CSIRO PUBLISHING

Reproduction, Fertility and Development

Volume 10, 1998
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PGR2025-00050

Reprod. Fertil. Dev., 1998, **10**, 165–72

Cloning and characterization of the cDNA encoding the PH20 protein in the European red fox *Vulpes vulpes*

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Abstract. The PH20 protein is thought to play a crucial role in mammalian fertilization. The fox *PH20* homologue has been cloned from a testis cDNA library and the deduced protein sequence shows high levels of homology to PH20 proteins isolated from other species. Unlike other PH20 proteins the fox protein does not appear to be membrane associated through a GPI-linkage nor does it show the presence of a transmembrane domain at the C-terminus of the protein. It is in this region that the proteins appear to be least conserved. Immunolocalization studies on fox sperm show that the PH20 protein is located on the inner acrosomal membrane. Transcription of *PH20* in the fox is seasonally regulated, with the mRNA expressed during those months when spermatogenesis is at its peak. The PH20 sequence described in this paper has been submitted to the Genbank database and has the accession number U41412.

Extra keywords: testis, sperm, immunocontraception.

Introduction

In Australia the European red fox *Vulpes vulpes* has a significant impact on native wildlife and agriculture. The development of an immunocontraceptive vaccine has been identified as a suitable tool that can be integrated into the long-term management of fox populations (for review see Bradley *et al*. 1997). In this regard several fox sperm proteins have been evaluated as candidate vaccine antigens (Beaton *et al*. 1994; Beaton *et al*. 1995; Bradley *et al*. 1996). Primakoff *et al*. (1988, 1997) found that when guinea-pigs were immunized with PH20 purified from guinea-pig sperm, 100% effective and reversible contraception was obtained in both sexes. The sera of these females also blocked sperm–egg binding *in vitro*. PH20 has been shown to have hyaluronidase activity in a number of species including human, guinea-pig and monkey (Gmachl *et al*. 1993; Lin *et al*. 1994; Cherr *et al*. 1996). A soluble hyaluronidase is known to be released upon the acrosome reaction, digesting the hyaluronic acid present between the cumulus cells and enabling the sperm to migrate through the cumulus layer of the egg (Lin *et al*. 1994). More recently Li *et al*. (1997) showed that some PH20 is also released during sperm capacitation in the monkey. While blocking of PH20 hyaluronidase activity prohibits the sperm from entering the cumulus cell layer, it does not prevent the sperm from binding the zona (Lin *et al*. 1994). Recent studies by Hunnicut *et al*. (1996*a*) have indicated the possibility of two separate functions for PH20: a hyaluronidase activity and a non-enzymatic function involved in secondary sperm–zona binding.

These observations led us to assess the fox PH20 homologue for use as an immunocontraceptive vaccine for the control of fox fertility. As a first step towards this evaluation we report the cloning of fox *PH20* and its expression and localization during spermatogenesis.

Materials and methods

Construction and screening of fox testis cDNA library

The testis cDNA library was constructed with the lambda Zap kit (Stratagene, La Jolla, CA, USA), and used polyA$^+$ RNA from a fox testis exhibiting full spermatogenesis (July). Approximately 5×10^5 recombinant plaques were screened with a rabbit *PH20* cDNA fragment under the following conditions: 6×SSPE (Maniatis *et al*. 1989), 0·1% SDS, 20 mM Tris pH 7·5, salmon sperm DNA, 1× Denhardt's solution. A number of hybridizing clones were obtained from which a full-length clone of 1·9 kb was used for further characterization.

DNA sequencing

Single-stranded DNA was prepared from exonuclease III nested deleted clones and sequenced with the Pharmacia Autoread^{TM} sequencing kit by the di-deoxy chain termination method. The reactions were separated on a 6% acrylamide gel and analysed with the Pharmacia LKB automated laser fluorescent (ALF) DNA sequencer.

Northern analysis

All procedures for the isolation and electrophoresis of fox-testis RNA are as previously described (Cohen *et al*. 1993). For Northern

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analysis the gels were transferred to Hybond N^+ (Amersham) in 50 mM sodium hydroxide. Prehybridization was performed in 3×PE buffer (Maniatis *et al*. 1989), 4% SDS, 1% BSA, 10% dextran sulfate, for 2–3 hours at 68°C. Random-primed probes were generated with the Amersham Megaprime™ kit, added to the hybridization solution and hybridized at 68°C for approximately 18 h.

The hybridized filter was washed at room temperature in 2×SSC, 0·1% SDS for 20 min followed by a 20 min wash at 68°C.

Sperm isolation

Caudal fox sperm were isolated from the epididymis by finely chopping the cauda in sperm buffer (30 mM Tris-HCl, pH 7·4, 103 mM NaCl, 5 mM KCl, 3 mM $MgCl_2$, 0·4 mM EDTA). The sperm were collected and washed by centrifugation at 200*g* for 5 min. Finally the sperm were resuspended in sperm buffer and used for the experiments described below.

Immunohistochemistry

Testis. Testis samples were fixed in Bouin's fixative for 18–24 h followed by dehydration in 70% ethanol before being embedded in paraffin blocks. Tissue sections were cut at 4-μm thickness, placed on microscope slides and air dried. The sections were dewaxed in xylene, then washed several times with water and finally with PBS buffer (10 mM phosphate buffer, 150 mM NaCl, pH 7·4). All subsequent procedures are as described by Cohen *et al.* (1993). Pre-immune serum was used as a negative control.

Sperm. Fresh caudal epididymal sperm were air dried onto glass slides and fixed with 80% methanol before rehydration in PBS. The subsequent manipulations were performed at room temperature in a humidified chamber. The sperm were first blocked in PBS containing 3% BSA for 1 h at room temperature. The PH20 antiserum was diluted 1 : 1000 in 1% BSA in PBS, added to the slides and incubated for 1 h. The slides were washed 3 times in PBS and a 1 : 60 dilution of the sheep anti-rabbit FITC-conjugated (Silenus) secondary antibody in PBS, 1% BSA added. Slides were again incubated for 1 h before a final wash in PBS. The slides were mounted in 90% glycerol/10% PBS, viewed and photographed under confocal microscopy (MRC-1000; Bio-rad). Negative controls included incubation of sperm samples with pre-immune serum.

Generation of antibodies to a PH20 peptide

Hydrophathy and surface probability plot analyses were used to identify a number of potential antigenic regions within the PH20 protein. One peptide region (encompassing amino acids 147–177) was initially selected for synthesis (Fig. 1). Rabbits were immunized subcutaneously with 100 μg of unconjugated peptide: peptide 1 (WDSWRPNWARNWRPKHIYKEQSIDLAQQQHI) in Freund's complete adjuvant, followed by two subcutaneous boosts of 100 μg each in Freund's incomplete adjuvant. The serum antibody responses were monitored by Western blot analysis against both sperm extracts and the PH20-maltose-binding fusion protein. Sera from these animals were used for the immunological studies at a dilution of 1 : 1000. All animal manipulations were in accordance with the CSIRO Wildlife and Ecology Animal Experimental Ethics Committee requirements and followed NHMRC guidelines.

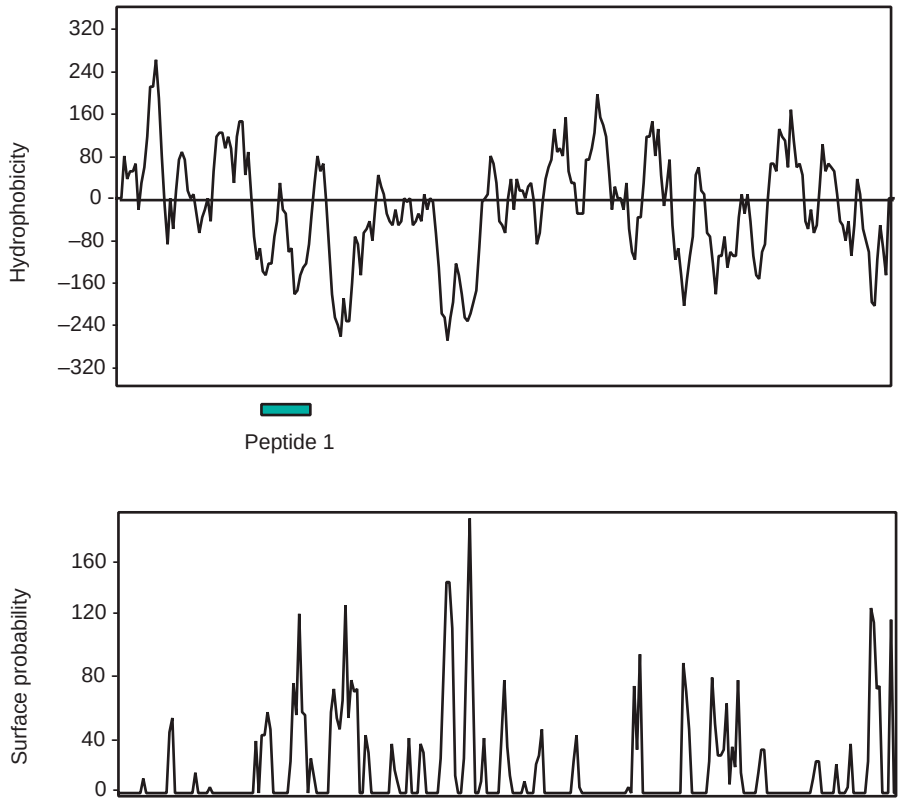


Fig. 1. Kyte-Doolittle hydrophobicity and surface probability analyses of fox PH20. The putative antigenic epitope, peptide 1 is represented by a box. Peptide 1 was used for the production of antisera used in the studies described.

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Western analysis

Proteins were extracted from caudal sperm in 10 mM CHAPS ([3-(3-cholamidopropyl)-dimethylammonio]-1-propane sulfonate; Sigma, St Louis, MO, USA) in PBS in the presence of protease inhibitors (200 μg mL^{-1} Aprotinin, 0·5 μg mL^{-1} Leupeptin, 0·5 μg mL^{-1} EDTA). Protein extracts were separated by SDS-PAGE electrophoresis (12% acrylamide) under both reducing and non-reducing conditions. The proteins were transferred onto PVDF (polyvinylidene difluoride) membrane (Millipore, USA) by the method of Towbin and Gordon (1984), and non-specific sites on the membrane blocked with 5% skim milk powder in TBS buffer (10 mM Tris-HCl, pH7·4, 50 mM NaCl) for 1 h at room temperature. Primary antibody was added in 1% skim milk powder in TBS and the filters incubated at room temperature for 1 h with gentle agitation. Unbound antibodies were removed by three 10-min washes in TBS. The second antibody, a 1:9000 dilution of anti-rabbit IgG peroxidase (Bio-Rad) in TBS containing 1% skim milk powder, was then added and the filter incubated for 1 h at room temperature. This incubation was again followed by three 10-min washes in TBS. Sites of antibody binding were visualized by incubation of the washed membranes in TBS containing 0·05% diaminobenzamidine and 0·01% H_2O_2. The reaction was terminated by washing the membrane in water.

Production of recombinant PH20

A fox PH20 recombinant fusion protein was produced by use of the maltose-binding protein (MBP) expression system (New England Biolabs). All subsequent procedures were performed according to the manufacturer's recommendations. The serum from rabbits immunized against the PH20 peptide was also tested against the fusion protein, thereby confirming the identity of the fusion protein and the specificity of the antibodies.

Results

Characterization of fox PH20

A 1·9-kb *PH20* clone was isolated from a fox testis cDNA library. The clone contains an untranslated 5′ leader sequence of 239 nucleotides. An open reading frame starts at nucleotide 240 with an ATG initiation codon and continues until the stop codon at nucleotide 1860 (Fig. 2). The open reading frame (1620 bp) encodes a protein of 540 amino acids with a predicted molecular weight of 62 kDa. Analysis of the 3′ untranslated region shows a consensus polyadenylation signal (AATAAA) at position 1860 which coincides with the stop codon.

Alignment of the deduced amino-acid sequence of fox PH20 to the proteins from six other species found in the Genbank database (Fig. 3), shows a high level of conservation between these species. Fox PH20 amino-acid sequence is 59% identical to human, 59% to monkey and 55% to rabbit, 51% to both guinea-pig and rat and 49% identical to mouse sequences.

Analysis of the amino-acid sequence of the fox PH20 protein reveals a hydrophobic N-terminal region, and there appears to be a signal sequence with a possible cleavage site between amino acids 34 and 35: CLT-QE (Nielsen *et al*. 1997).

The fox PH20 shows six potential Asn-glycosylation sites at amino acid residues 46, 103, 180, 226, 344 and 369, in common with the other PH20 proteins analysed (Fig. 2). Some putative sites for O-linked glycosylation can also be found at amino acid residues 56 and 273 (Gooley *et al*. 1991).

Recombinant PH20 from other species has been shown to possess hyaluronidase activity (Gmachl *et al*. 1993; Hou *et al*. 1996). In these species a number of potential hyaluronan binding sites have been identified in the N-terminal half of the protein. In the fox several putative hyaluronan binding domains are present at amino acids 121–128, 189–197, 197–205 and 280–288, although it is not known whether the expressed fox PH20 protein has hyaluronidase activity.

Expression of PH20 during spermatogenesis

The seasonal expression of *PH20* in the fox was studied by Northern blot analysis of testes taken at periods of both active and inactive spermatogenesis. The blot was probed with a 1·0-kb *EcoRI* fragment of fox *PH20* spanning the N-terminal region of the gene. A mRNA transcript of approximately 2·0 kb was detected in testis RNA samples taken from the months of May, June, July and August. No transcript was detected in RNA samples from testes sampled in April and September (Fig. 4).

Immunofluorescent-labelling studies were used to determine the localization of PH20 on fox sperm. These were performed on both air-dried and methanol-fixed caudal fox sperm with antiserum produced against the PH20 peptide. Antibody binding was restricted to the anterior head region, with intense fluorescence present within the acrosomal membrane compartment (Fig. 5). By contrast, no labelling was observed with air-dried sperm. In addition, no immunolabelling could be detected on testis sections from those months when spermatogenesis is most active (data not shown).

Immunological analyses by Western blot

A Western blot analysis of CHAPS-detergent extracts of whole-sperm proteins was conducted with serum raised against the fox PH20 peptide. Under reducing conditions a protein of approximately 60 kDa was identified on these blots (Fig. 6). Under non-reducing conditions a faster migrating band at 50 kDa was observed, suggesting probable disulfide-bond formation in the PH20 protein.

Discussion

In this study we report the cloning and partial characterization of the fox homologue to *PH20*. The clone was obtained initially through heterologous screening of a fox testis cDNA library, using a cDNA probe from rabbit *PH20*. A comparison of the sequence of fox PH20 with six PH20 proteins from other species shows a high level of sequence conservation. Fox PH20 shows the highest level of identity to human PH20, monkey

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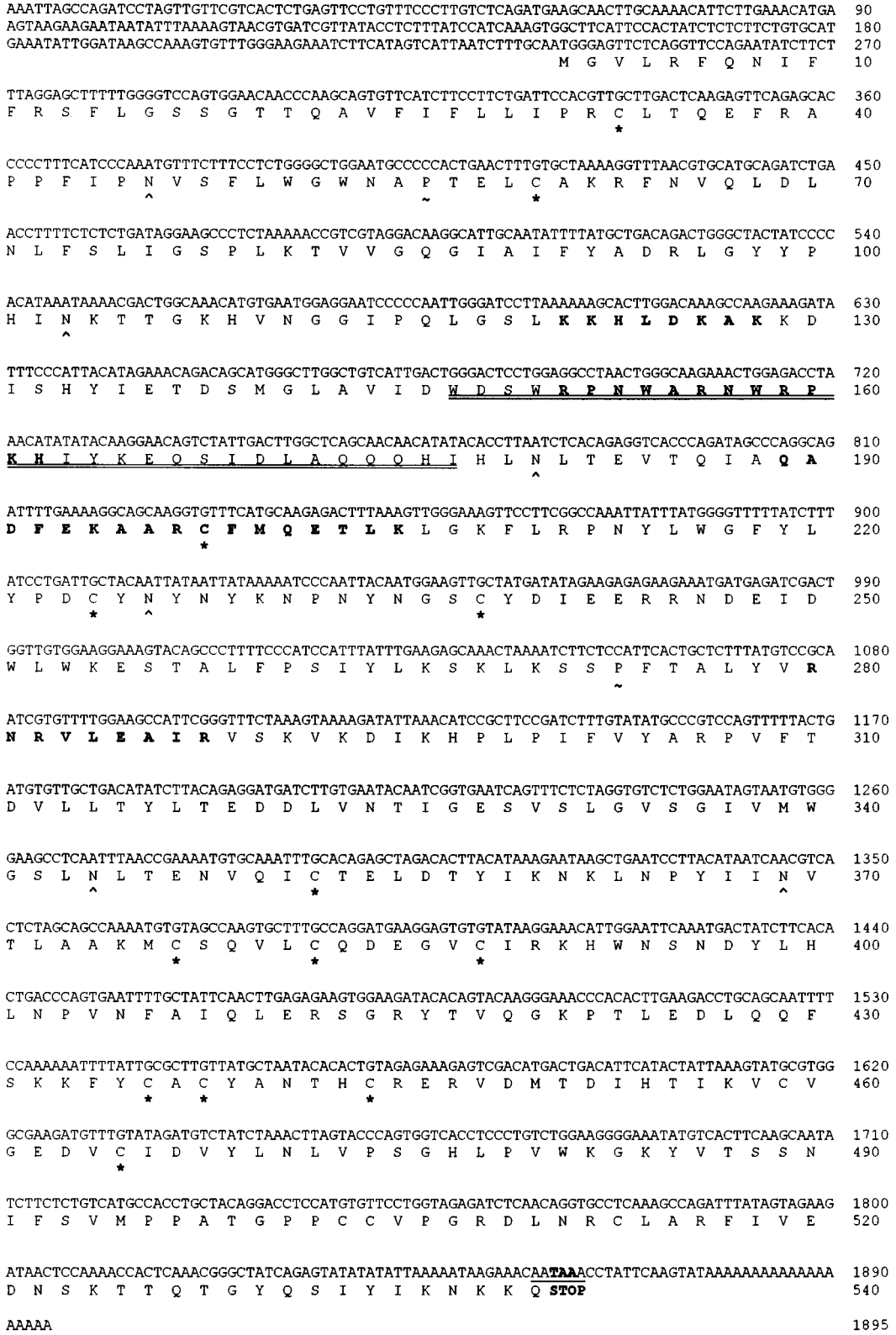


Fig. 2. Complete nucleotide and translated protein sequence of the cDNA encoding fox PH20. The polyadenylation signal is printed in bold and underlined. Potential N-linked glycosylation sites are indicated by ^. Potential O-linked sites are indicated by ~. Putative hyaluronan binding sites are printed in bold type. The peptide 1 used for the generation of anti-sera is double underlined. Conserved cysteine residues are indicated by *.

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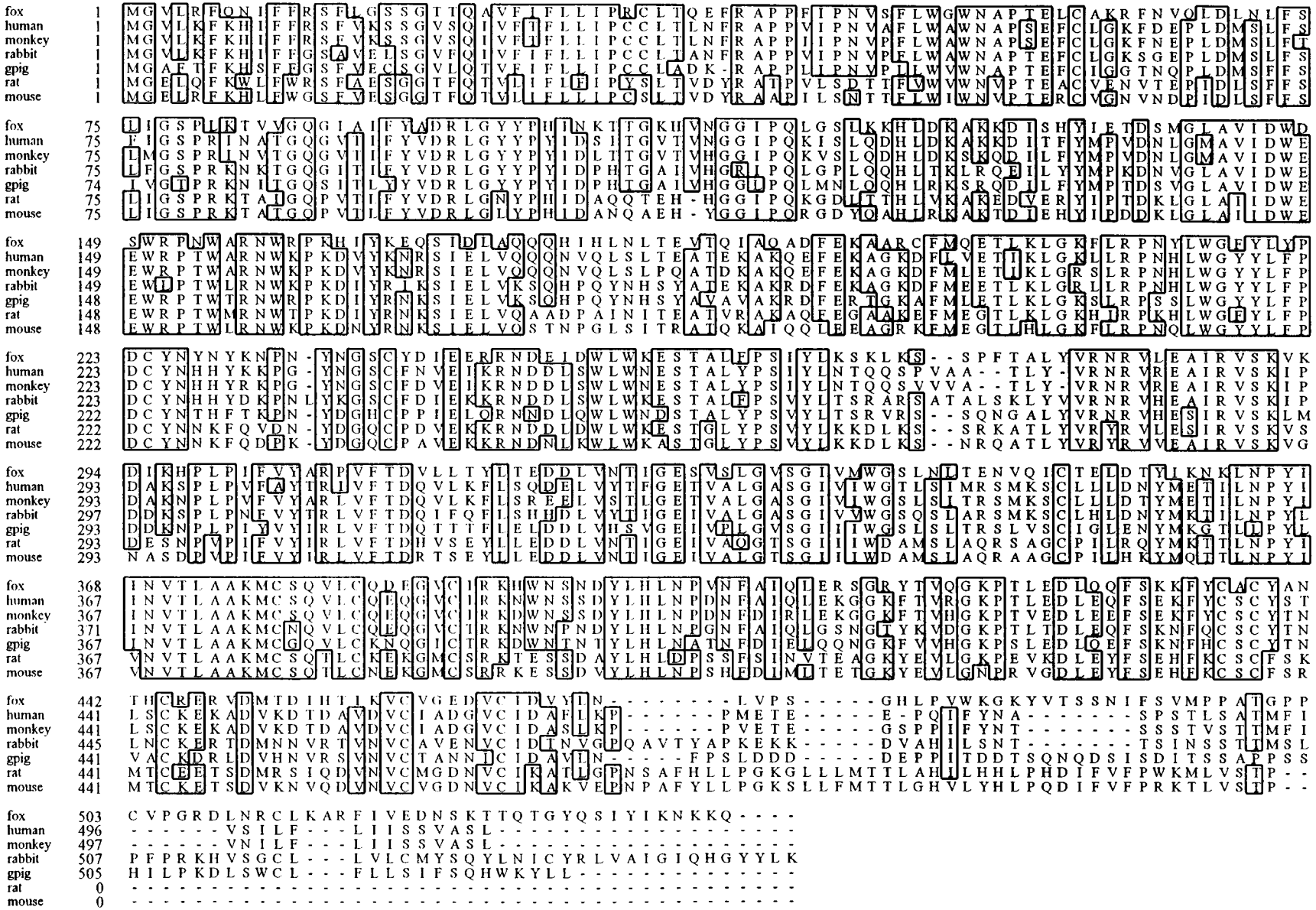


Fig. 3. Alignment of protein sequences from fox, human, monkey, rabbit, guinea pig, rat and mouse. Regions of amino-acid identity are boxed.

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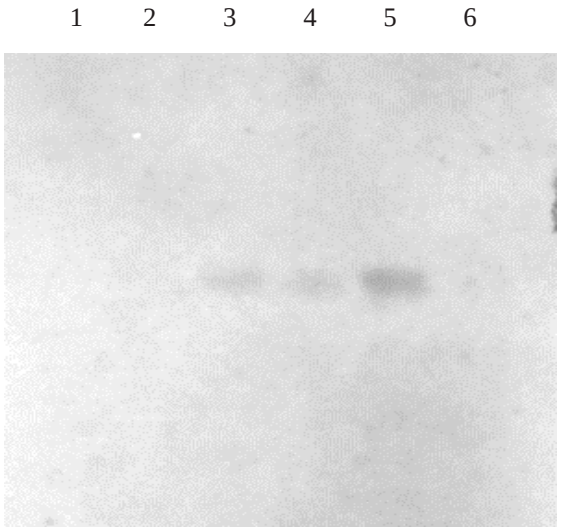


Fig. 4. Northern blot analysis illustrating the seasonal expression of fox *PH20* in fox-testis total RNA (10 μg) from the months April, May, June, July, August and September (Lanes 1–6 respectively). Hybridizations were performed in 3×PE, 4% SDS, 1% BSA, 10% dextran sulfate at 68°C, followed by stringent washes in 2×SSC, 0·1% SDS at 68°C.

and rabbit, with lower homology to guinea-pig, rat and mouse forms. The homology also extends to the spatial conservation of most of the cysteine residues found in all the species studied. Fox PH20 has 15 cysteine residues, of which 12 are conserved between the species studied in the analysis (Fig. 2). These residues are thought to be important in the formation of disulphide bonds necessary for the linking of the two functional domains proposed for the PH20 protein (Lathrop *et al*. 1990). The presence of disulfide-bond formation in fox PH20 is also indicated since this protein migrates faster under non-reducing than under reducing conditions.

The sequences appear to be most divergent at the C-terminus. This region is thought to be required for attaching the protein to the sperm membrane. Glycosyl-phosphatidylinositol-(GPI-) linked proteins often have a short hydrophobic C-terminal sequence, which serves as a signal for the GPI anchor (Furguson 1988). Short hydrophobic sequences have been found at the C-terminus of the human, guinea-pig and rabbit PH20 proteins. In human, monkey and guinea-pig, anchoring of the PH20 protein to the membrane has been shown to occur through a GPI linkage (Hunnicut *et al*. 1996*b*). Studies on the rat PH20 homologue 2B1, suggest that the PH20 protein is not membrane associated through an enzyme-cleavable GPI-linkage, but through a transmembrane domain (Hou *et al*. 1996). Fox PH20 contains neither a short hydrophobic C-terminal sequence indicating a possible GPI linkage (Fig. 1), nor a transmembrane domain at the far C-terminal region of the protein.

The glycosylated form of PH20 has been isolated from caudal guinea-pig sperm. Lathrop *et al*. (1990) showed that on digestion with an N-glycanase, guinea-pig PH20 migrated at a lower molecular weight as determined by SDS-PAGE, indicating that the protein is glycosylated. Fox PH20 protein also has a number of potential N-linked glycosylation sites where glycosylation may occur.

Studies on recombinant human, mouse, rat and monkey PH20 have shown that these proteins possess hyaluronidase activity (Gmachl *et al*. 1993; Lin *et al*. 1994; Hou

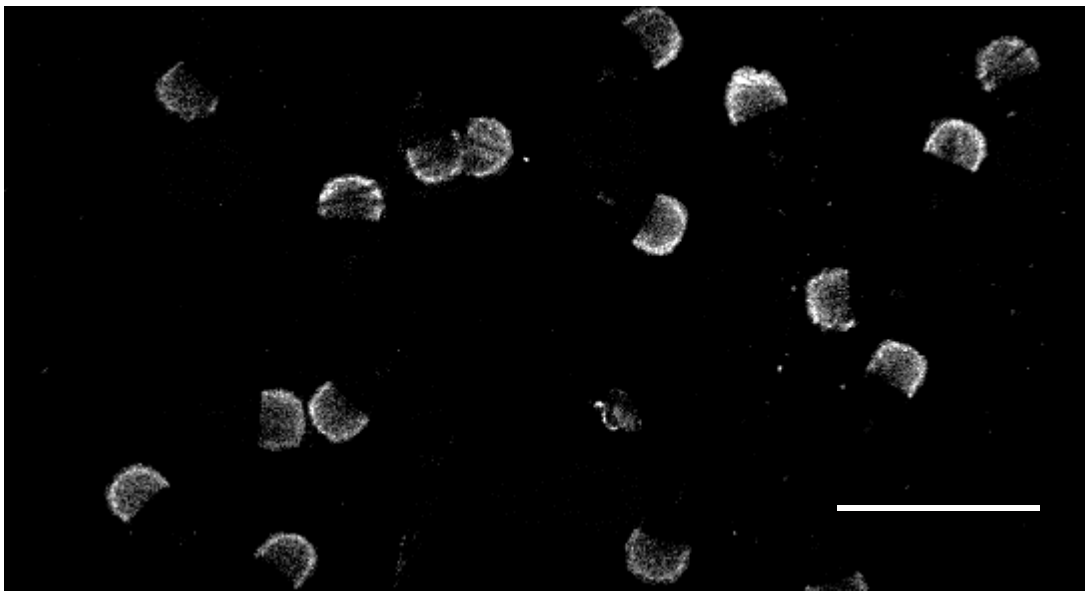


Fig. 5. Immunofluorescent localization of PH20 on methanol-fixed caudal fox sperm by the sera raised against the fox PH20 peptide (diluted 1 : 1000). Labelling on the sperm is localized to the inner acrosomal membrane. Bar, 20 μm.

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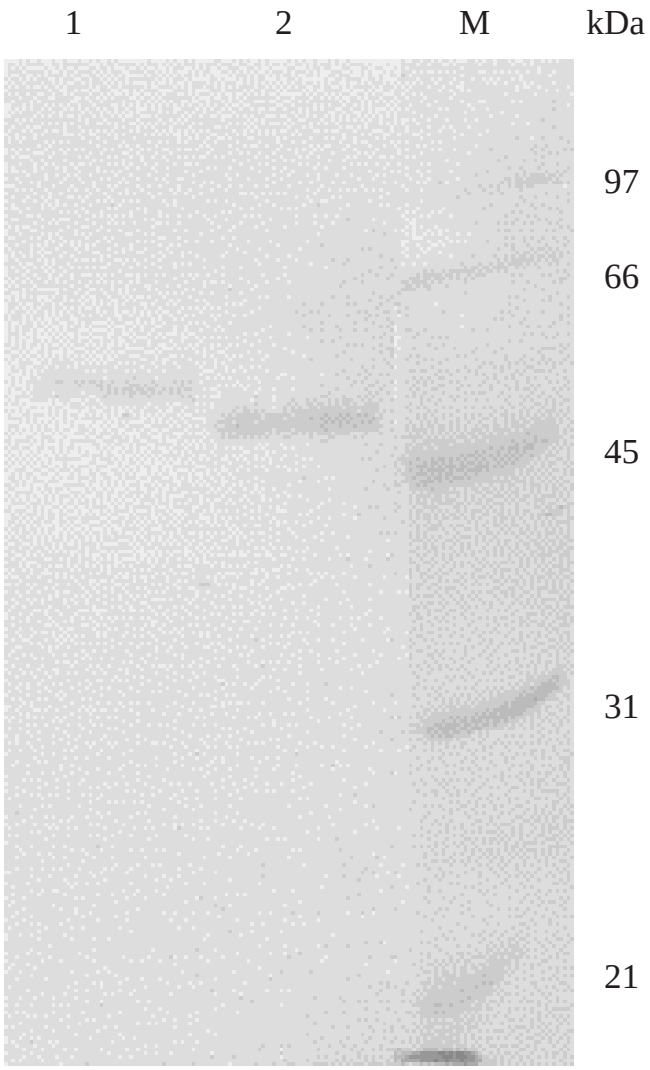


Fig. 6. Western blot analysis of fox PH20 on CHAPS extract of sperm under reducing (Lane 1) and non-reducing (Lane 2) conditions. The membrane was probed with anti-peptide serum raised in rabbits.

et al. 1996). All of these proteins contain a number of potential hyaluronan binding sites. Similarly, fox PH20 protein has a number of putative hyaluronan binding sites. One of these motifs (RPNWARNWR) is present between amino acids 151–159, and conforms to the binding motif BX_7B (where B is any basic amino acid and X any amino acid but not acidic and at least one basic) as originally proposed by Yang *et al*. (1994). This site is highly conserved between human, fox and monkey PH20 protein sequences but the rat and rabbit PH20 proteins show an amino acid change in the required basic residues. Alternative sites which fit the criteria of this motif are also present in several of PH20 sequences examined and in the fox can be found at amino acids 121–128, 189–197, 197–205 and 280–288 (Fig. 2). The last three were identified in the rat by Hou *et al*. (1996). However, several acidic residues can be found within these sites and these are thought to reduce the hyaluronan binding efficiency (Yang *et al*. 1994).

The guinea-pig PH20 protein has been analysed in more detail by Hunnicut *et al*. (1996*a*). They found that the protein is cleaved between the arginine (311) and serine (312) residues of the mature protein, giving rise to possibly two distinct functional domains, linked by a disulfide bridge. While the arginine and serine residues appear to be highly conserved in their relative positions in the six species examined (serine = alanine in mouse), the fox PH20 protein does not contain arginine and serine residues in these positions and therefore may not be cleaved at this position.

Recent studies have shown that the PH20 protein is located in different compartments in the sperm of different species (Myles and Primakoff 1997). For example, in mouse, human and monkey sperm it is located on the anterior or whole head, whereas in guinea-pig the protein appears to be localized on the posterior head of the sperm (Myles *et al*. 1984; Overstreet *et al*. 1995; Lin *et al*. 1994; cited in Myles and Primakof 1997). More recently Li *et al*. (1997) have shown that PH20 in the monkey can also be found on the inner acrosomal membrane. By contrast, the rat PH20 homologue, 2B1, is located on the tail and migrates to the head on capacitation (Jones *et al*. 1990).

Immunohistochemical localization of the fox PH20 protein by the PH20 peptide antiserum showed immunoreactivity on methanol-fixed sperm but not on air-dried sperm. Since methanol fixing permeabilizes the sperm, this observation indicates that the fox antigen is contained primarily within the intracellular acrosomal compartment of fox sperm.

Spermatogenesis in the European red fox is a seasonal event that in the southern hemisphere commences in mid-March (McIntosh 1963; Ryan 1976). Around this time there is a pronounced growth in size of the seminiferous tubule within the testis. Histological examination has shown that this size increase occurs simultaneously with cell differentiation in the testis and with the increase in number of spermatocytes within the tubules, with round and elongating spermatids evident by late April and sperm present within the tubule lumen by May (Cohen *et al*. 1993, Vandermark *et al*. 1993).

Northern analysis of *PH20* mRNA expression in fox testis sampled during the months of April to September identified a 2-kb transcript, consistent with expected size of the fox gene product. The *PH20* mRNA was first detected in May, albeit at a very low level. The *PH20* mRNA was then detected at higher levels in the months of July and August, as spermatogenesis reached a peak. No mRNA was detected in samples from the month of September, a finding consistent with histological

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studies which had shown that the tubules had regressed and spermatogenesis had undergone seasonal termination. Thus the temporal expression of PH20 reflects the seasonal nature of spermatogenesis in the fox.

Acknowledgments

This work was supported by the Australian Nature Conservation Agency (ANCA), CSIRO and the CRC for the Biological Control of Vertebrate Pest Populations. We thank Michael Holland for supplying the rabbit *PH20* clone, and Dr Peter Bird for his critical reading of the manuscript.

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Manuscript received 9 December 1997; accepted 2 July 1998

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