Zygote **7** (February), pp 27–35. © 1999 Cambridge University Press Printed in the United Kingdom

Cloning, sequencing and site of origin of the rat sperm receptor protein, ZP3

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Date submitted: 6.4.98. Date accepted: 4.6.98

Summary

The ZP3 gene encodes for a zona glycoprotein that serves as both a cell-specific binding site for capacitated spermatozoa and an inducer of acrosomal exocytosis during fertilisation. In this study we have determined the nucleotide sequence of rat ZP3 (accession no. Y10823), predicted primary amino acid structure and determined the cellular origin of this molecule within the ovary. Rat ZP3 was found to have an open reading frame of 1272 nucleotides encoding a polypeptide chain of 424 amino acids that was expressed exclusively by the actively growing oocyte population. Rat ZP3 exhibited 91%, 78% and 66% identity with the mouse, hamster and human homologues, respectively. Key features of mouse ZP3, including the number and location of cysteine and proline residues and *N*-linked glycosylation sites, were also conserved in the rat homologue. The putative *O*-linked glycosylation sites, a series of serine residues at $ZP3^{329–334}$, were also conserved in rat and mouse ZP3, although immediately downstream of this site the amino acid sequences deviated over a short stretch of amino acids. The hydropathicity profile revealed two hydrophobic domains. The first was associated with a putative N-terminal signal sequence which is unusual in the rat in possessing a proline residue at the –1 position relative to the signal cleavage site, a feature it shares with human and marmoset ZP3 but not mouse. The second hydrophobic domain was observed at the C-terminus downstream of a TGF-β type III receptor domain that appears to be common to all ZP3 sequences examined to date.

Keywords: Rat, Sperm receptor, Zona pellucida, ZP3

Introduction

The zona pellucida (ZP) is an extracellular glycoprotein coat that surrounds the mammalian oocyte and is involved in several unique biological functions during fertilisation and early embryonic development (Wassarman, 1990). It comprises three major protein species – ZP1, ZP2 and ZP3 – each of which is thought to have its own specific function (Wassarman *et al*., 1989). The current model, largely derived from experiments conducted in the mouse, suggests that the initial binding of spermatozoa to the surface of the zona pellucida is mediated through *O*-linked oligosaccharides on ZP3 (Florman & Wassarman, 1985; Kinloch *et al*., 1995) via receptors on the sperm plasma membrane, the identity of which is still uncertain (Bleil & Wasserman, 1990; Leyton *et al*., 1992; Youakim *et al*., 1994; Cheng *et al*., 1994). This primary interaction between the sperm surface and ZP3 induces an exocytotic event, the acrosome reaction (Bleil & Wassarman, 1983; Saling, 1991), which results in the release of lytic enzymes that are thought to be essential for sperm penetration through the zona pellucida. The acrosome reaction also exposes molecules associated with the inner acrosomal membrane that are thought to mediate 'secondary' zona binding through their interaction with another zona glycoprotein, ZP2 (Bleil *et al*., 1988). Once fertilisation has occurred, ZP2 plays a major role in blocking further zona penetration by becoming modified to ZP2f, a molecule that no longer expresses an affinity for spermatozoa (Bleil *et al*., 1981; Moller *et al*., 1990; Ducibella *et al*., 1993).

The primary amino acid structures of several ZP3 genes have been described including mouse (Kinloch *et al*., 1988, 1990), hamster (Ringuette *et al*., 1988),

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marmoset (Thillai Koothan *et al*., 1993) and human (Chamberlin & Dean, 1990). In species such as the mouse (Bleil & Wassarman, 1980; Bousquet *et al*., 1981; Salzmann *et al*., 1983; Kimura *et al*., 1994) and marmoset (Thillai Koothan *et al*., 1993) there is clear evidence that the ZP3 mRNA is transcribed exclusively in the oocytes of actively growing follicles. In other species, such as the rhesus monkey and human (Grootenhuis *et al*., 1996), ZP3 expression appears to be already activated in the oocytes of primordial follicles, while in yet others, such as the rabbit and pig, ZP3 genes appear to be transcribed by both the oocytes and granulosa cells with the growing follicle pool (Lee & Dunbar, 1993; Kolle *et al*., 1996).

The laboratory rat is a species of strategic importance to the fields of reproductive toxicology and contraceptive research and yet very little is known about the molecular structure of rat ZP3. In light of this deficiency, the present study was undertaken to clone and sequence the rat homologue of ZP3 and identify its site of expression.

Materials and methods

Rat ovarian ZP3 cDNA cloning

Total rat ovarian RNA was isolated from 50 mg of frozen tissue using RNeasy columns (Promega) according to the manufacturer's instructions. Poly(A+) mRNA was also isolated directly from crude homogenised rat ovarian tissue using oligo(dT) magnetic beads (Dynal) and left attached to the magnetic bead as a template for reverse transcription (RT). cDNA was synthesised from either total RNA with oligo(dT(15)) primer or poly(A+) mRNA attached to magnetic beads using Expand Reverse Transcriptase (Boehringer) for 2 h at 40 °C.

A cDNA library was made in Unizap vector and propagated in XL-1 Blue cells (Stratagene) as previously described (Einspanier *et al*., 1997). Regions of the rat ZP3 gene were amplified by polymerase chain reaction (PCR) using primers based on hamster (Kinloch *et al*., 1990) and mouse (Ringuette *et al*., 1988) conserved sequences, or primers synthesised to generated rat sequence (Table 1). Primers F18 (conserved mouse and hamster sequence) and R28 (rat sequence) gave a 853 base pair (bp) 5′ fragment, primers F11 and R10 (conserved mouse and hamster sequence) gave a 489 bp 3′ fragment and primers F18 and R10 gave a 1182 bp fragment corresponding to almost the entire coding region. The extreme 5′ sequence was obtained by PCR amplification from the constructed library using a gene-specific primer (R25 – rat sequence) and a vector-specific primer (T3). Similarly the 3′ sequence was obtained with primer F11 and the vector primer M13F

Table 1 Primer sequences used in PCR and sequencing

Primer	Sequence 5′–3′
F18	TGTCTCCTGCTGTGTGGAGGC
F11	CCTTGTGGATGGTCTATCTGAGAGC
R28	GGATCTGGTTAGCTGGAACGAC
R10	GCCAGGGTCAGGAATGCCACTG
R25	CTACGTCAGTATCCACTTGCTACG
T3	AATTAACCCTCACTAAAGGG
M13 Forward	TGACCGGCAGCAAAATG
T7	TAATACGACTCACTATAGGGCGA
SP6	ATTTAGGTGACACTATAGAATAC

(Table 1). Two microlitres of cDNA was amplified in a standard 50 µl PCR reaction with the following parameters: 95 °C for 1 min, 58 °C for 1 min, 72 °C for 1 min for 35 cycles followed by a final 7 min extension at 72 °C. PCR products were electrophoresed on 1% agarose gels, band excised and purified through a Qiagen PCR clean-up column. All purified products were ligated into pGEM T-easy cloning vector (Promega) at 4 °C overnight and transformed into XL-1 Blue competent cells.

Sequence analysis

All cloned fragments were sequenced on an ABI 737A automated sequencer using the Taq Terminator DNA Sequencing Kit (Perkin Elmer) and either T7 or SP6 sequencing primers (Table 1) or primers designed to internally generated rat sequences. Sequence data were analysed using a GeneJockey II (Biosoft, Cambridge).

RNA transcripts of rat ZP3

A 161 bp fragment of rat ZP3 was amplified from rat cDNA using primers F11 and R28. Purified PCR product was cloned into pGEM T-easy vector (Promega) containing T7 and SP6 promoters and sequenced to verify the correct PCR product and orientation of the insert. Purified plasmids were linearised with either *Sph*I or *Sac*I restriction enzymes (Promega). Sense and antisense riboprobes were transcribed from these promoters using the Riboprobe Combination System (Promega) and Dig-11-dUTP (Boehringer) as the detection label.

In situ hybridisation

All reagents and solutions were treated with 0.1% DEPC (diethylpyrocarbonate) water prior to use and processed according to Millar *et al.* (1993) with the following modifications. Rat ovaries were removed from 14-day-old pups and fixed in 4% paraformalde-

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hyde/PBS (phosphate-buffered saline) pH 7.4 for 4 h at room temperature. Tissues were processed and mounted in wax. Sections were cut and dried onto glass slides at 50 °C overnight. Fixed sections were dewaxed in xylene for 10 min until clear then rehydrated in 100%, 95% and 75% ethanol for approximately 30 s each. Tissues were permeabilised in 0.2 N HCl for 20 min, washed twice in DEPC water then incubated in proteinase K buffer (0.1 M Tris/HCl pH 8.0, 0.05 M EDTA) with 2 µg/ml proteinase K (Sigma) for 20 min at 37 °C. Proteinase K digestion was stopped by transferring the slides to 0.2% glycine/DEPC water at 4 °C for 10 min. Slides were washed in 80 mM triethanolamine buffer then acetylated in acetic anhydride (875 µl acetic anhydride/350 ml triethanolamine buffer) for 10 min at room temperature before rinsing in 4× STE buffer (40 mM Tris/HCl pH 7.4, 40 mM NaCl, 4 mM EDTA). Slides were prehybridised in buffer containing 50% formamide, 4× STE buffer, 1× Denhardt's solution, 0.125 mg/ml yeast tRNA, 0.125 mg/ml salmon sperm DNA for 2 h at 50 °C in a humidified oven. Slides were then hybridised overnight in fresh prehybridisation buffer containing 10% dextran sulphate and Dig-11-dUTP labelled sense or antisense riboprobe (preheated to 75 °C for 5 min) at 50 °C in a humidified oven. After hybridisation, sections were washed in 4× STE for 10 min then incubated in RNAse buffer (10 mM Tris pH 8.0, 500 nM NaCl, 1 mM EDTA) containing 20 µg/ml RNase A at 37 °C for 30 min. Slides were washed twice in 2× SSC (0.15 M sodium chloride and 0.015 M sodium citrate) then incubated in 0.1× SSC/30% formamide at 40 °C for 20 min before being transferred to TBS (100 mM Tris/HCl pH 7.5, 0.9% w/v NaCl) for 5 min.

Signal detection was achieved by blocking sections in TBS/sheep serum (1:5) for 30 min then incubating with a 1:200 dilution of anti-DIG AP antibody (Boehringer) in TBS/sheep (1:5) serum for 2 h at room temperature. Slides were washed twice in TBS for 15 min each then washed in 100 mM Tris/HCl pH 9.7, 100 mM NaCl, 50 mM $MgCl_2$ for 5 min. Colour development was obtained by incubating slides in 10 ml of 100 mM Tris/HCl pH 9.7, 100 mM NaCl, 50 mM $MgCl_2$ plus 45 µl nitroblue tetrazolium (NBT) and 35 µl 5-bromo-4-chloro-3-indolyl phosphate (BCIP) for 2–24 h at room temperature in a dark humid box. Colour development was stopped by immersing the slides in water. Sections were counterstained in haematoxylin then mounted with Pertex (Cellpath).

Results

Total RNA and poly(A+) mRNA isolated from rat ovaries were used for both cDNA synthesis and construction of a cDNA library. PCR primers designed on mouse and hamster ZP3 conserved sequences were successfully used to amplify the majority of the rat ZP3 gene in three fragments including nearly the full length coding region. The 5′ clone contained the sequence from nucleotide position 48 to 901, the 3′ clone contained the sequence from nucleotide 741 to 1230 and the large clone contained the sequence from nucleotide 48 to 1230. Sequence analysis was performed on both sense and antisense strands on all clones. Due to the difficulty in obtaining the very 5′ sequence of rat ZP3, this portion of the sequence was obtained by PCR of a fragment from the constructed library with a vector primer T3 and a gene-specific primer, R25. This gave a product of 371 bp that contained the ATG start site and 21 bp of untranslated sequence which also contained an exact Kozak consensus sequence (ACCATGG). Similarly the 3′ sequence was obtained using M13 forward primer and F11 which gave a stop codon and the poly(A) tail (Fig. 1).

The full sequence gives an open reading frame of 1272 bp that is identical in size to the mouse ZP3 and 6 bp longer than the hamster sequence. Similarities also exist with mouse, human and hamster in that rat ZP3 does not contain a 3′ untranslated region since the translational stop codon TAA coincides with the RNA polyadenylation signal AATAA (Kinloch *et al*., 1988, 1990; Ringuette *et al*., 1988; Chamberlin & Dean, 1990; Thillai Koothan *et al*., 1993).

The predicted rat ZP3 polypeptide consists of 424 amino acids with a predicted molecular mass of 46 815. A multiple alignment of rat, mouse, hamster and human sequences in shown in Fig. 2. The primary rat amino acid sequence has a high degree of homology with both the mouse (91%) and hamster (78.5%) homologues but less with the human (66%). The disparity between rat and mouse sequences is randomly distributed throughout the sequence with the exception of a small stretch of 8 amino acids immediately downstream of a series of 4 consecutive serine residues ($ZP3^{331–334}$) that are believed to represent the '*O*'-linked glycosylation sites responsible for sperm–zona recognition (Kinloch *et al*., 1995). Rat, mouse, hamster and human have five, five, three and three potential sites for *N*-linked glycosylation, respectively. Mouse and rat have identical *N*-glycosylation sites: hamster has all its three sites in the same place as rat and mouse although the most 3′ site has a different consensus sequence. Human has two out of three *N*-glycosylation sites in the same place, with the most 5′ site different and the most 3′ site having a different consensus sequence (Fig. 2).

Rat and mouse ZP3 sequences contain 17 and 16 cysteine residues respectively at identical positions except for the first cystine residue, which is absent in mouse. In addition, rat ZP3 shows the same abundance of serine and threonine residues as mouse ZP3 with all

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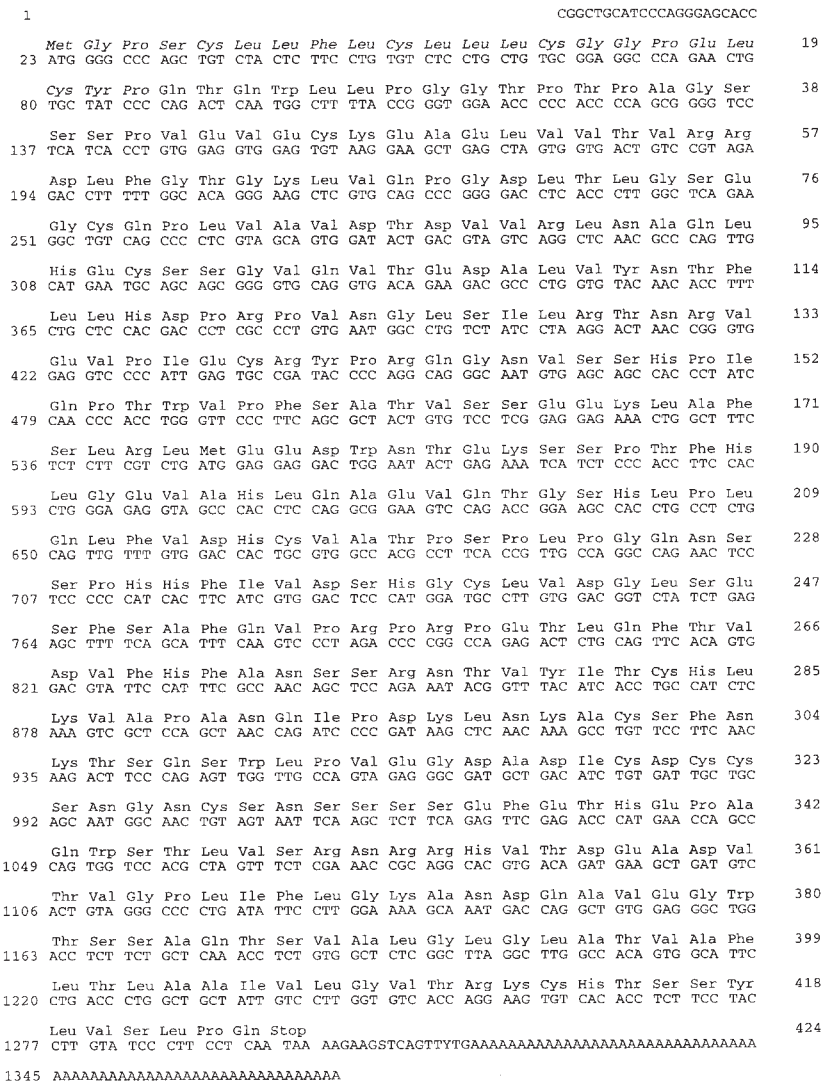

Figure 1 Primary sequence of rat ZP3 mRNA and polypeptide. The bottom line gives the nucloetide composition of rat ZP3 while the top line gives the amino acid sequence translated from a single open reading frame. The putative signal peptide is italicised. The nucleotide sequence is numbered on the left and amino acids on the right.

but one of the threonine residues (97%) and 39 of the 45 serine residues (87%) conserved. In addition, rat ZP3 is rich in proline residues compared with the average vertebrate protein and 94% of these are conserved in the mouse and rat ZP3 sequences (Kinloch *et al.*, 1990). Hydropathicity plots of rat ZP3 (Fig. 3) revealed the presence of two hydrophobic peaks, at the N- and C-termini of the molecule respectively, both of which appear to be a consistent feature of ZP3 in all species examined to date (Ringuette *et al.*, 1988; Thillai Koothan *et al.*, 1993). Homology with the ZP3 sequence from other species would suggest that the N-terminal hydrophobic domain falls within a signal peptide (Fig. 2) such that the mature rat ZP3 protein would commence with a glutamine residue as in the mouse, hamster and human homologues (Fig. 2). The predicted cleavage site for the rat ZP3 signal peptide is an unusual variant on the '3, –1 rule' developed by von Heijne (1986). This rule predicts that the residue at position –1 should be small, while the residue at position –3 must not be aromatic, large or polar. Furthermore proline should be absent from positions –3 through +1. Examination of the rat ZP3 sequence reveals that while position –3 is satisfied with a cysteine residue that is common to all ZP3 sequences examined to date, the proline in position –1 is unusual. Intriguingly, this unusual proline residue in close proximity to the cleavage site is also seen in human and marmoset, both of which terminate their ZP3 signal sequences with CYP, in exactly the same fashion as the rat homologue.

The C-terminal hydrophobic domain is down-

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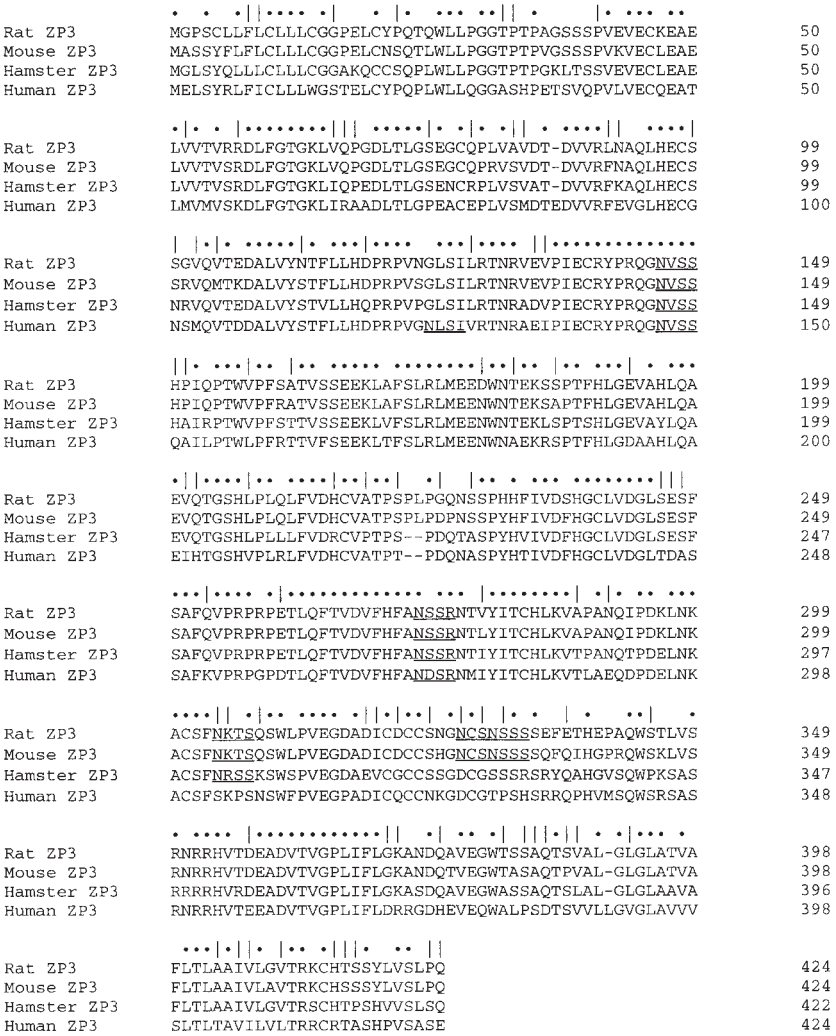

Figure 2 Multiple alignment of rat, mouse, hamster and human ZP3 sequences. Dots indicate amino acids that are completely conserved across all species; vertical lines indicate residues that are highly conserved. *N*-glycosylation sites are underlined.

stream of a TGF-β type III receptor-like region and immediately upstream of a short stretch of positively charged amino acids. Such an arrangement is typical of membrane-bound proteins featuring a hydrophobic transmembrane region followed by a short cytoplasmic tail (Bork & Sander, 1992). The significance of this arrangement is not clear at present but it may relate to the interaction between ZP3 and the other ZP glycoproteins or possibly the processing of the molecule on the surface of the oocyte.

In situ hybridisation analysis with an antisense RNA probe from rat ZP3 cDNA (nucleotides 741 to 901 bp) localised the mRNA to the oocyte of actively growing follicles and not any other cell type examined within the ovary, including the granulosa cells and primordial follicles. No signal was detected with the corresponding sense probe control (Plate 1, facing p. 32).

Discussion

The ZP3 sequence has been highly conserved during evolution giving a protein of 424 amino acids in the rat, mouse and human and 422 amino acids in the hamster (Ringuette *et al.*, 1988; Chamberlin & Dean, 1990; Kinloch *et al.*, 1990; Paterson *et al.*, 1995). Homologoues proteins of around 420 amino acids have even been detected in carp, goldfish and medaka (Chang *et al.*, 1996). Despite such conservation, detailed comparison of the primary amino acid sequences of these molecules reveals the expected phylogenetic relationships, for while closely related species show high levels of ZP3 sequence homology (carp and goldfish ZP3, 92%; rat and mouse ZP3, 91%) distantly related species to not (carp and mouse ZP3, 30%).

In terms of tissue specificity, ZP3 exhibits a degree of structural uniqueness (sperm/egg recognition)

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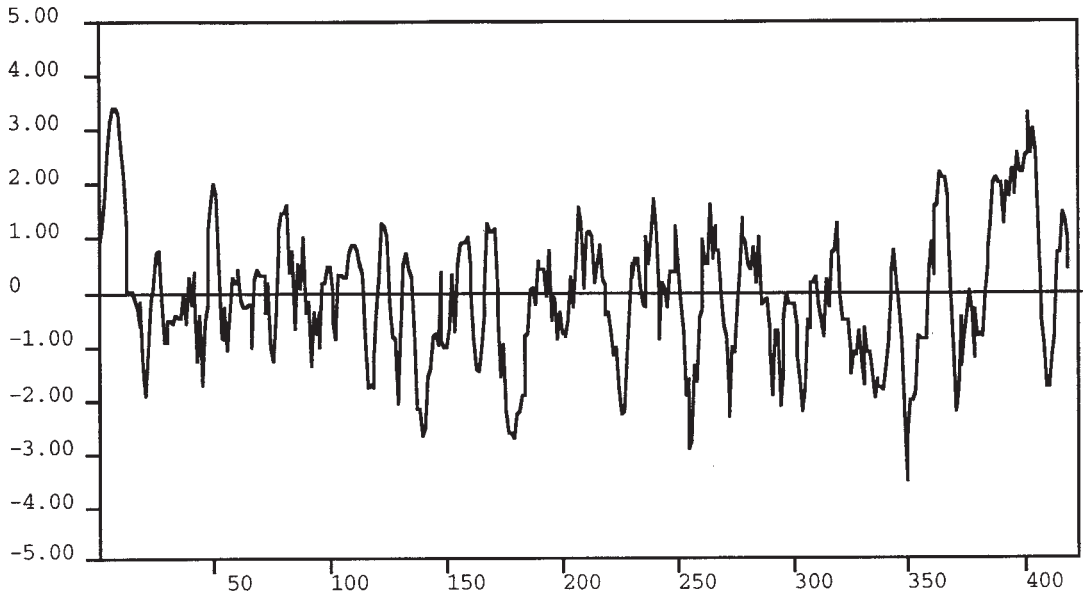

Figure 3 Hydropathicity plot of rat ZP3 (Kyte & Doolittle, 1982).

necessary for the support of a highly specialised biological function and the expression of specific, powerful epitopes. Despite such specificity, there is a 260 amino acid domain of this molecule which is shared with several other receptor-like molecules such as TGF-β receptor III, uromodulin and the major zymogen granule membrane glycoprotein GP-2 (Bork & Sander, 1992). This peptide domain may be involved in mediating the late stages of sperm–egg recognition, possibly through the involvement of receptors on the sperm surface of a type (tyrosine kinases) that generally associates with proteinaceous ligands (Leyton *et al.*, 1992). However, the initial stages of this recognition event are thought to be mediated by carbohydrate groups present on the surface of the zona pellucida.

Mouse ZP3 is known to contain specific serine/threonine (*O*-linked) oligosaccharides that represent an important binding site for spermatozoa (Bleil & Wassarman, 1988; Wassarman, 1990). Exon swapping and site-directed mutagenesis analyses have been used to demonstrate that the glycosylation of one or more of five serine residues clustered together in the peptide domain, $ZP3^{328–343}$, of exon 7 is required for this sperm binding activity. These serine residues occur upstream of a sequence of nine amino acids containing four cysteine residues, suggesting important structural constraints on the configuration of this region of the ZP3 molecule. Significantly, all these serine and cysteine residues are retained in rat ZP3, suggesting similarities in the structure of the sperm binding site consistent with the ability of mouse spermatozoa to bind to both mouse and rat zonae pellucidae (Aitken & Richardson, 1980). However, the fact that mouse spermatozoa cannot penetrate the rat zona pellucida also suggests that important differences in the biochemistry of sperm–egg interaction exist between these two species, possibly due to quantitative and qualitative differences in the specific configuration of the oligosaccharide side chains.

Glycosylation is certainly an important feature of the rat ZP3, for while the native molecule has a molecular mass of 115 kDa (Araki *et al.*, 1992) the amino acid sequence reported in this study predicts a polypeptide core of only 46 kDa. A majority of the oligosaccharide side chains associated with rat ZP3 must be *N*-linked because treatment of the native glycoprotein with *N*-glycanase, which removes all types of *N*-linked oligosaccharides, gave a proteinaceous core with a molecular mass approximating to the theoretical minimum (46 kDa). The fact that treatment of this deglycosylated molecule with *O*-glycanase induced an apparent further decrease of 2 kDa in the molecular mass of the glycopeptide suggests that *O*-linked oligosaccharide side chains are a feature of rat ZP3 (Araki *et al.*, 1992). However, the degree of *O*-linked glycosylation associated with rat ZP3 (2 kDa out of a 115 kDa molecule) is significantly less than that observed in the mouse homologue (9 kDa out of 85 kDa). Differences in the composition of these *O*-linked chains are also suggested by the observation that terminal galactose and fucose residues appear to be significant in mediating sperm–egg interaction in the mouse (Johnston *et al.*, 1988), whereas it is fucose, mannose and methyl-mannoside residues that appear to be of greater importance in the rat (Shalgi *et al.*, 1986).

The region of ZP3 covering the *O*-linked glycosyla-

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tion site ($ZP3^{318–352}$) is not just of importance in mediating sperm–egg recognition, it also contains major B- and T-cell epitopes relevant to the development of contraceptive vaccines (Gupta *et al*., 1994; Garza & Tung, 1995; Lou *et al.* 1995). In this context, it may be significant that there is a sequence of 8 amino acids immediately downstream of the 5 consecutive serine glycosylation sites ($ZP3^{335–342}$) where the sequence of rat ZP3 deviates significantly from every other species examined, sharing only a common histidine residue (His-339) with hamster, human and marmoset sequences and only 3 amino acids (Phe-336, His-339 and Pro-341) with the mouse ZP3 sequence. This unique sequence may be of importance in the development of defined peptide antigens for immunocontraceptive studies in the rat.

The demonstration that rat ZP3 is transcribed by oocytes present in the growing follicle population and not by primordial follicles or any somatic component of the ovarian follicles, may also be relevant to contraceptive vaccine development. In this respect the rat closely resembles the mouse (Kimura *et al*., 1994; Epifano *et al*., 1995) but differs from many other species examined. This immunocytochemical analyses employing an antibody raised against recombinant human ZP3 have demonstrated the presence of immunoreactive material in the primordial follicles of both human and rhesus monkey ovaries (Grootenhuis *et al*., 1996). Even in the marmoset 60% of primordial follicles were found to possess small islands of immunoreactive material (Grootenhuis *et al*., 1996). Furthermore in actively growing human and rhesus monkey follicles it was not just the ooplasm that stained for ZP3 antigens, the cytoplasm of the granulosa cells was also positive (Grootenhuis *et al*., 1996). The notion that both the oocyte and proximal granulosa cells can participate in the construction of a functional zona pellucida from the earliest stages of follicular development is supported by observations made in a number of other species. For example, bovine ovarian sections stained with antibodies against porcine ZP3α and ZP3β (the homologues of mouse ZP1 and ZP3, respectively) revealed weak expression of these antigens in primordial follicles but significant expression in both the oocyte and granulosa (particularly the corona radiata) cells of developing follicles (Sinowatz *et al*., 1995). It is possible that the inherent phagocytic activity of granulosa cells (Hartshorne, 1991) could account for the secondary uptake of zona antigens following the synthesis and release of these molecules by the oocyte. However, mRNA for porcine ZP3α has also been detected in both the oocyte and granulosa cells of developing follicles (Kolle *et al*., 1996). Moreover in the rabbit there is incontrovertible evidence that both granulosa cells and the oocyte are involved in the coordinate expression of a 55 kDa zona antigen, R55, the rabbit homologue of mouse ZP1 (Lee & Dunbar, 1993; Prasad *et al*., 1996; Grootenhuis *et al*., 1996).

In light of these data, the rat and the mouse would appear to be distinct from many other species in that ZP3 expression is confined to the oocytes of follicles recruited into the growing follicle pool. It is possible that these differences in the pattern of ZP3 expression between rodents and other species have an impact on the ovarian pathology observed following the induction of active immunity against ZP3. Thus in species such as the rabbit and marmoset monkey where ZP3 expression has been observed in primordial follicles, the development of immunity against this molecule is associated with a depletion of the primordial follicle population (Skinner *et al*., 1984; Paterson *et al*., 1992, 1996). However, in neither the rat (Aitken & Richardson, 1981; Aitken *et al*., 1981) nor the mouse (Rhim *et al*., 1992) has primordial follicle depletion been observed following the induction of immunity against ZP antigens. In the mouse there is clear evidence for a lymphocytic infiltration into the ovary following the induction of immunity against ZP3 (Rhim *et al*., 1992); however, the relationship between this response and primordial follicle loss has not been elucidated.

In conclusion, this study has defined the mRNA composition and primary amino acid structure of rat ZP3 and demonstrated conclusively that this gene is transcribed in the oocytes of actively growing follicles. The mRNA shows the characteristic absence of a 3′ non-coding region, which may be related to the developmentally regulated degradation of the ZP3 message in concert with oocyte maturation (Ringuette *et al*., 1988). The primary sequence predicts a 424 amino acid protein exhibiting a high level of homology with other rodent species with the exception of a specific region of the molecule adjacent to the sites of *O*-linked glycosylation that is probably linked to the expression of species-specific sperm receptor sites as well as the expression of unique ZP3 epitopes. The information contained in this paper should be of value in elucidating the biochemistry of conception in a species that is widely used as a model for the reproductive process.

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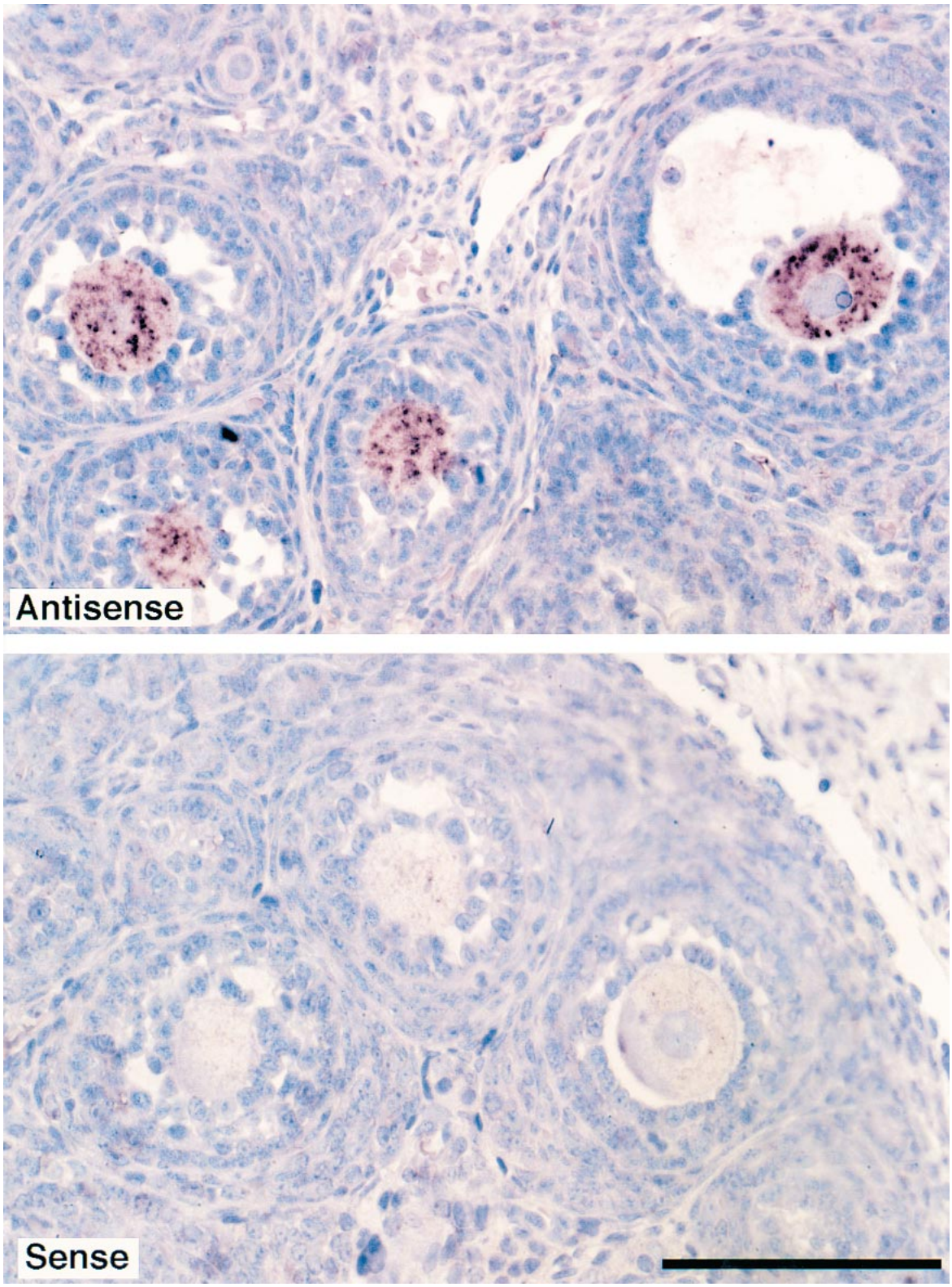

Plate 1 *In situ* hybridisation with antisense (top) and sense (bottom) riboprobes illustrating the localisation of the ZP3 transcripts within the rat ovary. Rat ZP3 mRNA is detected only in the oocytes of actively growing follicles. Scale bar represents 100 µm.

(*Facing p. 32*)

https://doi.org/10.1017/S0967199499000362 Published online by Cambridge University Press