Swann, Christine Anne; Hope, Rory Moulden; Breed, William Godfrey.
cDNA nucleotide sequence encoding the ZPC protein of Australian hydromyine rodents: a novel sequence of the putative sperm-combining site within the family Muridae, *Zygote*, 2002; 10 (4):291-299.

Zygote **10** (November), pp 291–299. © 2002 Cambridge University Press
DOI: 10.1017/S0967199402004021 Printed in the United Kingdom

cDNA nucleotide sequence encoding the ZPC protein of Australian hydromyine rodents: a novel sequence of the putative sperm-combining site within the family Muridae

Christine A. Swann, Rory M. Hope[1] and William G. Breed[2]*
Departments of Molecular Biosciences and Anatomical Sciences, University of Adelaide, South Australia

Date submitted: 13.2.02. Date accepted: 3.4.02

Summary

This comparative study of the cDNA sequence of the zona pellucida C (ZPC) glycoprotein in murid rodents focuses on the nucleotide and amino acid sequence of the putative sperm-combining site. We ask the question: Has divergence evolved in the nucleotide sequence of ZPC in the murid rodents of Australia? Using RT-PCR and (RACE) PCR, the complete cDNA coding region of ZPC in the Australian hydromyine rodents *Notomys alexis* and *Pseudomys australis*, and a partial cDNA sequence from a third hydromyine rodent, *Hydromys chrysogaster*, has been determined. Comparison between the cDNA sequences of the hydromyine rodents reveals that the level of amino acid sequence identity between *N. alexis* and *P. australis* is 96%, whereas that between the two species of hydromyine rodents and *M. musculus* and *R. norvegicus* is 88% and 87% respectively. Despite being reproductively isolated from each other, the three species of hydromyine rodents have a 100% level of amino acid sequence identity at the putative sperm-combining site. This finding does not support the view that this site is under positive selective pressure. The sequence data obtained in this study may have important conservation implications for the dissemination of immunocontraception directed against *M. musculus* using ZPC antibodies.

Keywords: Immunocontraception, Murid rodents, Species specificity, Zona pellucida

Introduction

An extracellular glycoprotein matrix, the zona pellucida, surrounds mammalian oocytes. This matrix plays a crucial role in both fertilisation and protection of the early conceptus. In the laboratory mouse (*Mus musculus*) a considerable amount of data on the zona pellucida and sperm–zona interactions have been accumulated (for review see Brewis & Wong, 1999; Prasad *et al*., 2000; Rankin & Dean, 2000; Rankin *et al*., 2000; Wassarman & Litscher, 2001). These data have provided a theoretical and experimental framework around which much of our present knowledge of how spermatozoa interact with the zona pellucida has been built.

In the laboratory mouse, the zona pellucida (ZP) consists of three sulfated and glycosylated proteins – ZPA, ZPB and ZPC – that are secreted by the growing oocyte. These three glycoproteins share a common domain, the 'ZP domain', a region of conserved cysteine residues, hydrophobicity and turn-forming residues (Bork & Sander, 1992). It has been proposed that the three glycoproteins assemble into an interconnecting fibrous protein complex, comprised of repeat units of ZPA and ZPC cross-linked by ZPB (Wassarman, 1988), and that the ZP domain plays an important role in the formation and maintenance of this structural arrangement (Wassarman, 1999).

During the process of fertilisation, in order for a spermatozoon to undergo sperm–egg fusion, it must first bind to, and penetrate, the ZP before it can enter the perivitelline space (Yanagimachi, 1994). In the laboratory mouse, ZPC has been identified as the

All correspondence to: Dr W.G. Breed, Department of Anatomical Sciences, University of Adelaide, South Australia 5005. Tel: +61 8 83035743. Fax: +61 8 83034398. e-mail: bill.breed@adelaide.edu.au
[1]Department of Molecular Biosciences, University of Adelaide, South Australia.
[2]Department of Anatomical Sciences, University of Adelaide, South Australia.
*Present address: Department of Anatomical Sciences, University of Adelaide, South Australia.

glycoprotein to which primary binding of spermatozoon occurs (Bleil & Wassarman, 1983). In the laboratory mouse, the process of sperm–zona pellucida (sperm–ZP) binding is mediated by oligosaccharides attached to specific *O*-linked (serine/threonine) residues on the glycoprotein surface of ZPC (for review see Wassarman, 1990; Shalgi & Raz, 1997). Exon swapping and site-directed mutagenesis experiments have identified a region of ZPC (Cys-328 to Gln-343) which contains the serine residues involved in primary sperm–ZP binding, named the 'sperm-combining site' (Kinloch *et al*., 1995; Chen *et al*., 1998). In particular, serine residues 332 and 334 have been found to be the critical *O*-linked oligosaccharide sites for primary sperm binding (Wassarman, 1999; Wassarman & Litscher, 2001).

Among different orders of mammals the region of the putative sperm-combining site has undergone considerable sequence divergence compared with the rest of ZPC (Wassarman & Litscher, 1995). Consideration of the synonymous/non-synonymous ratio of nucleotide acid substitutions suggests that this region is under positive selective pressure (Swanson *et al*., 2001). While it is the oligosaccharides on the ZPC polypeptide backbone that bind to the receptors on the sperm head surface, changes in the polypeptide primary structure in and around the sperm-combining site may influence the location and nature of the oligosaccharides added to the nascent ZPC (Nehrke *et al*., 1996).

Frequently, this process of primary sperm–ZP binding in the laboratory mouse is referred to as a species-specific phenomenon (Bleil & Wassarman, 1988; Wassarman, 1990, 1999; Cheng *et al*., 1994; Kinloch *et al*., 1995; Wassarman & Litscher, 1995, 2001; Liu *et al*., 1996; Snell & White, 1996; Shalgi & Raz, 1997; Wassarman *et al*., 1999), implying that the inability of sperm from one species to bind to the ZP of another species has the potential for being a reproductive isolating mechanism. However, the experimental evidence for species specificity of sperm–ZP binding is inconclusive (for review see Yanagimachi, 1994), and recently this assertion in relation to mammalian reproduction has been questioned (Jansen *et al*., 2001; Focarelli *et al*., 2001).

If primary sperm–ZP binding in the laboratory mouse is a species-specific phenomenon, and the site of primary sperm binding is the sperm-combining site, then it can be predicted that species specificity might be reflected in the amino acid sequence of this site, with closely related species exhibiting sequence divergence in order to facilitate a reproductive isolating mechanism.

To investigate the hypothesis of species specificity of sperm–ZP binding, this study compared the ZPC cDNA sequence, and in particular the region of the putative sperm-combining site, in five species of murid rodents. To that end, the complete cDNA coding sequence of the ZPC glycoprotein in two species of Australian hydromyine rodents, *Notomys alexis* and *Pseudomys australis*, and the putative sperm-combining site in a third, *Hydromys chrysogaster* (a species that diverged at the base of the hydromyine radiation; Watts & Baverstock, 1995), has been determined. These hydromyine rodents belong to a separate subfamily (the Hydromyinae) within the family Muridae, whereas the laboratory mouse (the model for sperm–ZP binding) and the laboratory rat, *Rattus norvegicus*, are members of the subfamily Murinae, whose wild ancestors evolved in Asia. The time of divergence of the hydromyine lineage from the murid in Asia is approximately 4 to 6 million years ago (Watts & Baverstock, 1995).

The aim of this comparative study was to determine the cDNA sequence of ZPC of these species of murid rodents with particular emphasis on the putative sperm-combining site; these findings may relate to the species specificity of sperm–ZP binding if this occurs.

Materials and methods

Nomenclature

The recommendations of Harris *et al*. (1994) for nomenclature of ZP genes and proteins are followed in this paper.

Animals

Notomys alexis (spinifex hopping mouse) females were supplied from a colony established at the University of Adelaide, whose wild ancestors were collected on Curtin Springs Station, in the south of the Northern Territory. *Pseudomys australis* (plains rat) females were supplied from a colony established by Dr C. Watts in 1968 and 1969, whose ancestors were collected from Marla Bore, northern South Australia and near Charlotte Waters in the south of the Northern Territory. All animals were originally housed in the Division of Animal Services, University of Adelaide and latterly in a private collection in the Adelaide Hills. The *Hydromys chrysogaster* (water rat) female was captured on the River Torrens in Adelaide, with permission from National Parks and Wildlife South Australia.

Total RNA

Animals used in this study were killed by asphyxiation with CO_2 and cervical dislocation. The ovaries were immediately removed, snap-frozen in liquid nitrogen,

and stored at –70 ºC. Total RNA was extracted from frozen ovarian tissue (approximately 50 mg per animal) using TriZOL (Invitrogen, Auckland), following the manufacturer's instructions.

cDNA synthesis

cDNA was synthesised from total RNA with either oligo(dT) primers or primer ZP3T17AP (Table 1) using Superscript II (Invitrogen, Auckland), following the manufacturer's instructions.

RT PCR

Regions of the *ZPC* gene were amplified by polymerase chain reaction (PCR) using either primers based on regions of conserved *ZPC* sequence in *M. musculus* and *R. norvegicus*, or primers designed from *N. alexis* and *P. australis* sequence as it became known (Table 1). All primers were purchased from GeneWorks (Adelaide)

PCR amplification was performed in a volume of 25 µl containing 1× *Taq* reaction buffer (10 mM Tris pH 8.3, 50 mM KCl and 0.1 mM dNTPs), 1.5 mM $MgCl_2$, 100 ng each of forward and reverse primer, 1 µg of cDNA and 0.5 U of *Taq* polymerase (BTQ-1 from GeneWorks, Adelaide). Reactions were cycled at 94 ºC for 30 s, 60 ºC for 45 s, and 72 ºC for 1 min using a PTC-2000 Peltier Thermal Cycler. Sequencing was performing using Big Dye Terminator chemistry and analysed on an ABI Prism 3700 DNA Automated sequencer. Forward and reverse sequences were assembled using BioEdit Sequence Alignment Editor (Hall, 1999).

PCR fragments not sequenced directly from the amplification products were cloned into pGEM-T $_{easy}$ (Promega, Sydney) and transformed into *E. coli* (strain DH5α) and subjected to blue/white colour selection. PCR fragments were radioactively labelled with ^{32}P using a Bresatec Gigaprime DNA labelling kit (GeneWorks, Adelaide) and used as probes in cloning experiments.

(RACE) PCR

To obtain full-length cDNAs, 5′-rapid amplification of cDNA ends (RACE) was performed using First Choice RLM-RACE (Ambion, Austin, TX) and following the manufacturer's instructions. Prior to (RACE) PCR, total RNA was treated with DNase I using DNA-free (Ambion, Austin, TX) following the manufacturer's instructions.

To obtain the 3′ end of the full-length transcript, cDNA was synthesised using the primer ZP3T17AP, and two-round PCR was performed using ZP3T17AP and then ZP3AP (a nested primer specific for the ZP3T17AP sequence) and a gene-specific primer. (Table 1)

Sequence comparison

ZPC cDNA and protein sequences were extracted from GenBank. Species names and accession numbers are: Cat (*Felis catus*) U05778; Cow (*Bos taurus*) U05775; Dog (*Canis familiaris*) U05780; Golden hamster (*Mesocricetus auratus*) M63629; Human (*Homo sapiens*) M60504; Bonnet monkey (*Macaque radiata*) X82639; Marmoset monkey (*Callithrix jacchus*) S71825; Laboratory mouse (*Mus musculus*) M20026; Laboratory rat (*Rattus norvegicus*) D78482; and Brandt's vole (*Microtus brandti*) AF304487.

Sequence alignments were carried out using the

Table 1 The sequences of forward and reverse primers utilised in this study and their corresponding position relative to laboratory mouse *ZPC* cDNA

Forward	5′	3′	mZPC position
FAZP	TGTCTCCTGCTGTGYGGAGGC		29–49
FA1ZP	GACGTGGTCAGGYTCAACGCC		258–279
FBZP	CCTTGTGGAYGGTCTATCTGAGAGC		717–741
FB1ZP[a]	ATACGCTCTATATCACCTGC		827–847
F5ZP[a]	GCCAAGCTATCTCCTCTTCC		6–26
Reverse			
RVAZP[a]	GGAGCAGAAAGGTACTGTACACC		349–326
RCZP	TCTGGTTAGCTGGCGCGAC		876–857
RD1ZP	GCCAGGGTCAGGAATGCCACTG		1208–1186
ZP3T17AP	GACTCGAGTCGACATCGATTTTTTTTTTTTTTTT		Poly T adapter
ZP3AP	GACTCGAGTCGACATCG		
gZP3CR[a]	GGAAGTCCACGATGAAGTGATAGG		709–686

[a]Designed from *N. alexis*/*P. australis* sequence as it became known.

CLUSTAL-W algorithm (Thompson *et al.*, 1994) and were edited and displayed using the computer program GeneDoc (Nicholas & Nicholas, 1997).

Results

Notomys alexis

Primers FAZP and RCZP, based on conserved *M. musculus* and *R. norvegicus* sequences, amplified a region of cDNA which was cloned and probed in a Southern blot analysis with a ^{32}P-labelled PCR fragment of size 160 bp (previously generated using the primers FBZP and RCZP). The probe hybridised to a fragment 810 base pairs in length. Primers FB1ZP and RD1ZP gave a 335 base pair fragment after PCR amplification. The terminal 3′ sequence of the cDNA was obtained by using primers ZP3AP and FBZP, generating a 497 base pair fragment. The 5′ end of the cDNA was obtained by (RACE) PCR using the primers supplied in the kit and RVAZP in a two-round PCR (following manufacturer's instructions). A 300 base pair fragment was obtained.

Pseudomys australis

The PCR primers FA1ZP and RCZP, designed on the basis of conserved ZPC regions in *M. musculus* and *R.norvegicus* rodents, amplified a 543 base pair cDNA fragment. Primers designed on the basis of *N. alexis* cDNA sequence, F5ZP and gZP3CR, gave a 668 bp fragment. The terminal 3′ sequence was obtained by using primers ZP3AP and FBZP, generating a 531 base pair fragment. The 5′ end of the cDNA was obtained by (RACE) PCR using the primers supplied in the kit in a two-round PCR, and RVAZP. A 137 base pair fragment was amplified.

Figs. 1 and 2 show the complete coding region of the ZPC cDNA for *N. alexis* and *P. australis* respectively. Both coding regions have an open reading frame of 1272 nucleotides and are identical in size to the coding region of the ZPC cDNA in the laboratory mouse *(M. musculus)* and laboratory rat *(R. norvegicus)*. The amino acid sequence is predicted to be 424 residues in length in respect of both hydromyine rodents.

Hydromys chrysogaster

Primers designed on the basis of conserved *M. musculus* and *R. norvegicus* sequences, FB1ZP and RD1ZP, amplified a 270 bp cDNA fragment that corresponded to the 3′ region of *ZPC* found in *N. alexis* and *P. australis* (Fig. 3).

Comparison of nucleotide and amino acid sequences

The degree of nucleotide sequence identity of the coding region of ZPC cDNA between *N. alexis* and *P. australis* is 98%. The predicted amino acid sequences are 96% identical. The two species of hydromyine rodents share 100% conserved cysteine residues with *M. musculus* and *R. norvegicus*, as well as hydrophobic regions, the furin cleavage site and the ZP domain. The two hydromyine rodents share an 88% and 87% level of amino acid sequence identity with *M. musculus* and *R. norvegicus* respectively.

The sperm-combining site

The predicted amino acid sequences corresponding to the putative sperm-combining sites are identical in the three species of hydromyine rodents. When this sequence is compared with that of various other mammalian species from different orders, it can be seen that in the murid rodents the first 7 residues of the putative sperm-combining site are fully conserved, including the five potential *O*-linked and the single potential *N*-linked, oligosaccharide sites (Fig. 4). Species distantly related to rodents show little sequence conservation in this region, with a variety of different arrangements of serine/threonine residues. Unique to the hydromyine rodents, however, are two additional potential *O*-linked oligosaccharide sites, Ser-336 and Ser-341. No cDNA sequences of ZPC in other species have serine/threonine residues at these positions.

Discussion

The process of speciation is a poorly understood phenomenon. However, a cause or consequence of speciation is the development of mechanisms that lead to reproductive isolation. One such mechanism that could prevent successful hybridisation between species is the inability of the sperm to bind to, and penetrate, the extracellular coat that surrounds the egg, the ZP. Certainly, for closely related externally fertilising species, reproductive isolation is evident at this level (Vacquier *et al.*, 1997). However, for internally fertilising species, such as mammals, selection to prevent hybridisation at that level may not occur due to the improbability of the sperm from one species reaching the site of fertilisation of another species' as a result of behavioural and/or morphological differences between species. Nevertheless, in spite of this, Wassarman *et al.*, working with the laboratory mouse, have repeatedly suggested that primary sperm–ZP binding is a species-specific event (Bleil & Wassarman, 1988; Wassarman, 1990, 1999; Moller *et al.*, 1990; Cheng

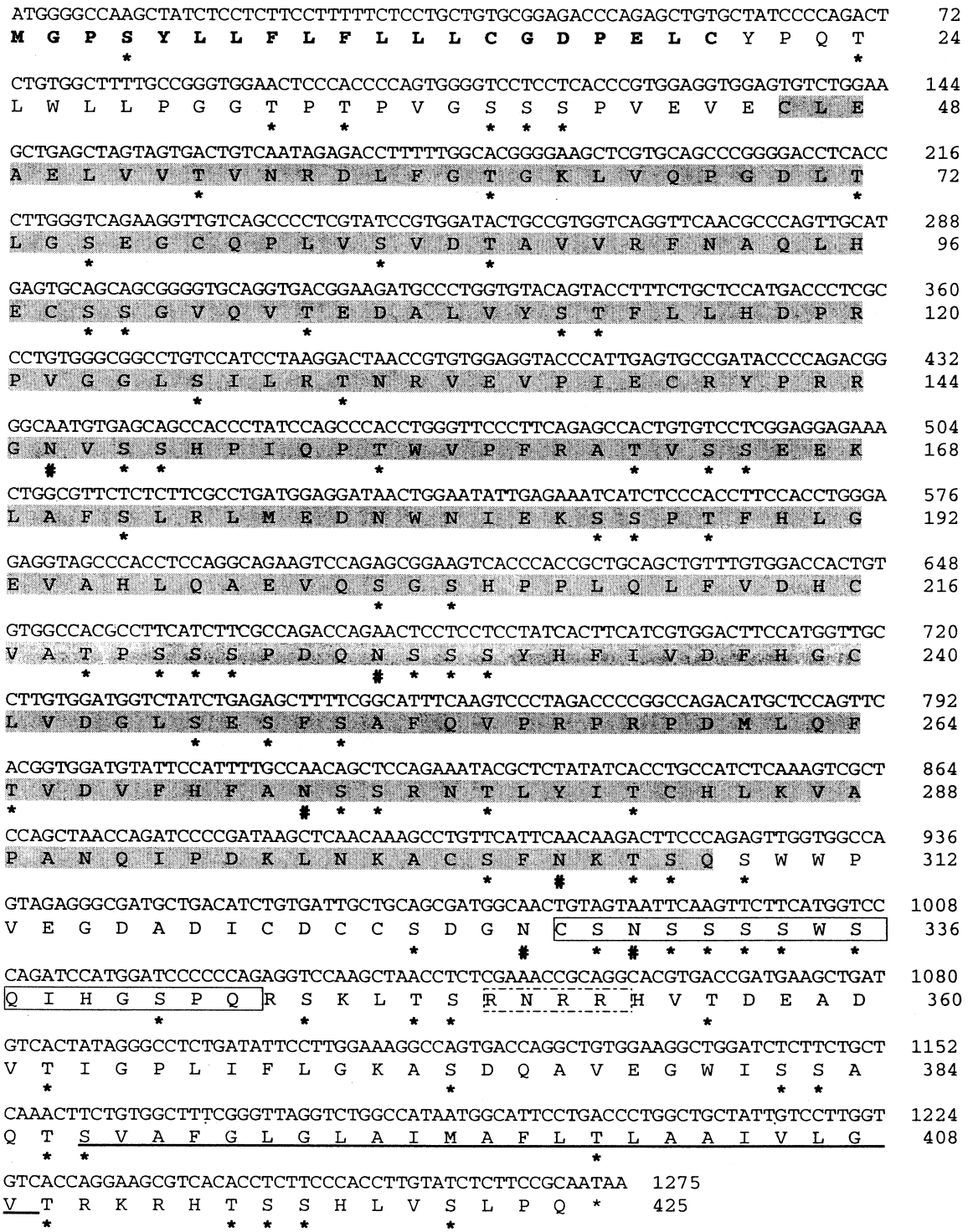

Figure 1 Nucleotide sequence of *Notomys alexis* ZPC cDNA and putative amino acid sequence (GenBank accession number AY078054) showing functional domains. Amino acids denoted in bold (residues 1 to 20) signify the putative peptide signal sequence. The ZP domain is shown in grey highlight. The putative sperm-combining site is boxed, and the furin cleavage sequence is shown in the dashed line box. The hydrophobic region is underlined. Potential *N*-linked oligosaccharide sites are denoted below the residue with a #, and potential *O*-linked oligosaccharide sites are denoted below the residue with an *.

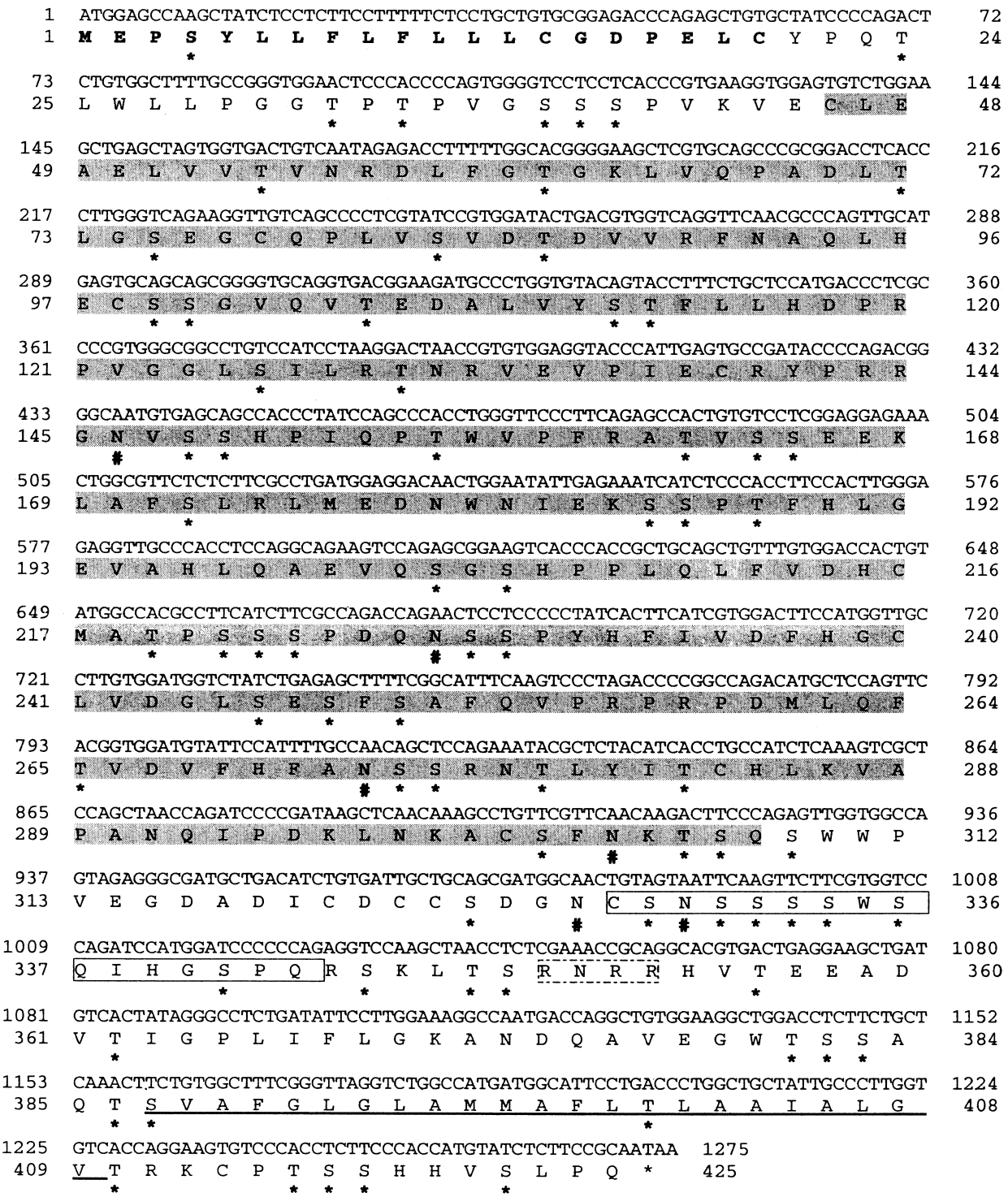

Figure 2 Nucleotide sequence of *Pseudomys australis* ZPC cDNA and putative amino acid sequence (GenBank accession number AY078055) showing functional domains. Amino acids denoted in bold (residues 1 to 20) signify the putative peptide signal sequence. The ZP domain is shown in grey highlight. The putative sperm-combining site is boxed, and the furin cleavage sequence is shown in the dashed line box. The hydrophobic region is underlined. Potential *N*-linked oligosaccharide sites are denoted below the residue with a #, and potential *O*-linked oligosaccharide sites are denoted below the residue with an *.

```
 880  CCCGATAAGCTCAACAAAGCCTGTTCGTTCAACAAGACTTCCCAGAGTTGGTGGCCAGTAGAGGGCGATGCT   951
 295  P  D  K  L  N  K  A  C  S  F  N  K  T  S  Q  S  W  W  P  V  E  G  D  A    318
                          *           *  *     *

 952  GACATCTGTGATTGCTGCAGCGATGGCAACTGTAGTAATTCAAGTTCTTCGTGGTCCCAGATCCATGGATCC  1023
 319  D  I  C  D  C  C  S  D  G  N  C  S  N  S  S  S  S  W  S  Q  I  H  G  S    342
                       *              *  #  *  *  *  *     *                 *

1024  CCCCAGAGGTCCAAGCTAACCTCTCGAAACCGCAGGCACGTGACTGAGGAAGCTGATGTCACTATAGGGCCT  1095
 343  P  Q  R  S  K  L  T  S  R  N  R  R  H  V  T  E  E  A  D  V  T  I  G  P    366
             *        *  *

1096  CTGATATTCCTTGGAAAGGCCAATGACCAGGCTGTGGAAGGCTGGACCTCTTCC  1149
 367  L  I  F  L  G  K  A  N  D  Q  A  V  E  G  W  T  S  S    385
```

Figure 3 Partial cDNA sequence of ZPC from *Hydromys chrysogaster* (GenBank accession number AY078056). The amino acid sequence in the box is the putative sperm-combining site. Potential *O*-linked oligosaccharide sites are denoted below the residue with an *. The nucleotides and amino acid residues are numbered according to their relative position on the cDNA sequence of *N. alexis*.

```
Notomys alexis              : C S N S S S S W S Q I H G S P Q
Pseudomys australis         : . . . . . . . . . . . . . . . .
Hydromys chrysogaster       : . . . . . . . . . . . . . . . .
Rattus norvegicus           : . . . . . . . E F E T . E P A .
Mus musculus                : . . . . . . . Q F . . . . P R .
Mesocricetus auratus        : . G S . . R . R Y . A . . V S .
Microtus brandti            : . . S . R Y . R P R A . A V A -
Felis catus                 : . G L Q G R . . R L S . L D R P
Canis familiaris            : . G L P G R . R R L S . L E R G
Bos taurus                  : . G I . G R . M R L S . R E G -
Homo sapiens                : . G T P . H . R R . P . V M S .
Callithrix jacchus          : . G T P . H A R R . P . V V S L
Macaque radiata             : . G T P . H . R R . P . V V S .
```

Figure 4 Amino acid sequence comparison of the putative sperm-combining site of ZPC among different species of mammals (residues Cys-328 to Gln-343). The residues highlighted in grey are the potential *O*-linked glycosylation sites.

et al., 1994; Kinloch *et al.*, 1995; Wassarman & Litscher, 1995, 2001; Liu *et al.*, 1996; Wassarman *et al.*, 1999). If this is the case it may be predicted that the amino acid sequence of the region of ZPC, which contains the *O*-linked oligosaccharide sites necessary for primary sperm–zona binding, may differ significantly between closely related species. A comparison of the ZPC sequence of murids thus tests this hypothesis.

The Australasian hydromyine rodent diverged from a common ancestor with the Asian murids at least 4 to 6 million years ago (Watts & Baverstock, 1995). The level of amino acid sequence identity of ZPC between *N. alexis* and *P. australis* suggests that the three-dimensional structure, and function, of ZPC in these two species of hydromyine rodents is very similar despite differences in ZP thickness (McGregor *et al.*, 1989) and sperm head morphology (Breed, 1997). Furthermore, *H. chrysogaster*, the third species of hydromyine rodent, which diverged at the base of the hydromyine radiation from the lineage that gave rise to *Pseudomys* and *Notomys* (Watts & Baverstock, 1995), shares a 100% level of amino acid sequence identity with *N. alexis* and *P. australis* between residues Cys-328 and Gln-343, the putative sperm-combining site. These three species share a unique arrangement of potential *O*-linked oligosaccharide sites within this region (Ser-336 and Ser-341) not shared with *M. musculus* and *R. norvegicus* (Phe-336 and Pro-341) nor seen in other mammalian species (Fig. 4).

While the particular regulation of glycosylation within different species is poorly understood, it may be that the arrangement of *O*-linked glycosylation sites influences the oligosaccharides present on the ZP surface. This in turn could affect the specificity of sperm–ZP binding (Wassarman, 1990). However, among the three closely related species of hydromyine rodents there are no amino acid differences in the putative sperm-combining site. It follows, therefore, that since there is no amino acid substitution in this region amongst hydromyine rodents then there is no support for the hypothesis of species specificity of sperm–ZP binding based on sequence homology of ZPC.

The high level of divergence at the putative sperm-combining site between distantly related species, and the claimed positive selection acting upon this region (Swanson *et al.*, 2001), suggest that this region of ZPC may be subject to more changes than the rest of the gene. There may be other selective pressures upon ZPC

that are not, as yet, known. However, this study has demonstrated that the sequence of the ZPC among closely related murids is certainly not under positive selective pressure, at least within the hydromyine murid rodents.

The determination of the complete coding sequence of the ZPC cDNA of two species of hydromyine rodents and the 3′ end of the same cDNA for a third hydromyine species, could have important implications for the development and practical application of an immunocontraceptive vaccine based on antibodies to the ZPC glycoprotein for controlling populations of wild house mice (Lou *et al*., 1995; Zhang *et al*., 1997; Jackson *et al*., 1998; Ylönen, 2001). Sequence identity between *M. musculus* and these hydromyine species, which are native rodent species of Australia, should be taken into account when consideration is given to dissemination of a vaccine within which *M. musculus* ZPC is incorporated to suppress fertility of populations of the introduced house mouse, *M. musculus*.

To further extend this comparative study, sperm–ZP binding experiments could be conducted whereby the sperm from one species is incubated with the oocytes of a closely related species. The present finding would predict that there would be no selectivity of sperm from one species of hydromyine rodents binding to the ZP of another. However, since in laboratory mice the *O*-linked sugar components are critical for sperm–ZP binding, a study of oligosaccharide ligands on ZPC of the hydromyine species needs to be performed to further determine the interspecific similarity in the sperm-combining site of these murid rodents.

Acknowledgements

We thank Chris Leigh, Scott Spargo, and David Wheeler for their invaluable technical assistance.

References

Bleil, J.D. & Wassarman, P.M. (1983). Sperm–egg interactions in the mouse: sequence of events and induction of the acrosome reaction by a zona pellucida glycoprotein. *Dev. Biol.* **95,** 317–24.

Bleil, J.D. & Wassarman, P.M. (1988). Galactose at the nonreducing terminus of *O*-linked oligosaccharides of mouse egg zona pellucida glycoprotein ZP3 is essential for the glycoprotein's sperm receptor activity. *Proc. Natl. Acad. Sci. USA* **85**, 6778–82.

Bork, P. & Sander, C. (1992). A large domain common to sperm receptors (Zp2 and Zp3) and TGF-β type III receptor. *FEBS Lett.* **300**, 237–40.

Breed, W.G. (1997). Evolution of the spermatozoon in Australasian rodents. *Aust. J Zool.* **45**, 459–78.

Brewis, I.A. & Wong, C.H. (1999). Gamete recognition: sperm proteins that interact with the egg zona pellucida. *Rev. Reprod.* **4**, 135–42.

Chen, J., Litscher, E.S. & Wassarman, P.M. (1998). Inactivation of the mouse sperm receptor, mZP3, by side-directed mutagenesis of individual serine residues located at the combining site for sperm. *Proc. Natl. Acad. Sci. USA* **95**, 6193–7.

Chen, A., Le, T., Palacios, M., Bookbinder, L.H., Wassarman, P.M., Suzuki, F. & Bleil, J.D. (1994). Sperm–egg recognition in the mouse: characterization of sp56, a sperm protein having specific affinity for ZP3. *J. Cell Biol.* **125**, 867–78.

Focarelli, R., La Sala, G.B., Balasini, M. & Rosati, F. **(**2001). Carbohydrate-mediated sperm–egg interaction and species specificity: a clue from the *Unio elongatulus* model. *Cells Tissues Organs* **168,** 76–81.

Hall, T.A. (1999). BioEdit: a user-friendly biological sequence alignment editor and analysis program for Windows 95/98/NT. *Nucleic Acids Symp. Ser.* **41,** 95–8.

Harris, J.D., Hibler, D.W., Fontenot, G.K., Hsu, K.T., Yurewicz, E.C. & Sacco, A.G. (1994). Cloning and characterization of zona pellucida genes and cDNAs from a variety of mammalian species: the ZPA, ZPB and ZPC gene families. *DNA Sequence* **4,** 361–93.

Jackson, R.J., Maguire, D.J., Hinds, L.A. & Ramshaw, I.A. (1998). Infertility in mice induced by a recombinant ectromelia virus expressing mouse zona pellucida glycoprotein 3. *Biol. Reprod.* **58,** 152–9.

Jansen, S., Ekhlasi-Hundrieser, M. & Töpfer-Petersen, E. (2001). Sperm adhesion molecules: structure and function. *Cells Tissues Organs* **168,** 82–92.

Kinloch, R.A., Sakai, Y. & Wassarman, P.M. (1995). Mapping the mouse ZP3 combining site for sperm by exon swapping and site-directed mutagenesis. *Proc. Natl. Acad. Sci. USA* **92,** 263–7.

Liu, C., Litscher, E.S., Mortillo, S., Sakai, Y., Kinloch, R.A., Stewart, C.L. & Wassarman, P.M. (1996). Targeted disruption of the *mZP3* gene results in production of eggs lacking a zona pellucida and infertility in female mice. *Proc. Natl. Acad. Sci. USA* **93,** 5431–6.

Lou, Y., Ang, J., Thai, H., McElveen, F. & Tung, K.S.K. **(**1995). A zona pellucida 3 peptide vaccine induces antibodies and reversible infertility without ovarian pathology. *J. Immun.* **155,** 2715–20.

McGregor, L., Flaherty, S.P. & Breed, W.G. (1989). Structure of the zona pellucida and cumulus oophorus in three species of native Australian rodents. *Gamete Res.* **23,** 279–87.

Moller, C.C., Bleil, J.D., Kinloch, R.A. & Wassarman, P.M. (1990). Structural and functional relationships between mouse and hamster zona pellucida glycoproteins. *Dev. Biol.* **137**, 276–86.

Nehrke, K., Hagen, F.K. & Tabak, L.A. (1996). Charge distribution of flanking amino acids influences *O*-glycan acquisition *in vivo*. *J. Biol. Chem.* **271**, 7061–5.

Nicholas, K. & Nicholas, H.B. Jr **(**1997). GeneDoc: a tool for editing and annotating multiple sequence alignments. Distributed by the authors: *EMBNEW NEWS* **4,** 14.

Prasad, S.V., Skinner, S.M., Carino, C., Wang, N., Cartwright, J. & Dunbar, B.S. (2000). Structure and function of the proteins of the mammalian zona pellucida. *Cells Tissues Organs* **166,** 148–64.

Rankin, T. & Dean, J. (2000). The zona pellucida: using molecular genetics to study the mammalian egg coat. *Rev. Reprod.* **5**, 114–21.

Rankin, T., Soyal, S. & Dean, J. (2000). The mouse zona pellucida: folliculogenesis, fertility and pre-implantation development. *Mol. Cell. Endocrinol*. **163**, 21–5.

Shalgi, R. & Raz, T. **(**1997). The role of carbohydrate residues in mammalian fertilization. *Histol. Histopathol.* **12,** 813–22.

Snell, W.J. & White, J.M. (1996). The molecules of mammalian fertilisation. *Cell* **85**, 629–37.

Swanson, W.J., Yang, Z., Wolfner, M.F. & Aquadro, C.F. (2001). Positive Darwinian selection drives the evolution of several female reproductive proteins in mammals. *Proc. Natl. Acad. Sci., USA* **98,** 2509–14.

Thompson, J.D., Higgins, D.G. & Gibson, T.J. (1994). CLUSTAL-W: improving the sensitivity of progressive multiple sequence alignment through sequence weighting, position-specific gap penalties, and weight matrix choice. *Nucleic Acids Res.* **22,** 4673–80.

Vacquier, V.D., Swanson, W.J. & Lee, Y.H. (1997). Positive Darwinian selection on two homologous fertilising proteins: what is the selection process driving their divergence? *J. Mol. Evol.* **44,** S15–22.

Wassarman, P.M. (1988). Zona pellucida glycoproteins. *Annu. Rev. Biochem.* **57,** 415–42.

Wassarman, P.M. (1990). Profile of a mammalian sperm receptor. *Development* **108**, 1–17.

Wassarman, P.M. (1999). Mammalian fertilization: molecular aspects of gamete adhesion, exocytosis and fusion. *Cell* **96,** 175–83.

Wassarman, P.M. & Litscher, E.S. (1995). Sperm-egg recognition mechanisms in mammals. *Curr. Top. Dev. Biol*. **30,** 1–19.

Wassarman, P.M. & Litscher, E.S. (2001). Towards the molecular basis of sperm and egg interaction during mammalian fertilization. *Cells Tissues Organs* **168**, 36–45.

Wassarman, P.M., Chen, J., Cohen, N., Litscher, E., Liu, C., Qi, H. & Williams, Z. (1999). Structure and function of the mammalian egg zona pellucida. *Mol. Dev. Evol*. **285**, 251–8.

Watts, C.H.S. & Baverstock, P.R. (1995). Evolution in the Murinae (Rodentia) assessed by microcomplement fixation of albumin. *Aust. J. Zool*. **43,** 105–18.

Yanagimachi, R. (1994). Mammalian fertilization. In *The Physiology of Reproduction* (ed. E. Knobil & J.D.Neill), pp. 189–317. New York: Raven Press.

Ylönen, H. (2001). Rodent plagues, immunocontraception and the mouse pox virus. *Trends Ecol. Evol*. **16**, 418–20.

Zhang, X., Lou, Y.-H., Koopman, M., Doggett, T., Tung, K.S.K. & Curtiss, R. III (1997). Antibody responses and infertility in mice following oral immunization with attenuated *Salmonella typhimurium* expressing recombinant murine ZP3. *Biol. Rep.* **56**, 33–41.