces cerevisiae*, *cdc2* and *CDC28*, respectively, which belong to the serine(threonine) kinase category (44, 49). This new gene (*mak*) was expressed mainly in testicular cells at and after meiosis, suggesting that it plays an important function in spermatogenesis.

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MATERIALS AND METHODS

Rats and mice. All rats and ICR mice used in this study were purchased from Shizuoka Laboratory Animal Center (Hamamatsu, Shizuoka, Japan). Mutant mice and their littermate controls, except for T37H, T16H, and their littermate controls, were purchased from Jackson Laboratory (Bar Harbor, Maine). T37H, T16H, and their littermate controls were maintained in our laboratory. The genotypes of the mutant mice are as follows, with control genotypes in parentheses, except for T37H: *X*/*X Sxr*/+ (*X*/*Y* +/+ or *X*/*Y Sxr*/+), *qk*/*qk* (+/+ or *qk*/+), *T*(*X*;16)/*Y* (*X*/*Y*), and *T*(*X*;4)/*Y*.

Pregnant-mare serum gonadotropin (5 U; Sankyo) was administered intraperitoneally to stimulate follicle development in 8-week-old ICR mice, and ovaries were recovered 24 or 48 h later.

Germ cell fractionation. Male germ cells were fractionated by using a unit gravity velocity sedimentation system STA-PUT (Johns Scientific, Toronto, Canada) in 2 to 4% (wt/vol) bovine serum albumin gradients (45). The purity of fractions was determined with a microscope, and pooled fractions more than 80% pure were used for RNA preparation.

RNA extraction, blot analysis, and probes. Tissues and cells were homogenized in 4.2 M guanidine thiocyanate (Fulka), and total cellular RNA was prepared as described by Glisin et al. (21). Poly(A)$^+$ RNA was isolated from oligo(dT) columns (4).

Total or poly(A)$^+$ RNA was electrophoresed in 1% agarose gel containing 2.2 M formaldehyde and 20 mM sodium phosphate buffer (pH 7.2) (34). RNA was transferred to nitrocellulose (Schleicher & Schuell, Inc.) in 20× SSC (1× SSC is 0.15 M NaCl and 0.015 M sodium citrate, pH 7.4). Prehybridization was performed overnight at 37°C in 50% formamide–3× SSC–50 mM Tris hydrochloride (pH 7.5)–20 μg of tRNA (Sigma Chemical Co.) per ml–20 μg of boiled salmon sperm DNA (Sigma) per ml–1 mM EDTA–0.02% bovine serum albumin–0.02% polyvinylpyrrolidone–0.02% Ficoll. Hybridization was performed for 40 h at 37°C in this solution containing a ^{32}P-labeled DNA probe. Filters were finally washed at 50°C for 30 min in 0.1× SSC and 0.1% sodium dodecyl sulfate.

All DNA and cDNA fragments used in this study as

probes were electrophoretically purified from agarose gels and labeled by using hexadeoxynucleotide random primers with [α-^{32}P]dCTP (Amersham Corp.) (18). The *mak* cDNA probe was a 0.8-kilobase (kb) *Sac*I-*Bam*HI fragment (see Fig. 3A). The H-*ras* probe was a 460-base-pair *Eco*RI fragment from pBS-9 (16). The K-*ras* probe was a 1-kb *Eco*RI fragment from pHiHi3 (17). The *abl* probe was a 1.9-kb *Sac*I-*Hin*dIII fragment from pABsub3 (22). The *mos* probe was a 0.8-kb *Ava*I-*Hin*dIII fragment from pMS1 (41).

Genomic DNA and cDNA libraries. Human and rat genomic DNA libraries were constructed as follows. Human placental DNA partially digested with *Alu*I and *Hae*III was ligated with Charon 4A *Eco*RI arms with *Eco*RI linkers, and rat testis DNA partially digested with *Sau*3AI was ligated with EMBL3 *Bam*HI arms (Promega Biotec) as described by Maniatis et al. (34). By using bacteriophage λ gt10 as a vector, a rat testis cDNA library was constructed as described by Gubler and Hoffman (24), with a slight modification. To generate the blunt ends of cDNA fragments, mung bean nuclease was used instead of T4 DNA polymerase.

mak genomic DNA and cDNA clones were isolated from libraries by using a method described by Benton and Davis (8). A human genomic *Hin*fI-*Hin*fI DNA fragment 0.8 kb long was found to encode an exon sequence (see Fig. 1). By using this DNA as a probe, a rat genomic *Hin*dIII-*Sph*I fragment 1.3 kb long was isolated and used for Northern (RNA) blot analysis. The rat testis cDNA library was also screened with this 1.3-kb rat genomic DNA fragment.

DNA sequencing. DNA fragments were subcloned into pUC118 (Takara Shuzo) and sequentially and unidirectionally deleted with exonuclease III (27). DNA sequences were determined by the method of Sanger et al. (47).

Western blotting. For Western blotting (immunoblotting), cell lysates of testis tissue were prepared by using a lysis buffer containing 50 mM Tris hydrochloride (pH 7.4), 0.15 M NaCl, 1% Triton X-100, 1% deoxycholate, 0.1% sodium dodecyl sulfate, 20 mM EDTA, and 200 U of aprotinin per ml. The lysates were separated by sodium dodecyl sulfate-polyacrylamide gel electrophoresis and transferred to a nylon filter. A rabbit antiserum to a synthetic peptide corresponding to the 20 amino acids of the *mak* carboxy terminus was purified by oligopeptide affinity column chromatography and used for immunoblotting. Specific bands were detected by incubating the filters with ^{125}I-labeled protein A-Sepharose. *Sac*I-*Eco*RI *mak* cDNA (the *Sac*I site was digested to form a blunt end) containing the entire coding region was inserted into the *Hin*cII-*Eco*RI site on pUC19 plasmid DNA, and the β-galactosidase-*mak* fusion protein was induced in *Escherichia coli* by isopropyl-β-D-thiogalactopyranoside. This fusion protein consists of 5 amino acids from the amino terminus of β-galactosidase, 8 amino acids from the multiple cloning site in plasmid pUC19, 11 amino acids from the 5′ noncoding region of *mak*, and 622 amino acids from the *mak* coding region. The cell lysates of *E. coli* clones that express the fusion protein were also used for Western blot analysis.

RESULTS

Human DNA sequence cross-hybridized with the v-*ros* oncogene. A human genomic DNA library was screened with a v-*ros* DNA fragment under a hybridization condition of very low stringency (35). In one clone, we found a 133-base-pair contiguous open reading frame surrounded by consensus splice acceptor and donor sites (Fig. 1A). The deduced amino acid sequence consisted of 43 amino acids and contained the residues highly conserved in the kinase domains

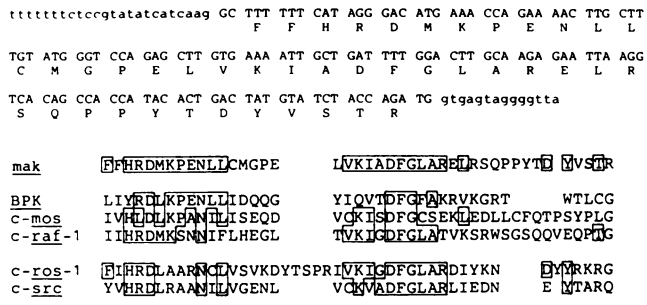


FIG. 1. Nucleotide and deduced amino acid sequences of an exon of the human *mak* gene. The primary structure of a human *mak* DNA fragment is shown (top of figure). The deduced amino acid sequence of a portion of the human *mak* gene is compared with those of protein kinase genes (bottom of figure). Amino acids identical to those of the *mak* gene product are boxed. BPK, Bovine cyclic AMP-dependent protein kinase.

of the protein kinase family (Fig. 1B). By Southern blot analysis using this DNA fragment as a probe, discrete single bands were detected in rat and mouse DNAs (data not shown), suggesting that this new gene is phylogenetically conserved in mammalian species. In fish and *Drosophila melanogaster* genomic DNAs, we also detected faint bands cross-hybridizing with the *mak* sequence. However, it is not clear whether these bands are the real homolog of the *mak* gene.

Since mRNAs transcribed from this gene were not detected in any of more than 10 kinds of human tumor cell lines by Northern blot analysis (data not shown), we considered that this gene might be expressed in a strictly restricted manner. As will be described later, this gene is highly expressed in testis germ cells. Thus, we designated this gene *mak* (male germ cell-associated kinase).

Molecular cloning of rat *mak* cDNA. To examine this new gene in detail, a genomic *mak* fragment was isolated from a rat genomic DNA library by cross-hybridization with human *mak* DNA. Total cellular RNAs were prepared from various organs of 8-week-old rats, and expression of rat *mak* was screened by using the rat genomic *mak* fragment. Two discrete bands, 3.8 and 2.6 kb, were detected only in testis tissue. The entire coding sequence of the *mak* gene was isolated from an 8-week-old-rat testis cDNA library. Figure 2 shows a typical result of Northern blot analysis with the rat *mak* cDNA fragment as a hybridization probe. The 2.6-kb transcript of *mak* in testis tissue was four to six times more abundant than the 3.8-kb *mak* transcript. The structures of three overlapping cDNAs are indicated in Fig. 3A. The nucleotide and deduced amino acid sequences were shown in Fig. 3B.

The 2.6-kb *mak* cDNA contained a 1,866-base-pair contiguous open reading frame initiating at ATG (nucleotide residues 237 to 239 in Fig. 3B), and its predicted product consisted of 622 amino acids. Since a stop codon was found upstream from the ATG codon in the same reading frame (residues 174 to 176 in Fig. 3B) and other open reading frames could encode the predicted products of very few amino acids, the ATG sequence found at residues 237 to 239 in Fig. 3B seemed most likely to be the translation initiation codon of the *mak* gene. Within the amino-terminal 300-amino-acid sequence, the predicted product had protein kinase consensus motifs, including lysine for the ATP-binding site (amino acid residue 33) and a Gly-X-Gly-X-X-Gly stretch (residues 11 to 16) (6). Comparison of the nucleotide and deduced amino acid sequences in an exon

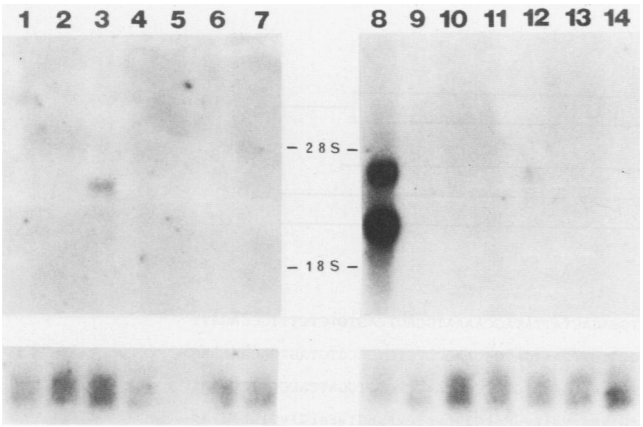


FIG. 2. Tissue-specific expression of the rat *mak* gene. Four-microgram samples of poly(A)$^+$ RNA prepared from various tissues were electrophoresed and transferred to nitrocellulose filters. The filters were hybridized with a rat *mak* cDNA (top) or v-H-*ras* DNA (bottom) fragment as a probe. The RNA samples used were prepared from brain (lanes 1 and 10), heart (lanes 2 and 11), lung (lanes 3 and 12), liver (lanes 4 and 13), kidney (lanes 5 and 14), placenta (lane 6), muscle (lane 7), testis (lane 8), or salivary gland (land 9) tissue. Tissues were prepared from 20-day-old embryos (lanes 1 to 5 and 7) or 4-week-old (lanes 8 to 14) rats.

(nucleotide residues 595 to 727) of human and rat *mak* genes revealed 92% homology at the nucleotide level and a complete match at the amino acid level. Thus, the *mak* gene appears to be structurally highly conserved among mammalian species.

The amino acid sequence downstream from the kinase domain had a stretch with high proline and glutamine contents (50%; amino acid residues 306 to 370 in Fig. 3B). A similar stretch has been found in other genes, such as the human mineralocorticoid receptor and c-*jun* genes, and is thought to serve as an intramolecular hinge region (3, 10). The carboxyl region of the *mak* gene further downstream of the proline- and glutamine-rich region showed no significant homology with other known genes. Thus, the predicted product of the *mak* gene can be divided into three domains: the amino-terminal protein kinase domain, the proline- and glutamine-rich domain, and the carboxy-terminal domain.

Comparison of the predicted product of the *mak* gene with those of other protein kinase genes in the kinase domain. The protein kinase domain of the *mak* gene product was compared with those of other protein kinase gene products. The *mak* kinase domain was most homologous to cell cycle control genes *cdc2* and *CDC28* in *S. pombe* and *S. cerevisiae*, respectively (29, 33), and to *CDC2*Hs, which is a functional homolog of *cdc2* and *CDC28* in humans (32) (Table 1). The amino acid sequence in the *mak* kinase domain was approximately 50% homologous (including both identical and conservative amino acids) to those of *CDC2*Hs, *cdc2*, and *CDC28*, whereas the homologies between *mak* and other protein-serine(threonine) kinase genes, c-*mos*, c-*raf*-1, cyclic AMP-dependent protein kinase α, protein kinase C (β form), and testis-specific phosphorylase kinase (PSK-C3) (11, 13, 25, 48, 52), were as low as those between *mak* and protein-tyrosine kinase genes such as c-*src* and c-*ros*-1 (Table 1) (1, 35). Furthermore, in the alignment of the deduced amino acid sequences, the putative translation initiation sites were identical among the *mak*, *CDC2Hs*, and *cdc2* genes (Fig. 4). Since the products of *cdc2* and *CDC28* genes are shown to possess serine(threonine)-specific protein kinase activity (44, 49), the *mak* gene also appears to be a member of the protein-serine(threonine) kinase gene family. In addition, the *mak* gene was highly homologous, but not identical, to the kinase domain of a newly isolated gene, PSK-J3, although the entire sequence of the PSK-J3 gene has not been described (26) (Table 1).

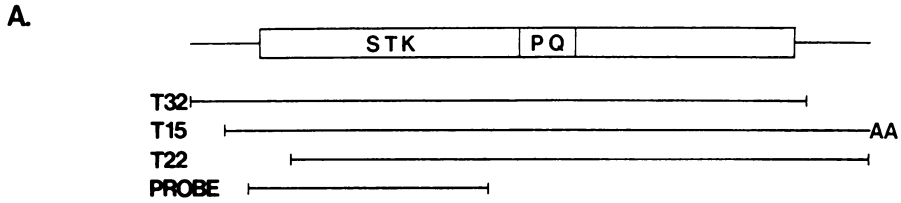
Tissue-specific expression of the *mak* gene. Northern blot analysis (Fig. 2) demonstrated that two discrete bands of the *mak* gene, 2.6 and 3.8 kb, were found in 4-week-old testis tissue. In addition, a very faint 3.8-kb band was detected in embryonal and 4-week-old lung tissues but no bands were seen in other tissues examined (Fig. 2, legend). Since the probe used contained the entire kinase domain of *mak* (Fig. 3A) and the size of the faint band detected in lung tissue was not 2.6 kb but only 3.8 kb, we thought it possible that this faint band was the transcript of another protein kinase gene. This was confirmed by no detectable cross-hybridization of the band with a 3′-noncoding sequence of the *mak* cDNA. Since the *mak* gene was expressed in a strictly tissue-specific manner, the *mak* gene product may play a role in spermatogenesis.

Expression of the *mak* gene in germ cells of testes. Three approaches were used to elucidate which type of cells in testis tissue would express *mak* transcripts. First, expression of *mak* was analyzed in germ cells fractionated with STAPUT by using a continuous albumin gradient (see Materials and Methods). It is well known that germ cells at various stages of spermatogenesis can be separated from each other on the basis of differences in size and shape. The testicular cells were separated into three fractions, and the *mak* mRNA in the fractionated cells was examined. The highest expression of the *mak* gene was observed in germ cells in the fraction which included spermatogonia; primary spermatocytes at the leptotene, zygotene, and early pachytene stages; and secondary spermatocytes (Fig. 5A, lane 2). The fractionated germ cells at the late pachytene stage and round spermatids expressed smaller amounts of *mak* mRNA. The testicular cells at the late stage of spermatogenesis, such as elongated spermatids, contained only a small amount of 2.6-kb *mak* mRNA (data not shown).

To confirm the reliability of fractionation of testicular germ cells with STAPUT, expression of c-K-*ras* was examined, since c-K-*ras* was previously reported to be expressed at the pachytene stage in testicular germ cells of mice (50). A 2.1-kb c-K-*ras* transcript in rats was detected in the fractions (Fig. 5A, lanes 2 and 3) which were expected to contain the germ cells at the pachytene stage.

The results described above indicated that (i) the *mak* gene is expressed mainly in testicular germ cells and (ii) the highest level of *mak* mRNA was detected in a fraction containing spermatogonia and the cells at the early stage of meiotic division cycle 1. To distinguish the two possibilities that expression of the *mak* gene is associated with mitotic division of spermatogonia or with a later stage of spermatogenesis, including the meiotic process, we examined the time course of the appearance of *mak* transcripts in testis tissue after birth.

It is well established that the number of spermatogonia in testis tissue increases through mitosis after birth, whereas spermatogenesis by meiosis starts after puberty, 20 to 24 days after birth in rats. The 3.8-kb *mak* transcript first appeared at 20 to 22 days after birth and gradually increased (Fig. 5B). The 2.6-kb transcript appeared slightly later than the 3.8-kb transcript, 24 to 26 days after birth, and dramatically increased between 26 days and 5 weeks. Both tran-



B.

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1                                                    TCTGAATATGTGGAGACTATTATAGCAAAATGGACTCAGTGTCTCTTTGCCAGATT

57   CCTTATTGCTGTATCCCATGAGCCTACCTGACCTTGAGGAAGAGCCGTGTTTAATGATGACAAGCCTGTCTCCCGTGTAGTGACAAAAAG

147  GAGGAGGACCACAAGGAAAGACGGATTTAGATTTCTACTCAGCAAGGAAGCGAGCTCTGTCAGCAACCTCTCTGGATTACCCTTCCCAAG

1    MetAsnArgTyrThrThrMetArgGlnLeuGlyAspGlyThrTyrGlySerValLeuMetGlyLysSerAsnGluSerGlyGluLeuVal
237  ATGAACCGATATACAACCATGAGGCAGCTGGGGGACGGCACGTACGGGAGTGTGCTTATGGGCAAGAGCAATGAGTCTGGGGAGCTGGTG

31   AlaIleLysArgMetLysArgLysPheTyrSerTrpAspGluCysMetAsnLeuArgGluValLysSerLeuLysLysLeuAsnHisAla
327  GCAATCAAGAGGATGAAGAGAAAGTTCTATTCTTGGGATGAGTGTATGAACTTGCGAGAAGTTAAGTCCCTGAAGAAACTCAATCATGCC

61   AsnValIleLysLeuLysGluValIleArgGluAsnAspHisLeuTyrPheIlePheGluTyrMetLysGluAsnLeuTyrGlnLeuMet
417  AATGTGATTAAACTAAAAGAAGTTATCAGAGAAAATGACCATCTTTATTTTATATTTGAATATATGAAAGAAAACCTCTATCAGCTAATG

91   LysAspArgAsnLysLeuPheProGluSerValIleArgAsnIleMetTyrGlnIleLeuGlnGlyLeuAlaPheIleHisLysHisGly
507  AAAGACAGAAACAAACTGTTCCCTGAGTCAGTCATCAGAAATATTATGTATCAAATACTACAGGGGCTGGCATTTATCCACAAACATGGC

121  PhePheHisArgAspMetLysProGluAsnLeuLeuCysMetGlyProGluLeuValLysIleAlaAspPheGlyLeuAlaArgGluLeu
597  TTTTTTCACAGGGACATGAAACCTGAGAATTTGCTTTGCATGGGTCCAGAGCTGGTGAAGATTGCTGATTTTGGACTTGCGAGAGAATTA

151  ArgSerGlnProProTyrThrAspTyrValSerThrArgTrpTyrArgAlaProGluValLeuLeuArgSerSerValTyrSerSerPro
687  AGATCACAGCCACCATATACTGACTATGTGTCTACCAGATGGTACCGTGCTCCTGAAGTTTTGCTAAGGTCTTCAGTGTACAGCTCTCCC

181  IleAspValTrpAlaValGlySerIleMetAlaGluLeuTyrThrPheArgProLeuPheProGlyThrSerGluValAspGluIlePhe
777  ATTGACGTGTGGGCCGTGGGAAGTATAATGGCCGAGCTATATACGTTTAGACCGCTTTTCCCAGGGACCAGTGAAGTTGATGAGATCTTT

211  LysIleCysGlnValLeuGlyThrProLysLysSerAspTrpProGluGlyTyrGlnLeuAlaSerSerMetAsnPheArgPheProGln
867  AAAATTTGCCAAGTGTTAGGGACTCCCAAGAAAAGTGACTGGCCGGAGGGGTACCAGCTGGCATCCTCCATGAACTTCCGCTTTCCCCAG

241  CysIleProIleAsnLeuLysThrLeuIleProAsnAlaSerSerGluAlaIleGlnLeuMetThrGluMetLeuAsnTrpAspProLys
957  TGCATTCCTATAAACCTGAAAACTCTCATTCCCAATGCCAGTAGTGAGGCTATTCAGCTTATGACAGAAATGCTTAACTGGGATCCAAAG

271  LysArgProThrAlaSerGlnAlaLeuLysHisProTyrPheGlnValGlyGlnValLeuGlyProSerAlaHisHisLeuAspAlaLys
1047 AAACGGCCAACTGCAAGCCAGGCACTGAAGCACCCATATTTTCAAGTCGGTCAGGTATTGGGCCCTTCTGCACACCATCTGGATGCAAAA

301  GlnThrLeuHisLysGlnLeuGlnProProGluProLysProSerSerSerGluArgAspProLysProLeuProAsnIleLeuAspGln
1137 CAGACTTTGCACAAGCAGCTGCAGCCTCCAGAGCCAAAGCCATCTTCCTCTGAACGGGATCCTAAGCCTTTGCCAAACATCCTTGATCAG

331  ProAlaGlyGlnProGlnProLysGlnGlyHisGlnProLeuGlnAlaIleGlnProProGlnAsnThrValValGlnProProProLys
1227 CCTGCCGGGCAGCCCCAGCCAAAACAGGGCCACCAACCACTGCAGGCCATTCAGCCACCACAGAACACAGTGGTTCAGCCACCTCCAAAG

361  GlnGlnGlyHisHisLysGlnProGlnThrMetPheProSerIleValLysThrIleProThrAsnProValSerThrValGlyHisLys
1317 CAGCAGGGTCACCATAAGCAACCACAAACGATGTTTCCAAGTATCGTCAAAACCATACCAACGAATCCAGTCAGCACAGTAGGCCATAAG

391  GlyAlaArgArgArgTrpGlyGlnThrValPheLysSerGlyAspSerCysAspAsnIleGluAspCysAspLeuGlyAlaSerHisSer
1407 GGGGCCCGGAGACGGTGGGGTCAGACAGTCTTCAAGTCTGGAGACAGCTGTGACAACATCGAGGACTGCGACTTGGGAGCCTCCCACTCC

421  LysLysProSerMetAspAlaPheLysGluLysLysLysLysGluSerProPheArgPheProGluAlaGlyLeuProValSerAsnHis
1497 AAGAAGCCGAGCATGGATGCCTTCAAGGAAAAAAAAGAAGAAGGAGTCTCCATTTCGGTTTCCAGAAGCAGGACTCCCAGTCTCCAACCAC

451  LeuLysGlyGluAsnArgAsnLeuHisAlaSerLeuLysSerAspThrAsnLeuSerThrAlaSerThrAlaLysGlnTyrTyrLeuLys
1587 TTGAAGGGGGAAAATAGAAATTTACATGCATCCTTAAAATCTGACACAAACTTGTCAACTGCTTCAACCGCTAAGCAGTACTATTTGAAA

481  GlnSerArgTyrLeuProGlyValAsnProLysAsnValSerLeuValAlaGlyGlyLysAspIleAsnSerHisSerTrpAsnAsnGln
1677 CAATCAAGATACCTTCCGGGTGTGAACCCCAAGAACGTGTCTTTGGTAGCTGGCGGCAAGGATATAAATTCACACTCTTGGAATAATCAG

511  LeuPheProLysSerLeuGlySerMetGlyAlaAspLeuAlaPheLysArgSerAsnAlaAlaGlyAsnLeuGlySerTyrSerAlaTyr
1767 CTATTTCCTAAGTCTCTGGGATCCATGGGGGCGGACCTCGCTTTCAAGAGGAGTAACGCAGCAGGGAACCTTGGGAGCTACAGCGCTTAC

541  SerGlnThrGlyCysValProSerPheLeuLysLysGluValGlySerAlaGlyGlnArgIleHisLeuAlaProLeuGlyAlaSerAla
1857 AGCCAGACGGGATGCGTGCCTTCCTTTCTCAAGAAAGAAGTGGGATCAGCTGGCCAGAGGATCCACTTGGCGCCTCTGGGTGCGTCGGCT

571  AlaAspTyrThrTrpSerThrLysThrGlyArgGlyGlnPheSerGlyArgThrTyrAsnProThrAlaLysAsnLeuAsnIleValAsn
1947 GCCGACTATACCTGGAGCACAAAAACTGGCCGAGGCCAGTTTTCAGGACGAACTTACAATCCCACAGCCAAAAATCTCAATATTGTGAAC

601  ArgThrGlnProValProSerValHisGlyArgThrAspTrpValAlaLysTyrGlyGlyHisArg
2037 CGCACACAGCCAGTCCCCTCGGTGCACGGGAGGACAGACTGGGTGGCTAAGTACGGAGGCCACCGGTAGGAGAAGTGGAAGCCTGAAGCA

2127 TTGCTCCGTAGAGGACAATCACGCCCCTTGATCCTGGGAGACGTCTACAGAGTCTATTTCTACCGAGTTCCACAGGACGCACACAACCGT

2217 GGGCGCCTCAGAGACCGGAAGTCAGCTCCCGATTTCTTCCTTTTCCGGAAATGCAATGCATTTTCTTAACTTGTGGCCAGCAGTGCTGAT

2307 GCAGCACCGTGCTGAAACTTTTGAGCGGGACTTTTAAAGAGTATTGAATAAATTATTTGCCAAAGTAAAAAAAAAAAAAAAAAA
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FIG. 3. Molecular cloning of rat *mak* cDNA. (A) The predicted structure, three overlapping cDNA fragments of the rat *mak* gene, and a 0.8-kb *Sac*I-*Bam*HI fragment used as a probe in this study are shown. The coding region is indicated by an open box. STK and PQ represent the kinase domain and the proline- and glutamine-rich domain in the *mak* gene product, respectively. Clone T15 contained a poly(A) stretch with 18 adenosine residues at its 3′ end (AA). (B) The nucleotide (residues 1 to 2390) and deduced amino acid (residues 1 to 622) sequences of the rat *mak* gene are presented. The numbers of nucleotides and amino acid residues are indicated to the left. Three-letter amino acid codes are used. The glycines and the lysine which form the ATP-binding site (6) are indicated by closed circles. The consensus sequence of the poly(A) addition signal, AATAAA, is underlined.

TABLE 1. Homology of the deduced amino acid sequence in the kinase domain between the *mak* gene and other protein kinase genes

Gene	% Identity	% Homology[a]
*CDC2*Hs	39	51
cdc2	38	49
CDC28	35	47
PSK-J3	38	48
c-*mos*	16	24
c-*raf*-1	19	25
BPK	22	30
PKC	19	25
PSK-C3	29	40
c-*src*	19	26
c-*ros*-1	20	27

[a] Homologous amino acids include identical and conservative residues described by Barker and Dayhoff (5), such as isoleucine, leucine, and valine; serine and threonine; arginine and lysine; and aspartic acid and glutamic acid.

scripts kept a high level until at least 9 weeks. These results strongly suggest that the *mak* gene plays a role in and/or after the meiotic process rather than in the mitotic phase in testis tissue.

Expression of the *mak* gene in mutant strains of mice defective in spermatogenesis. To confirm the possible involvement of the *mak* gene in or after meiosis, a third approach was used. *mak* expression was investigated in testes of mice bearing mutations that affect or block spermatogenesis at known stages. Total RNA was prepared from whole testes of four strains of mice and of the appropriate control mice and hybridized with rat *mak* cDNA as a probe.

Sex reversal (*Sxr*-carrying) mice have small testes completely devoid of germ cells because of failure of spermatogonium replication (12). T16H and T37H mice have X-autosome translocations that result in male sterility due to a cell-lethal effect at the pachytene stage (28). The testes of quaking (*qk*-carrying) mice are about half the size of those of normal mice and have defects of final sperm head shaping. They virtually lack production of mature spermatozoa (7). A 3.7-kb transcript of the *mak* gene was weakly expressed in testes of T16H and T37H mice, and both 3.7- and 2.6-kb transcripts were strongly expressed in *qk-qk*-carrying mice. (Fig. 6). However, no mRNA was detected in the testes of mice with *Sxr*. As a control, Fig. 6 also showed testis-specific 4-kb c-*abl* mRNA, which was reported to correlate with the presence of spermatids (39). As expected, testis-specific c-*abl* mRNA was detected only in testes of quaking mice. These findings further support the possibility that the *mak* gene is expressed mainly in germ cells at and/or after the pachytene stage (Table 2).

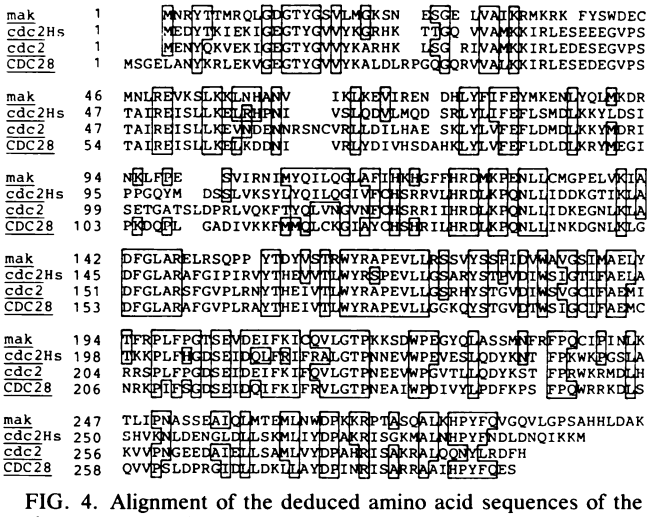

FIG. 4. Alignment of the deduced amino acid sequences of the *mak*, *CDC2*Hs, *cdc2*, and *CDC28* gene products. Amino acids identical to those in the *mak* gene product are boxed. The numbers of amino acid residues are indicated to the left. The predicted *mak* gene product shown here includes only amino acid residues 1 to 300.

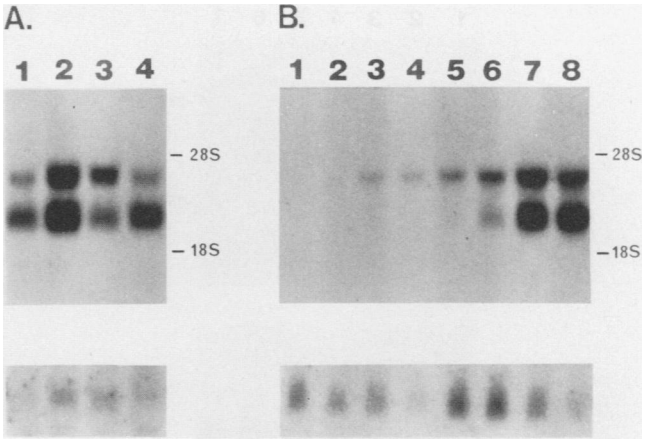

FIG. 5. *mak* transcripts in fractionated male germ cells and their appearance in testis tissue as a function of age. (A) Total RNA (20 μg) prepared from fractionated male germ cells (see Materials and Methods) was electrophoresed and transferred to a filter. The filter was first hybridized with *mak* cDNA (top) and then with a v-K-*ras* DNA fragment (bottom) after removal of the first probe. The total RNA used was prepared from whole testes (lane 1); from fractionated testicular germ cells including spermatogonia, primary spermatocytes at the leptotene, zygotene, and early pachytene stages, and secondary spermatocytes (lane 2); from primary spermatocytes at the late pachytene stage (lane 3); or from round spermatids (lane 4). The band of the c-K-*ras* transcript (bottom) was 2.1 kb long. (B) Total RNAs (20 μg) prepared from whole testes in 16 (lane 1), 18 (lane 2), 20 (lane 3), 22 (lane 4), 24 (lane 5), and 26 (lane 6)-day-old and 5 (lane 7) and 9 (lane 8)-week-old rats were electrophoresed and transferred to filters. The filters were hybridized with a *mak* cDNA (top) or v-H-*ras* DNA (bottom) fragment as a probe. The c-H-*ras* transcript used as a control was 1.4 kb long.

The *mak* gene products in testis tissue. Some of the transcripts expressed in testis tissue have recently been shown not to be efficiently translated (19). To examine whether the *mak* gene is translated in rat testis tissue, we prepared antiserum to the synthetic peptide corresponding to the carboxy-terminal 20-amino-acid residues of the predicted *mak* gene product.

Western blotting analysis showed that this antiserum could recognize 60- to 66-kilodalton proteins in testes of 8-week-old rats but not in those of 18-day-old rats (Fig. 7, lanes 1 and 2). This antiserum also recognized a β-galactosidase-*mak* fusion protein expressed in *E. coli* (Fig. 7, lane 5). These results strongly suggest that the 60- to 66-kilodalton proteins are the translation products of the *mak* transcripts. An about 44-kDa band was detected in both testis tissue and *E. coli* expressing the *mak* products with this antiserum. This peptide might be the proteolytic cleavage product within these cells.

Since this antiserum recognized only the denatured form of the *mak*-encoded proteins, we could not examine the protein kinase activity associated with the *mak* gene products.

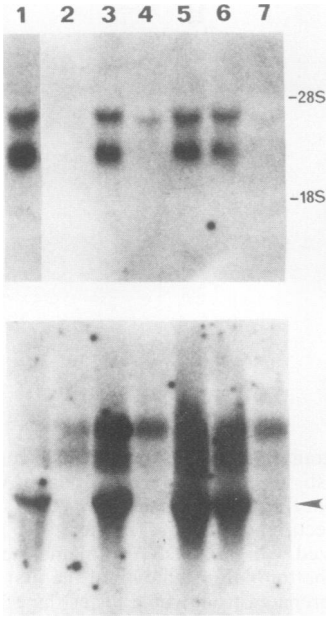

FIG. 6. *mak* transcripts in testes obtained from mutant mice with genetic lesions that affect spermatogenesis at different stages. Total RNAs (20 μg) prepared from whole testes of littermate controls of *Sxr*-carrying mice (lane 1), *Sxr*-carrying mice (lane 2), littermate controls of T16H mice (lane 3), T16H mice (lane 4), littermate controls of *qk*-carrying mice (lane 5), *qk*-carrying mice (lane 6), and T37H mice (lane 7) were analyzed by Northern blotting by using probes for *mak* (top) and c-*abl* (bottom) genes. The experimental conditions were essentially the same as those described in the legends to Fig. 2 and 5. The testis-specific 4-kb transcript of the c-*abl* gene (39) is indicated by an arrowhead.

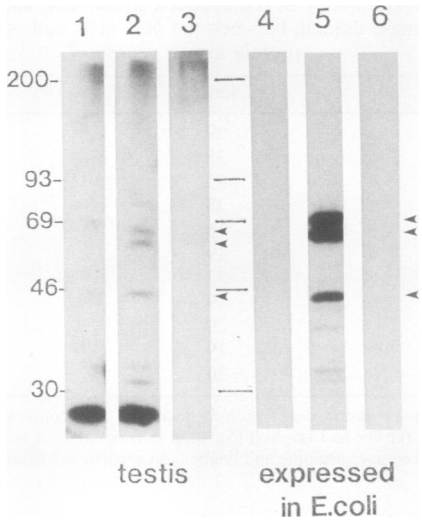

FIG. 7. Western blot analysis of *mak* gene products. Cell lysates of testis tissue (lanes 1 to 3) and *E. coli* (lanes 4 to 6) were separated by sodium dodecyl sulfate-polyacrylamide gel electrophoresis and transferred to a nylon filter. The filter was incubated with an affinity-purified antiserum to the carboxy-terminal 20 amino acid residues of the *mak* gene product, and the bands were detected with ^{125}I-labeled protein A-Sepharose. Lanes: 1, 18-day-old-rat testis; 2 and 3, 8-week-old-rat testis; 4, *E. coli* transformed with control pUC19 plasmid DNA; 5 and 6, *E. coli* transformed with *mak* cDNA-containing pUC19 DNA. In lane 3 and 6, the antiserum was preincubated with the synthetic 20-mer oligopeptide for blocking. The approximately 25-kilodalton band in lanes 1 and 2 appeared to be due to a nonspecific cross-reaction. The numbers on the left indicate molecular sizes in kilodaltons. The arrowheads indicate possible *mak* gene products.

Expression of the *mak* gene in ovary tissue. It was shown that another serine(threonine) kinase gene, c-*mos*, was expressed at a high level in oocytes of mice (23, 40), and the c-*mos* product has recently been demonstrated to play a crucial role in meiotic maturation of oocytes in the amphibian *Xenopus laevis* (46). Since the *mak* gene appears to encode serine(threonine) kinase and to be involved in spermatogenesis, the relationship between the *mak* gene and oogenesis was examined.

Ovaries of adult rats contain a relatively small number of oocytes, and the developmental stages of oocytes are diverse. Therefore, expression of the *mak* and c-*mos* genes was tested by using RNAs prepared from ovaries of younger mice, which are relatively abundant in oocytes, and from those of gonadotropin-stimulated mice, which contain many uniformly growing oocytes. c-*mos* transcripts were detected in both normal and gonadotropin-stimulated ovaries, as previously reported (23). Although *mak* transcripts were detected in 1 μg of total RNA prepared from testes of ICR mice, they were not detected in 20 μg of total RNA prepared from normal or gonadotropin-stimulated ovaries (data not shown). These findings suggest that the *mak* gene is not expressed in oocytes from the dictyotene stage to maturation division stage 2 before ovulation.

TABLE 2. Expression of *mak* and c-*abl* genes in testes of mutant mice

Gene	Gene expression in the following mutants[a]:		
	Sxr-carrying mice	T16H mice	*qk-qk*-carrying mice
mak	−	±	+
abl	−	−	+

[a] Gene expression: +, positive; ±, slight; −, negative. Expression of *abl* indicates detection of the testis-specific 4-kb transcript of the c-*abl* gene (38). *Sxr*-carrying, T16H, and qk-qk-carrying mice are deficient in maturation at the spermatogonium, mitotic division 1, and spermatid stages, respectively.

DISCUSSION

We isolated a novel gene designated *mak* which was expressed mostly in testicular germ cells at and after the meiotic differentiation stage. Since a protein kinase domain of the predicted *mak* gene product is similar to *S. pombe cdc2*, *S. cerevisiae CDC28*, and human *cdc2*Hs gene products, the *mak* gene appears to be closely related to the *cdc2-CDC28-cdc2*Hs gene family. However, the structure of the *mak* gene product seems to be unique in the protein kinase superfamily that includes the *cdc2* group. The *cdc2*, *CDC28*, and *cdc2*Hs gene products contain about 300 amino acids and consist mainly of a protein kinase domain, whereas the *mak* gene product is about twice as large as the products of the *cdc2* family and contains a proline- and glutamine-rich region and a further downstream carboxy-terminal region. These regions might regulate *mak* kinase activity or function to localize the *mak* gene product at a proper position within the cell.

The highest expression of the *mak* gene was observed in a fraction containing primary and secondary spermatocytes at the meiotic phase. One interesting possibility is that the *mak* gene product has a role in the meiotic process. At least two

protein kinase genes have been demonstrated to be directly involved in the meiotic process. The *ran1*$^+$ (*pat1*$^+$) gene in *S. pombe* encodes a kinase domain in its amino-terminal region. Thus, we compared the *mak* gene with the *ran1*$^+$ (*pat1*$^+$) gene at the levels of structure and gene expression. However, the homology between their protein kinase domains was only 30% at the amino acid level. Moreover, *ran1*$^+$ (*pat1*$^+$) kinase is expressed constitutively and is considered to suppress the initiation of meiosis (30, 37). Inactivation of *ran1*$^+$ (*pat1*$^+$) kinase activity by association with the *mei3*$^+$ product allows cells to enter the meiotic stage (38). In contrast, the *mak* gene is expressed mainly in testicular germ cells in and after meiosis. These results suggest that the *mak* gene is not a functional homolog of the *ran1*$^+$ (*pat1*$^+$) gene in mammals, although it is not clear how the meiotic process in spermatogenesis is related to that in *S. pombe*.

Another protein kinase gene involved in meiosis is the c-*mos* proto-oncogene. Sagata et al. (46) demonstrated a positive function of c-*mos* in oocyte maturation of *X. laevis*. Since the c-*mos* gene is also expressed in oocytes in mice (23, 40), it seems likely that the c-*mos* gene in mammalian species has a role in oocyte maturation as in *X. laevis*. On the basis of the different expression patterns of *mak* and c-*mos* in testes and ovaries, the *mak* gene seems to be involved mainly in spermatogenesis but not in oogenesis. However, since the oocytes in the ovaries examined in this study had passed the dictyotene stage, which is a resting stage between prophase and metaphase, and the *mak* gene appeared to be expressed in germ cells at and after the pachytene stage of the prophase in spermatogenesis (Fig. 5A), we cannot rule out the possibility that the *mak* gene is expressed in earlier stages of oogenesis which have been completed in female embryos.

Two species of *mak* transcripts, 2.6 and 3.8 kb long, are expressed in testis tissue. We did not obtain the full-length 3.8-kb cDNA of the *mak* gene. However, we obtained *mak* cDNA fragments corresponding to the 3′ noncoding region which hybridized only with 3.8-kb *mak* transcripts (data not shown). Thus, we suggest that an AATAAA stretch (43) present at nucleotide residues 2353 to 2358 is used for the poly(A) addition signal to form a 2.6-kb mRNA and that the major difference between the 3.8-kb and 2.6-kb transcripts may be due to a difference between the sizes of the 3′ noncoding sequences generated by alternative poly(A) additions.

In testes of both rats and mice, two discrete bands were detected by hybridization with the kinase domain of *mak* cDNA as a probe. The higher band appeared earlier than the lower one did after birth (Fig. 5B) and was found in the RNA obtained from the testes of T16H and T37H mice, whose spermatogenesis is stopped during the first meiotic division (Fig. 6). These findings suggest that the large transcript of the *mak* gene is expressed in germ cells of earlier stages in the spermatogenic lineage and that the physiological function of the 3.6-kb product is different from that of the 2.6-kb product.

The primary structure of the kinase domain of the *mak* gene product has a similarity to the *cdc2-CDC28* gene family compared with other serine(threonine) or tyrosine protein kinase gene products. Recent studies have shown that the *cdc2*Hs gene product, a 34-kilodalton serine(threonine) protein kinase, is directly involved in the mitotic phase of the mammalian cell cycle (14). Furthermore, a similar protein is a component of MPF (maturation- or M-phase-promoting factor) in *Xenopus* oocytes (15, 20) and is the M-phase-specific histone H1 kinase in starfish oocytes (2, 31). However, the maturation-promoting factor(s) in spermatogenesis has not been extensively studied. It would be interesting to see whether the *cdc2*Hs protein kinase itself also functions in spermatogenesis or whether another protein homologous to p34 protein kinase, such as the *mak* gene product, has a similar role in the maturation process. However, since the degree of similarity between the *mak* and the *cdc2* and *CDC28* gene products is not extremely high, it seems also possible that the biological function of the *mak*-encoded protein is not related to that of *cdc2*- and *CDC28*-encoded proteins but is necessary for the specific protein phosphorylation reaction involved in spermatogenesis.

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