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Spermatid-specific expression of protamine 1 in transgenic mice

(germ cell/spermiogenesis/gene transfer)

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ABSTRACT **Protamines are abundant basic proteins involved in the condensation of sperm chromatin. In the mouse, protamine genes are transcribed postmeiotically in round spermatids. We have cloned and sequenced the mouse protamine 1 gene. Ten lines of transgenic mice harboring marked protamine 1 sequences were generated by microinjection of fertilized eggs. Transcription of the transgene is restricted to round spermatids and in several cases exceeds that of the endogenous gene. The cis-acting sequences required for tissue-specific protamine expression reside on a 2.4-kilobase restriction fragment. Prospects for using transgenic mice to address fundamental questions of male germ-cell development are discussed.**

Protamines are small, highly basic proteins that replace histones and testis basic proteins during development of mature spermatozoa (1). In mammals, extensive disulfide crosslinking of protamine results in the formation of a compact chromatin structure devoid of transcriptional activity. Two protamine variants have been identified in the mouse, protamine 1 (mP1) and protamine 2 (mP2), differing in size, amino acid composition, and relative abundance (1, 2). The functional significance of these differences is not known.

An analysis of protamine expression during prepubertal development of the mouse testis (3), as well as in isolated spermatogenic cell types (4, 5), indicates that mP1 and mP2 are specifically transcribed in haploid, round spermatids. The existence of haploid gene expression has important biological ramifications in that segregation and subsequent expression of genes after meiosis could potentially result in phenotypic differences between individual spermatozoa. The extent to which this occurs is not known, although it has been postulated as a mechanism explaining transmission-ratio distortion, the non-Mendelian inheritance of specific alleles (6). A complication of this model is that spermatids develop as a syncytium, interconnected by cytoplasmic bridges about 1 μm in diameter (6). This architecture is thought to assure the synchronous development of clonally derived spermatids. Information transfer between spermatids could also minimize or obviate the consequences of postmeiotic gene transcription; genetically haploid spermatids may be functionally diploid with respect to genes expressed after meiosis. The absence of distinguishing genetic markers specifically expressed in the haploid stages of spermatogenesis has precluded an investigation into the role of cytoplasmic bridges in maintaining an equal distribution of gene products between individual spermatids.

Protamine synthesis is also regulated at the level of translation. Although mP1 mRNA is first detected in round spermatids, it is stored in inactive ribonucleoprotein particles for up to 8 days until these cells differentiate into elongating spermatids (7). Concomitant with the recruitment of mP1 mRNA onto polysomes is extensive deadenylylation of the transcript (7). Similar events are observed in the synthesis of the trout protamines, although these genes are active during meiosis (8).

The isolation of a cDNA encoding mP1 has been described (5). Using sequences derived from this cDNA, we cloned and characterized the mP1 gene. To develop a system in which to examine the regulation and consequences of postmeiotic gene expression in the mouse testis, we introduced marked copies of this gene into mice by microinjection of fertilized eggs. Expression of the transgene during male germ-cell development, as well as in a variety of other tissues, was monitored.

MATERIALS AND METHODS

Cloning and Sequencing of the mP1 Gene. C57BL mouse spleen DNA was partially digested with endonuclease *Mbo* I and cloned into the *Bam*HI site of λ phage EMBL3. About 300,000 recombinant phage were screened with a pair of radiolabeled complementary oligonucleotides corresponding to nucleotides (nt) 232–287 of the mP1 cDNA (5). Recombinant phage DNA was isolated by standard procedures. Regions encompassing the mP1 gene were subcloned into M13mp18 or -19 vectors (9) and sequenced by the chain-termination method (10). The sequence between the *Nco* I site at nt −90 relative to the start of transcription and the *Bgl* II site at nt +625 was determined on both strands.

Plasmid Construction and Microinjection. The sequence 5′ CAGTCTCAGGATCCACCATG 3′ was inserted into a nuclease S1-treated *Nco*I site at nt +95 by using complementary oligonucleotides to differentiate between marked and endogenous protamine transcripts. In addition, a 237-base-pair (bp) *Bcl* I–*Bam*HI restriction fragment of simian virus 40 (SV40) was inserted into the unique *Bgl* II site at nt +625 to identify the transgene. The resultant construct, mP1′−4.8, extending from an *Sst* I site at −4.8 kilobases (kb) to the 3′ *Sal* I site (Fig. 2), was separated from vector sequences and injected into the male pronucleus of fertilized mouse eggs as described (11). mP1′−.88 was generated by isolating an *Acc* I–*Sal* I restriction fragment from mP1′−4.8. Transgenic mice harboring mP1′−4.8 or mP1′−.88 sequences were identified by "tail dot" analysis (12) using a nick-translated SV40 probe. Transgene copy number was determined by comparing the hybridization of wild-type and transgenic spleen DNA to a nick-translated mP1 probe in a dot hybridization assay.

RNA Extraction and Analysis. RNA used for primer extension and blot analyses was extracted by homogenization in guanidinium isothiocyanate, followed by precipitation with lithium chloride as described (13). Total nucleic acids extracted from tissues by use of $NaDodSO_4$ and proteinase K (14) were used for solution hybridization analysis.

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Abbreviations: mP1 and mP2, mouse protamines 1 and 2; SV40, simian virus 40; nt, nucleotide(s).

For blot hybridization analysis, RNA was denatured in a formamide/formaldehyde buffer and then electrophoresed in a 1% agarose gel (15). Electrophoretically separated RNAs were blotted onto nitrocellulose and hybridized to the end-labeled oligonucleotide specific for the mP1′−4.8 and mP1′−.88 transcripts. Hybridization was performed at 42°C in a solution containing 6× SSPE (20× SSPE = 3.6 M NaCl/200 mM NaP_i/20 mM EDTA, pH 7.4), 5× Denhardt's solution (1× = 0.02% Ficoll/0.02% polyvinylpyrrolidone/0.02% bovine serum albumin), 0.1% $NaDodSO_4$, and 100 μg of denatured herring sperm DNA per ml. Blots were washed at 55°C in 5× SSPE/0.1% $NaDodSO_4$/0.1% $NaPP_i$.

Primer-extension analysis was performed by hybridizing RNA to an end-labeled oligonucleotide primer complementary to mP1 sequences between nt +99 and nt +119. This primer hybridizes to both endogenous and transgene mP1 transcripts but yields extension products that can be electrophoretically distinguished due to the oligonucleotide insertion at nt +95 in the transgene. Hybridizations were carried out at 42°C for 90 min in 30 μl of 660 mM NaCl/22 mM Tris Cl, pH 8/4.4 mM EDTA containing ≈20 fmol of end-labeled primer and 2 μg of testis RNA. Reaction mixtures were then diluted to 80 μl with water. After precipitation with ethanol, nucleic acids were suspended in 30 μl of 100 mM KCl/100 mM Tris Cl, pH 8.3/10 mM $MgCl_2$/10 mM dithiothreitol/500 μM each dNTP plus bovine serum albumin at 250 μg/ml and 7 units of avian myeloblastosis virus reverse transcriptase (Life Sciences, St. Petersburg, FL) and incubated at 42°C for 30 min. Reaction products were precipitated with ethanol, denatured in 50% formamide at 100°C, and analyzed by electrophoresis in an 8% acrylamide/8 M urea sequencing gel.

Solution hybridization analysis of total nucleic acid was carried out as previously described (16), using the end-labeled oligonucleotide specific for mP1′−4.8 mRNA.

RESULTS

Cloning and Characterization of the mP1 Gene. Two complementary oligonucleotides corresponding to a portion of the 3′ untranslated region of the mP1 cDNA were used to screen a C57BL mouse genomic library constructed in λ phage EMBL3. Approximately 300,000 recombinant phage were analyzed and one positive clone was identified. Southern blotting (17) experiments revealed that the mP1 gene is single-copy (data not shown).

Appropriate M13 subclones encompassing the mP1 gene were sequenced (Fig. 1). Two discrepancies between this sequence and that of the mP1 cDNA (5), located in the 3′ untranslated region, are underlined. These nucleotide variations are most likely due to strain polymorphisms between CD-1 and C57BL mice.

The point of transcription initiation was identified by primer extension using an oligonucleotide complementary to the 5′ untranslated region of mP1 (data not shown). A consensus "TATA" homology is present at nt −30 relative to this point. The exon/intron boundaries were deduced from the mP1 cDNA sequence (5) and consensus splice signals (18). The 3′ end of the mP1 transcript was determined previously (5).

Expression of mP1 Following Gene Transfer into Mice. To determine if cloned sequences flanking the mP1 structural gene could properly direct its expression to round spermatids of the mouse testis, transgenic mice harboring these sequences were generated. A 237-bp *Bcl* I–*Bam*HI restriction fragment of SV40 was cloned into a unique *Bgl* II site downstream of the mP1 gene, and a pair of oligonucleotides (see *Materials and Methods*) was inserted into the *Nco* I site at position +95. The resultant construct, mP1′−4.8 (Fig. 2), was used to create six lines of transgenic mice. Mice carrying

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-560 gtctagtaat  gtccaacacc  tccctcagtc  caaacactgc
-520 tctgcatcca  tgtggctccc  atttatacct  gaagcacttg
-480 atggggcctc  aatgttttac  tagagcccac  ccccctgcaa
-440 ctctgagacc  ctctggattt  gtctgtcagt  gcctcactgg
-400 ggcgttggat  aatttcttaa  aaggtcaagt  tccctcagca
-360 gcattctctg  agcagtctga  agatgtgtgc  tttcacagtt
-320 acaaatccat  gtggctgttt  cacccacctg  cctggccttg
-280 ggttatctat  caggacctag  cctagaagca  ggtgtgtggc
-240 acttaacacc  taagctgagt  gactaactga  acactcaagt
-200 ggatgccatc  tttgtcactt  cttgactgtg  acacaagcaa
-160 ctcctgatgc  caaagccctg  cccacccctc  tcatgcccat
-120 atttggacat  ggtacaggtc  ctcactggcc  atggtctgtg
                                 NcoI
-80  aggtcctggt  cctctttgac  ttcataattc  ctaggggcca
-40  ctagtatcta  taagaggaag  agggtgctgg  ctcccaggcc
+1   acagcccaca  aaattccacc  tgctcacagg  ttggctggct
+41  cgacccaggt  ggtgtcccct  gctctgagcc  agctcccggc
+81  caagccagca  CCATGGCCAG  ATACCGATGC  TGCCGCAGCA
                 NcoI
+121 AAAGCAGGAG  CAGATGCCGC  CGTCGCAGGC  GAAGATGTCG
+161 CAGACGGAGG  AGGCGATGCT  GCCGGCGGAG  GAGGCGAAgt
+201 aagtagaggg  ctgggctggg  ctgtgggggg  tgtggggtgc
+241 gggacttggg  catgtctggg  agtccctctc  accacttttc
+281 ttacctttct  agGATGCTGC  CGTCGCCGCC  GCTCATACAC
+321 CATAAGGTGT  AAAAAATACT  AGatgcacag  aatagcaagt
+361 ccatcaaaac  tcctgcgtga  gaattttacc  agacttcaag
+401 agcatctcgc  cacatcttga  aaaatgccac  cgtccgatga
+441 aaaacaggag  cctgctaagg  aacaatgcca  cctgtcaata
+481 aatgttgaaa  actcatccca  ttcctgcctc  ttggtccttg
+521 ggcttgggga  ggggtgcgcg  gatgtggtta  gggaacatga
+561 ctggtcaaat  gggaagggct  tcaaaagaat  tcccaatatt
+601 gactaccaag  ccacctgtac  agatct
                            BglII
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FIG. 1. Sequence of the mP1 gene. The TATA sequence, the point of transcription initiation, and the relevant restriction sites are underlined. The two nucleotide differences between this sequence and that of an mP1 cDNA clone are located in the 3′ untranslated region and are underlined. The transcribed portion of the gene (nt 1–495) is shown in bold type. Translated regions are shown in uppercase print. The mP1 amino acid sequence is indicated (standard one-letter amino acid symbols) above the exon sequences.

the transgene were identified using an SV40 probe, and expression of this gene relative to the endogenous protamine 1 gene was determined using a primer-extension protocol. The insertion of the oligonucleotides enabled us to electrophoretically separate extension products derived from endogenous and transgene mP1 transcripts by using a primer 3′ to the insertion. As shown in Fig. 3, mP1′−4.8 is expressed at variable levels in the adult testis of all six transgenic lines.

Blot hybridization analysis of RNA from various tissues isolated from 6-week-old mice of line 1688-6 showed that mP1′−4.8 expression in this line is restricted to the testis (Fig. 4). In addition, the same tissues of adult male mice from lines 1567-5, 1688-4, and 1694-7 were analyzed for mP1′−4.8 expression by a quantitative solution hybridization protocol (16). In each case, expression above background (background was equivalent to 10 molecules of mRNA per cell) could only be detected in the testis, where the endogenous mP1 transcript is present at about 3000 molecules per cell (unpublished results).

The time at which a transcript appears during the first wave of spermatogenesis can be used to identify the spermatogenic cell type in which transcription initiates (1, 3). Expression of mP1′−4.8 in line 1567-5 was monitored as a function of neonatal development. As shown in Fig. 5, no expression of the transgene was observed in testis samples from 10- and

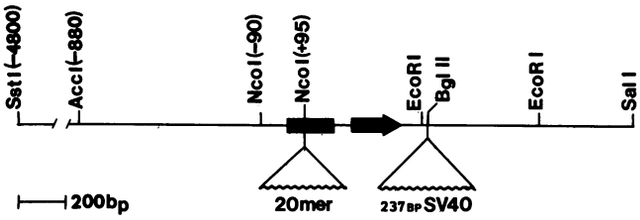

FIG. 2. Restriction map of mP1′−4.8. Relevant restriction sites of the mP1 gene and their position relative to the point of transcription initiation are indicated. The SV40 and oligonucleotide insertions used to identify the transgene and its transcript, respectively, are depicted.

19-day-old males. Expression was detected at day 25, however. This pattern of expression parallels that of the endogenous mP1 gene (3) and is indicative of round-spermatid-specific mP1′−4.8 transcription.

To further localize the cis-acting sequences required for efficient, tissue-specific mP1 transcription, expression of mP1′−.88 was monitored in four transgenic males. In two of these males, levels of the marked transcript exceeded those of the endogenous mP1 message (Fig. 6). As demonstrated for mP1′−4.8, mP1′−.88 mRNA was not detected in other tissues (data not shown).

DISCUSSION

In the mouse, several components of mature spermatozoa, such as the protamines, are encoded by genes transcribed during the haploid stages of spermatogenesis (19). We have cloned and characterized the mP1 gene and found that it is specifically expressed in round spermatids following gene transfer into mice. Using defined mP1 sequences, it should be feasible to target the expression of various gene products to male germ cells in transgenic mice.

cDNAs encoding protamine variants from several sources have been described in detail (5, 20, 21). However, protamine gene structure has only been examined in the trout (22). None of the seven trout protamine genes contain introns (22). DNA sequence analysis of the mP1 gene revealed the presence of a 90-bp intron. In addition, a considerable degree of sequence

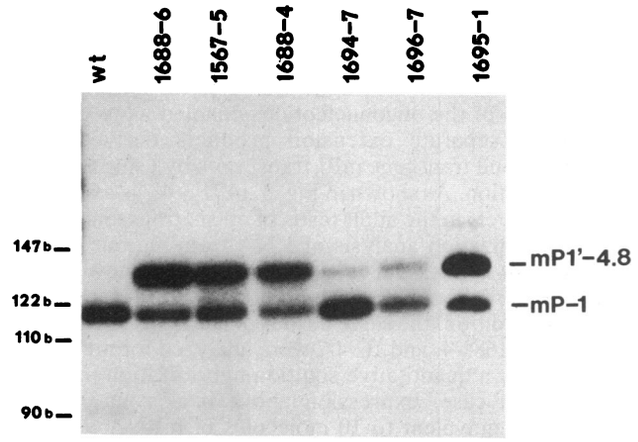

FIG. 3. Expression of mP1′−4.8 relative to the endogenous mP1 gene. Primer-extension analysis of adult testis RNA from wild-type (wt) and mP1′−4.8 transgenic mice was performed. Equal amounts of RNA were analyzed. The sizes (in bases, b) of various *Msp* I pBR322 restriction fragments used as size markers are shown. The difference in size between mP1′−4.8 and mP1 extension products is due to the 20-bp oligonucleotide insertion in the transgene. The 1688-4, 1694-7, and 1696-7 testis samples were from founder males. The remaining samples were from males of established lines.

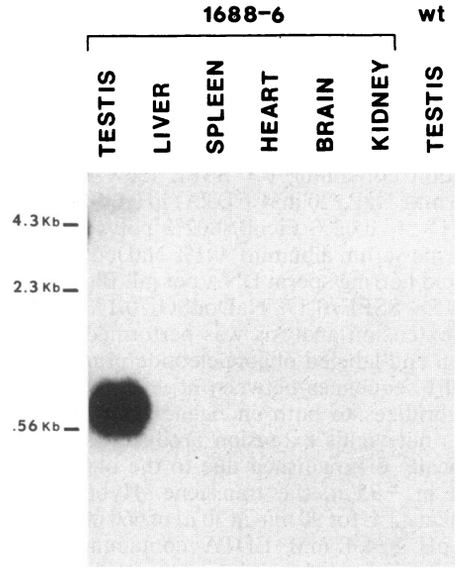

FIG. 4. Tissue specificity of mP1′−4.8 expression. Total RNA samples (10 μg) from various tissues of an adult male of the 1688-6 line and from testis of an adult wild-type (wt) male were examined by blot hybridization analysis using an oligonucleotide probe specific for mP1′−4.8. The integrity of RNA in all lanes was established by hybridizing the blot to an actin probe (data not shown). Size markers at left indicate positions of various *Hin*dIII λ restriction fragments.

identity 5′ to the TATA homology is observed between mP1 and the gene encoding the major protamine variant in mouse, mP2 (P. A. Johnson, J.J.P., P. C. Yelick, R.D.P., and N. B. Hecht, unpublished data). It is tempting to speculate that homologies between nontranscribed portions of these two related genes reflect a functional significance of these sequences in transcriptional regulation.

The mp1′−4.8 construct is expressed in the adult testis of six independent transgenic mice (Fig. 3). In four cases,

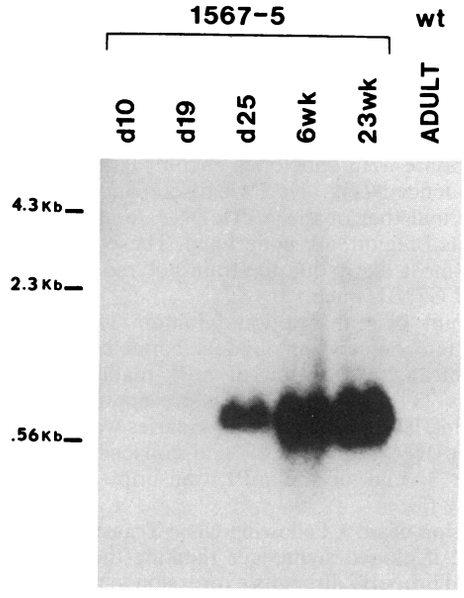

FIG. 5. Expression of mP1′−4.8 during testis development. Total testis RNA samples (10 μg) from immature (10-, 19-, and 25-day-old) and adult (6- and 23-week-old) males of the 1567-5 line and from an adult wild-type (wt) mouse were examined by blot hybridization analysis using an oligonucleotide probe specific for mP1′−4.8. The integrity of RNA in all lanes was established by hybridizing the blot to an actin probe (data not shown).

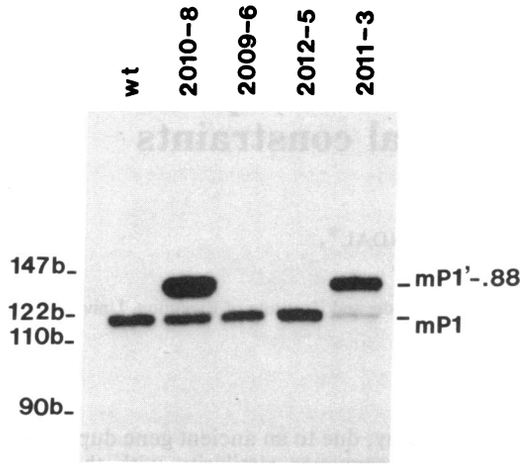

FIG. 6. Expression of mP1'−.88 relative to the endogenous mP1 gene. Primer-extension analysis of total adult testis RNA was performed as described for Fig. 3. The 2009-6 and 2010-8 testis samples were from founder males. The remaining samples came from males of established lines. wt, Wild type.

accumulation of the marked transcript exceeds that of the endogenous mP1 message. This observation is most easily explained by the presence of multiple active copies of the transgene. The endogenous mP1 gene is single-copy, whereas these four mice harbor between 4 and 20 copies of the marked gene (data not shown). The low level of expression observed in two mice could be a consequence of integration into an unfavorable chromosomal location. Alternatively, as pedigrees from these two animals were not established, the possibility exists that the founder males were germ-line mosaics (23), with only a fraction of the spermatids carrying the transgene.

One of our goals is to specifically direct gene products to round spermatids by using mP1 regulatory sequences. Thus it was necessary to establish not only that mP1'−4.8 was efficiently transcribed in the adult testis but also that its expression was restricted to the appropriate cell type within this tissue. As shown in Fig. 5, mP1'−4.8 message was not present in day-19 testis but was present by day 25; hence we conclude that expression of this construct is restricted to round spermatids.

These data suggest that the cis-acting sequence requirements for high-level tissue-specific protamine expression are fully contained within mP1'−4.8. To further delineate these requirements, mP1'−.88 was tested. Although only two of four animals expressed this transgene in the adult testis, levels of the marked message exceeded those of the endogenous mP1 transcript (Fig. 6). Thus, a maximum of 2.4 kb of DNA sequence, of which only 880 bp is 5' to the start of transcription, is necessary for efficient protamine gene transcription. RNA blot analysis, similar to that shown in Fig. 4, showed that mP1'−.88 is specifically transcribed in the testis (data not shown). The reduced frequency of expression may be a reflection of an increased sensitivity of the deletion construct to neighboring chromosomal sequences.

As there are no continuous spermatid cell-culture systems, one is restricted to transgenic mice to delineate the cis-acting requirements for postmeiotic expression in the mouse testis. Preliminary results with an mP1–human growth hormone fusion gene suggest that sequences located 3' to the *Bgl* II site at nt +625 are not required for efficient testis-specific mP1 gene transcription.

We have generated several lines of transgenic mice that express levels of a marked mP1 mRNA exceeding those of endogenous mP1. These males are fertile and properly transmit the transgene, suggesting that increased levels of mP1 mRNA are not deleterious to spermatid maturation. It is formally possible that the oligonucleotide insertion into the 5' untranslated region of the transgene prevents translation. However, the heterogeneity in size of mP1'−4.8 mRNA (compare day 25 vs. adult, Fig. 5) suggests that some of the transcripts are deadenylylated and are most likely entering the pool of total mP1 mRNA available for translation. Perhaps posttranslational regulatory events operate to assure a precise ratio between mP1 and mP2 deposited onto DNA. The two variants have considerably different amino acid contents (1), paralleling those observed between the human protamine variants (24), and may have distinct roles in the DNA-packaging process. Alternatively, the presence of two protamine variants, at a specific ratio, may not be necessary for proper sperm function. In the rat and several other mammalian species, only one protamine variant, resembling mP1 in amino acid composition, is found in mature sperm (1).

While the contribution of haploid gene expression to the process of spermatogenesis in mouse is appreciated, its role in dictating the identities and fates of genetically distinct spermatids is unclear (1, 6, 19). The ability to target gene products specifically to round spermatids in transgenic mice by use of mP1 regulatory sequences will provide valuable insights into both the regulation and consequences of postmeiotic gene expression in the mouse testis.

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