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Oocyte-Specific Expression of Mouse *Zp-2*: Developmental Regulation of the Zona Pellucida Genes

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The zona pellucida surrounds all mammalian oocytes and plays a vital role at fertilization and in early development. The genes that code for two of the mouse zona proteins (ZP2 and ZP3) represent a developmentally regulated set of genes whose expression serves as markers of mouse oocyte growth and differentiation. We previously characterized the single-copy *Zp-3* gene and showed that its expression is oocyte specific and restricted to a narrow window of oocyte development. We now define the *Zp-2* gene transcript and show that it is coordinately expressed with *Zp-3* only during the 2-week growth phase of oogenesis that occurs prior to ovulation. Like *Zp-3*, the expression of *Zp-2* is restricted to oocytes, and, although not detectable in resting oocytes, both ZP2 and ZP3 transcripts accumulate to become very abundant messengers in 50-μm-diameter oocytes. Ovulated eggs contain ZP2 and ZP3 transcripts which are 200 nucleotides shorter than those found in growing oocytes and have an abundance of less than 5% of the peak levels. In an attempt to understand the molecular details associated with the developmentally regulated, tissue-specific gene expression of the zona genes, the *Zp-2* genetic locus has been characterized and its 5′ flanking sequences have been compared with those of *Zp-3*. Both genes contain three short (8- to 12-base-pair) DNA sequences of 80 to 88% identity located within 250 base pairs of their transcription start sites.

At birth the mouse ovary contains 10,000 to 15,000 primordial oocytes (29), the vast majority of which are in the prophase of the first meiotic division. During the reproductive life of the female, cohorts of these resting oocytes (10 to 15 μm in diameter) enter into a 2-week growth phase which culminates in meiotic maturation and subsequent ovulation (3, 11). Virtually nothing is known about the signals that induce this growth and differentiation, but one potential marker of these phenomena is the expression of the zona pellucida genes.

The zona pellucida surrounds all mammalian oocytes; in the mouse it is composed of three sulfated glycoproteins, ZP1, ZP2, and ZP3 (7, 45). The mouse zona proteins are coordinately synthesized in the growing oocyte (8, 45) and are secreted to form a filamentous zona matrix (39) in which ZP2 and ZP3 complex into copolymers cross-linked by ZP1 (24). At fertilization, sperm initially bind to ZP3 via O-linked oligosaccharide side chains (9, 10, 21). Following the induction of the sperm acrosome reaction on the surface of the zona, ZP2 acts as a secondary sperm receptor that is necessary for the maintenance of sperm binding to the egg (6). ZP2 is proteolytically cleaved after fertilization (5, 36), and this modification, along with presumed changes in ZP3, are postulated to play an important role in the postfertilization block to polyspermy.

The genes that code for the mouse zona pellucida represent a remarkable set of developmentally regulated, oocyte-specific genes which specifiy products vital to fertilization and early mammalian development. We have previously characterized *Zp-3* (14, 41, 42), a single-copy mouse gene that is expressed uniquely during the growth and differentiation of oocytes (40). We now report the isolation and characterization of *Zp-2*, a second mouse zona pellucida gene, and characterize its tissue-specific expression.

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MATERIALS AND METHODS

Screening and isolation of mouse ZP2 cDNA and genomic DNA clones. A λgt11 ovarian cDNA library was screened (6×10^6 recombinant bacteriophages) initially with anti-zona polyclonal antisera (42), and positive clones were rescreened with IE-3, a monoclonal antibody specific for ZP2 (20). A single positive 927-base-pair (bp) cDNA insert was subcloned into the *Eco*RI cloning site of the Bluescript KS (+) vector, pZP2.1 (Fig. 1, nucleotides [nt] 22 to 948), and the insert was used to rescreen the library. Two overlapping clones were identified and subcloned: pZP2.2 contained 1,723 bp (Fig. 1, nt 460 to 2182) and pZP2.3 contained 1,179 bp plus 39 adenosines (Fig. 1, nt 1023 to 2201). A near-full-length ZP2 cDNA (Fig. 1, nt 22 to 2201) was constructed and designated pZP2.4.

A recombinant λJ1 mouse genomic library (15) was screened (1.25×10^6 recombinant bacteriophages) with the *Eco*RI insert of pZP2.4. The presence of the entire *Zp-2* locus was confirmed by rescreening positive clones with a 5′-(*Eco*RI-*Sty*I fragment of pZP2.1; nt 22 to 258) and a 3′ (*Hin*cII-*Eco*RI fragment of pZP2.2; nt 2017 to 2182)-specific probe.

Isolation of RNA. Total RNA was isolated from tissue dissected from 3-week-old NIH Swiss mice, and poly(A)$^+$ RNA was obtained by fractionation of total RNA by oligo(dT) column chromatography (42). Total RNA was isolated from 15-, 40-, 50-, and 65-μm oocytes obtained from the ovaries of 3-day-old, 1-week-old, 2-week-old, and 3-week-old female mice, respectively (26). Ovulated eggs were isolated from the oviducts of 6-week-old female mice 16 h after human chorionic gonadotropin administration (25), and RNA was prepared as above. Granulosa cells were isolated free of oocytes from ovarian follicles (3-week-old mice), and total RNA was prepared.

Northern (RNA) blot analysis. RNAs were electrophoresed in 3% formaldehyde–1.2% agarose gels (18), transferred to GeneScreen Plus membrane (Du Pont, NEN Research Prod-

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                                        30                                  60                                  90
CAC CTC GGC GCT TTG GTG GTA CCT TCC AAC ATG GCG AGG TGG CAG AGG AAA GCA TCT GTA AGC TCT CCG TGC GGC AGG AGC ATC TAC AGG
                                        Met Ala Arg Trp Gln Arg Lys Ala Ser Val Ser Ser Pro Cys Gly Arg Ser Ile Tyr Arg

                                   120                 ↓               150                                 180
TTT CTT TCC CTC TTA TTC ACC CTT GTG ACT TCA GTG AAC TCA GTA AGC CTT CCT CAG TCC GAG AAT CCT GCC TTC CCA GGC ACT GTC ATT
Phe Leu Ser Leu Leu Phe Thr Leu Val Thr Ser Val Asn Ser Val Ser Leu Pro Gln Ser Glu Asn Pro Ala Phe Pro Gly Thr Leu Ile

                                   210                                 240                                 270
TGT GAC AAA GAC GAA GTG AGA ATT GAA TTT TCA AGC AGA TTT GAC ATG GAA AAA TGG AAT CCT TCT GTG GTG GAT ACC CTT GGT AGT GAA
Cys Asp Lys Asp Glu Val Arg Ile Glu Phe Ser Ser Arg Phe Asp Met Glu Lys Trp Asn Pro Ser Val Val Asp Thr Leu Gly Ser Glu

                                   300                                 330                                 360
ATT TTG AAC TGC ACT TAT GCT CTG GAC TTG GAA AGG TTC GTC CTG AAG TTC CCT TAC GAG ACC TGC ACT ATA AAA GTG GTT GGT GGA TAC
Ile Leu Asn Cys Thr Tyr Ala Leu Asp Leu Glu Arg Phe Val Leu Lys Phe Pro Tyr Glu Thr Cys Thr Ile Lys Val Val Gly Gly Tyr

                                   390                                 420                                 450
CAG GTG AAC ATC AGA GTG GGG GAC ACC ACC ACT GAT GTG AGA TAT AAA GAT GAC ATG TAT CAT TTC TTC TGT CCA GCT ATT CAA GCA GAG
Gln Val Asn Ile Arg Val Gly Asp Thr Thr Thr Asp Val Arg Tyr Lys Asp Asp Met Tyr His Phe Phe Cys Pro Ala Ile Gln Ala Glu

                                   480                                 510                                 540
ACC CAT GAG ATT TCA GAA ATT GTT GTC TGC AGG AGA GAT CTA ATA TCT TTT TCT TTC CCA CAA CTT TTC TCT AGG CTT GCT GAT GAA AAC
Thr His Glu Ile Ser Glu Ile Val Val Cys Arg Arg Asp Leu Ile Ser Phe Ser Phe Pro Gln Leu Phe Ser Arg Leu Ala Asp Glu Asn

                                   570                                 600                                 630
CAG AAT GTA TCT GAG ATG GGA TGG ATT GTT AAG ATT GGC AAT GGT ACA AGA GCC CAC ATT CTG CCC TTG AAG GAT GCC ATA GTA CAA GGA
Gln Asn Val Ser Glu Met Gly Trp Ile Val Lys Ile Gly Asn Gly Thr Arg Ala His Ile Leu Pro Leu Lys Asp Ala Ile Val Gln Gly

                                   660                                 690                                 720
TTT AAT CTT CTG ATT GAC AGC CAG AAA GTG ACT CTC CAC GTG CCA GCC AAT GCT ACT GGA ATA GTT CAC TAT GTG CAA GAG AGC AGC TAT
Phe Asn Leu Leu Ile Asp Ser Gln Lys Val Thr Leu His Val Pro Ala Asn Ala Thr Gly Ile Val His Tyr Val Gln Glu Ser Ser Tyr

                                   750                                 780                                 810
CTC TAT ACT GTG CAG CTG GAG CTC TTG TTC TCA ACC ACT GGG CAG AAG ATC GTC TTC TCA TCA CAC GCT ATC TGC GCA CCA GAT CTT TCT
Leu Tyr Thr Val Gln Leu Glu Leu Leu Phe Ser Thr Thr Gly Gln Lys Ile Val Phe Ser Ser His Ala Ile Cys Ala Pro Asp Leu Ser

                                   840                                 870                                 900
GTG GCT TGT AAT GCT ACA CAC ATG ACT CTC ACT ATA CCA GAA TTT CCT GGG AAG CTA GAG TCT GTG GAC TTT GGA CAA TGG AGC ATC CCT
Val Ala Cys Asn Ala Thr His Met Thr Leu Thr Ile Pro Glu Phe Pro Gly Lys Leu Glu Ser Val Asp Phe Gly Gln Trp Ser Ile Pro

                                   930                                 960                                 990
GAG GAC CAA TGG CAT GCC AAT GGA ATT GAC AAA GAA GCA ACA AAT GGC TTG AGA TTG AAT TTC AGA AAA TCT CTC CTG AAA ACT AAA CCC
Glu Asp Gln Trp His Ala Asn Gly Ile Asp Lys Glu Ala Thr Asn Gly Leu Arg Leu Asn Phe Arg Lys Ser Leu Leu Lys Thr Lys Pro

                                  1020                                1050                                1080
TCT GAA AAA TGT CCA TTC TAC CAG TTC TAC CTC TCT TCA CTC AAG CTG ACC TTC TAC TTC CAA GGG AAC ATG CTA TCC ACA GTG ATA GAT
Ser Glu Lys Cys Pro Phe Tyr Gln Phe Tyr Leu Ser Ser Leu Lys Leu Thr Phe Tyr Phe Gln Gly Asn Met Leu Ser Thr Val Ile Asp

                                  1110                                1140                                1170
CCT GAG TGC CAC TGT GAG TCA CCA GTC TCT ATA GAT GAA CTG TGT GCA CAG GAT GGG TTT ATG GAC TTT GAG GTC TAC AGC CAC CAA ACA
Pro Glu Cys His Cys Glu Ser Pro Val Ser Ile Asp Glu Leu Cys Ala Gln Asp Gly Phe Met Asp Phe Glu Val Tyr Ser His Gln Thr

                                  1200                                1230                                1260
AAA CCC GCA CTG AAC CTG GAC ACC CTC CTG GTG GGA AAT TCC TCT TGC CAG CCT ATT TTC AAG GTG CAG TCT GTG GGG CTT GCA AGG TTT
Lys Pro Ala Leu Asn Leu Asp Thr Leu Leu Val Gly Asn Ser Ser Cys Gln Pro Ile Phe Lys Val Gln Ser Val Gly Leu Ala Arg Phe

                                  1290                                1320                                1350
CAC ATA CCT CTG AAT GGA TGT GGA ACA AGG CAG AAA TTT GAA GGT GAT AAA GTC ATC TAT GAG AAT GAA ATA CAT GCT CTC TGG GAA AAC
His Ile Pro Leu Asn Gly Cys Gly Thr Arg Gln Lys Phe Glu Gly Asp Lys Val Ile Tyr Glu Asn Glu Ile His Ala Leu Trp Glu Asn

                                  1380                                1410                                1440
CCA CCC TCC AAC ATT GTA TTC AGA AAC AGC GAG TTC AGG ATG ACA GTA AGA TGC TAT TAC ATC AGA GAC AGT ATG CTA CTA AAT GCC CAT
Pro Pro Ser Asn Ile Val Phe Arg Asn Ser Glu Phe Arg Met Thr Val Arg Cys Tyr Tyr Ile Arg Asp Ser Met Leu Leu Asn Ala His

                                  1470                                1500                                1530
GTC AAA GGA CAT CCT TCT CCA GAG GCC TTT GTA AAG CCA GGC CCA CTG GTG TTG GTC CTA CAA ACA TAC CCA GAC CAA TCC TAC CAA CGG
Val Lys Gly His Pro Ser Pro Glu Ala Phe Val Lys Pro Gly Pro Leu Val Leu Val Leu Gln Thr Tyr Pro Asp Gln Ser Tyr Gln Arg

                                  1560                                1590                                1620
CCT TAC AGG AAG GAT GAG TAC CCT CTA GTG AGG TAC CTC CGC CAG CCA ATC TAC ATG GAA GTG AAG GTC TTG AGC AGG AAC GAT CCC AAC
Pro Tyr Arg Lys Asp Glu Tyr Pro Leu Val Arg Tyr Leu Arg Gln Pro Ile Tyr Met Glu Val Lys Val Leu Ser Arg Asn Asp Pro Asn

                                  1650                                1680                                1710
ATC AAG CTG GTC TTA GAT GAC TGC TGG GCA ACT TCT TCT GAG GAC CCG GCC TCT GCG CCT CAG TGG CAG ATT GTC ATG GAT GGC TGT GAA
Ile Lys Leu Val Leu Asp Asp Cys Trp Ala Thr Ser Ser Glu Asp Pro Ala Ser Ala Pro Gln Trp Gln Ile Val Met Asp Gly Cys Glu

                                  1740                                1770                                1800
TAT GAA CTG GAC AAC TAC CGC ACT ACT TTC CAC CCA GCT GGC TCC TCT GCA GCC CAT TCC GGT CAC TAC CAG AGG TTT GAT GTG AAG ACT
Tyr Glu Leu Asp Asn Tyr Arg Thr Thr Phe His Pro Ala Gly Ser Ser Ala Ala His Ser Gly His Tyr Gln Arg Phe Asp Val Lys Thr

                                  1830                                1860                                1890
TTT GCC TTT GTA TCA GAG GCA CGG GGG CTC TCC AGC CTG ATC TAC TTC CAC TGC AGT GCC TTG ATC TGT AAC CAA GTC TCT CTT GAC TCC
Phe Ala Phe Val Ser Glu Ala Arg Gly Leu Ser Ser Leu Ile Tyr Phe His Cys Ser Ala Leu Ile Cys Asn Gln Val Ser Leu Asp Ser

                                  1920                                1950                                1980
CCT CTG TGC TCT GTG ACT TGC CCT GCA TCA CTG AGG AGC AAA CGA GAG GCC AAC AAA GAA GAC ACA ATG ACG GTT AGC CTT CCA GGA CCT
Pro Leu Cys Ser Val Thr Cys Pro Ala Ser Leu Arg Ser Lys Arg Glu Ala Asn Lys Glu Asp Thr Met Thr Val Ser Leu Pro Gly Pro

                                  2010                                2040                                2070
ATT CTC TTG CTG TCA GAT GTC TCT TCA TCC AAA GGT GTT GAC CCC AGC AGC TCT GAG ATT ACC AAG GAT ATT ATT GCC AAG GAT ATT GCT
Ile Leu Leu Leu Ser Asp Val Ser Ser Ser Lys Gly Val Asp Pro Ser Ser Ser Glu Ile Thr Lys Asp Ile Ile Ala Lys Asp Ile Ala

                                  2110                                2130                                2160
TCT AAA ACA CTG GGT GCT GTG GCT GCA CTA GTG GGC TCA GCT GTC ATT CTA GGC TTC ATC TGT TAC CTG TAT AAG AAA AGA ACT ATA AGG
Ser Lys Thr Leu Gly Ala Val Ala Ala Leu Val Gly Ser Ala Val Ile Leu Gly Phe Ile Cys Tyr Leu Tyr Lys Lys Arg Thr Ile Arg

                                  2190
TTC AAT CAC TGA TTG GAC TTG CAA ATA AAG AGA CTG CAG TC
Phe Asn His
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ucts) and probed with a nick-translated ^{32}P-labeled cDNA insert from pZP2.1, pZP2.4, or pZP3.2 (42). As a positive control the blots were reprobed with a ^{32}P-labeled actin probe (42).

ZP2 and ZP3 RNA transcripts used as standards were synthesized from pZP2.4 and pGEM3.1, respectively. pZP2.4 was linearized with *Bam*HI, and RNA transcripts (2,320 nt) were transcribed from the T3 promoter by using an RNA transcription kit (Stratagene Inc.). ZP3 transcripts (800 nt) from pGEM3.1 were synthesized as previously described (40). The numbers of ZP2 and ZP3 transcripts per oocyte were calculated by comparing the band intensities (as determined by densitometry of autoradiographs) of the ZP2 and ZP3 transcripts in oocytes with the band intensities of the ZP2 and ZP3 standards, correcting for the difference in length between the mRNAs and the standards.

DNA sequencing. DNA sequence was obtained with Gemseq (Promega Biotec) and Sequenase (U.S. Biochemical Corp.) sequencing kits by using synthetic oligonucleotides based on ZP2 cDNA sequences as primers. ZP2 cDNA inserts from pZP2.1, pZP2.2, and pZP2.3 were sequenced, and the identity of these clones was confirmed by comparing the amino acid translation of the cDNA sequences with amino acid sequences determined from an isolated trypsin-digested ZP2 peptide and the N terminus of ZP2 isolated from the zona (Fig. 1).

A 2.1-kbp DNA fragment from the *Zp-2* genomic insert containing the 5′-flanking regions and the first two exons of *Zp-2* and a 3.3-kbp DNA fragment from the insert containing the 3′ flanking region and the last four exons of *Zp-2* were subcloned into pBluescript and designated pGZP2.1 and pGZP2.2, respectively. Both genomic subclones were sequenced, and the sequence of the remaining exons was determined by using the *Zp-2* recombinant phage DNA as a template.

Amino acid sequencing of ZP2 protein. ZP2 (20 μg) was isolated by electroelution from sodium dodecyl sulfate-gels and carboxyamidomethylated (42). After removal of sodium dodecyl sulfate by filtration through Extractigel-D (Pierce Chemical Co.), the extracted protein was lyophilized, suspended (0.25 mg/ml in 0.2 M ammonium acetate [pH 8.5]), and digested with trypsin (1:25, wt/wt) for 2 h at 37°C. The sample was acidified to a final concentration of 1% trifluoroacetic acid and the tryptic peptides separated by reverse-phase high-pressure liquid chromatography (46). The amino acid sequence of a single peptide was determined by D. Atherton, Rockefeller University, by using gas phase microsequencing. N-terminal amino acid sequences were initially determined by Applied Biosystems, Inc., and subsequently confirmed by M. Moos, National Institutes of Health.

S1 nuclease analysis. The single-stranded 5′ genomic probe for the S1 nuclease analysis was prepared by using a synthetic oligonucleotide primer complementary to map positions 62 to 78 of ZP2 cDNA. A 350-bp *Bsp*MI fragment of pGZP2.1 was used as a template along with Taq polymerase in a DNA thermocycler by using the above primer. A 0.2-ng portion of the resulting 108-nt single-stranded fragment (end labeled and gel purified) was hybridized to 0.5 μg of mouse ovarian poly(A)$^+$ RNA or 10 μg of mouse liver RNA to 5× the $C_0t_{1/2}$ and digested with 800 U of S1 nuclease (13). The digestion products were analyzed on a 6% polyacrylamide sequencing gel. Primer extension assays were performed as previously described (41).

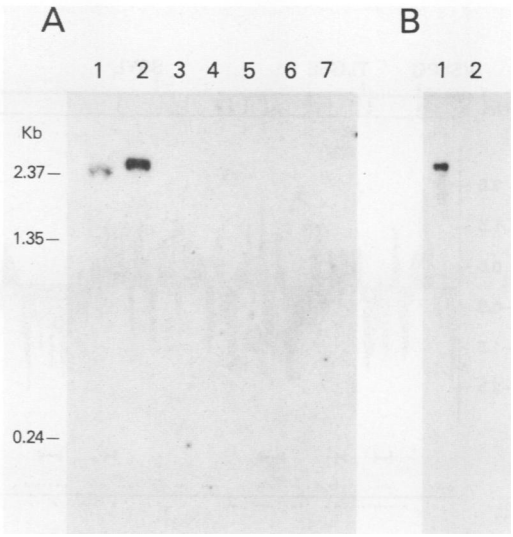

FIG. 2. Oocyte-specific expression of *Zp-2*. (A) Autoradiograph of a Northern blot analysis of mouse tissues probed with ^{32}P-labeled insert from pZP2.1. Lanes: 1, 10 μg of total ovarian RNA; 2, 1 μg of poly(A)$^+$ ovarian RNA; 3, 10 μg of ovarian poly(A)$^-$ RNA; 4 to 7, 10 μg of total RNA from brain, heart, liver, and testes, respectively. (B) Autoradiograph of Northern blot analysis of total RNA from 50 growing oocytes (lane 1) and granulosa cells from 25 follicles (lane 2) isolated from 3-week-old mice and probed as in panel A. Numbers to the left are molecular size markers in kilobases.

Determination of intron size. The sizes of the introns were determined by sequencing with synthetic oligonucleotide primers used in cDNA sequencing or by polymerase chain reactions. *Zp-2* recombinant phage genomic DNA was used as the template for polymerase chain reactions primed with synthetic oligonucleotides that mapped at positions at either side of each intron. We performed 25 cycles of polymerase chain reaction and determined the sizes of products on agarose and polyacrylamide gels.

RESULTS

Characterization of the ZP2 mRNA and the ZP2 protein. The structure of the cytoplasmic ZP2 mRNA (Fig. 1) was deduced from the nucleic acid sequence of near-full-length cDNAs and a genomic clone containing exon 1. The 5′ end of the mRNA was defined by the protection of a 78-nt fragment after S1 nuclease digestion of a 108-nt single-stranded probe derived from genomic DNA (map positions −30 to 78) which had been hybridized to ovarian poly(A)$^+$ mRNA (data not shown). The 2,201-nt ZP2 mRNA has a very short 5′ untranslated region of 30 nt and a similarly short (32-nt) 3′

FIG. 1. Structure of the ZP2 mRNA and protein. The nucleic acid sequence of near-full-length cDNAs and exon 1 of *Zp-2* were used to deduce the structure of the ZP2 mRNA and resultant protein. The initiation and termination codons are boxed, and the polyadenylation signal is overlined. The single 2139-nucleotide open reading frame is translated into protein in line 2. The 34-amino-acid signal peptide is indicated by the wavy line, and the arrow points to the signal peptidase cut site. Amino acid sequences which were experimentally determined by isolation and direct sequencing of an N-terminal and an internal ZP2 peptide are underlined with a dashed line. The seven potential N-linked glycosylation sites [Asn-X-(Thr/Ser)] are underlined with a solid line.

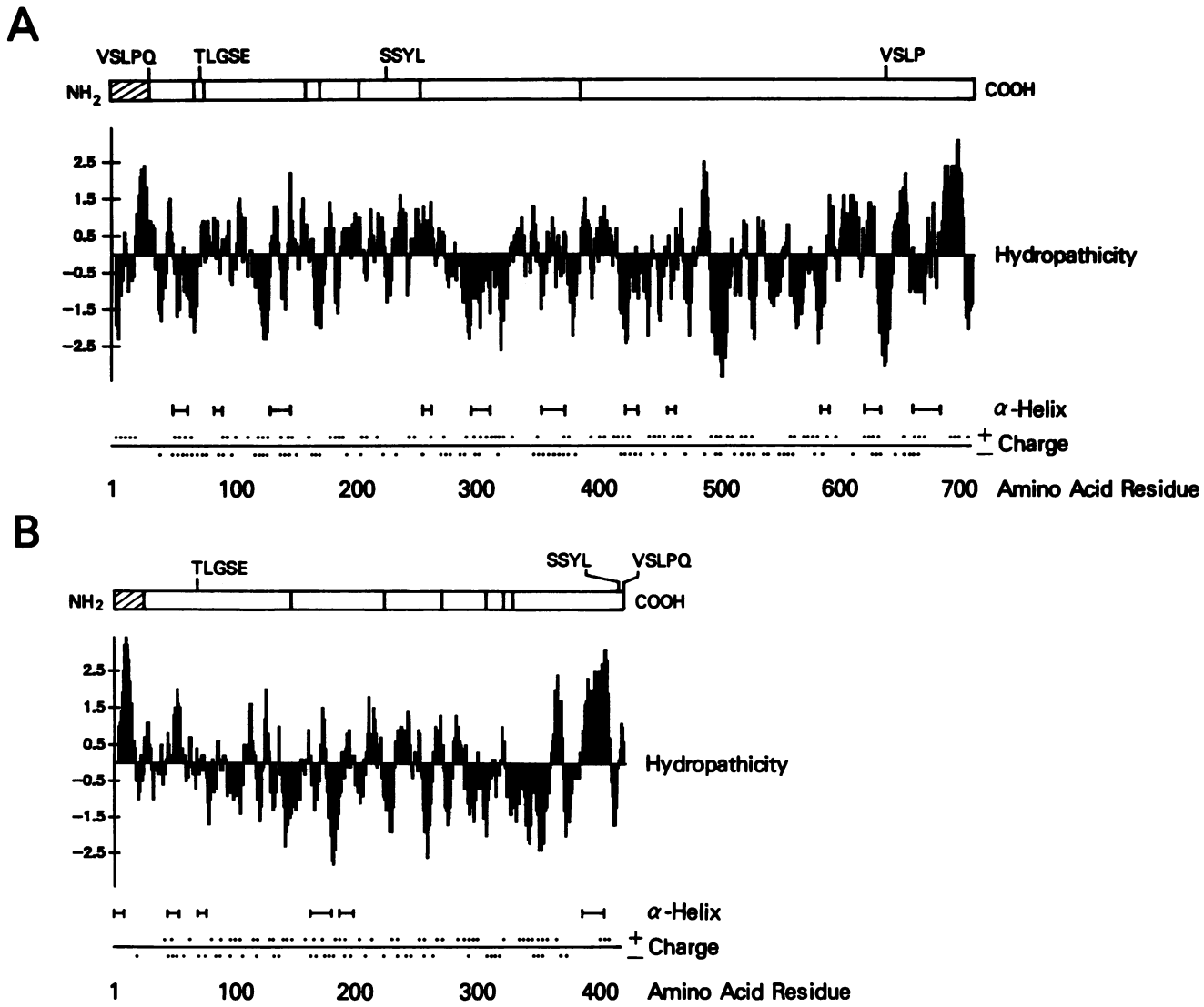

FIG. 3. Secondary structure of the deduced ZP2 and ZP3 proteins. (A) Schematic representation of the ZP2 mRNA with a single open reading frame containing a signal peptide (▨) and seven potential N-linked glycosylation sites (|). The hydropathicity of the protein was determined by the Kyte and Doolittle algorithm (33), and the α-helical structure was determined by the method of Garnier et al. (22). (B) Same as panel A, but for ZP3. The three amino acid identities in the ZP2 and ZP3 polypeptides are indicated by the single-letter amino acid code above each protein: VSLPQ, Val-Ser-Leu-Pro-Gln beginning at residues 35 and 645 (contains only the first four residues) in ZP2 and 420 in ZP3; TLGSE, Thr-Leu-Gly-Ser-Glu beginning at residue 76 in ZP2 and 72 in ZP3; SSYL, Ser-Ser-Tyr-Leu, beginning at residue 228 in ZP2 and 416 in ZP3.

untranslated region (Fig. 1). Although the nucleic acid sequence is distinctly different from that of ZP3, this same motif of short 5′ and 3′ untranslated regions is preserved in both zona transcripts (41). Oligo(dT)-purified ZP2 transcripts migrate on Northern blots with a molecular size of 2.4 kilobases (Fig. 2), which suggests that ZP2 mRNA (like ZP3) isolated from growing oocytes contains an approximately 200-nt poly(A) tail. The comigration of rRNA with ZP3 may account for the slight difference in mobility of ZP3 transcripts detected in total and poly(A)$^+$ ovarian RNAs (Fig. 2).

The ZP2 mRNA has a single open reading frame of 2,139 nt which codes for a polypeptide of 80,217 daltons representing 713 amino acids (10.8% acidic, 9.5% basic, 10.2% aromatic, and 34.8% hydrophobic). The amino acid sequence contains seven Asn-X-(Ser/Thr) sequences which are potential N-linked glycosylation sites (Fig. 1 and 3A). The first 34 amino acids of the open reading frame are absent from the N-terminal amino acid sequence obtained from sodium dodecyl sulfate-polyacrylamide gel electrophoresis-purified secreted ZP2 protein and presumably represent a signal peptide. The amino acids at the −1 and −3 positions from the presumptive signal peptidase cleavage site are Ser and Asn, respectively; these are in accordance with the (−3, −1) rule proposed by von Heijne (48, 49). The resultant secreted protein would have a molecular mass of 76,373 daltons.

No overall sequence similarity was detected between ZP2 and ZP3 at either the nucleic acid or the amino acid level. However, there are two 5-amino-acid regions that are identical in both polypeptides (Fig. 3). The most striking one (Val-Ser-Leu-Pro-Gln) is located at the exact N terminus of

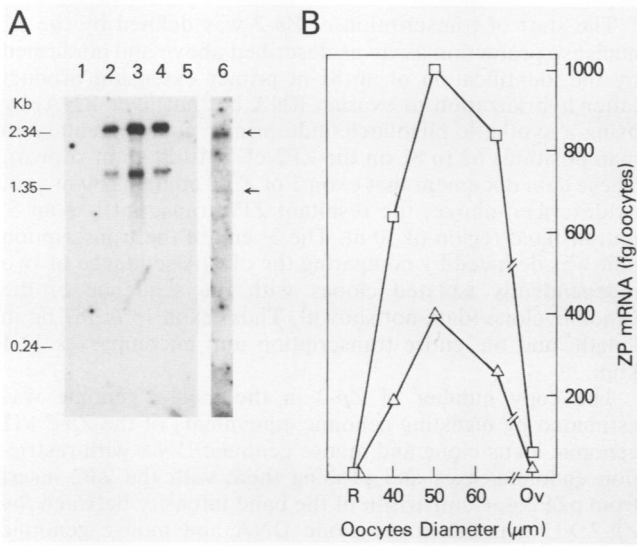

FIG. 4. Developmental expression of *Zp-2* and *Zp-3* during oogenesis. (A) Autoradiograph of Northern blot analysis of RNA isolated from mouse oocytes or eggs probed with ^{32}P-labeled insert from pZP2.4 and from pZP3.2 (42). Lanes indicate stage of oocyte development: 1, 800 resting oocytes; 2, 200 oocytes of diameter 40 μm; 3, 200 oocytes of diameter 50 μm; 4, 200 oocytes of diameter 65 μm; 5, 200 ovulated eggs; 6, 800 ovulated eggs. Blots were exposed at −70°C for 4 days (lanes 1 to 5) or 10 days (lane 6). Numbers to the left represent molecular size markers. Arrows to the right of lane 6 indicate ZP2 and ZP3 transcripts. (B) Quantitation of the abundance of ZP2 (□) and ZP3 (△) transcripts during oogenesis based on densitometry of hybridization signals in panel A and the hybridization signal obtained by using increasing amounts of synthetic ZP2 and ZP3 transcripts. Abbreviations: R, resting oocytes; OV, ovulated eggs.

the secreted ZP2 protein and at the exact carboxyl terminus of the ZP3 protein. Four of these five amino acids (Val-Ser-Leu-Pro) are also present a second time in ZP2 at position 645. Another 5-amino-acid identity (Thr-Leu-Gly-Ser-Glu) is present at a comparable distance from the amino terminus of ZP2 (amino acid residue 76) and ZP3 (amino residue 72). Based on a comparison with the frequencies of amino acids in 500 proteins (16), the probabilty that these 5-amino-acid identities arose by chance is 1×10^{-5} and 2.6×10^{-5}, respectively. There is an additional 4-amino-acid identity between the two proteins (Ser-Ser-Tyr-Leu) positioned at amino acid residue 228 in ZP2 and 416 in ZP3. Otherwise, although there is an increase in the number of glycine (85%) and alanine (55%) residues, the amino acid composition of ZP2 appears comparable to that of other proteins (16), and there does not appear to be a pronounced clustering of a particular amino acid along the polypeptide backbone. Neither ZP2 nor ZP3 have a high degree of predicted α-helical structure (22), and hydropathicity plots of the two polypeptides (33) indicate that ZP2 has a very hydrophobic region near its carboxy terminus (Fig. 3), as previously described for ZP3 (41). These hydrophobic regions, along with the short identical amino acid regions, may be important for the interaction of zona polypeptides in the extracellular zona matrix.

Oocyte-specific expression of ZP2 transcripts. To examine the tissue-specific expression of *Zp-2*, we performed Northern blot analysis on poly(A)$^+$ RNA from ovary and total RNA from ovary, brain, heart, liver, and testes. A single poly(A)$^+$ transcript with a molecular size of 2.4 kilobases was detected in ovarian RNA (Fig. 2A). To determine whether this expression could be localized further, we isolated total RNA from oocytes and granulosa cells derived from the ovaries of 3-week-old female mice. Using Northern blots probed with a ZP2 cDNA, we detected a single transcript in oocyte RNA, but not in granulosa cell RNA (Fig. 2B). As controls, the above Northern blots were also reprobed with a labeled β-actin cDNA and actin transcripts were detected (data not shown). Thus, *Zp-2* appears to be expressed only in oocytes, making it, along with *Zp-3*, a member of a set of oocyte-specific genes.

The accumulation of ZP2 transcripts during oocyte growth was investigated by isolating total RNA from resting (15-μm), 40-, 50-, and 65-μm-diameter oocytes as well as from ovulated eggs. After electrophoresis in formaldehyde-agarose gels and membrane transfer, the RNA was probed with ZP2- and ZP3-specific cDNA inserts (Fig. 4A). Known amounts of synthetic ZP2 and ZP3 RNA transcripts electrophoresed on the same gels were used as standards to quantitate the levels of ZP2 and ZP3 transcripts (data not shown). Neither zona transcript was detected in resting oocytes (Fig. 4A), even when the RNA from four times as many oocytes was examined. However, substantial amounts of ZP2 and ZP3 transcripts were detected in 40-μm-diameter oocytes (Fig. 4A), and the amount of ZP2 transcripts increased to a maximum of 1,000 fg per oocyte while the amount of ZP3 transcripts increased to a maximum level of 400 fg per oocyte in 50-μm oocytes (Fig. 4B). Based on earlier estimations (17, 27), the peak values of ZP2 and ZP3 represent 1% and 0.4%, respectively, of the total poly(A)$^+$ content of growing oocytes. The ZP2 and ZP3 transcripts accumulated in parallel, and ZP2 was present in approximately twofold molar excess throughout oocyte growth.

As oocyte growth continued, the number of both zona transcripts decreased, and in ovulated eggs there was less than 5% of their peak levels. To better visualize the ZP2 and ZP3 transcripts in ovulated eggs, RNA was isolated from 4 times as many eggs and after hybridization the Northern blot was exposed for 2.5 times as long (Fig. 4A, lane 6). The sizes of both ZP2 and ZP3 transcripts were distinctly decreased in ovulated eggs from their sizes in growing oocytes. The

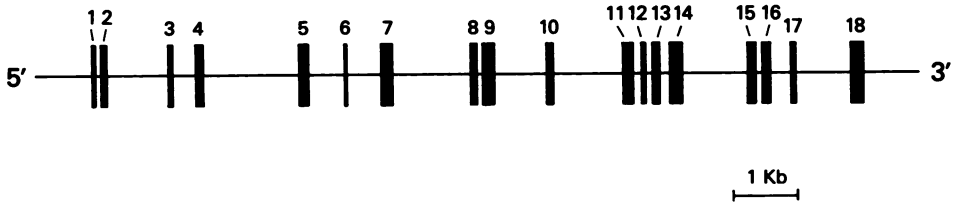

FIG. 5. Schematic representation of the intron-exon map of *Zp-2*. Dark bars represent each of the 18 exons, which range in size from 45 to 190 bp and are separated by 17 introns ranging in size from 81 to 1490 nt. The entire transcription unit is 12.1 kbp long.

TABLE 1. Exon and intron sizes of mouse *Zp-2* gene

Exon	Position	Length (nt)	Intron	Length (nt)
1	1–80	80	1	90[a]
2	81–169	89	2	960
3	170–253	84	3	356[a]
4	254–348	95	4	1490
5	349–498	150	5	580
6	499–543	45	6	530
7	544–705	162	7	1230
8	706–802	97	8	95[a]
9	803–987	185	9	800
10	988–1114	127	10	1080
11	1115–1296	182	11	88[a]
12	1297–1388	92	12	97[a]
13	1389–1513	125	13	139[a]
14	1514–1703	190	14	1030
15	1704–1839	136	15	81[a]
16	1840–1936	97	16	314[a]
17	1937–2014	78	17	850
18	2015–2201	187		

[a] Exact length determined by sequencing.

magnitude of the decrease is roughly 200 nt, which corresponds to the estimated length of their poly(A) tails.

Genomic locus and exon-intron mapping of *Zp-2*. A genomic clone containing a 14.4-kbp insert was isolated from a λJ1 mouse library and included the entire mouse *Zp-2* locus as well as 1.5 kbp of 5′-flanking regions and 0.8 kbp of 3′-flanking regions. Oligonucleotide primers, used to sequence the ZP2 cDNA, were used to sequence the *Zp-2* gene, and 18 exons ranging from 45 to 190 bp were identified (Fig. 5; Table 1). The nucleotide sequence of the *Zp-2* exons was identical to that determined for the ZP2 cDNA. The sizes of the introns, which ranged from 81 to 1,490 bp, were determined either by direct sequencing or by analyzing polymerase chain reaction products primed with synthetic oligonculeotides that mapped to regions flanking the introns (Table 1). Each exon (except exon 1) is preceded by a splice acceptor consensus sequence (12), and each (except exon 18) is followed by a splice donor consensus sequence (Table 2).

TABLE 2. Immediate flanking sequences of *Zp-2* exons

5′-Flanking sequence[a]	Exon no.	3′-Flanking sequence[a]
	1	GTGAGGCATTCT
TCTCCTATCCAG	2	GTATGTCTCTTG
TCTGTTGTCCAG	3	GTATGTAGTACG
TGTCTTCTGTAG	4	GTAAGCAAGTGT
GTTTGTACACAG	5	GTAAGTGATGTG
TTTTTTTTCCAG	6	GTAAGAATACAA
TGAATTTTGAAG	7	GTAGGTTTGAAA
TCTGACCCCCAG	8	GTGAGGCCTGAG
CGTTTTGTACAG	9	GTATGTTTGTTA
CTCTCGTTCCAG	10	GTAAGCATGTTT
GTCCACTTACAG	11	GTGAGTAGCAAC
CCAACATTGAAG	12	GTGTGATGCAAA
ACATTTTTCTAG	13	GTGAGGTGTCGC
TGTTAATTGCAG	14	GTATGTACTCCC
CCTGTTTCCTAG	15	GTATGTGATCAA
TTCCTCTTCCAG	16	GTAAAAATTCAA
CTCCCACCACAG	17	GTAAACATTTAG
CTTTCTTTGTAG	18	

[a] The splice acceptor and splice donor consensus sequences are in bold letters.

The start of transcription of *Zp-2* was defined by the S1 nuclease protection assay as described above and confirmed by the identification of an 81-nt primer extension product (after hybridization to ovarian RNA but not liver RNA) by using a synthetic oligonucleotide primer complementary to map positions 62 to 81 on the ZP2 cDNA (data not shown). These data document that exon 1 of *Zp-2* contains 80 nt and, as described above, the resultant ZP2 transcript has an 5′ untranslated region of 30 nt. The 3′ end of the transcription unit was deduced by comparing the cDNA sequence of two independently isolated clones with the sequence of the genomic clone (data not shown). Thus, exon 18 is 187 bp in length, and the entire transcription unit encompasses 12.1 kbp.

The copy number of *Zp-2* in the mouse genome was estimated by digesting genomic equivalents of the *Zp-2* λJ1 genomic locus clone and mouse genomic DNA with restriction endonucleases and probing them with the ZP2 insert from pZP2.4. Comparison of the band intensity between the *Zp-2* λJ1 genomic locus clone DNA and mouse genomic DNA suggested that *Zp-2* is present in the mouse genome in low copy number (data not shown). Mouse genomic DNA and the *Zp-2* genomic recombinant phage DNA were digested with four different restriction enzymes and examined by Southern blot analysis. Examination of the banding patterns of the restriction enzyme-digested genomic DNA indicated that all bands observed could be accounted for in the restriction enzyme-digested λJ1 DNA, thus providing evidence that there is only one copy of *Zp-2* in the mouse genome (data not shown).

5′-Flanking region of *Zp-2*. The *Zp-2* 5′-flanking region contains a TATAA box at −31 and a CCAAT box at −69 bp from the transcription start site. Several novel tandem repeats have been identified in the *Zp-3* locus (14, 31), the only other oocyte-specific gene that has been found in mice. However, similar repeats were not detected in the first 1.5 kbp of the 5′-flanking region or in the 0.8-kbp 3′-flanking region of *Zp-2*. A comparison of the 5′-flanking sequences of *Zp-2* and *Zp-3* detected three homologous regions, ranging in size from 8 to 12 bp, within the first 250 bp of the transcription start site. The three elements, GTGAAAGGGTGG (at bp −63), ATTCTGGT (at bp −194), and ACTCACCTGG (at bp −219), are 80 to 88% conserved between the two oocyte-specific genes. Although present in other genes, they are rarely located in the 5′-flanking regions and are not related to other *cis* elements that have been reported in the literature. Provocatively, similar sequences are also found at approximately the same distance upstream of the transcription start site of the human *Zp-3* gene (M. E. Chamberlin, Ph.D. thesis, Johns Hopkins University, Baltimore, Md., 1989). Their role in the oocyte-specific expression of the zona genes remains to be determined.

DISCUSSION

Zp-2 and *Zp-3* encode two proteins that are secreted and participate in the formation of the mouse zona pellucida. The expression of the two zona genes is oocyte specific and developmentally regulated. Although unrelated to one another by sequence and located on separate chromosomes (34), the transcripts of these two genes accumulate coordinately during the growth phase of oogenesis prior to ovulation. At present, the regulation of their expression is poorly understood. From our investigations we hope to learn more about the mechanisms of oocyte-specific gene expression

and to make use of these findings to begin to determine the signals that induce resting oocytes to enter into growth and differentiation.

During mouse oogenesis there is a rapid accumulation of poly(A)$^+$ RNA, and full-grown oocytes contain 85 to 90 pg of poly(A)$^+$, which represents 20% of the total RNA (1, 2). Approximately one-fourth of the poly(A)$^+$ RNA is found on polysomes, while the rest accumulates as stable maternal RNA for use later in oogenesis (1, 17). Although the expression of a number of genes has been examined during mouse oogenesis, to date only *Zp-2* and *Zp-3* appear to be expressed exclusively in oocytes (40, 41, 43) (see above). Neither ZP2 nor ZP3 transcripts are detected in resting, primordial oocytes but quickly accumulate as abundant mRNAs when oocytes enter into their growth phase. At their peak levels, ZP2 and ZP3 mRNAs represent approximately 1% and 0.4%, respectively, of the total poly(A)$^+$ RNA. Although turnover studies have not been reported, given the great abundance of the zona transcripts we speculate that both ZP2 and ZP3 are quite stable during oogenesis. Throughout oocyte growth the molar ratio of ZP2 to ZP3 transcripts is 2:1, although the reported protein ratio is approximately 1:1 (50). This suggests that posttranscriptional regulation may play an important role in *Zp-2* and *Zp-3* gene expression.

It is noteworthy that both ZP2 and ZP3 mRNA have short 5′ (30 and 29 nt, respectively) and 3′ (32 and 16 nt, respectively) untranslated regions. This motif appears to be evolutionarily conserved in that the size of ZP3 mRNA is the same in at least three mammalian species (41). Short 5′ and 3′ untranslated regions, although not unknown (4, 28, 30, 38) are unusual, and they may be important for the processing of ZP2 and ZP3 transcripts. The stability of bulk oocyte poly(A)$^+$ RNA decreases during meiotic maturation and subsequent ovulation such that more than 50% of the poly(A)$^+$ RNA is either deadenylated or degraded (37). This composite observation reflects widely divergent processing of individual mRNAs: some are deadenylated, some are degraded (37), and some are polyadenylated (26, 37). The decline of the ZP2 and ZP3 transcripts during this stage of oogenesis is dramatic and places them in the class of mRNAs which are degraded during meiotic maturation and ovulation.

The poly(A)$^+$ ZP2 and ZP3 transcripts are actively translated into protein in growing oocytes (8, 45) and thus appear to associate with the polysome fraction of RNA. The subsequent decrease in zona protein synthesis observed in the latter stages of oocyte growth (45) corresponds to a decline in the abundance of the two zona transcripts. The low level or absence of zona protein synthesis in fully grown oocytes (8) and ovulated eggs (45) correlates temporally with a precipitous decline in the abundance of ZP2 and ZP3 transcripts to less than 5% of the peak levels. Furthermore, the zona transcripts detected in ovulated eggs are 200 nt shorter [the approximate length of their poly(A) tail] than that observed in growing oocytes, suggesting that deadenylation may have occurred during meiotic maturation and ovulation. The presence of a poly(A) tail has been associated with the translatability of a number of mouse oocyte mRNAs including tissue plasminogen activator, hypoxanthine phosphoribosyltransferase, actin, and α-tubulin (2, 24, 35), and similar observations have been made with *Spisula* (44) and *Xenopus* (19, 35) oocytes. Thus, it appears that the absence of ZP2 and ZP3 zona protein synthesis after ovulation may be due both to the low level of zona transcripts and to an inability to translate them in their shortened, presumably deadenylated, form.

Both ZP2 and ZP3 are heavily glycosylated proteins (7, 45). The 713-amino-acid peptide of ZP2 contains seven potential N-linked glycosylation sites, six of which have been reported to be derivatized (23), and more than 100 potential O-linked glycosylation sites. Incubation of growing oocytes in the presence of tunicamycin inhibits N-linked glycosylation and results in the detection of an 81,000-dalton protein (23), which is larger than the 76,373-dalton polypeptide chain deduced from DNA sequence, a discrepancy that may result from O-linked glycosylation. Both the ZP2 and ZP3 proteins have a signal peptide which directs their secretion to the extracellular matrix of the zona pellucida, and there may be additional signals to direct the processing of the intracellular proteins. Although neither polypeptide chain contains previously described intracellular trafficking signals (32, 47), the three regions of amino acid identity in ZP2 and ZP3 may provide such functions.

Electron microscopy and biochemical analysis of the zona pellucida suggest that ZP2 and ZP3 are arranged into long filaments which are cross-linked by ZP1 (24). Little is known about the protein domains that modulate these interactions, but the aforementioned amino acid identities may be important, as may the very hydrophobic region at the C terminus of each polypeptide. After the initial binding of sperm to the zona pellucida at fertilization, ZP2 acts as a secondary sperm receptor (6). Following fertilization, ZP2 is proteolytically cleaved, releasing a 23,000-dalton glycopeptide which is bound via a disulfide bond(s) to the larger, 90,000-dalton moiety (5, 36). Candidate proteases responsible for this cleavage have recently been identified (36), and this biochemical modification of ZP2 is associated with the postfertilization block to polyspermy.

Both *Zp-2* and *Zp-3* are present in single copy in the mouse genome, and although they are located on separate chromosomes (34), their expression is coordinately regulated and tissue specific. If the transcription of zona genes is governed by a common factor or factors, the identical regulatory regions may be identified in the control regions of the two genes. Both genes have a TATAA box in their 5′-flanking region, and *Zp-2*, but not *Zp-3*, has a CCAAT box at −69 from the transcription start site. Novel tandem repetitive sequences have been identified at the 5′-flanking regions of *Zp-3* (14, 31), but there are no similar sequences upstream of the *Zp-2* transcription start site. However, both zona genes contain three short sequence similarities (8 to 12 bp) arranged at comparable distances from the transcription start site in the first 250 bp of the 5′-flanking region. Studies are under way to determine whether these DNA sequences have a functional role in the expression of *Zp-2* and *Zp-3*. These investigations may lead to the identification of common factors which regulate the coordinate expression of the oocyte-specific zona pellucida genes.

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