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Male-enhanced antigen gene is phylogenetically conserved and expressed at late stages of spermatogenesis

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ABSTRACT **The male-enhanced antigen gene (*Mea*) was previously isolated from a mouse testicular cDNA library by using a pool of specific antisera against the serological H-Y antigen. The present studies characterize the human and mouse cDNAs and indicate that the *MEA* gene is conserved at both nucleic acid and protein levels. The corresponding mRNA encodes proteins of 18–20 kDa. The phylogenetic conservation could be extended to other mammalian species by Southern blot analysis. Although the *Mea* gene was transcribed as a 1-kilobase mRNA in most tissues, it was expressed at the highest level in adult testis. The testis-enhanced expression of the *Mea* gene was associated with germ cell development at late stages of spermatogenesis. Chromosome walking experiments identified two linked genes, A and B, located within 38 kilobases of human genomic sequence. Like the *MEA* gene, genes A and B were coordinately transcribed in the testis, which suggests that *MEA* and genes A and B are members of a gene family. *In situ* hybridization studies localized the *MEA* gene to the short arm of human chromosome 6 at band p21.1–21.3, close to the major histocompatibility complex locus. The genetic conservation and testis-specific expression of the *MEA* gene support the hypothesis that it plays an important role in mammalian spermatogenesis and/or testis development.**

The male-enhanced antigen gene (*Mea*) was initially isolated from a mouse prokaryotic expression cDNA library by using a pool of specific antisera against the serological H-Y antigen (1, 2). Although the *Mea* gene can be considered a candidate gene for the H-Y antigen, its exact identity has yet to be established independently. Preliminary results from molecular analysis indicated that the *Mea* gene is genetically conserved in all mammalian genomes examined and is expressed at relatively high levels in the testis and only at basal levels in the ovary (2, 3). We surmise that the *Mea* gene may be important for the development and physiology of the mammalian testis. We have now isolated the complete cDNAs for both the mouse *Mea* and the human *MEA* genes from the respective testicular cDNA libraries. Molecular characterization of the *Mea* gene showed a phylogenetic conservation and a haploid-enhanced expression pattern in the mouse testis. *In situ* hybridization localized the *MEA* gene to the short arm of human chromosome 6 at band p21.1–21.3. We have also demonstrated that *MEA* belongs to a gene cluster that is preferentially expressed in mammalian testis. Hence, the present results further corroborate our supposition that the *MEA* and other linked genes are vital for normal spermatogenesis in adults.‡

MATERIALS AND METHODS

Construction of cDNA Libraries. Poly(A)$^+$ RNA was isolated from testes of BALB/c mice (Simonsen Laboratories, Gilroy, CA) and fresh human surgical biopsy tissues (Cooperative Human Tissue Network, Birmingham, AL) as described (4, 5). cDNAs were synthesized from the poly(A)$^+$ RNA according to a modified protocol of Gubler and Hoffman (6, 7). Several cDNA libraries, some selected for various size ranges, were constructed in the bacteriophage vectors, λgt10 and λgt11 (8). The initial numbers of recombinant phages in these cDNA libraries usually ranged from 1 to 25 × 10^6 plaque-forming units before amplification.

Isolation of *Mea* Genes. Three *Mea* cDNAs were isolated independently from a mouse testis cDNA library as described (7, 8). Larger clones were isolated from both mouse and human testis cDNA libraries in λgt10 or λgt11 by the procedure of Benton and Davis (9) and were identified by hybridization with the mouse *Mea* cDNA probe.

DNA Sequence Analysis. The cDNA clones were subcloned into the single-stranded M13 bacteriophages and sequenced by the chain-termination reaction using deoxyadenosine 5'-[α-[^{35}S]thio]triphosphate as tracer (10). All cDNAs were analyzed in both orientations and with overlapping fragments. The corresponding nucleotide and amino acid sequences were analyzed with a set of computer programs (Department of Biochemistry, University of California, San Francisco) and a Microgenie sequence analyzer (Beckman).

Southern and Northern Hybridizations. Testes and other tissues from mice and rats were obtained freshly from sacrificed animals. Tissues from rabbit, guinea pig, bull, and dog were purchased from Pel-Freez Biologicals. Mutant mice, XX*Sxr* and XX*Sxr'*, were obtained from A. McLaren (Medical Research Council, Mammalian Development Unit, London); *Tfm*/Y and *qk*/*qk* were purchased from The Jackson Laboratory. DNA and RNA were isolated from the tissues according to established procedures (4, 5). DNA from the great apes was isolated from peripheral blood provided by the Yerkes Regional Primate Research Center (Atlanta). Purified DNAs were digested with *Eco*RI, size-fractionated by 0.8% agarose gel electrophoresis, and analyzed by Southern hybridization. Total or poly(A)$^+$ RNAs were denatured by glyoxylation and were analyzed by Northern blotting as described (5). DNA probes were labeled with ^{32}P to specific activities of 0.5–2 × 10^9 cpm/μg by nick-translation reactions (Bethesda Research Laboratory) and were used in both Southern and Northern hybridizations. Filters were hybridized with their respective probes (2 × 10^6 per ml) in a buffer containing 50% (vol/vol) formamide at 41°C for 20 hr and washed in 0.1× SSC/0.1% SDS at 50°C for 30 min (1× SSC = 0.15 M NaCl/0.015 M sodium citrate).

Isolation of Cosmids and Linked Gene cDNA. The human genomic sequence harboring the *MEA* gene was isolated from a recombinant cosmid library constructed in the vector pCV108 (5). The cosmid library was screened at high density using the human *MEA* cDNA as a probe. Four recombinant cosmids harboring *MEA* sequences were isolated. One

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‡The sequences reported in this paper have been deposited in the GenBank data base (accession no. M27937 and M27938).

cosmid, designated Cos*MEA*-A, was labeled with ^{32}P by nick-translation, denatured, and reassociated with total human DNA to subtract out the repetitive sequences; it was then used as a probe for Northern analysis. To isolate the linked genes, total insert from Cos*MEA*-A was purified from the vector sequence, labeled, and subtracted with human DNA as described above and used as a probe for hybridization in cDNA library screening. Duplicate filters were hybridized with the human *MEA* cDNA probe. Recombinant bacteriophages that hybridized positively to the cosmid probe, but not to the cDNA probe, were further purified and analyzed. The identity of the cDNA was established by Northern analysis of testicular RNA.

DNA-Mediated Gene Transfer. Human cosmids harboring the *MEA* and linked genes were transferred to Chinese hamster ovary (CHO) and human HeLa cells by the calcium phosphate precipitation method and were selected with the antibiotic G418 (5). Transformed cells were pooled and propagated to two confluent T-150 flasks in G418 medium and harvested for both RNA and DNA preparations as described (4, 5).

Chromosome *in Situ* Hybridization. The human *MEA* cDNA probe was labeled by oligonucleotide priming with ^3H-labeled deoxynucleotides to a specific activity of 3×10^8 cpm/μg. *In situ* hybridization to normal human chromosomes followed the method of Harper and Saunders (11) as modified by Cannizzaro and Emanuel (12). Slides were exposed for 1 week and all silver grains on or touching chromosomes were scored.

RESULTS AND DISCUSSION

Molecular Isolation and Sequence Determination of the *MEA* Gene. In the absence of purified serological H-Y antigen and its amino acid or nucleotide sequence, we used the prokaryotic hybrid protein expression and antibody-binding approach to isolate candidate genes for this antigen (2, 8). We screened a mouse testicular cDNA library constructed in λgt11 vector with a pool of specific mouse antisera. Three independent cDNA clones derived from the same mRNA formed hybrid proteins that were consistently recognized by the H-Y antisera. They contained inserts ranging from 400 to 700 base pairs that detected a 1-kilobase (kb) transcript in Northern analysis of poly(A)$^+$ RNA derived from adult mouse testis. Several cDNA clones harboring longer inserts were subsequently isolated by hybridization screening of both mouse and human cDNA libraries constructed in λgt10. The nucleotide sequences of these clones were completely determined (Fig. 1). The human and mouse cDNAs contained inserts of 832 and 841 nucleotides, respectively, excluding the poly(A) track. The human cDNA contained three translation initiation codons (ATG) at its 5′ end at amino acid positions 1, 14, and 24. The translated proteins at these initiation codons contained 185, 172, and 161 amino acids and have calculated molecular masses of 20, 18.5, and 18.2 kDa, respectively. It is still uncertain which of these initiation codons is utilized *in vivo*. However, the initiation sequence flanking the third methionine seems to match more closely (9 of 10 bases) the consensus initiation codon sequence (GCCACCATGG) (13, 14) than those from the first two methionines (7 of 10 bases). Interestingly, unlike the human cDNA, the mouse cDNA sequence does not include the first methionine codon. Furthermore, two insertions were observed in the mouse protein at positions 26 (proline) and 79 (glutamic acid). These results are consistent with those from repeated sequence determinations of the same and other *Mea* cDNA clones from the mouse library. Both human and mouse cDNAs contained the same termination codon (TGA) and putative polyadenylylation signal (AATAAA) at similar po-

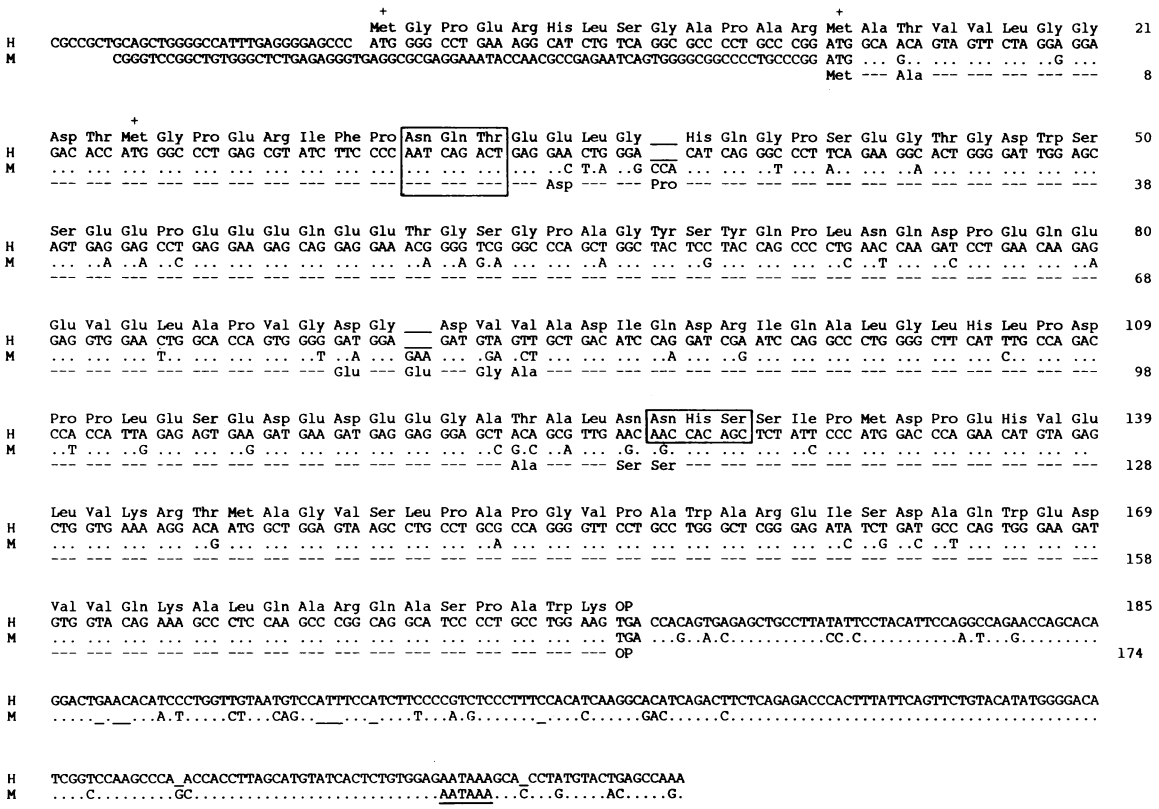

FIG. 1. Nucleotide and corresponding amino acid sequences of the human *MEA* (H) and mouse *Mea* (M) cDNAs. Three initiation codons (+) are identified at the 5′ end of the human cDNA. Two putative N-glycosylation sites in the human protein are boxed. The polyadenylylation site is underlined.

sitions in their 3′ untranslated regions. The deduced mRNA sequence for the mouse *Mea* gene encodes proteins of 174 and 164 amino acids from the first and second methionine with calculated molecular masses of 18.6 and 18.3 kDa, respectively. In addition to several third-base substitutions, the human *MEA* and mouse *Mea* genes share at least 90% homology in the protein and mRNA coding sequences. Two putative N-glycosylation sites (Fig. 1, boxed) are present at amino acid positions 32–34 (Asn-Gln-Thr) and 127–129 (Asn-His-Ser) of the human sequence. However, only the first site is conserved in the mouse. It is not known whether these sites are actually glycosylated *in vivo*. Extensive search of several data banks (including GenBank and Protein Identification Resource) did not reveal any significant homology to other genes at either the DNA or protein level, suggesting that the *MEA* gene is a newly isolated sequence.

The presence of three in-frame initiation (ATG) codons at the 5′ end of the human *MEA* mRNA is quite unusual. According to the scanning model of eukaryotic translation, the first ATG serves as the initiation site for protein synthesis (13). However, sequence analysis of many vertebrate mRNAs indicates that the flanking sequences are important for the successful initiation of translation (14). For the consensus sequence (GCC)GCC$\frac{A}{G}$CCATGG derived from this survey, 97% of vertebrate mRNAs contain a purine at position −3 (the A of the ATG codon is at +1) and most frequently it is an adenine. Studies using site-directed mutation of this sequence indicate that the nucleotide A at position −3 is most important for a strong initiation of translation. Furthermore, the periodical spacing of G at positions +4, −3, −6, and −9 seems to enhance the initiation process (15). Of the three ATG codons identified in the 5′ end of the human *MEA* mRNA, the third ATG site contains the highest homology (90%) to the consensus sequence and maintains the above essential features for a strong initiation site for translation. The two upstream ATG sites do not have these features and would be weak or leaky initiation sites. Multiple uptstream ATG codons are rare in most vertebrate mRNAs, although they are present in over two-thirds of protooncogenes and related growth factors (13, 14). Studies of experimentally altered transcription sequences in several systems indicated that, despite the reduced efficiency, internal ATG codons can also be used as translation initiation sites (16, 17). Furthermore, in-frame ATG codons in the rat insulin-like growth factor (IGF-1), the human glucocerebrosidase and β-hexosaminidase B genes had also been demonstrated to be capable of initiating protein synthesis (18–20). Hence, multiple translation initiations of *MEA* transcripts would be a likely *in vivo* event that probably produces polymorphic MEA proteins. Additional experiments are needed to clarify this possibility.

Phylogenetic Conservation and Chromosomal Location of the *MEA* Gene in Mammals. The high homology between the human *MEA* and mouse *Mea* cDNAs at the nucleic acid and protein levels suggests that the *MEA* gene is highly conserved in these two species. This genetic conservation extended to other mammalian species, such as orangutan, gorilla, chimpanzee, dog, rat, bull, rabbit, and guinea pig, by Southern analysis using either mouse or human cDNA as a probe. Under stringent hybridization conditions, the mouse cDNA probe hybridized to these mammalian DNA samples with unique patterns similar to single-copy sequences (Fig. 2). The similarity in restriction fragment sizes between humans and the great apes is especially striking and suggests that DNA sequence conservation may be extended to the flanking sequences of the *MEA* gene in these species.

As evidenced by the positive hybridization of the *MEA* probe to both male and female DNAs, this gene is probably not located on the unique portion of the Y chromosome.

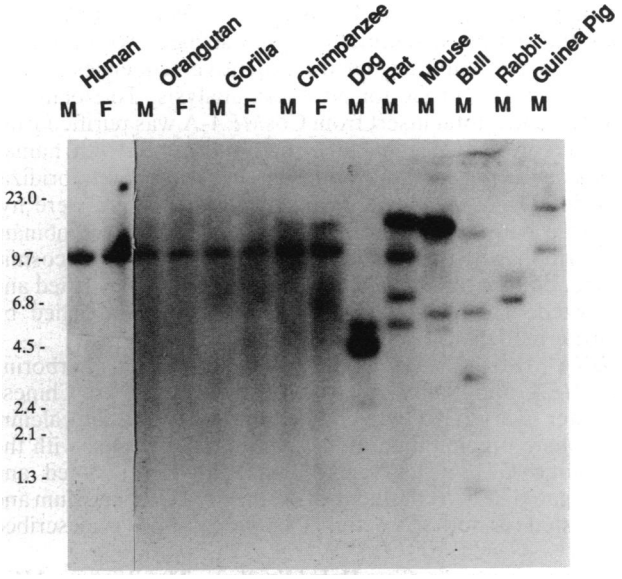

FIG. 2. Conservation of *MEA*-like sequences in mammalian genomes. Male (M) and female (F) DNA isolated from various mammalian tissues were analyzed by Southern blotting and hybridized with the mouse *Mea* cDNA probe under stringent conditions.

Preliminary studies using chromosome dot hybridization techniques had mapped the human *MEA* gene to chromosome 6 (2). Southern blot analysis of DNA derived from somatic cell hybrids harboring different portions of this chromosome had assigned the *MEA* gene to region p23–q12 (21). To confirm this assignment and further sublocalize the *MEA* gene, we performed *in situ* hybridization of ^{3}H-labeled human cDNA to metaphase chromosomes. A total of 489 metaphase cells were scored, of which 90 cells with autoradiographic grains were analyzed. Fig. 3 shows the grain distribution on an idiogram of chromosome 6 from results of a detailed study of 13 cells with grains on this chromosome. Of the 119 grains in these metaphase cells (≈9.2 grains per cell), 23 (19%) were located on chromosome 6. Of these, 78% were clustered on the short arm. About 55% of the grains on the short arm were located on bands p21.1–21.3, thus mapping the *MEA* gene to this region of chromosome 6. Preliminary chromosome mapping of the *Mea* gene in the mouse genome has assigned the functional gene to chromosome 17 (21).

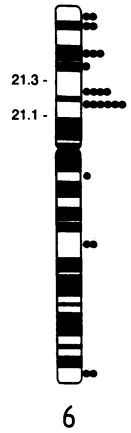

FIG. 3. Idiogram of human chromosome 6 illustrating the grain distribution on this chromosome from *in situ* hybridization analysis. The relative high density of grains on band p21.1–21.3 hence maps the *MEA* gene to this region of chromosome 6.

Stage-Specific Expression of the *Mea* Gene During Spermatogenesis. Previous studies established that the mouse *Mea* gene is expressed at high levels in the testis and is barely detectable in the ovary. Most other tissues, such as brain, kidney, heart, and liver, expressed the *Mea* gene at slightly higher levels than that of the ovary. Little or no difference was detected between male and female RNA samples of the same tissues (2). *Mea* gene expression was further demonstrated in a Northern blot of mRNA derived from various mammalian testes (Fig. 4 *Left*). The *Mea* gene was transcribed in developing mouse testes as early as the 6th day postpartum (Fig. 4 *Center*) and showed substantially higher levels of expression in adult testis. Such an increase correlates very well with the progression of testicular maturation and the appearance of germ cells at later stages of spermatogenesis. To confirm the stage-specific expression of the *Mea* gene, we analyzed the *Mea* transcripts in the testes of several mutant mice whose spermatogenic processes were blocked at different stages (Fig. 4 *Right*). The XX sex-reversed (XX*Sxr* and its variant XX*Sxr'*) mice harbor a portion of the sex-determining region of the Y chromosome on their paternal X chromosome and hence develop testes (22). However, they are sterile because of depletion of primary spermatogonia and there are no spermatogenic cells in their testes (23). The X chromosome-linked testicular feminizing mutation (*Tfm*/Y) is related to a defect of the androgen receptor. Spermatogenesis in the hemizygous males is blocked at the first meiotic division. Spermatids are absent in the *Tfm*/Y testes (24). The quaking phenotype (*qk*/*qk*) is caused by a mutation on chromosome 17, and the development of mature spermatids is blocked in homozygous males (25). Northern blot analysis of the testicular RNA derived from the XX*Sxr*, XX*Sxr'*, and *Tfm*/Y mice showed the same low levels of *Mea* transcription as in the ovary (Fig. 4 *Right*). However, the amount of *Mea* transcripts in the testes of the *qk*/*qk* males was comparable to that of normal males. These observations suggest that the *Mea* gene is preferentially expressed at the haploid stages of spermatogenesis.

Identification of a Testis-Specific *MEA* Gene Cluster. To isolate the genomic sequences harboring the structural gene for *MEA*, we used the human *MEA* cDNA as hybridization probe to screen a human genomic library constructed in the cosmid vector pCV108 (5). Four recombinant cosmids were isolated from a screening of 300,000 colonies. These cosmids harbored an average of 38–42 kb of human DNA and together

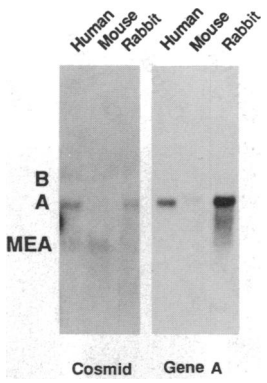

FIG. 5. Identification of a testis-specific gene cluster. (*Left*) Northern blot of testis RNA isolated from human, mouse, and rabbit with the entire human Cos*MEA*-A used as probe. In addition to the *MEA* transcript, two other RNA species of 2.1 (gene A) and 2.9 (gene B) kb were detected. (*Right*) Rehybridization of the same filter with isolated gene A cDNA probe.

overlapped ≈60 kb of sequences flanking the *MEA* gene. To determine whether any other gene(s) is linked to the *MEA* gene within the human DNA inserts, we used the entire cosmid as a hybridization probe in Northern analysis of RNA isolated from human, mouse, and rabbit testes (Fig. 5 *Left*). Interestingly, in addition to the expected *MEA* band, two other poly(A)$^+$ RNA species were detected in the human testis, indicating that two other genes were also present within the 38 kb of human DNA. These two genes, tentatively identified as genes A and B, produced mRNA of 2.1 and 2.9 kb, respectively (Fig. 5 *Left*). Positive hybridization was also observed in RNA samples from mouse and rabbit testes. Both genes A and B seemed to differ from the *MEA* gene, since previous hybridization of the same blot with the *MEA* probe did not identify these two mRNA species.

Based on this information, we initiated experiments to isolate these two linked genes by using a differential hybridization scheme. Duplicate sets of filters containing plaques from a human testicular cDNA library constructed in λgt10 were hybridized separately with the human *MEA* cDNA and cosmid probes. The plaques that showed signals with the cosmid but not with the cDNA probe must have sequences other than the *MEA* gene that were responsible for the specific hybridization. Eight cDNA clones harboring inserts

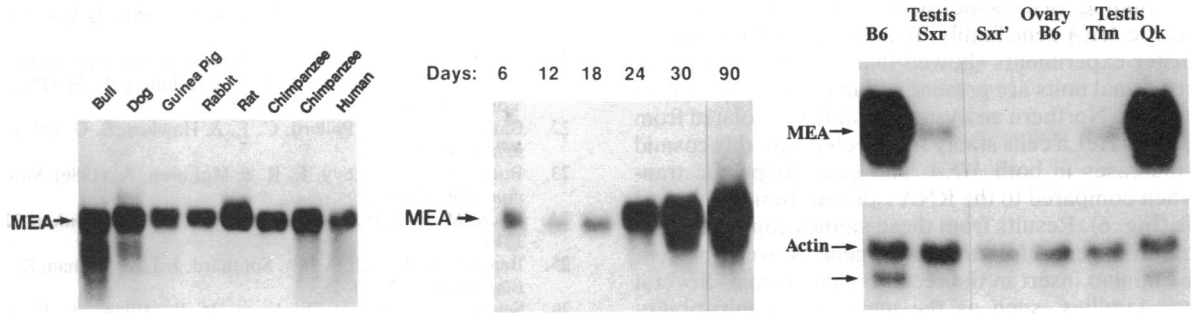

FIG. 4. (*Left*) Expression of *MEA* gene in mammalian testes. Poly(A)$^+$ RNA (2 μg) isolated from testes of bull, dog, guinea pig, rabbit, rat, two chimpanzees, and human were analyzed by Northern hybridization. The RNA filter was hybridized with the human *MEA* cDNA and washed extensively under stringent conditions. Detection of specific RNA bands in all testis samples indicates that the *MEA* gene is conservatively expressed in this mammalian organ. (*Center*) Northern analysis of RNA isolated from developing mouse testes. Ten micrograms of total RNA from the testes of mice at 6, 12, 18, 24, 30, and 90 days was analyzed by Northern hybridization using the mouse *Mea* cDNA as a probe. The amount of *Mea* transcripts increases as the testes develop to maturity (30 days). This increase in *Mea* gene expression parallels the appearance of germ cells at later stages of spermatogenesis. (*Right*) Northern analysis of RNA isolated from adult testes of mutant mice. Six micrograms of total RNA was loaded per sample. Normal C57/B6 testis (lane 1) and ovary (lane 4) were used as controls. Lane 2, Sxr, XX*Sxr* sex-reversed mice; lane 3, Sxr', XX*Sxr'*, a variant of Sxr; lane 5, Tfm, *Tfm*/Y testicular feminizing mutation; lane 6, Qk, *qk*/*qk* quaking mutation. (*Upper*) Autoradiogram of the filter probed with the mouse *Mea*. Spermatogenesis was blocked at early stages in the Sxr, Sxr', and Tfm testes and showed low levels of *Mea* transcripts. (*Lower*) Autoradiogram from rehybridizing the filter with human actin probe, indicating that a comparable amount of RNA was loaded per lane. Lower arrow indicates spermatid-specific actin transcripts in normal (lane 1) and Qk (lane 6) testes.

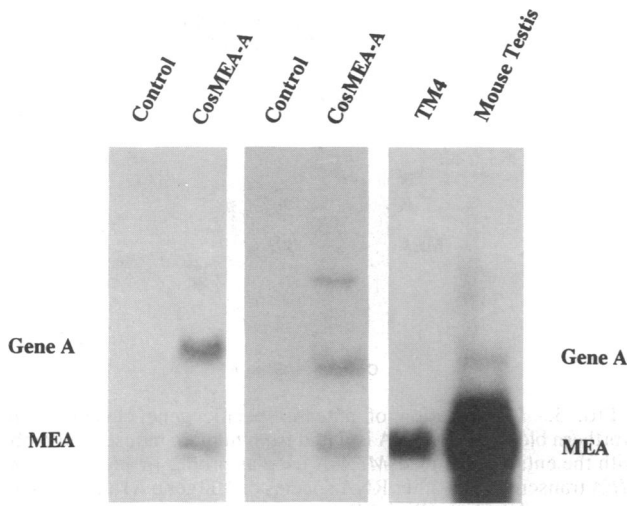

FIG. 6. Northern blot analysis of RNA isolated from CHO (*Left*) and HeLa (*Center*) cells harboring the Cos*MEA*-A DNA, and positive control cells, TM4 (a mouse Sertoli cell line) and mouse testis (*Right*). Thirty micrograms of total RNA isolated from control and transformant cells was analyzed. The filters were first hybridized with the human *MEA* cDNA probe and then rehybridized with the gene A probe. The resulting autoradiograms were superimposed for the composite shown. CHO and HeLa cells express both genes at low levels (1, 2). A weakly hybridized gene A band was observed in the mouse testis RNA (see also Fig. 5); however, a strong hybridization was detected with the corresponding mouse gene A probe.

of various sizes were isolated from a screening of 250,000 pfu. The inserts from all eight cDNA clones cross-hybridized with the largest 1.3-kb insert, indicating that they were all derived from the same mRNA. Rehybridization of the previous Northern filter with this 1.3-kb cDNA probe showed that these cDNAs were derived from gene A transcripts (Fig. 5 *Right*). Furthermore, an additional cosmid isolated from a mouse genomic library also contained sequences that hybridized to both the *MEA* and gene A cDNA probes, suggesting that these two genes are closely linked in the mouse genome (data not shown). These data indicate that the *MEA* gene is a member of a gene cluster that specifically and coordinately expresses in mammalian testes.

The identification of the two other linked genes, A and B, which are expressed coordinately with the *MEA* gene, is an important finding that demonstrates the possibility of a testis-specific *MEA* gene family. In addition, DNA-mediated gene transfer experiments showed that both *MEA* and gene A transcriptional units are present within the 38 kb of human genomic DNA. Northern analysis of total RNA isolated from either CHO or HeLa cells stably transfected with this cosmid showed increases in both *MEA*- and gene A-specific transcripts when compared to the RNA isolated from the control host cells (Fig. 6). Results from these studies confirmed that both structural genes for *MEA* and gene A were present within the human insert in the recombinant cosmid. Several other gene families, such as the major histocompatibility complex and tumor necrosis factor α and β mapped to this region of human chromosome 6, also exhibit similar arrangement of their gene members (26).

Irrespective of whether or not the *Mea* gene is the serological H-Y, it possesses some important properties such as phylogenetic conservation and tissue-specific expression that suggest it serves a vital function in mammalian testis. Furthermore, its possible relationship to other interesting loci mapped to the mouse chromosome 17 and/or human chromosome 6 should also be considered. These loci include the T/t complex, whose genes are essential for normal embryogenesis and spermatogenesis and are expressed during fetal development and in adult testis (27–29), and the dominant autosomal locus T-associated sex-reversal (*Tas*), whose mutation is lethal in homozygous individuals and affects the sexual differentiation of heterozygous XY carriers (30, 31). Although we do not know whether *Mea* is also involved in such developmental processes, preliminary analysis indicates that it is expressed in fetal mouse tissues, including the testes (32). The molecular properties and chromosomal locations of the *Mea* and linked genes surely place them in the portion of the mammalian genome that is essential for embryogenesis, spermatogenesis, and sex determination.

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