Identification and Characterization of the Regulated Pattern of Expression of a Novel Mouse Gene, \textit{meg1}, during the Meiotic Cell Cycle\textsuperscript{1}

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Abstract

The gene designated \textit{meg1} (meiosis expressed gene) is a new mouse gene identified during a search for mammalian genes potentially involved in meiotic processes. Two classes of complementary DNAs were isolated from an adult mouse testis complementary DNA library, which shared the same 3' end including the entire putative coding region but differed in their 5' ends. Only one of these complementary DNA classes appeared to correspond to the very abundant 0.75-kilobase testicular transcript of \textit{meg1}. Sequence analysis predicts a 10.8-kilodalton protein which is highly charged and lysine rich. It is also relatively rich in potential phosphoacceptor amino acids (~17%), several of which are located in phosphorylation consensus sequences. The pattern of expression of \textit{meg1} was studied utilizing a combined Northern blot and \textit{in situ} hybridization analysis. Of the adult tissues examined, \textit{meg1} transcripts were detected exclusively in testis. Analysis of mRNA from testes of two germ cell deficient mutant strains did not reveal significant levels of \textit{meg1} transcripts. Analysis of RNA from enriched populations of spermatogenic cells from adult testes and localization by \textit{in situ} hybridization revealed that \textit{meg1} transcripts are most abundant in pachytene spermatocytes. These results suggest a role for \textit{meg1} during germ cell differentiation, possibly during meiotic prophase.

Introduction

The existence of sexually reproducing species is dependent upon a process called meiosis, in which the number of chromosomes in the gametes is reduced by half, without any loss of essential genetic information. At the chromosomal level, cells undergoing meiosis perform a very defined and evolutionarily conserved sequence of events including chromosome duplication, pairing and recombination of homologous chromosomes, and reductional and equational divisions. Our understanding of the meiotic process in general, and of that process in mammals in particular, is mainly at the descriptive level. The sequence of events has been detailed with microscopic observations, and classical genetic analysis had been used to document the process of recombination. Little is known about the regulation of the various stages of meiosis and of the factors that participate in this regulation.

Insight into some regulatory mechanisms, operating in both the mitotic and the meiotic cell cycles, has been obtained from the recent identification of key cell cycle regulatory genes such as the \textit{cdc}2 gene in yeast and its homologues in higher organisms, and the various members of the cyclin family (1, 2). With respect to the regulation of earlier meiotic stages, such as the transition from mitotically dividing cells to entry into meiosis and progression through the first meiotic prophase, the largest body of information has accumulated from studies on the yeast \textit{Saccharomyces cerevisiae} (3–15).

In higher organisms, and mammals in particular, much less is known. A good model system for studying mammalian meiosis is mouse spermatogenesis. A detailed, morphologically characterized developmental schedule of the germ cell differentiation has been established (16, 17), mutant strains of mice exist in which the cells are arrested at specific stages of spermatogenesis (18–20), and enriched populations of spermatogenic cells at different stages of differentiation are accessible via cell separation techniques (21). Many mouse genes which display a unique pattern of expression during spermatogenesis have been described (22). It has been shown that an intensive transcriptional activity takes place in meiotic spermatocytes, especially in mid and late pachytene stages, but a considerable proportion of the RNAs (and proteins) synthesized during this stage are stored in the cell for subsequent use (23–25). Several genes exhibit expression only in postmeiotic stages, such as the protamine 1 and 2 and the transition protein (TP) genes (26), and as such are more likely to be associated with the dramatic changes that occur during spermiogenesis rather than in meiotic events. To date, none of the genes has been correlated with a specific meiotic event.

In this paper, we describe the identification and characterize the pattern of expression of a new mouse gene, \textit{meg1},\textsuperscript{2} that was identified during a search for mammalian genes potentially involved in meiotic processes.

Results

Identification of \textit{meg1}. Given the conservation of both the meiotic process itself and of several key molecules thus far identified, such as \textit{p34}\textsuperscript{cdk2} and cyclins, we hy...
pothesized that other genes important in regulating entry into meiosis in yeast might have evolutionarily conserved counterparts in mammals. The initial goal at the outset of this study was to search for murine genes structurally homologous to the yeast meiosis regulating gene, RME1 (7, 8). Since the deduced amino acid composition of the RME1 protein contains three zinc fingers in its carboxy terminus (7), an RME1 clone (SpΔ-117) was hybridized, under low stringency conditions, to a Southern blot of eight uncharacterized putative zinc finger containing testicular cDNA clones. One of these clones, 3.2 kb in size, gave a positive signal (data not shown). This clone contained an internal EcoRI site, yielding two fragments of ~1.5 kb and 1.7 kb [designated 1.5 and 1.7, respectively (Fig. 1a)]. To determine whether this positive signal was due to sequence similarities to RME1 other than within the conserved zinc finger regions, a Southern blot of the 1.5 and 1.7 fragments was hybridized with different regions of the RME1 clone, SpΔ-117. Both the 1.5 and 1.7 fragments hybridized with the complete SpΔ-117 probe at relatively high stringency conditions [final wash at 0.5× SSC-0.1% SDS at 42°C (Fig. 1b)]. When a 350-bp BglII-EcoRI fragment of SpΔ-117, which does not contain the zinc finger domain, was used as a probe, only the 1.5-kb band could be detected (Fig. 1b). Conversely, only the 1.7-kb band was detected by the 750-bp EcoRI-EcoRI fragment of SpΔ-117, which contains the zinc finger domain (Fig. 1b). These results suggested that the 1.7 fragment contained the zinc finger domain and that the hybridization by SpΔ-117 with the 1.5-kb fragment reflected homology to RME1 outside the conserved zinc finger domain. However, as discussed below, only a portion of this prediction was borne out.

A preliminary characterization of the transcripts corresponding to the cDNAs was performed by Northern blot hybridization analysis of total testis RNA, with the 1.5 and 1.7 inserts as probes. The 1.5 probe detected a very abundant transcript of 0.75 kb and another relatively weakly hybridizing transcript of 3.2 kb (Fig. 1c). The 1.7 probe detected only the 3.2-kb transcript (Fig. 1c). To further characterize these clones and their relationship to one another, direct DNA sequence analysis was undertaken, initially of the 1.5-kb fragment. This analysis revealed that the 1.5-kb fragment contained a stretch of (G)15A(C)9 0.55 kb from the 3′ end (Fig. 1a). A 264-bp-long ORF, initiating with an ATG within a canonical translation initiation consensus sequence (27, 28), was observed in this 0.55-kb fragment beginning 102 bp 3′ to the GC region on one strand. However, a second undisputed ORF (at least 700 bp long), again initiating with an ATG, was located 220 bp 5′ to the GC region on the complementary strand (Fig. 1a). These results suggested the possibility that the 1.5 fragment represented, in fact, two independent cDNAs that were artifically linked at their ends during the generation of the testis cDNA library.

To further examine this possibility, the 1.5 fragment was digested with BamHI to generate two fragments of about 0.75 kb each, which were used as probes for Northern blot hybridization of total testis RNA. The 3′ 0.75-kb fragment, which contains the GC region, was designated 0.71, and the 5′ fragment was designated 0.71I (Fig. 1a). Probe 0.71 detected only the abundant 0.75-kb transcript, whereas the 0.71I probe detected only the 3.2-kb transcript (Fig. 1d). These results further supported the notion that the 1.5 fragment represented cDNAs of two independent genes. Further sequence and expression analysis has revealed that the 5′ portion of the 1.5 fragment (0.71I) contains a portion of a cDNA that corresponds to a 3.2-kb transcript that contains a zinc finger domain. These studies will be presented in a subsequent report. The focus of this study is the characterization of the gene, represented by the 3′ fragment (referred to as clone 0.71), that gives rise to the very abundant 0.75-kb transcript in testis. For reasons that will be more apparent from the results presented below, this gene has been designated meg1 (meiosis expressed gene 1).

To determine whether meg1 represents a single gene or is a member of a family of several related genes, Southern blot hybridization analysis of EcoRI digested mouse genomic DNA was performed. Genomic DNA from male and female mice from two strains was examined, using clone 0.71 as a probe. Two bands of ~9.5 kb and 4.4 kb were observed, independent of the sex or strain of the source of DNA (data not shown). We conclude that meg1 is not linked to the Y chromosome and is not likely to be a member of a multigene family. In addition, clone 0.71 was hybridized under low stringency conditions to a blot that consisted of EcoRI digested genomic DNA from human, rat, raffles snake, chicken, frog (Xenopus), fish (Oryzias latipe), sea urchin, Drosophila, and yeast (Saccharomyces), to obtain preliminary information regarding its evolutionary conservation. Weakly hybridizing bands of about 7.0 kb and 4.0 kb and of 7.0 kb and 2.3 kb could be detected in human and rat, respectively (data not shown). No such apparent bands were detected under these conditions in the other organisms tested (data not shown). These results, although suggesting the existence of a closely related gene in other mammals, do not rule out the possibility that genes homologous to meg1 exist in lower organisms as well.

Characterization of the meg1 cDNA(s). To confirm that clone 0.71 corresponds to a bona fide cDNA representative of the meg1 transcript, it was used as a probe to screen a second, independently generated adult mouse testis cDNA library. Four clones were isolated and designated 1a2, 2a2, 1a2, and 2c2 (Fig. 2a). DNA sequence analysis revealed that the longest clone (1a2, 684 bp) shared 100% identity over 447 bp with the 3′ end of clone 0.71 (not including the poly(A) tail), including all of the ORF. However, the sequence diverged in the 219 bp corresponding to the 5′ region upstream of the putative ATG. Clone 2c2 (372 bp long) was similar to 1a2, including its 5′ end. Clone 2a2 (532 bp long) was similar to 0.71, including its entire 5′ end, except for 19 bp flanking the GC region. Finally, clone 1a2 (297 bp long) was similar to the 3′ end of clones 0.71, 2a2, and 1a2 (Fig. 2a). These five independent clones therefore share the same 3′ end, including the whole ORF, but have two different 5′ ends (Fig. 2a). The possibility of cloning artifacts accounting for these differences was

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4 The abbreviations used are: cDNA, complementary DNA; bp, base pairs; cAMP-PK, cyclic AMP dependent protein kinase; cGMP-PK, cyclic GMP dependent protein kinase; PKC, protein kinase C; CaM kinase II, calcium/calmodulin protein kinase II; kb, kilobase(s); ORF, open reading frame; PBS, phosphate buffered saline; poly(A)1, polyadenylated; SDS, sodium dodecyl sulfate; SSC, standard saline citrate.

4 P. S. Burke and D. J. Wolgemuth, in press.
considered unlikely since it was improbable that totally independent clones from different cDNA libraries would show the same artifact.

To address the question of whether both of these classes of cDNAs corresponded to the previously detected 0.75-kb transcript (Fig. 1d), the different 5' ends as well as the common 3' end of the cDNA clones were used as probes to hybridize to blots of total testis RNA. The prediction was that the 5' end of the corresponding cDNA would detect the 0.75-kb transcript. An EcoRI-Sacl fragment from clone 11a2 (209 bp) was used as a probe representing the entire 5' end of this clone (Figs. 2a and 3a), whereas a BamHI-Nael fragment from clone 0.71 (231 bp) was used as a probe representing the second 5' end (Figs. 2a and 3b). The 473-bp Sacl-Xhol fragment of clone 11a2 was used as a probe for the common 3' end (Fig. 2a). As can be seen in Fig. 2b, both the common 3' probe and the unique 5' probe from clone 0.71 detected the 0.75-kb transcript. This transcript was not detected by the unique 5' probe of clone 11a2, even after longer exposure and reduced washing stringency conditions. However, a very faint band of about 3.0 kb was observed after exposing the film for 3 days. When the entire clone 11a2 was used as a probe, a similar 3.0-kb band as well as faint 1.87-kb, 4.7-kb, and 6.5-kb bands were detected after prolonged exposure in total testis RNA, in addition to the broad 0.75-kb band Fig. 2c). None of the bands other then the 0.75-kb transcript was observed in testis poly(A)* RNA (Fig. 2c). The 1.87-kb and 4.7-kb bands in Fig. 2c might be attributable to nonspecific probe binding to the ribosomal bands. The 3.0-kb as well as the 6.4-kb transcripts could represent an unprocessed precursor of meg1 mRNA since they were detected by both cDNAs (2a2 and 11a2) in total RNA after prolonged exposure (data not shown) but not in poly(A)* RNA.

Sequence analysis of clone 11a2 provided additional data, suggesting that it represents a nontranslated RNA. First, clone 11a2 has in its 5' end an ORF of 114 bp, initiating with an ATG at position 101 (Fig. 3a). Such upstream ORFs are believed to reduce significantly the efficiency of translation of the main initiation site (27, 29). Secondly, 11a2 contains two complementary stretches of 9 bp each, upstream of the ATG yielding the longest ORF of the cDNA [positions 119–127 (Fig. 3a) and 85–93 (Fig. 3b)], which could possibly form secondary structures. If such structures do occur, an inhibition of translation of the main ORF could result, especially since one of these stretches is located only 5 bp upstream of the major ATG and includes part of the translation initiation consensus sequence which is believed to be part of the ribosomal recognition site (28). Therefore, the structural and expression data together suggest that clone 2a2, rather then 11a2, represents the cDNA corresponding to the translatable mRNA of this gene.

Fig. 1. a, schematic representation of the 3.2-kb putative zinc finger containing clone that hybridized with the yeast gene RME1 (clone SPΔ-117). An internal EcoRI site divides the clone into two fragments designated 1.7 and 1.5. A BamHI site divides the 1.5 clone into two fragments designated 0.71 and 0.71I. A GC stretch located in the 5' portion of 0.71 is indicated, as well as two ORFs located on complementary strands and oriented opposite to one another. b, Southern blot hybridization of clones 1.7 and 1.5 with three different restriction fragments of the RME1 clone, SPΔ-117. A BglII/Xhol (B/X) fragment corresponds to the entire SPΔ-117 clone. The BglII/EcoRI (B/E) and EcoRI/EcoRI (E/E) fragments correspond to the non-zinc-finger containing domain and to the zinc finger containing domain of RME1, respectively. Exposure time, 24 h. c, Northern blot hybridization of mouse total testis RNA (20 μg) to clones 1.5 and 1.7 as probes (exposure time, 16 h). d, Northern blot hybridization of mouse total testis RNA (20 μg) to clones 0.71 and 0.71I as probes (exposure time, 16 h).
The 3' untranslated region common to all of the clones has two unique characteristics: it is relatively AT rich, and it lacks the consensus AATAAA polyadenylation signal. Instead, a closely related sequence, CATAAA, which has been observed as a polyadenylation signal in 1% of polyadenylated RNAs of vertebrates (30), is located 20 bp upstream the poly(A) tail and substitutes for the canonical sequence.

A search through the GeneBank/EMBL database did not reveal any DNA sequences with homology to meg1. A search through the SwissProt database for any related known proteins revealed no similarity between the putative meg1 protein and the proteins in the database. The database was also researched for similarity between the 38-amino acid potential product of the short ORF in the 5' end of clone 1a2, but no homologous proteins were identified. Thus, meg1 is likely to be a new gene which is not significantly related to any of the known genes represented in the databases searched.

Sequence Characterization of the Putative meg1 Protein. The meg1 cDNA has a 264-bp ORF that putatively encodes a basic protein of 88 amino acids with an estimated pI of 9.16 and a calculated molecular weight of 10,800. The deduced amino acid composition (Fig. 3b) revealed several interesting characteristics. First, meg1 protein is highly charged, with 39.7% charged amino acids, including 13.64% lysine, 7.9% arginine, 1.1% histidine, 11.4% glutamic acid, and 5.7% aspartic acid. The lysine residues are predominantly distributed within the protein in pairs (amino acids 7,9; 15,16; 57,58; 82,84). In addition, there are pairs of arginines (amino acids 61,62) and lysine/arginine (amino acids 71,73). Secondly, the predicted meg1 protein contains a high proportion of potential phosphoacceptor amino acids (>17% of the total amino acid complement), including 8 tyrosine residues (representing >9% of the total amino acids), 4 serines (all of which are within the first 18 amino acids at the amino terminus), and 3 threonine residues. Third, meg1 contains 9 valine residues, 4 of which are clustered between amino acids 41–56, with a second cluster of 4 residues at the carboxy terminus between amino acids 80–87. In a search for functional consensus sequences, no calcium binding sites (31), ATP binding sites (32), zinc binding sites (33), or glycosylation sites (34) could be detected. However, as discussed later, several consensus sequences for phosphorylation of proteins (35) were observed.

Tissue Specificity of meg1 Expression. To determine the tissue distribution of meg1 expression in the mouse, total RNA was isolated from 10 adult tissues, including testis, epididymis, ovary, spleen, lung, liver, kidney, heart, brain, intestine, and from placenta (from day 12 conceptuses), and analyzed by Northern blot hybridization analysis, using clone 2a2 as probe. The very abun-
dant 0.75-kb transcript was detected exclusively in testis RNA (Fig. 4). No signal was detected in any other tissue, even after longer exposures (data not shown). The filter was washed and reprobed with fl-actin to confirm RNA integrity (Fig. 4). Since liver RNA showed a very faint signal with fl-actin, this blot was reprobed once again with glyceraldehyde-3-phosphate dehydrogenase as a probe. Liver RNA integrity was verified by the appearance of the expected 1.4-kb band (data not shown). In addition, no expression of meg1 transcripts was detected in total RNA from embryos day 12.5 and day 15.5 postcoitum (data not shown) or in poly(A)* RNA from testes of day 7 postnatal animals (Fig. 2c). Thus, among the tissues examined, the meg1 transcript was expressed exclusively in the samples tested.

**Fig. 3.** a, nucleotide sequences of the 5' end of clone 11a2; b, nucleotide sequences of the entire clone 2a2. The deduced amino acid sequences of the short ORF in the 5' end of 11a2 and of the longest ORF in clone 2a2 are shown. The EcoRI, Nael, and SacI restriction sites are indicated. The ACG sequence in clone 2a2 at the junction point between clone 2a2 and 11a2 is boxed, with an arrow indicating the exact junction point (the next nucleotide of clone 11a2 would be the G on the right side of the arrow). Complementary stretches of 9 bp each within clone 11a2 are marked with dashed lines. The putative CATAAA polyadenylation signal is underlined.

**Fig. 4.** Northern blot hybridization analysis of total RNA (20 μg/sample) from 11 adult tissues, using clone 2a2 as a probe (exposure time, 16 h). The ethidium bromide staining of the gel suggests that samples were relatively equally loaded. RNA integrity was verified by washing the filter and reprobing it with fl-actin as a probe (bottom panel; exposure time, 24 h).

animals. The 0.75-kb transcript was therefore most abundant in germ cells. The trace levels of 0.75-kb transcripts detected in the mutant testes after prolonged exposure (Fig. 5a) may reflect transcripts from the few partially differentiated germ cells that can occasionally be found in the seminiferous tubules of mutant mice (37) or may result from very low levels of transcripts in somatic cells.

To determine which spermatogenic cells express the 0.75-kb transcript, enriched populations of meiotic prophase spermatocytes (predominantly in the pachytene stage of meiosis), postmeiotic early spermatids, and a mixture of residual bodies and cytoplasmic fragments from elongating spermatids were obtained (21). Total RNA was isolated from the various cell types and analyzed by Northern blot hybridization with the 2a2 clone as a probe (Fig. 5b). The 0.75-kb transcript was very abundant in RNA from the meiotic prophase cells. Denstometric analysis revealed that the levels of the 0.75-kb transcript decreased slightly (~20%) in the early spermatid fraction. The presence of meg1 transcripts in the residual bodies fraction suggests that this mRNA is not degraded rapidly, since transcriptional activity ceases...
prior to stage 9 of spermiogenesis (39). This pattern of decreasing abundance of the 0.75-kb transcript from meiotic prophase through early spermatids to elongating spermatids suggests that meg1 is predominantly transcribed in cells at meiotic prophase and is slowly turned over once the meiotic divisions are completed. However, the possibility that this gene is actively transcribed in haploid early spermatids as well cannot be ruled out.

In Situ Hybridization Analysis. To further define the cellular expression of meg1, we performed a series of in situ hybridization experiments on testis sections from normal animals and atrichosus mutants (homozygous as well as heterozygous). Antisense and sense RNA probes driven from clone 2a2 were used for experimental and control hybridizations, respectively. An intense hybridization signal was observed in all tubules from normal adult testes, independent of the stage of their seminiferous epithelium cycle [as described by Oakberg (40); Fig. 6, a and b]. Examination of the cellular distribution revealed that this signal was exclusive to the meiotic spermatocytes and early spermatids (Fig. 6, c and d). No signal above background was noted in the spermatogonial cells in the periphery of the tubules, nor in the elongated spermatids which are located just adjacent to the lumen (Fig. 6, a and b; c and d). No signal above background was detected in the interstitial regions of the adult testis (Fig. 6, a and b), nor when sense oriented probes were used (data not shown). A similar pattern of meg1 localization was observed in heterozygous mutants (+/at) testes (data not shown). In sections from homozygous mutant (at/at) testes, which are deficient in germ cells, no signal above background could be detected (Fig. 6, e and f), in agreement with the Northern blot hybridization analysis (Fig. 5a).

Discussion

In this study, we describe the identification of a novel mouse gene, meg1, which exhibits a unique pattern of expression during germ cell differentiation. A very abundant meg1 transcript of 0.75 kb was detected in total testis RNA from adult mice, but not in poly(A)* RNA from testes of 7 postnatal mice. Transcripts of meg1 were not observed in RNA from 10 other adult tissues or in total RNA from midgestation embryos. Moreover, this transcript was not detected in testes of two germ cell deficient mutant strains, except for trace amounts that were detected by Northern blot analysis under conditions of increased amounts of RNA and prolonged exposure. These results indicate that meg1 not only is expressed in a testis specific pattern but is also primarily germ cell specific as well. Analysis of RNA from the enriched populations of spermatogenic cells revealed that meg1 was most abundant in the meiotic cells, a pattern that was confirmed by in situ analysis.

The meg1 mRNA levels appeared to decrease in the haploid cells. This expression pattern of meg1 is distinct from that observed for other genes also expressed in spermatocytes, such as phosphoglycerate kinase 2 (41) or some acrosomal specific proteins (25), whose mRNAs appear to be stored for postmeiotic translation and function. The expression pattern of meg1 would be more characteristic of genes associated with meiotic events. Moreover, analysis of the temporal synthesis pattern of proteins constituting the spermatozoa (25) did not reveal labeled proteins of a size corresponding to that of the predicted meg1 protein. The simplest interpretation would be that the meg1 protein is not present in spermatozoa.

Although speculative, comparison of meg1 with other germ cell specific, size related proteins, with several common structural features, might give a clue as to its possible function. The predicted meg1 protein is a small, basic (lysine rich) protein, which is also rich in potential phosphoacceptor amino acids (~17%), mainly tyrosine (~9%). It is reminiscent of the transition proteins TP1 and TP2, which are germ cell specific, small (54 and 117 amino acids, respectively), basic (lysine and arginine rich), DNA associated proteins that are believed to function in DNA condensation (26). TP1 and TP2 contain many phosphoacceptor residues, mainly serine and threonine (42, 43). The tyrosine residues present within the transition proteins have been shown to intercalate between the DNA bases, possibly inducing conformational changes as well as nicks in native DNA (44). Such interactions might be of significance during meiosis as well as during the chromatin remodeling of postmeiotic spermatids. The pattern of expression of meg1 versus the exclusively haploid expression of TP1 and TP2 (26, 45) might suggest roles during meioic versus postmeiotic events, respectively. Of other size related, testis specific genes that share some similarities with meg1, the rat RT7 (46) deduced product is 90 amino acids long and is serine and threonine rich (10% each). However, this gene is
also a haploid expressed gene, and its function is not known.

As mentioned above, p11meg1 is relatively rich in potential phosphoacceptor amino acids (~17%). We examined meg1 for consensus phosphorylation sequences most frequently recognized by various serine/threonine protein kinases (35). Four protein kinases were noted to have the potential to phosphorylate meg1-protein (Table 1). Furthermore, all 4 serines in the meg1 protein, as well as threonine at amino acid 65, lie within such consensus sequences (Table 1). This would argue in favor of meg1 being phosphorylated, especially at residues S11 and/or T65, which could potentially be phosphorylated by three different kinases: cAMP-PK, cGMP-PK, and PKC for S11 and cAMP-PK, PKC, and CaM kinase II for T65 (Table 1). Interestingly, two isozymes of cAMP-PK have been re-
ported in mouse spermatogenic cells: type I is present in pachytene spermatocytes and in round spermatids, whereas type II is present in round and elongating spermatids (47, 48). The relatively high percentage of tyrosine residues in meg1 suggests that meg1 might be phosphorylated on one or more tyrosine residues. Interestingly, the tyrosine positioned at amino acid 38 lies within an EGF receptor tyrosine kinase consensus phosphorylation site [X[E/D]Y*X (49)]. None of the other tyrosine residues in meg1 are located within sequences recognized by specific tyrosine kinases, as listed by Pearson and Kemp (49). With respect to potential tyrosine kinases, the putative tyrosine kinase lerT might be of special interest. It has been shown that lerT has a testis specific transcript and that it is predominantly expressed in spermatocytes at the mid and late pachytene stage (50, 51). The phosphorylation site of lerT, however, has not been determined. Other potential tyrosine kinases shown to be expressed during spermatogenesis include the protooncogenes c-abl (52) and pim-1 (53).

An additional structural characteristic of the predicted meg1 protein is that the basic residues, mainly lysine, are predominantly distributed along the protein in pairs. Pairs of basic amino acids have been shown to play an important role in the recognition of specific sequences by various serine/threonine protein kinases (35). Moreover, pairs of basic amino acids seem to be important in designating specific domains of the protein. For example, pairs of lysine and arginine residues separate the component peptides of the bovine corticotropin-β-lipotropin precursor (54) and bracket all enkephalin sequences in mouse proenkephalin [opioid precursor (55)]. This suggests that creating small basic zones along the protein might be important in recognition sites for enzymes or other cellular components.

Two cDNA classes were isolated in this study. The two cDNA classes, represented by clones 2a2 and 11a2, shared the same 3' end, including the entire putative ORF, but differed in their 5' end. Clone 2a2 was considered to represent the translatable 0.75-kb mRNA, since its 5' end region detected the 0.75-kb mRNA, whereas the 5' end of clone 11a2 did not. The fact that clones 2a2 and 11a2 represent different 5' ends does not favor the possibility that 11a2 is an unprocessed precursor of meg1, in which case both 5' ends were expected to be included in the precursor RNA. Although we cannot exclude the possibility that the 5' end of clone 11a2 represents an intron and that this clone, as well as clone 2c2, resulted from an incomplete reverse transcription of a precursor RNA during the generation of the mouse cDNA library, we suggest an alternative promoter model as the most likely explanation for the alternative 5' exons (56). Alternative promoters resulting in somatic specific transcript and germ cell specific transcript have been observed in other genes, such as the proenkephalin gene (55) and the c-mos gene (57). An AGG sequence, which was found in clone 2a2 at a site corresponding to the junction point between clone 2a2 and 11a2 [positions 66–68 (Fig. 3b)], is consistent with the sequence expected at a joining point of two exons (58, 59). This suggests that 2a2 is a postsplcing product and therefore potentially transcribed from a more upstream promoter. This is further supported by the presence of the sequence CTCCAGAGATA at the 3' region of the 5' end (Fig. 3a) that shows good homology to the consensus acceptor sequence (T/C)G(A/T)AG (58, 59). A reservation to this model would be the extra nucleotide A at the 3' end of the proposed acceptor sequence (position 219 [Fig. 3a]). Although it is possible that this A was added after splicing via RNA editing processes (60), this point requires further investigation. Confirmation of the mode of generation of the different RNA species can be achieved by analyzing the 5' region of meg1 in genomic clones, which is currently under investigation.

In conclusion, meg1 is a novel mouse gene with a unique pattern of expression in male germ cells that makes it a candidate for being involved in meiotic processes. It will be interesting to examine the expression of meg1 during specific developmental stages of postnatal tests at both the RNA and protein levels as well as to examine the comparable stages of meiosis of female germ cells to determine the generality of its potential function.

Materials and Methods

Source of Tissues and Cells. Swiss Webster mice (Charles River, Wilmington, DE), 60 days of age, were used as a source of normal adult mouse tissues for all experiments, except those using mouse mutant strains. For studies on

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germ cell deficient testes, heterozygous and homozygous littermates from two mouse mutant strains, white spotted (W) and atrichosis (at), were purchased from the Jackson Laboratory (Bar Harbor, ME). Enriched populations of germ cells in specific stages of spermatogenesis were separated by sedimentation at unit gravity as described by Wolgemuth et al. (21). Tissues for DNA or RNA isolation were frozen in liquid nitrogen immediately upon dissection and stored at −70°C until used. All animals were sacrificed by cervical dislocation.

Probes. The following probes were used: (a) SpΔ-117, a 1.3-kb BglII-XhoI fragment of the yeast Saccharomyces cerevisiae RME1 gene (a gift from Dr. A. P. Mitchell, Columbia University, New York, NY), which consists of 700 bp from the 3' end of the coding region of the gene and an additional 600 bp from the 3' untranslated end of the gene (7); (b) pA1, a 2.0-kb chicken β-actin cDNA (61); (c) pRGAPDH-1, a 1.4-kb PstI-PstI fragment from the rat glyceraldehyde-3-phosphate dehydrogenase gene (62) (obtained from Drs. R. K. Assoian and L. Scotto, Columbia University); and (d) clones that were isolated during this study and fragments of these clones were also used as probes as specified in the text. All DNA probes were labeled with [α-32P]dCTP (DuPont, Wilmington, DE) by the random priming labeling technique, to a specific activity of greater than 10^6 cpm/μg, using the Amersham Multiprimer DNA labeling kit. Sense and antisense RNA probes for in situ hybridization experiments were generated from linearized clones constructed in pluBEScript SK (Stratagene, La Jolla, CA), by using either T7 or T3 RNA polymerase (Promega Biotech, Madison, WI). RNA probes were labeled with [α-35S]UTP (DuPont), to a specific activity of greater than 10^6 cpm/μg, and hydrolyzed to an approximate size of 0.1 kb, according to Cox et al. (63).

Southern Blot Hybridization Analysis. Mouse genomic DNA or cloned DNA was digested with appropriate restriction enzymes, electrophoresed on 0.8% agarose gels, blotted onto nitrocellulose filters (Schleicher & Schuell, Keene, NH) according to standard procedures (64), and cross-linked to the filters by baking for 2 h at 80°C in a vacuum oven. Prehybridization (3–4 h) and hybridization (15–20 h) took place at 37°C or 42°C for low or high stringency hybridization conditions, respectively, in 43% formamide, 5X SSC, 1X Denhardt's, 50 mM or 20 mM NaPO₄ (for low or high stringency, respectively), 0.1% SDS, and 100 μg/ml sonicated and denatured salmon sperm DNA. Dextran sulfate was added to the hybridization solution to a final concentration of 10%, as well as labeled probe to a final concentration of 1–2 × 10^6 cpm/ml. For low or high stringency washing conditions, final washes of filters, 20 min each, consisted of: 1X SSC-0.1% SDS at 37°C, or 0.5X SSC-0.1% SDS at 65°C, respectively. Filters were then air dried and exposed to Kodak XAR film with an intensifying screen at −70°C for the times indicated in the text.

RNA Isolation and Northern Blot Analysis. RNA was isolated using the LiCl precipitation method described by Cathala et al. (65). Poly(A)+ RNA was selected by oligo-dT cellulose chromatography according to Aviv and Leder (66). RNA samples were electrophoresed on denaturing 1% agarose-2.2 M formaldehyde gels, blotted onto Nytran membranes (Schleicher & Schuell) for 8–12 h according to standard procedures (64), and UV cross-linked to the membranes (120 mJ/cm²) with a Stratallinker UV cross-linker (Stratagene). Prehybridization was performed at 42°C for 3–8 h in 50% formamide, 5X SSC, 5X Denhardt's solution, 50 mM NaPO₄, 0.1% SDS, and 300 μg/ml sonicated and denatured salmon sperm DNA. Hybridization, at 42°C for 15–20 h, was performed in 50% formamide, 5X SSC, 1X Denhardt's solution, 20 mM NaPO₄, 10% dextran sulfate, and 100 μg/ml sonicated and denatured salmon sperm DNA. Final washes, 20 min each, consisted of: 0.1X SSC-0.1% SDS at 65°C, and 0.1X SSC at 65°C. Filters were exposed to autoradiographic film at −70°C for the indicated times. Densitometry analysis of autoradiograms utilized the JAVA software (Jandel Scientific, Corte Madera, CA), after normalizing RNA levels from the ethidium bromide image.

Library Screening. Approximately 4 × 10^5 plaques from an amplified adult mouse testis cDNA library, constructed using the Stratagene ZAP-cDNA synthesis kit (provided by D. L. Chapman, Columbia University) were screened with the clone 0.71 as a probe (see "Results"), according to standard procedures (64). The filters (duplicates for the first two plaque purification rounds) were washed in 50 mM Tris (pH 8.0), 1 mM NaCl, 1 mM EDTA, and 0.1% SDS at 42°C for 1 h. Prehybridization, hybridization, and posthybridization washes were identical to those described for low stringency Southern hybridizations except that they were performed at 42°C. Positive clones were purified by three rounds of plaque selection. Inserts from the tertiary screen were excised in vivo according to the protocol of Stratagene. Resulting phagemids contained the cDNA of interest at the EcoRI and Xhol site of pBuescript SK(−).

DNA Sequencing. The chain termination method (67) was utilized for sequencing. For each clone, both strands were fully sequenced using the T3 and T7 promoter primers (Promega Biotech) and the Perkin Elmer Cetus AmpliTaq sequencing kit, according to the manufacturer's protocol. Sequences were analyzed using the IBI Pasteil Sequence Analysis Software (68) and the sequence analysis programs for the VAX (69).

In Situ Hybridization. For in situ hybridization analysis of testicular tissues, frozen sections were used. Samples were fixed immediately upon dissection at 70°C in 4% paraformaldehyde (in PBS, pH 7.4) at 4°C, washed in PBS, and thereafter kept in 30% sucrose at 4°C until embedding. Samples were frozen in Tissue-Tek medium (Miles, Elkart, IN) with liquid nitrogen, and 6–8-μm sections were cut on a cryostat and placed on Tespa-treated slides, which were then heated to 37°C for 2 h and stored at −70°C with desiccant until further use. Prior to hybridization, the slides were allowed to destore for 30 min at room temperature. The sections were then treated as follows: postfixation in 4% paraformaldehyde (20 min), PBS washes (2X 5 min each), proteinase K treatment for 8 min (20 μg/ml in 5 mM EDTA-50 mM Tris-HCl, pH 7.5), PBS wash (5 min), relaxation in 4% paraformaldehyde (5 min), incubation in 0.1 m triethanolamine, pH 8.0, with acetic anhydride added to 0.25%, PBS and 0.85% saline washes (5 min each), and dehydration in increasing ethanol concentrations (50–95%, 2 min each and 2X 100%, 2 min each). Prehybridization and hybridization were performed using the procedure described by Jaffe et al. (70). The slides were viewed on a Leitz photomicroscope under bright field, dark field, and epiluminescence optics. Photographs were taken using Kodak Technical Pan film.
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References


