Mouse Zp1 Encodes a Zona Pellucida Protein Homologous to Egg Envelope Proteins in Mammals and Fish*

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Zp1 encodes one of the three major glycoproteins of the zona pellucida, an extracellular matrix that surrounds growing oocytes, ovulated eggs, and preimplantation embryos. The mouse gene is composed of 12 exons ranging in size from 82 to 364 base pairs and spans 6.5 kilobase pairs on chromosome 19 (2.13 ± 1.5 centimorgans distal to D19B1r1). The Zp1 exon map is similar to ZPB, a human orthologue, and an E-box (CANNTG), implicated in oocyte-specific gene expression of mouse Zp2 and Zp3, is similarly located upstream of the transcription start site. The single copy Zp1 gene encodes a 623-amino acid protein, the carboxyl-terminal half of which is significantly similar to a corresponding region of the amino acid protein, the carboxyl-terminal half of the ZP1 gene. Among vertebrates, different reproductive strategies have evolved based on mating behavior, gamete structures, and the specificity of recognition molecules on the surface of sperm and eggs. In all vertebrates, however, a prerequisite to successful fertilization is penetration of sperm through an acellular envelope surrounding ovulated eggs. In mammals, capacitated sperm bind in a seemingly non-site-directed manner to the zona pellucida. Following the induction of the acrosome reaction and release of lytic enzymes, sperm penetrate the zona and fuse with the egg’s plasma membrane, triggering the postfertilization block to polyspermy (1). In contrast, most fish sperm lack an acrosome and penetrate the vitelline envelope surrounding fish eggs via a discrete micropyle (2). Most commonly, the micropylar channel is sufficiently narrow to permit the passage of a single sperm, and subsequent fusion with the plasma membrane induces the cortical granule reaction, resulting in a block to polyspermy (3). It has become increasingly clear that the proteins of the zona pellucida are conserved among eutherian mammals and that the proteins of the vitelline envelope are conserved among teleostean fish. More recently, it has become apparent that, although critical for specialization, the proteins from the mammalian egg envelope are distinctly related to those of the teleostean envelope.

The mouse zona pellucida contains three major glycoproteins: ZP1, ZP2, ZP3. Genes encoding the latter two zona proteins have been characterized. Zp2 is composed of 18 exons (4), of which six encode a 241-amino acid domain reported as 28% identical with the wtf protein of the white flounder teleost (5). Zp3 contains eight exons (6, 7), of which the first six encode a tandem-like, 193-amino acid domain that is 33% identical with ZI-3, a major component of the inner layer of the egg envelope of a second teleost, Oryzias latipes (8). Although similar structural domains are present in egg envelope proteins of teleosts and eutherian mammals, the site of synthesis is quite different in these two classes of vertebrates. In mice, the three zona genes (Zp1, Zp2, Zp3) are transcribed exclusively in growing oocytes (4, 9, 10), and the resultant zona proteins are secreted to form the extracellular matrix. In contrast, there is growing evidence that proteins of the two teleost egg envelopes are produced in the liver after stimulation with estrogens and then transported to the egg where they form the vitelline envelope (5, 8).

We have previously reported the characterization of mouse Zp2 and Zp3 genes. We now report the characterization of mouse Zp1 and compare the encoded protein to other egg envelope proteins in mammals and fish.

MATERIALS AND METHODS

Screening a Mouse Genomic Library—A 18 × 10^6 bacteriophage of a λ 129/5v mouse genomic library (Stratagene) were screened by plaque hybridization (11) with a 32P-labeled mouse ZP1 cDNA (10). Phage DNA was isolated and digested with NotⅠ, and the insert was subcloned into SuperCos 1 cosmids (Stratagene). Cosmid DNA was amplified in XL1-Blue MR cells (Stratagene) grown overnight at 30 °C (LB broth, 50 μg/ml ampicillin) and purified with a Plasmid Maxi kit (Qiagen). The sequence of the genomic insert was determined by MSeSO-modified deoxy chain termination (12) using α-35S-dATP (Amersham Corp.) and the Sequenase sequencing kit (version 2.0; U.S. Biochemical Corp.). Both strands of the coding regions were sequenced. Sequence analysis was performed using the Genetic Computer Group (program manual for the Wisconsin Package, version 8, 1994) and PCGene (IntelliGentics) computer software.

Intron-Exon Map Determination—The transcription start site was determined by S1 nuclease protection. A 5′ genomic fragment, −197 to 94, was amplified by polymerase chain reaction using isolated Zp1 as a template, and synthetic oligonucleotides were derived from the genomic sequence. After subcloning the fragment into TO cloning vector (Invitrogen), the resultant plasmid (pMdzP1.5) was linearized with HindⅠI. A 32P-end-labeled synthetic oligonucleotide primer complementary to map position 40−62 of ZP1 cDNA was used in an asymmetric polymerase chain reaction to generate a single-stranded 5′ genomic probe (381 nucleotides, 0.5 ng of this probe was hybridized to 30 μg of mouse ovarian RNA or mouse liver RNA and digested with 300 units of S1 nuclease (13). The digestion products were analyzed on 6% polyacrylamide sequencing gels; sequencing reactions with the same primer were used as molecular weight markers. The remaining boundaries of exons were determined by comparison of the genomic sequence with that of ZP1 cDNA (10).

The sizes of introns were determined by DNA sequencing or polym...
erase chain reaction in a Perkin Elmer GeneAmp PCR System 9600 using Zp1 exon-specific forward and reverse oligonucleotide primers. The reaction conditions were as described by the Taq polymerase protocol (Perkin Elmer): 25 cycles of 95 °C for 15 s, 50 °C for 45 s, and 72 °C for 1.5 min. The first cycle was preceded by 5 min at 99 °C, and the last cycle was followed by a 7-min extension at 72 °C. The polymerase chain reaction products were analyzed by agarose gel electrophoresis.

Southern blot Analysis—30 μg of mouse 129Sv genomic DNA (Jackson Laboratory) and 0.05 μg of the Zp1 cosmid were digested with restriction enzymes (BamHI, EcoRV, NcoI, KpnI, and HindIII). The digested samples were separated on a 0.7% agarose gel and transferred to a Nytran membrane (Schleicher & Schuell). A ZP1 cDNA fragment digested samples were separated on a 0.7% agarose gel and transferred to a Nytran membrane (Schleicher & Schuell). A ZP1 cDNA fragment was subcloned into SuperCos I. After digestion, Southern blots were prepared and probed with 32P-labeled ZP1 cDNA (six examples are shown in Fig. 2A, lanes 3–8). Haplotyping analysis (Fig. 2B) of the 94 animals detected two animals that had a recombination event between the proximal D19Bir1 locus (2.13% recombination frequency) and 1 animal that had a recombination event between the more distal D19Bir3 locus (1.06% recombination frequency). Thus, the Zp1 locus maps to mouse chromosomes 19, 2.1 ± 1.5 cM distal to the D19Bir1 locus (Birkenmeier anonymous DNA fragment 1 on chromosome 19, see Ref. 14) and 1.06 ± 1.06 cM proximal to the D19Bir3 locus (Birkenmeier anonymous DNA fragment 1 on chromosome 19) (Fig. 2C).

The single genetic locus and the simple digestion patterns seen in the preliminary studies designed to detect polymorphisms at the Zp1 locus suggested that Zp1, like Zp2 and Zp3, is a low copy number gene. Six restriction enzymes recognizing hexanucleotide sites (SacI, BamHI, EcoRV, NcoI, KpnI, and HindIII) were used to digest isogenic samples of genomic DNA, and the Zp1 fragment was subcloned into SuperCos I. After digestion, Southern blots were prepared and probed with 32P-labeled ZP1 cDNA (Fig. 3). All of the restriction enzyme fragments detected in genomic DNA (three examples are shown in Fig. 3A) were present in the subcloned genomic fragment digests (Fig. 3B). These observations are consistent with there being only one copy of Zp1 in the mouse genome.

Conservation of the Mouse Zp1 Coding Region—The mouse Zp1 gene obtained from 129Sv mice encodes a polypeptide chain of 623 amino acids (Fig. 4A). There are five nucleotide differences from the cDNA sequence obtained from NIH Swiss mice (10). Two differences between the mouse strains are silent, the remaining differences result in three amino acid substitutions. Of these substitutions, two are conservative changes at amino acid residue 445 (Val → Leu) and 486 (Arg → Lys); the third substitution at residue 246 (Thr → Ala) is not. The effect of these polymorphisms is uncertain, as both 129Sv and NIH Swiss mice are fertile and appear to have normal zonae pellucidae. Similar polymorphisms have not been detected in different strains of mice at the Zp2 (4) or Zp3 loci (6, 7, 17). As noted above, the 623-amino acid mouse ZP1 protein is considerably

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1 The abbreviations used are: bp, base pair(s); kb, kilobase pair(s); cm, centimorgans.

2 L. Rowe, personal communication.
longer than orthologues found in other mammals (16, 18, 19),
most of which are approximately 540 residues in length. The
effect of this difference on the structure or function of the
mouse zona remains to be determined.

In addition to the conservation among mammals, the ZP1
protein also contains a domain that is present in other zona
pellucida proteins of the same species as well as in other egg
envelope proteins from very disparate species (Fig. 4
B). Within
348 amino acids of ZP1 (residues 268–623) that align with
mouse ZP2 (residues 363–713), 47% of the amino acids are
similar, 32% are identical. In Zp1, this region is encoded by
exons 5–12 and, in Zp2, by exons 11–18. Although each gene
is located on a different chromosome, it appears that the domain
encoded by these eight exons comes from a common ancestral
gene. It further appears that much of this ancestral gene was
present 650 million years ago. A similar (albeit slightly
smaller) domain is present in an egg vitelline envelope protein
present in white flounder (5), where it is encoded by exons 2–7
of the wf-gene that correspond to exons 5–9 plus exon 11 of
mouse Zp1 (Fig. 4 B).

Conservation in the Zona Gene Promoters—
Earlier analysis
of mouse Zp2 and Zp3 promoters had identified a 12 bp DNA
sequence (element IV) that was necessary and sufficient to
promote high levels of expression of reporter gene constructs
microinjected into growing oocytes. This element binds a puta-
tive transcription factor, ZAP-1 (Zona Activating Protein), that
is relatively abundant in oocytes but not in eight other tis-
sues, including granulosacells and testis (20, 21). Developmen-
tally, ZAP-1 DNA binding activity is observed prior to birth,
when ZP2 transcripts are first detected in primordial mouse
oocytes (22).

To investigate the possibility that a common regulatory
pathway controls the expression of the three zona genes, we
have determined the DNA sequence of 250 bp of the mouse
Zp1 promoter. An ATATAA box was identified 230 bp up-
stream of the transcription start site, but no CAAT box was
detected. Comparison of the Zp1 promoter sequence with a data base of the
binding sites for known transcription factors identified a mul-
titude of potential binding sites. However, none were also pres-
et at comparable positions in the Zp2 and Zp3 promoters
except for a consensus E-box sequence (CANNTG) located at
−218 bp from the transcription start site (Fig. 5). In Zp2 and
Zp3, the E-box forms the core of the aforementioned element
IV. Clustered 6-bp mutations in it inhibit reporter gene activity
and prevent the formation of the ZAP-1 complex (20). The Zp1
E-box (CAGcTG) is located at virtually the identical position in
the Zp2 promoter (−216 bp) and is located similarly to the
critical E-box in the Zp3 promoter (−181 bp).

![Fig. 2. Chromosome localization. A, Southern blot analysis of genomic DNA (4 μg) probed with 32P-labeled ZP1 cDNA after digestion with HindIII: parental controls C57BL/6J (lane 1) and M. spreitus (lane 2); back-cross progeny [(C57BL/6J × SPRET/Ei)F1] × SPRET/Ei) animals 89–94, (lanes 3–8). Numbers to the left indicate molecular weight markers (kb). B, haplotype analysis of the genomic DNA from 94 lines of back-cross progeny (F1 × SPRET/Ei) of the BSS panel from the Jackson Laboratory. Dark and light squares indicate the presence of the C57BL/6J and M. spreitus alleles, respectively. Gray squares indicate animals untyped for Vidlr. Previously mapped loci bracket Zp1 and are indicated at the left. The distances between loci are indicated at the bottom in cM ± standard deviation (S.E.). The numbers at the bottom of each column of squares indicate the number of progeny with the particular haplotype. C, schematic representation of mouse chromosome 19. Centromere is a filled circle at the top of the vertical line. Relevant loci are listed on the right. Numbers on the left indicate the approximate map distance between selected loci in cM.

![Fig. 3. Restriction Enzyme Analysis of Zp1. A, Southern blot of 129Sv mouse genomic DNA (10 μg) probed with 32P-labeled ZP1 cDNA after digestion with SacI (lane 1), BamHI (lane 2), and EcoRV (lane 3). Numbers to the left indicate molecular weight markers (kb). B, same as panel A except that each lane contains the 18.5 kb of Zp1 fragment subcloned into SuperCos I (0.05 μg). None of the enzymes cleave within the 7.6-kb vector.](image-url)
The zona is composed of three distinct glycoproteins, each of growing oocytes, ovulated eggs, and preimplantation embryos. The zona pellucida is an extracellular matrix that surrounds growing oocytes, ovulated eggs, and preimplantation embryos. The zona is composed of three distinct glycoproteins, each of which is conserved among eutherian mammals (differences in nomenclature complicate correspondence). Several of the genes encoding the zona proteins have been characterized. The exon-intron maps and coding sequence of mouse Zp1, Zp2 or Zp3, there is evidence of common ancestry between classes. The mouse Zp1 protein contains a 348-amino acid domain that is 47% similar to mouse Zp2 and is encoded by eight exons in both mouse Zp1 and Zp2. A similar domain was first noted by comparing R55 (rabbit orthologue of mouse ZP1) to mouse ZP2, although the genetic locus of the rabbit gene was not reported (18). As is most common in cases of partial gene duplication and exon shuffling (26–29), the 5' ends of this 348-amino acid domain encoded by mouse Zp1 and Zp2 are bounded by type 1 introns (i.e. the intron begins after the first residue of the codon) and the open reading frame is maintained with the nonconserved exons. The sequence conservation, coupled with the alignment of 10 cysteine residues, suggests that the three-dimensional structure of the two proteins in their respective zona matrices may be conserved as well. In addition to conservation among mammals within each class (e.g. Zp1, Zp2 or Zp3), there is evidence of common ancestry between classes. The mouse Zp1 protein contains a 348-amino acid domain that is 47% similar to mouse Zp2 and is encoded by eight exons in both mouse Zp1 and Zp2. A similar domain was first noted by comparing R55 (rabbit orthologue of mouse ZP1) to mouse ZP2, although the genetic locus of the rabbit gene was not reported (18). As is most common in cases of partial gene duplication and exon shuffling (26–29), the 5' ends of this 348-amino acid domain encoded by mouse Zp1 and Zp2 are bounded by type 1 introns (i.e. the intron begins after the first residue of the codon) and the open reading frame is maintained with the nonconserved exons. The sequence conservation, coupled with the alignment of 10 cysteine residues, suggests that the structural aspects of this domain are similar in the two zona proteins. These data indicate that the eight exons come from a common ancestral gene that has been duplicated in mammals and reutilized by exon shuffling. Although related, each of the mouse zona genes has been mapped to a distinct chromosome. In this manuscript we locate Zp1 to the proximal portion of chromosome 19, 21.1 ± 1.5 cm distal to D19Bir1 (an anonymous DNA fragment). We have previously located mouse Zp2 and mouse Zp3 on chromosome 7 (11.3 ± 0.3 cm distal to Tyr) and chromosome 5 (9.2 ± 2.9 cm distal to Gus), respectively (30).

A slightly smaller portion of the ZP1/ZP2 domain encoded by Zp1 exons 5–9 plus exon 11 has been identified in the wfl gene of white flounder, a distantly related aquatic vertebrate (5). Although the wfl protein appears to be part of the fish egg envelope, the expression of the wfl gene is restricted to the liver where it is inducible with estrogens. Outside of this 275-amino acid domain, the fish protein is quite dissimilar. It does not include a furin proteolytic site important in the processing of partial gene duplication and exon shuffling (26–29), the 5' ends of this 348-amino acid domain encoded by mouse Zp1 and Zp2 are bounded by type 1 introns (i.e. the intron begins after the first residue of the codon) and the open reading frame is maintained with the nonconserved exons. The sequence conservation, coupled with the alignment of 10 cysteine residues, suggests that the structural aspects of this domain are similar in the two zona proteins. These data indicate that the eight exons come from a common ancestral gene that has been duplicated in mammals and reutilized by exon shuffling. Although related, each of the mouse zona genes has been mapped to a distinct chromosome. In this manuscript we locate Zp1 to the proximal portion of chromosome 19, 21.1 ± 1.5 cm distal to D19Bir1 (an anonymous DNA fragment). We have previously located mouse Zp2 and mouse Zp3 on chromosome 7 (11.3 ± 0.3 cm distal to Tyr) and chromosome 5 (9.2 ± 2.9 cm distal to Gus), respectively (30).

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**DISCUSSION**

The zona pellucida is an extracellular matrix that surrounds growing oocytes, ovulated eggs, and preimplantation embryos. The zona is composed of three distinct glycoproteins, each of which is conserved among eutherian mammals (differences in nomenclature complicate correspondence). Several of the genes encoding the zona proteins have been characterized. The exon-intron maps and coding sequence of mouse Zp2, human ZP2, and the pig homologue (4, 21, 23), and of mouse Zp3, human ZP3, and hamster ZP3 (6, 7, 24, 25) are well conserved. A third human zona gene, ZPB, has recently been reported (16). Because it is distinct from human ZP2 and ZP3, we reasoned that it is the orthologue of mouse Zp1. The recent cloning of mouse Zp1 cDNA from an expression library (10) has confirmed this hypothesis. A near full-length ZP1 cDNA was used as a probe to isolate Zp1 from a 129Sv genomic library. The amino acid sequence, represented with single-letter code, is shown on the first line. Dashes indicate that the sequence is not conserved, coupled with the alignment of 10 cysteine residues, suggests that the structural aspects of this domain are similar in the two zona proteins. These data indicate that the eight exons come from a common ancestral gene that has been duplicated in mammals and reutilized by exon shuffling. Although related, each of the mouse zona genes has been mapped to a distinct chromosome. In this manuscript we locate Zp1 to the proximal portion of chromosome 19, 21.1 ± 1.5 cm distal to D19Bir1 (an anonymous DNA fragment). We have previously located mouse Zp2 and mouse Zp3 on chromosome 7 (11.3 ± 0.3 cm distal to Tyr) and chromosome 5 (9.2 ± 2.9 cm distal to Gus), respectively (30).

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A Ginsberg, unpublished observations.
(encoded by the last exon of each mouse zona gene). A recent report has identified a second, different, protein domain present in mammal and fish egg envelope proteins (8). A 261-amino acid sequence present in mouse ZP3 (encoded by Zp3 exons 1-6) is 33% identical with LS-F, a precursor of ZI-3, an egg envelope glycoprotein of O. latipes (medaka). Like the white flounder protein (from which it is distinct), LS-F transcripts are uniquely present in the liver where they are inducible with estrogen. Thus, although domains of vitelline envelope and zona pellucida proteins have been conserved for at least 650 million years, the control mechanisms for their expression have not. It appears that in fish, the two major glycoproteins are synthesized in the liver and transported to the egg where they form the inner egg envelope. The three mammalian zona proteins (one of which, Zp1, is ancestrally related to a second, Zp2) are synthesized exclusively in the oocyte (10).

Conservation among the zona genes extends to their promoters and may account, in part, for their coordinate and oocyte-specific expression. The sequence of the promoter region of mouse Zp1 was determined and compared with the promoters of mouse Zp2 and Zp3 (4, 7). Approximately 200 nucleotides upstream of the transcription start site of each gene is a canonical E-box sequence (CANNTG) (20) that has been described as a binding site for a class of transcription factors known as basic helix-loop-helix proteins (31). These factors commonly bind as heterodimers; one subunit is a ubiquitously expressed protein (E2A, HEB, E2-2), and the other is a tissue-specific protein. Using reporter gene constructs microinjected into growing oocytes, we find that cluster mutations of the E-box in either the Zp2 or Zp3 promoter, dramatically reduce reporter gene expression. Using gel mobility shift assays with synthetic oligonucleotides (40 bp) centered on the CANNTG binding site of either the Zp2 or Zp3, we can detect ZAP-1 in oocytes but not granulosa cells (20). The appearance of the ZAP-1 complex in oocytes is coincident with the detection of ZP2 transcripts in the prenatal ovary (22). It will be of interest to determine if similar investigations detect functional ZAP-1 binding to the E-box in the mouse Zp1 promoter.

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