DISSERTATION

CLONING AND EXPRESSION OF A PORCINE ZONA PELLUCIDA GENE: AN APPROACH TO IMMUNOCONTRACEPTION

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CLONING AND EXPRESSION OF A PORCINE ZONA PELLUCIDA GENE: AN APPROACH TO IMMUNOCONTRACEPTION

Submitted by

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WE HEREBY RECOMMEND THAT THE DISSERTATION PREPARED UNDER OUR SUPERVISION BY GREGORY KENNETH FONTENOT ENTITLED CLONING AND EXPRESSION OF A PORCINE ZONA PELLUCIDA GENE: AN APPROACH TO IMMUNOCONTRACEPTION BE ACCEPTED AS FULFILLING IN PART REQUIREMENTS FOR THE DEGREE OF DOCTOR OF PHILOSOPHY.

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ABSTRACT OF DISSERTATION

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ABSTRACT

Immunization with native porcine zona pellucida (ZP) proteins has been shown to induce infertility in females of several species and is thus a potentially valuable method of contraception. However, more extensive testing and commercialization of such a vaccine has been hampered by the limited availability of ZP proteins from natural sources. Availability of recombinant ZP proteins should simplify production of a practical ZP vaccine. The objective of this research was to clone a porcine ZP gene and use it to develop a recombinant ZP vaccine for use in pet animals.

Polyadenylated RNA isolated from swine ovary was used to generate a cDNA library in the bacteriophage lambda gt11. This library was screened immunologically for ZP sequences using a polyclonal antiserum raised against solubilized porcine ZP. One immunoreactive clone, PZP, contained an insert of approximately 2.6 kb and was characterized further. The three Eco RI fragments constituting PZP were

isolated and subcloned into a plasmid vector. The amino acid sequence deduced from the nucleotide sequence of PZP is considered to represent 305 residues from the carboxy-terminal end of the ZP protein. A putative N-glycosylation site is present at residue 288 of this polypeptide. Comparison of the deduced amino acid sequence of PZP with deduced protein sequences from all of the other ZP proteins published to date failed to reveal significant homology.

To confirm that PZP represented a ZP mRNA, a 418 bp fragment of the cDNA was expressed as a fusion protein in E. coli and used to hyperimmunize a rabbit. Antibodies to the PZP fusion protein bound to ZP surrounding porcine oocytes, stained ZP in sections of porcine ovary and immunoprecipitated a porcine ZP protein that was tentatively identified as ZP2.

The PZP fusion protein was preliminarily evaluated as a vaccine in rabbits. Two groups of four adult rabbits were immunized three or four times with PZP2 fusion protein emulsified in either one of two adjuvants. Reproductive function was evaluated eight and sixteen weeks after initial immunization. In comparison to control rabbits, no effect of vaccination on reproductive function was observed.

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REVIEW OF THE LITERATURE

INTRODUCTION

Overpopulation of dogs and cats is an escalating problem in many areas of the world. Populations of dogs and cats in the United States, estimated to be approximately 50 million each, have been relatively stable during the last decade (Vet health care market for dogs and cats 1984), but only because approximately 20 million of these animals are euthanized or die from exposure, starvation or trauma each year (Hodge 1984; Nassar et al. 1984; Beck 1974). Most of those that die are healthy animals that have either been lost or purposely abandoned by their owners (Beck 1974). Dogs and cats have very efficient reproductive systems, and stabilization of pet populations by interfering with birth rate would require interception of approximately 4 million pregnancies each year.

In addition to the animal suffering that results from pet overpopulation, stray animals also generate a large economic burden to society and create substantial public health problems. Communities in the United States spend over $500 million annually to operate animal control programs (Moulton 1988). Stray animals are responsible for

1-3 million bites per year, resulting in an estimated annual expenditure of $90-270 million for medical bills and for 30,000 cases of rabies prophylaxis (Flowers 1979). It was calculated that in Houston, Texas in 1974, 38.6 tons of feces and 94,950 gallons of urine were excreted daily by stray dogs and cats (Flowers 1979).

It is clear that alternative methods for pet contraception are needed to humanely suppress populations of dogs and cats. One promising strategy that has been investigated in several species, but implemented with none, is based on immunizing animals against proteins of the zona pellucida (ZP). It has been repeatedly and consistently demonstrated that females immunized against these proteins develop an immune response that elicits infertility by blocking ovulation or fertilization. To date, the major impediment to development of a practical ZP vaccine has been the expense of obtaining those proteins from their natural source. With the advent of recombinant DNA technology, it is now possible to isolate the genes encoding these proteins and to develop a vaccine composed of recombinant protein. The studies described in this dissertation focus on isolating a gene for one of the porcine ZP proteins and on initial attempts to develop and test a recombinant ZP vaccine in rabbits.

BIOLOGY OF THE ZONA PELLUCIDA

Oogenesis, Folliculogenesis and Development of the Zona Pellucida

Oogenesis is a complex and lengthy series of genetic and morphologic changes that results in the transformation of immature, diploid gonocytes into highly specialized, haploid germ cells capable of being fertilized. Interesting differences in the timing of these events exist among species, but the fundamental changes that occur are common to all mammals (Mauleon and Mariana 1977; Levasseur 1979). Oogenesis is initiated in mammals prior to birth, when a small number of primitive gonocytes begin to proliferate mitotically to populate the ovary with oogonia. Following completion of its last mitotic division, the oogonium enters a final S phase and DNA is replicated in preparation for meiosis. Primary oocytes formed by mitotic divisions of oogonia enter the first meiotic prophase, but become arrested in the diplotene phase of prophase, also known as the "dictyate" stage. The total number of oocytes within the ovaries increases to a peak before birth, but this pool is rapidly depleted throughout the remainder of prenatal and postnatal life by the process of atresia (Baker 1971; Baker 1972a,b). Starting just before puberty, maturation of the endocrine system initiates a continuing series of cycles in which groups of oocytes grow and exit from meiotic arrest.

A very small number of these oocytes develop into mature gametes capable of being fertilized.

Intimately associated with oogenesis is the process of folliculogenesis, which is the association of somatic cells with the oocyte to form an ovarian follicle, and the development of that follicle to a state capable of undergoing ovulation. Shortly after oocytes are formed, they become surrounded by a single layer of epithelial cells to form primordial follicles.

The ZP is formed around the oocyte after the primordial follicle begins to grow and develop into a more mature stage; it is not found surrounding stationary, non-growing oocytes. Ultrastructural aspects of ZP development have been described for the rat (Kang 1974). The ZP initially appears as patches of amorphous material in the broadened perivitelline area of oocytes that are surrounded by a single layer of flattened granulosa cells (Type 3a follicles; classification of Pedersen and Peters 1968). By the time granulosa cells attain a cuboidal shape (Type 3b follicles), the ZP is completely formed. Terminal formation of the ZP occurs in type 4 follicles, where the ZP is surrounded by two thick rows of granulosa cells which send microvilli and cytoplasmic projections through the ZP to contact the oocyte. As described below, the ZP is composed of several glycoproteins that form a loose network of filaments making the ZP permeable to relatively large macromolecules such as enzymes and immunoglobulins (Sellens

and Jenkinson 1975). Electron micrographs of fully-formed, fixed and sectioned ZP show that the randomly arranged strands are in appearance like other extracellular matrixes (Guraya 1974; Dumont and Brummett 1985). The filaments of the ZP are composed of structural repeats formed by the association of each of the proteins making up the ZP (Greve and Wassarman 1985).

Function in Fertilization and Early Embryonic Development

Normal fertilization requires rigid control over a complex series of interactions between oocyte and spermatozoa. The ZP plays a crucial role in fertilization as this is where the sperm first adheres to the egg and through which it penetrates prior to fusion with the membrane of the oocyte (Gwatkin 1977; Yanagimachi 1977; Wassarman 1987a). Binding is species-specific and mediated via sperm receptors in the ZP. Although mating between two very different species of modern animals is usually inhibited by behavioral differences today, placement of species-specific sperm receptors in a location proximal to the membrane of the oocyte may have been an driving force of evolution in the development of the ZP. Ancestral marine animals that evolved a mechanism for preventing interspecific fertilization would surely have been more successful in producing viable offspring. That is, oocytes from animals without a method to recognize sperm of their

own species would likely be fertilized promiscuously, and often fail to develop.

The sperm ligand recognizing the species-specific receptor in the ZP is located on the plasma membrane. The identity of this ligand is currently unknown (Cornwall et al. 1991). After binding to the surface of the ZP, a sperm must undergo the acrosome reaction to enable it to penetrate the ZP and reach the vitelline membrane of the egg. The acrosome reaction is also initiated by binding of the sperm to a ZP protein, and can be artificially induced by exposure of sperm to solubilized ZP proteins in vitro (Wassarman et al. 1985 a,b).

Once a sperm binds to the ZP, it must penetrate that structure to reach the plasma membrane of the oocyte. Penetration of the ZP is dependent upon both enzymatic (acrosomal enzymes) and mechanical (motility of sperm) mechanisms. Acrosomal enzymes released in the vicinity of the sperm head during the acrosome reaction progressively degrade a tract in the ZP, aided substantially by the propulsive force of the sperm flagellum (Yanagimachi 1988).

Following penetration of the ZP, the sperm binds to and fuses with the plasma membrane of the oocyte. In mammals, the equatorial segment of the sperm head first binds to the plasma membrane of the oocyte, followed by engulfment of the anterior portion of the sperm head and incorporation by fusion of sperm membrane into the plasma membrane of the

oocyte (Yanagimachi and Noda 1970; Bedford et al. 1979; Yanagimachi 1981).

Sperm-oocyte membrane fusion stimulates resumption and completion of the second meiotic division of the oocyte, and ultimately, in establishment of blocks to polyspermy. Binding and fusion of the fertilizing sperm to the vitelline membrane stimulates the cortical reaction, an exocytotic event in which cortical granules surrounding the periphery of the oocyte fuse to the plasma membrane and release their contents into the perivitelline space. Cortical granules are membrane-bound organelles containing calcium, serine proteases and sulfated mucopolysaccharides (Schuel 1978). These substances diffuse into the ZP and induce the zona reaction, a process that serves as the fundamental block to polyspermy in most mammals. One effect of the zona reaction is a dramatic increase in the "hardness" of the ZP, which in reality is probably a change in the susceptibility of the ZP to proteolysis by acrosomal enzymes. The net effect is that sperm that have partially penetrated are stopped within the ZP as it "hardens". Secondly, fertilized eggs fail to bind additional sperm, indicating that the zona reaction destroys sperm receptors (Wassarman et al. 1985a,b). Finally, solubilized ZP proteins isolated from fertilized eggs and cleaving embryos no longer induces the acrosome reaction when mixed with sperm in vitro (Bleil and Wassarman 1980a; Wassarman et al. 1985a,b). Among the mammals that have been studied, the rabbit has a different mechanism for preventing

polyspermy; their oocytes do not undergo a classical zona reaction and many sperm can penetrate the ZP without polyspermy resulting.

After fertilization and until hatching of the embryo, the zona pellucida aids in the maintenance of cell-to-cell contact of the cleaving blastomere and protects the developing embryo as it travels down the oviduct to the uterus (Mintz 1962). At least in mice, hatching of the embryo out of the zona pellucida results from the production of an embryo protease called strypsin (74,000 Mr), a trypsin-like protease produced by the cells of the mural trophectoderm (Wassarman et al. 1984; Perona and Wassarman 1986). Mural trophectoderm is localized to one area of the developing embryo and its production of strypsin and the resulting proteolysis causes hatching of the embryo through one small area of the zona pellucida.

Zona Pellucida Proteins

Protein characteristics of ZP glycoproteins suggest that they represent a unique class of extracellular molecules and are not related to other components of extracellular matrices, including laminin, fibronectin, entactin, collagen IV, and heparin sulfate (East and Dean 1984). The unique nature of ZP proteins is to be expected due to the specialized roles that each play in fertilization and early embryonic development. Detailed characterization of ZP proteins has been largely restricted to those from

mice and pigs, although data on basic protein composition is available from several other species.

Mouse Zona Pellucida Proteins - The ZP of the laboratory mouse has a mass of approximately 3 ng and is composed of three glycoproteins designated ZP1, ZP2 and ZP3 (Bleil and Wassarman 1980a; Shimizu et al. 1983; Wassarman et al. 1986; Wassarman 1987b,c,d). Small quantities of additional molecules, such as hyaluronic acid and glycosaminoglycans, are present in the ZP, but probably not integral components. The three glycoproteins already identified appear to account for the mass of protein present in the mouse ZP.

Each of the three mouse ZP glycoproteins appears to be a unique gene product, as they are very different in nucleic acid, amino acid and oligosaccharide composition, glycopeptide maps, immunological reactivities, and biological functions (Bleil and Wassarman 1980a; 1982; Shimizu et al. 1983; Wassarman 1987a).

In the mouse, ZP2 is the major component of the ZP, representing 65% of its mass. ZP3 contributes approximately 25%, with ZP1 making up the remaining 10%. The final structure of the mouse ZP is composed of 2-3 um long filaments with a width of 7 nm, showing a structural repeat every 14-15 nm. The structural repeats appear to be formed by heterodimers of ZP2 and ZP3. The exact role of ZP1 in the structure of the ZP is not totally clear, but available evidence suggests that dimers of ZP1 may interact with

ZP2:ZP3 heterodimers to crosslink filaments into a three dimensional network (Greve and Wassarman 1985; Kuno et al. 1986; Wassarman 1987b,d).

Each of the ZP glycoproteins displays considerable heterogeneity when examined after electrophoresis in polyacrylamide, due to differential glycosylation (Shimizu et al. 1983; Wassarman et al. 1986). The mean apparent molecular weights of native ZP1, ZP2 and ZP3 are 200, 120 and 83 KDa, reflecting the substantial burden of carbohydrate added to polypeptides of 75, 81 and 44 KDa, respectively. Each of the ZP proteins is modified after translation to contain complex-type asparagine-linked (N-linked) oligosaccharides (Greve et al. 1982; Salzmann et al. 1983; Wassarman et al. 1986), and at least ZP2 and ZP3 contain serine/threonine-linked (O-linked) oligosaccharides (Florman and Wassarman 1985; Wassarman et al. 1985a; Wassarman et al. 1986). The N- and O-linked oligosaccharides have important roles in the function of individual ZP glycoproteins.

The primary sperm receptor in the mouse resides on ZP3 (Florman and Wassarman 1985; Wassarman et al. 1985a,b; Bleil and Wassarman 1986; Wassarman 1987a,b,c). Sperm receptor activity was demonstrated by incubating spermatozoa with purified ZP3 protein and showing that this treatment prevented sperm from binding to other ZP-encased oocytes. ZP3 isolated from the ZP of fertilized ova did not cause this effect, confirming that fertilization results in

destruction of sperm receptor activity. Sperm receptor activity is not directly attributable to the polypeptide chain of ZP3, but to a specific class of O-linked oligosaccharides which, when released from the ZP3 protein, retain the ability to bind to sperm (Florman and Wassarman 1985). Removal of N-linked oligosaccharides had no effect on sperm binding, but ZP3 that was been depleted of O-linked oligosaccharides did not retain sperm receptor activity.

In addition to bearing the sperm receptor, ZP3 is also the component of the ZP that induces sperm to undergo the acrosome reaction. ZP3 purified from ovarian and unfertilized, ovulated oocytes very effectively induces the acrosome reaction, whereas ZP1 and ZP2, or ZP3 purified from embryos, are ineffective (Wassarman et al. 1985a,b; Wassarman 1987d). The ability of ZP3 to induce the acrosome reaction is dependent on both its O-linked oligosaccharides and peptide chain (Wassarman et al. 1985a).

The spermatozoal ligand that binds ZP3 is effectively lost from sperm during the acrosome reaction, but sperm must remain bound to and then penetrate the ZP. Maintenance of sperm binding after the acrosome reaction appears to be function of ZP2, the secondary sperm receptor, and it appears that ZP2 binds a spermatozoal ligand located in the inner acrosomal membrane (Bleil and Wassarman 1986; Wassarman 1987d).

Following fusion of the egg and sperm, changes are induced in the structure of the ZP that establish the block

to polyspermy; collectively, these changes are known as the zona reaction. Binding of a spermatozoon to the plasma membrane of the oocyte induces exocytosis of cortical granules with diffusion of their contents into the perivitelline space and ZP (Schuel 1985). Enzymatic activity derived from cortical granules hydrolyze O-linked oligosaccharides on ZP3, destroying sperm receptors and the ability of ZP3 to induce the acrosome reaction. Cortical granule enzymes also induce limited proteolysis of ZP2, generating smaller peptides that become covalently attached to glycoproteins by intramolecular disulfide bonds, resulting in a "hardened" zona (Bleil et al. 1981). The hardened zona has a decreased solubility in low pH and reduced susceptibility to proteases and reducing agents (Bleil et al. 1981). The net effect of these changes in ZP2 and ZP3 is to prevent additional sperm from binding the ZP and to stop those sperm that have partially penetrated.

Mouse ZP at first appear as a patchy material around the oocyte in type 3a-3b follicles, which are observed by the end of the first week after birth (Peters 1969). ZP1, ZP2, and ZP3 account for 5-10% of totals protein synthesized, and at least 90% of glycoproteins secreted by growing oocytes (Greve et al. 1982). Several lines of evidence support the contention that, in the mouse, ZP glycoproteins are synthesized exclusively by the oocyte, without detectable contribution from follicular cells (Greve et al. 1982). Isolated oocytes, cultured in vitro without

follicle cells, synthesize and secrete all three glycoproteins and cultured follicular cells fail to synthesize detectable quantities of any of the three ZP glycoproteins (Bleil and Wassarman 1980b; Wassarman et al. 1986). Calculations based on rates of synthesis of ZP glycoproteins by oocytes in vitro suggest that oocytes alone are capable of synthesizing the mass of the ZP (Bleil and Wassarman 1980a). Polyclonal and monoclonal antibodies directed against the ZP detect those glycoproteins only in oocytes, not in surrounding follicular cells (Greve et al. 1982; East and Dean 1984; Wassarman et al. 1986). Finally, cDNA probes for mouse ZP2 and ZP3 have been used for in situ hybridization to clearly localize the corresponding mRNAs only to oocytes (Philpott et al. 1987).

Porcine Zona Pellucida Proteins - The ZP of the pig contains approximately 30 ng of protein (Hedrick and Wardrip 1986). Solubilized porcine ZP has consistently been resolved into four glycoproteins by SDS-PAGE under reducing conditions, but different groups have utilized different names for the same molecular species (Table 1.1: Dunbar et al. 1981; Sacco et al. 1981; Subramanian et al. 1981; Hedrick and Wardrip 1987; Sacco et al. 1989). The nomenclature of Sacco and coworkers (1981) is used in the remainder of this dissertation.

Table 1.1. Nomenclature for Porcine ZP Glycoproteins

Mean Apparent Mr				
82-118	58-96	40-74	20-25	Reference
ZP3	ZP2	ZP1		Dunbar et al. 1981
ZP1	ZP2	ZP3	ZP4	Sacco et al. 1981; Subramanian et al. 1981
ZP1 (ZP2+ZP4)		ZP3		Hedrick and Wardrip, 1987
ZP1 (ZP2+ZP4)		ZP3 (alpha and beta)		Sacco et al. 1989

Deglycosylation of these four glycoproteins resulted in resolution of five polypeptides with average apparent Mr of 65,000-70,000 (ZP1), 50,000-55,000 (ZP2), 36,000-40,000 (ZP3 alpha), 32,000-37,000 (ZP3 beta), and 15,000 (ZP4). It is now thought that ZP2 and ZP4 are proteolytic products of ZP1 (Yurewicz et al. 1986; Yurewicz et al. 1986; Hedrick and Wardrip 1987; Henderson 1987).

ZP3 is the major component of the porcine ZP, comprising as much as 70-80% of total glycoprotein (Yurewicz et al. 1987; Hedrick and Wardrip 1987). Sacco and coworkers (1987b) have shown an association of sperm receptor activity with purified ZP3. Upon deglycosylation, ZP3 is seen to be composed of two different peptides (Yurewicz et al. 1986; Hedrick and Wardrip 1987; Henderson et al. 1987). ZP3 alpha, the more acidic form (Mr=37,000, unreduced), and ZP3 beta, the more basic form (32,000, nonreduced), appear to be distinct and unique polypeptides. Differences are observed in peptide maps of ZP3 alpha and beta, the amino terminus of one is blocked while the other is not and the two peptides

can be distinguished from each other immunologically. ZP3 alpha has been shown to be the sperm receptor in the ZP of the pig (Sacco et al. 1989). Functions of ZP3 beta have not as yet been defined, but ZP1 has been shown to be the primary substrate for acrosin, the acrosomal enzyme of the sperm needed for penetration of the ZP (Brown and Cheng 1985; Dunbar et al. 1985; Urch et al. 1985).

In contrast to the mouse, pig ZP proteins may be synthesized in follicular cells as well as the oocyte. A battery of monoclonal antibodies developed against porcine ZP proteins were used to immunohistochemically localize ZP proteins in sections of porcine ovary sections and significant staining of granulosa cells was observed (Takagi et al. 1989). Staining was first observed in the primordial follicle stage. In more advanced follicles, cumulus or preovulatory, staining of the follicular cells around the ZP was not observed, suggesting that synthesis of ZP proteins by follicular cells is stage specific. Definitive evidence for follicular cell synthesis of porcine ZP proteins will likely require localization of the mRNAs for those proteins by in situ hybridization.

Zona Pellucida Proteins of Other Species - In addition to the mouse and pig, the basic composition of ZP has been determined for humans, squirrel monkeys and rabbits (Sacco et al. 1981). Human ZP are made up of at least three different glycoproteins with molecular weight ranges of 80,000-120,000, 73,000, and 59,000-65,000. In the squirrel

monkey four glycoproteins are seen with molecular weights of 63,000-78,000, 63,000-70,000, 47,000-51,000, and 43,000-47,000. Rabbit ZP are made up of three glycoproteins with molecular weights of 100,000-118,000, 83,000-110,000, and 80,000-92,000. More specific information on the composition and biologic functions of ZP proteins in these species is not yet available.

Molecular Biology of the Zona Pellucida

Nucleotide sequence data and information on genomic organization of ZP genes are currently available only from mice, hamsters, rabbits and humans. Partial amino acid sequences have been derived for some porcine ZP proteins (Yurewicz et al. 1987), but cloning of pig ZP genes has not been reported.

Mouse Zona Pellucida Genes - As mouse oocytes pass through the growing phase prior to ovulation, they go from a resting diameter of 12-15 um to 75-80 um. During that time, each oocyte synthesizes an estimated 90 pg of polyadenylated RNA, representing approximately 19% of the total RNA transcribed (Bachvarova 1985). The bulk of these oocyte mRNAs (75%) appear to be very stable, with the remainder having a mean half life of 6 days (DeLeon et al. 1983). During meiotic maturation and ovulation the stability of these mRNAs is lost, resulting in persistence of only 10% of the population to the two-cell stage (Clegg and Piko 1983; Bachvarova et al. 1983). Messenger RNAs representing ZP proteins are not detected in resting oocytes, but both ZP2

and ZP3 transcripts subsequently increase in abundance, reaching a peak of 0.1-0.2% of total mRNA (Ringuette et al. 1986; Philpott et al. 1987; Liang et al. 1990). Expression of ZP3 parallels ZP2 at approximately one third of ZP2 levels. Following ovulation and completion of meiosis, the abundance of ZP transcripts drops to 15% of peak values and with this drop zona protein biosynthesis ceases (Bleil and Wassarman 1980b; Shimizu et al. 1983).

The first ZP gene to be isolated from any species was that encoding mouse ZP3. Complementary DNA clones of ZP3 were isolated by screening ovarian cDNA libraries prepared in lambda gt11 with monoclonal antibodies to the sperm receptor (Ringuette et al. 1986). Sequences from overlapping cDNAs and genomic clones (Chamberlin and Dean 1989) were combined to show that the full length ZP3 cDNA was 1317 bp with a poly (A) tail of 200-300 bp. Following a 29 bp 5′ untranslated region is a single open reading frame encoding ZP3, including a 22 amino acid signal sequence. At the 3′ end of the gene is a 16 bp untranslated region containing the termination codon as part of the polyadenylation signal (Ringuette et al. 1988). The 5′ and 3′ untranslated regions, determined by S1 analysis, are unusually, but not uniquely short. For instance, the 5′ regions on immunoglobulin variable-region genes have been shown to be equally short (Kelly et al. 1982), and the mouse thymidylate synthetase mRNA completely lacks a 3′ untranslated region (Hehn et al. 1986).

A genomic clone of ZP3 was isolated by using the cDNA clone to probe a mouse genomic library (Chamberlin and Dean 1989). The 8 exons in this gene span approximately 8.6 Kb. A novel 54 bp tandem sequence was repeated six times in the 5′ flanking region 507 bp upstream of the transcription start site, and five times in the seventh intron of this gene. These blocks of repeats are present in the mouse genome at approximately 500 copies, possibly associated specifically expressed in oocytes.

The mouse ZP2 gene was isolated from a lambda gt11 library by immunoscreening with a monoclonal antibody specific for ZP2. Utilizing strategies similar to those successful in obtaining a full length clone of ZP3, overlapping cDNAs as well as genomic clones were used to obtain the full length sequence of ZP2. It was determined that a single open reading frame of 2139 bp encoded the ZP2 protein (Liang et al. 1990). Confirmation that this clone represented ZP2 was obtained by comparing its deduced amino acid sequence with that obtained by direct sequencing of isolated ZP2 peptides. Like ZP3, ZP2 mRNA has very short 5′ and 3′ untranslated regions of 30 and 32 nucleotides. Northern analysis using the ZP2 clones as probes revealed expression of ZP2 mRNA in oocytes, but not in follicular cells, brain, heart, liver and testis. The sensitivity of those assays to detect ZP2 mRNA in non-oocyte cell types was low, in that the target was total RNA. The ZP2 mRNA was determined to be 2400 bases in length, suggesting that ZP2

transcripts isolated from growing oocytes contain a poly(A) tail of roughly 200 bases. Northern analysis using RNA from isolated oocytes at different developmental stages showed that resting and ovulated eggs are did not synthesize detectable quantities of ZP2 mRNA, and that level of expression of this mRNA peaked in 50 um oocytes.

A genomic clone of mouse ZP2 was obtained by screening a lambda J1 genomic library with the cDNA clone of ZP2 (Liang et al. 1990). The lambda clone was 14.4 kb in length, which included the entire ZP2 coding region, as well as 1.5 kb of 5′-flanking region and 800 bp of 3′-flanking region. Sequence analysis revealed that the ZP2 gene contains 18 exons ranging from 45 to 190 bp in length and that the exons were identical to the previously sequenced cDNA. Comparisons of band intensities between digested genomic equivalents of lambda J1 and mouse genomic DNA, as well as examination of banding patterns of restriction enzyme-digested genomic DNA demonstrated that there is only one copy of the ZP2 gene in the mouse genome.

Comparisons of nucleic acid and protein sequences of mouse ZP2 and ZP3 showed no significant homology (Liang et al. 1990). ZP2 did not possess the tandem repeats seen in ZP3, but both genes contain three short sequences similarities. These 8-12 bp regions are arranged in the first 250 bp of the 5′-flanking region and may play a role in the expression of these two oocyte-specific genes.

Human Zona pellucida genes- The human homolog of the mouse sperm receptor has recently been identified (Chamberlin and Dean 1990). A human genomic library in Charon 4A was screened with pZP3.2, a cDNA clone of the mouse sperm receptor (Ringuette et al. 1988). The single copy, 18.3 Kb, human sperm receptor gene is organized into 8 exons. These 8 exons are very similar to the 8 exons of the mouse sperm receptor, with the coding regions of the two genes being 74% identical.

Hamster zona pellucida genes- Also recently identified has been the hamster sperm receptor (Kinloch et al. 1990) Genomic sequences were isolated from a hamster genomic library prepared in lambda Dash (Stratagene) screened with sequences encoding the mouse sperm receptor (Moller et al. 1990). The hamster gene is also organized in a similar manner as the mouse sperm receptor. Eight exons make up the gene with exons II, III, V, VI, and VII being identical to the corresponding mouse sperm receptor exons. Exons IV contains a 6 bp deletion compared to the mouse gene and exon VIII has non-coding regions which differ slightly in sequence from its mouse counterpart.

Rabbit zona pellucida genes- The most recently identified ZP gene has been a rabbit cDNA encoding a 55 KDa ZP protein (Schwoebel et al. 1991). A cDNA library constructed from 6-week-old rabbit ovaries was screened with polyclonal antibodies prepared against solubilized rabbit zona pellucida proteins separated by two-dimensional PAGE

and affinity purified on pig ZP. The deduced amino acid sequence of this rabbit ZP cDNA contained two small regions of homology to mouse ZP2, consisting of 86 and 56 residue tracts of 48 and 58% similarity.

Conservation of Zona Pellucida Gene and Protein Sequences - All mammalian oocytes are surrounded by a ZP and in each species characterized to date, the ZP is made up of only a few glycoproteins. Due to the similarity in function of the ZP among species, one would expect that genes encoding ZP proteins must be to some extent homologous. Ringuette and coworkers (1986; 1988) investigated this hypothesis by hybridizing radiolabled cDNA for the mouse ZP3 gene to digested genomic DNA from diverse species of mammals and other vertebrates. Distinct patterns of hybridization were observed with DNA from humans, mice, rats, rabbits, pigs, dogs and cattle, and interestingly also from chickens, although that signal was weak in comparison to mammals. Hybridization was not detected between mouse ZP3 and DNA from sea urchins, clawed toad, trout, fruit flies or yeast. These results suggested that the gene encoding ZP3 is unique to mammals, and that a related gene exists in chickens. They also indicate that there is considerable homology among mammals in at least one ZP gene. Additionally, Northern analysis with mouse ZP3 probe was performed using ovarian polyadenylated RNA from mice, rats, rabbits, dogs, cows and chickens. No signal was seen with chicken RNA, but hybridization was seen with RNA from each of the mammalian

species. The size of the RNA from these animals that hybridized to mouse ZP3 was approximately 1.4 kb in all cases.

IMMUNOCONTRACEPTIVE POTENTIAL OF ZONA PELLUCIDA PROTEINS

The population of humans and their pets is growing at an ever increasing and alarming rate. World population is currently increasing by greater than 220,000 per day and by the end of the century, global population is forecast to reach 6.3 billion, double the figure of 1960 (Population Statistics Division, OPCS 1987). Where there are humans so will be their pets. In the United States, one of the most affluent and educated countries on the planet, 20 million cats and dogs are euthanized in shelters or die as strays of starvation or disease every year (Olson et al. 1986). In third world countries, in which 80% of the world's population lives, excessive numbers of stray animals imposes a very significant burden to finances and to public health programs.

The principles of developing vaccines to prevent or interfere with pregnancy are similar in many respects to those used to develop vaccines to protect against infectious disease, but there are strategic differences in approach. Vaccines designed to provide long-term protection against infectious disease agents often are aided by "natural" boosting during the individual's lifetime, and are targeted against non-self antigens. Vaccines to induce infertility

are necessarily directed against self antigens and may not be naturally boosted, making immunity relatively short in duration. Several targets have been investigated as sites of action for immunocontraceptive vaccines, including sperm antigens (Herr et al. 1990), human chorionic gonadotropin (Talwar et al. 1988; Jones et al. 1988) and the ZP (reviewed by Henderson et al. 1988).

Several features of the ZP make it an attractive target for development of contraceptive vaccines in animals and man. First, the fundamental importance of the ZP in fertilization suggests that binding of antibodies to ZP proteins may interfere with any of a number of critical processes, including binding of sperm to the ZP, induction of the acrosome reaction and sperm penetration of the ZP. Indeed, vaccines composed of a limited number or combination of ZP epitopes may allow induction of an immune response that specifically inhibits only a subset of these events. Secondly, it appears that ZP proteins are expressed exclusively in the ovary making it unlikely that ZP vaccines will induce crossreactive autoimmune disease in other organ systems. Finally, immune reactions against the ZP do not seem capable of harming the embryo. ZP vaccines will therefore not be classified as abortifacients, which may make them more readily accepted for use in humans than vaccines that neutralize chorionic gonadotropin.

The ZP is made up of several components, each of which has the potential of being a target of a contraceptive

vaccine. Research is currently underway in a number of laboratories to identify contraceptive epitopes on the ZP, both on native forms of the proteins and on proteins devoid of carbohydrates. These findings will be necessary for the development of a practical and effective contraceptive vaccine because the final form of the vaccine will most likely be produced by recombinant DNA technology. Expression of ZP proteins in bacteria would be an attractive approach to vaccine production, but such proteins would be devoid of glycosylation, which might significantly reduce their efficacy. Proteins produced in yeast are glycosylated, but not in exactly the same manner as occurs by synthesis in mammalian cells. If such patterns of glycosylation are sufficient, a ZP vaccine produced in yeast would have significant cost savings in comparison to a ZP vaccine composed of proteins expressed in mammalian cell culture.

Inhibition of Sperm Binding In Vitro by Antibodies to the Zona Pellucida

The concept of using ZP proteins as a vaccine for the prevention of fertilization first derived from studies in mice in which it was demonstrated that antisera against ovarian homogenates inhibited fertilization in vitro (Shivers et al. 1972; Jivek and Polock 1975; Tsunoda and Chang 1976a,b; Tsunoda 1977; Tsunoda and Chang 1978). It was observed that anti-ovarian antibodies were able to block the attachment to and penetration of sperm through the ZP.

Subsequent studies demonstrated that the same inhibitory effects could be obtained by immunization with isolated ZP.

Prevention of sperm binding by anti-ZP antiserum occurs either by direct binding to sperm receptors, as with specific monoclonal antibodies raised against the receptor, or by formation of an immunoprecipitate on the surface of the ZP occluding the sperm receptor. The precipitation formed on the surface of the ZP by polyclonal antibodies is due to the antibody's cross-linking ability which, by a process of stearic hinderance, blocks sperm receptor sites (Aitken et al. 1981). Monoclonal anti-ZP antibodies have shown a very limited capacity to form precipitates on the ZP, and appear to inhibit fertilization either by binding to the sperm receptor itself (Sacco et al. 1984; Ringuette et al. 1986, Millar et al. 1989) or by binding to antigenic sites close enough to the sperm receptor to sterically inhibit the binding of sperm (Isojima et al. 1984; Koyama et al. 1985; East et al. 1984; 1985).

Numerous in vitro studies using oocytes of various species with antisera against ZP antigens have supported the concept of using the ZP as a target for immunocontraception (Table 1.2).

Table 1.2. In vitro reaction of various species of oocytes to antibodies raised against the ZP.

Oocyte Species	Ab Procedure	PPT Formation	Inhibit Sperm Binding	Reference
Hamster	rabbit anti-hamster ovary	++++	Yes	Shivers et al. 1972
	rabbit anti-rat ovary	++	Yes	Tsunoda and Chang 1976
Mouse	rabbit anti-mouse ovary	++++	Yes	Jilek and Pavlok 1975
	rabbit anti-rat ovary	+++	Yes	Tsunoda and Chang 1976
	rabbit anti-MZP	++++	Yes	Tsunoda 1977 Tsunoda and Chang 1978
	rabbit anti-PPZA	+	No	Sacco et al. 1981
Rat	Rabbit anti-rat ovary	++++	Yes	Tsunoda and Chang 1976
	rabbit anti-MZP	+++	Yes	Aitken et al. 1981
	rabbit anti-PZPA	+	Yes	Sacco et al. 1981
Rabbit	Rabbit anti-PZP	++	No	Sacco et al. 1981
	sheep anti-RZP	++++	NT	Maresh and Dunbar 1987
	sheep anti-RZPI	+++	NT	Maresh and Dunbar 1987
	sheep anti-RZPII	++++	NT	Maresh and Dunbar 1987
Pig	rabbit anti-PZP	++++	NT	Sacco et al. 1981
	human autoimmune	++	NT	Caudel et al. 1987
	sheep anti-RZP	+++	NT	Marsh and Dunbar 1987
	rabbit anti-PZP3	++++	Yes	Sacco et al. 1989
	rabbit anti-PZP3 EBDG	+++	Yes	Sacco et al. 1989
	rabbit anti-PZP3 DG	++	No	Sacco et al. 1989
	rabbit anti-ZP3 alpha EBDG	++	Yes	Sacco et al. 1989
	rabbit anti-ZP3 beta EBDG	++	No	Sacco et al. 1989
Human	rabbit anti-PZP	+++	Yes	Sacco et al. 1981
	mouse monoclone anti-PZP	NT	Yes	Hasegawa et al. 1988
Marmoset	rabbit anti-marmoset ovary	++++	Yes	Shivers et al. 1978
	rabbit anti-human ovary	+++	Yes	Shivers et al. 1978

Oocyte Species	Ab Procedure	PPT Formation	Inhibit Sperm Binding	Reference
Squirrel monkey	rabbit anti-PZP	+++	Yes	Sacco et al. 1981
Dog	dog anti-DZP	++	No	Mahi-Brown et al. 1982
	dog anti-PZP	++++	Yes	Mahi-Brown et al. 1982
	sheep anti-RZP	++	Yes	Maresh and Dunbar 1987
	rabbit anti-PZP	++	NT	Maresh and Dunbar 1987
	mouse monclone RZP	+	NT	Maresh and Dunbar 1987
	mouse monoclone PZP	+	NT	Maresh and Dunbar 1987
	mouse monoclone PZPI	+	NT	Maresh and Dunbar 1987
	mouse monoclone PZP	NT	Yes	Bamezai et al. 1988
Cat	sheep anti-RZP	+	NT	Maresh and Dunbar 1987
	sheep anti- PZP	+	NT	Maresh and Dunbar 1987
	mouse monoclone RZP	+++	NT	Maresh and Dunbar 1987
	mouse monoclone PZPI	++	NT	Maresh and Dunbar 1987

NT= Not Tried
PZP= Pig Zona Pellucida
DZP= Dog Zona Pellucida
DG= Complete deglycosylation
RZP= Rabbit Zona pellucida
EBDG= Partial deglycosylation

Antibodies developed against ovarian homogenates from rats and mice bind to ZP of rat, mouse, and hamster oocytes, forming immunoprecipitates that inhibit in vitro fertilization (Shivers et al. 1972; Jivek and Pavlock 1975; Tsunoda and Chang 1976a,b). Antibodies against ovarian homogenates did not inhibit the development of embryos, but did inhibit hatching of mouse embryos (Shivers 1974) and implantation of hamster embryos (Dudkiewicz et al. 1975).

Subsequent studies focused on production of antibodies to isolated mouse ZP. ZP isolated from cumulus-free mouse

oocytes were used to immunize rabbits and the resulting antisera were added to media for in vitro fertilization (Tsunoda 1977; Tsunoda and Chang 1978; Aitken et al. 1981). Rabbit anti-mouse ZP bound strongly to ZP of mice and rats, and inhibited binding of sperm and fertilization. When added after fertilization, antibodies against isolated ZP had no effect on development, hatching, or implantation of mouse embryos (Tsunoda and Whittingham 1982). The antiserum used in the Tsunoda and Whittingham (1982) study was raised against only isolated ZP. Earlier studies showing an inhibition of hatching or implantation used antisera against whole ovarian homogenates. Such antisera contained antibodies to numerous contaminating ovarian proteins which undoubtedly interfered with development of the fertilized embryos.

Production of antisera to mouse ZP proteins requires thousands of oocytes that have to be individually isolated from superovulated mice, making it difficult and expensive to obtain large amounts of protein. An alternative source of ZP protein that would provide large amounts of material was needed for further characterization of contraceptive epitopes. Due to the relative ease of obtaining porcine ovaries, much of the research on ZP proteins and immunizations began to utilize porcine-derived proteins. Procedures have been developed for isolation of porcine ZP by mincing ovaries and collection of oocytes on a series of nylon screens of progressively smaller mesh size. Using

this basic scheme, milligram quantities of ZP proteins can be isolated in a relatively short period of time (Dunbar and Raynor 1980; Sacco et al. 1981; Yurewicz et al. 1983; Hedrick and Wardrip 1986). In addition to the relative ease of obtaining ZP proteins, other factors make porcine proteins an attractive source of immunogens. Even though ZP antigens are tissue specific they are not species specific, and antibodies produced against the ZP of one species, such as the pig, can bind to ZP from other species (Sacco 1978; Shivers et al. 1978; Gwatkin and Williams 1978; Sacco et al. 1981). Antisera developed to pig ZP show relatively extensive crossreactivity with ZP of humans, squirrel monkeys, rabbits, cats, and dogs (Sacco 1977; Shivers and Dunbar 1977; Takai et al. 1981; Sacco et al. 1981; Maresh and Dunbar 1987).

Antisera to chromatographically-purified porcine ZP proteins was shown to bind to ZP of several species. The relative degree of cross reactivity was pig > human > squirrel monkey > rabbit > rat > mouse, as determined by percentage of precipitations of ^{125}I-labeled ZP proteins. When oocytes from these species were incubated with the antiserum in vitro, sperm binding was inhibited for squirrel monkeys and human, but not for rabbits or mice (Sacco et al. 1981).

Marsh and Dunbar (1987) produced a variety of monoclonal and polyclonal antibodies to rabbit and pig ZP to determine the extent of similarity of zona proteins among

species. Antibodies produced against solubilized rabbit ZP recognized ZP from other species in the order of rabbit >> pig > dog > cat > rat. Antibodies against solubilized pig ZP recognize other ZP in the order of pig > dog > rabbit > cat > rat. In addition to antibodies against whole ZP, antisera formed against isolated ZP proteins from rabbits (ZP1 and ZP2) and pigs (ZP3), and monoclonal antibodies against solubilized rabbit ZP and pig ZP1 were produced that bound to pig, dog and cat zonae. Collectively these data suggest that ZP proteins of the pig, dog and cat are more closely related to each other than to rabbit and rat.

Recently, several monoclonal antibodies against pig ZP antigens have been produced that cross react with human and dog ZP proteins. Hasegawa and coworkers (1981) developed a monoclonal antibody that cross reacted with porcine and human zonae and used this antibody to purify the 92 KDa protein of pig ZP. This purified protein was then used as an antigen to produce an anti-ZP antiserum that inhibited sperm binding in vitro. Bamezai and associates (1988) generated a monoclonal antibody against pig ZP that inhibited sperm binding in a canine in vitro fertilization system.

Antibodies that have been shown to cause an effect on sperm binding in vitro are valuable tools for the identification and isolation of genes encoding contraceptive epitopes on ZP proteins. Through the use of DNA technology,

such genes could then be used to produce recombinant vaccines.

Native ZP proteins are glycosylated and it is important to determine whether non-glycosylated ZP proteins are effective contraceptive antigens. If they are, ZP proteins produced in E. coli would be predicted to be effective vaccines. Sacco et al. (1989) performed a study which addresses the importance of which proteins should be used as immunogens and the degree of modifications needed for such a protein to be effective as a contraceptive immunogen. The 55 KDa family of porcine ZP (ZP3) was used as the source of antigen. Once thought to be a single protein, ZP3 is now known to be composed of two proteins: ZP3-alpha, the sperm receptor, and ZP3-beta, for which a function has yet to be defined. Antisera were produced against native ZP3 as well as to partially and completely deglycosylated ZP3. Additional antisera were generated against partially deglycosylated ZP3-alpha and ZP3-beta. Each of these antisera produced a precipitate on the surface of pig ZP, but inhibition of sperm binding was seen only with antiserum against native ZP3 (both fully glycosylated and partially deglycosylated) or against partially deglycosylated ZP3-alpha. These results confirmed that the sperm receptor of the pig is located on ZP3 alpha and that glycosylation of the receptor plays an important part of its function.

Inhibition of Fertility by Immunization Against Zona Pellucida Proteins

Infertility has been induced in several species by either passive or active immunization against ZP proteins (Table 1.3).

Table 1.3. In vivo reaction of various species to active or passive immunizations with ZP

Species	Immunization	Disruptions	Effects on Fertility	Reference
Mouse	rabbit anti-mouse ovary	ND	infertile	Jilek and Pavlock, 1975
	mouse ovary	ND	decreased fertility	Tsunoda and Chang 1976b
	rabbit anti-MZP	ND	infertile	Tsunoda and Chang 1978
	mouse monoclone ZP2, ZP3	normal	infertile 80 days	East et al., 1984
	synthetic sperm binding epitope ZP3	normal	Different levels of infertility avg=16-36 wk	Millar et al., 1989
Rat	rabbit anti-mouse ovary	ND	infertile	Tsunoda and Chang 1976a,b
	rat ovary	ND	no significant infertility	Tsunoda and Chang 1976b
Hamster	rabbit anti- hamster ovary	ND	infertile	Oikawa and Yanagimachi 1975
Rabbit	rabbit zonae	normal	fertile	Wood et al. 1981
	Pig ZP	ovarian function disrupted	infertile	Wood et al. 1981 Skinner et al. 1984
Dog	Dog ZP	normal	fertile	Mahi-Brown et al. 1982
	Pig ZP	abnormal cycle	infertile	Mahi-Brown et al. 1982
	Pig ZP or purified pig ZP fraction	abnormal cycle increased FSH, LH, ovarian cystes	infertile	Mahi-Brown et al. 1985 Mahi-Brown et al. 1988
Human	Naturrally occuring anti-ZP antibodies	normal	infertile	Caudle et al., 1987

Species	Immunization	Disruptions	Effects on Fertility	Reference
Squirrel monkey	Pig ZP3 (55 Kda)	abnormal, reversable	infertile, but resersable	Sacco et al., 1983 Sacco et al., 1987 Sacco et al., 1989
Bonnet monkey	Pig ZP3 (55 Kda)	adjuvant dependent abnormality	ND	Upadhyay et al. 1989
Babbon	PZPI,PZPII	abnormal	ND	Dunbar et al., 1989
Horse	Pig ZP	normal	infertility, reversable	Liu et al. 1989

Early studies demonstrated that immunization with ovarian homogenates reduced reproductive performance in rats and mice (Tsunoda and Chang 1976b). Immunization of rats with 3 injections per week for seven weeks with 0.5 ml of rat ovarian homogenate (26.5 mg/ml) reduced the number of pregnancies to 71% of controls for the first pregnancy and 80% of controls for the second pregnancy. Litter size was not significantly reduced in those animals becoming pregnant. Mice immunized in the same manner with 0.5 ml of mouse ovarian homogenates (8.4-19.8 mg/ml) had a pregnancy rate only 13% that of controls; in this case, there was also a significant decrease in litter size. Infertility in these animals was caused by an inhibition of sperm binding. Oocytes recovered from treated mice had a decreased ability to be fertilized in vitro and were seen to be covered with a precipitate on the outer surface of the zona. Treatment did not have an effect on the response of mice to superovulation.

Mice immunized passively with a rabbit antisera against mouse ovarian homogenate had a dramatically reduced fertilization rate in vivo (0.6% fertilization of 206 oocytes observed) (Jilek and Pavlok 1975). The inhibition of fertility was deduced to be due to prevention of sperm binding and penetration of the ZP, as zona-free oocytes were fertilized normally in vitro in the presence of the anti-ovarian antiserum.

Antiserum directed at the ZP is just as effective as anti-ovarian antiserum in producing inhibition of fertilization (Tsunoda and Chang 1978). Oocytes obtained from mice passively immunized with rabbit anti-mouse ZP antiserum were covered with a precipitate. This effect persisted for up to 30 days and during that period, fertility was nil (0 oocytes fertilized of 329 recovered). Fertility returned after the precipitate was no longer present.

East et al. (1984) developed monoclonal antibodies to different antigenic determinants on mouse ZP2 and ZP3. Administration of these antibodies to mice resulted in coating of the surface of the zona as seen by immunofluorescence staining. When administered prior to mating, oocytes became coated with antibody and were not fertilized. If administered 2 days after mating 2-cell embryos also become coated, but developed and implanted normally. One administration of the monoclonal antibody

resulted in infertility that persisted an average of 80 days (44-117 days).

Isolation of a cDNA encoding mouse ZP3 made it possible to more precisely define the sperm-binding domain on that protein. Millar and coworkers (1989) cloned random fragments of ZP3 cDNA into the expression vector lambda gt11, and screened the resulting epitope library with a monoclonal antibody previously shown to inhibit fertilization. Sequence analysis of eight independent phage isolated from immunoreactive plaques revealed a common 24 bp sequence which was deduced to encode a seven residue peptide that functioned as the sperm-binding domain. A 16 amino acid peptide containing this seven amino acid epitope was synthesized and conjugated to keyhole limpet hemocyanin. Sixteen female random-bred Swiss mice were immunized intraperitoneally with 100 ug of the peptide conjugate emulsified in an equal volume of Freund's complete adjuvant and boosted with equal doses in Freund's incomplete adjuvant at 10-14 day intervals; circulating antibodies to the peptide were detected in an enzyme-linked immunosorbant assay. The antibody response plateaued after three immunizations. Ovaries from four animal were stained and shown to be saturated with immunoglobulin. The remaining 12 animals were housed continuously with fertile males and manifest differing periods of infertility. Three of twelve animals, those with the lowest titers of ZP antibodies, gave birth within the same time period as control animals. In

the remaining 9 animals a contraceptive effect was observed that lasted 16-36 weeks. Three of these animals, those with intermediate titers of antibodies to ZP3, gave birth after 16-24 weeks. Those animals with the highest titers of antibodies to ZP3 remained infertile for the duration of the study (9 months).

Antibodies raised against the sperm binding epitope of mouse ZP3 of mice did not bind to ZP of hamster, guinea pig, cat or dog oocytes (East et al. 1985). This epitope would therefore be of no use as a vaccine for the prevention of pregnancy in the these species

As described in the previous section, experiments conducted in vitro have suggested that porcine ZP proteins might be an effective source of antigen for immunizing and inducing infertility in diverse non-rodent species. These findings have largely been supported by subsequent experiments in which reproductive function was evaluated after immunization of rabbits, dogs, primates and horses with porcine ZP proteins. Wood and coworkers (1981) conducted fertility studies utilizing rabbits immunized with either porcine or rabbit proteins. To determine effects on fertility, two female rabbits each were immunized with 300 ug of intact heat-solubilized or detergent-disassociated pig zonae and intact or heat-solubilized rabbit ZP, all in Freund's complete adjuvant, boosts were administered at weeks 4, 9 and 25 with 150, 75 and 75 ug of protein in Freund's incomplete adjuvant. Only animals immunized with

intact or heat-solubilized porcine ZP failed to give birth after insemination.

In addition to determining fertility, Wood and coworkers (1981) examined effects of anti-ZP antibodies on the early events of fertilization. Twenty-four female rabbits were immunized as described previously (n = 4/group), inseminated and ova collected by flushing oviducts 29-34 hours later. A dramatic finding, not anticipated at the start of this study, was that no normal embryos or oocytes were recovered from those animals immunized with either intact or heat-solubilized porcine ZP. Of the eight animals immunized with these preparations, only one had what appeared to be ovulation sites and corpora lutea.

Skinner et al. (1984) conducted similar studies with rabbits, and demonstrated that immunization with porcine ZP proteins disrupted ovarian function and led to changes in hormonal responses and cellular differentiation. Immunized animals failed to form functional corpora lutea or show elevations in serum concentrations of progesterone in response to injections of hCG. They was also had increased serum concentrations of FSH and LH, suggesting interference with negative feedback. The altered hormonal responses were evident only after week 20 following primary immunization. By week seven, numbers of primary, secondary and tertiary follicles were reduced, and by week 23 few growing follicles were present. In animals examined 40-48 weeks after immunization, the number of primary follicles were reduced

from an average of 11 in control animals to 2 in treated animals, and the ovaries from immunized rabbits contained numerous clusters of normal appearing follicular cells devoid of zona-encased oocytes. The abnormalities in folliculogenesis appeared to occur during the stage at which ZP proteins were normally being synthesized and secreted, leading to disruption in assembly of the ZP. These studies demonstrate that immunization with ZP proteins can lead to infertility not only from inhibition of sperm binding, but also by alteration of ovarian cellular differentiation.

Two studies have been conducted to investigated porcine ZP immunogens in dogs. In the first study (Mahi-Brown et al. 1982), bitches were immunized monthly for 4-6 months with heat-solubilized canine or porcine ZP; 2000 ZP equivalents in Freund's complete adjuvant were administered initially, with boosts of 1000 ZP in Freund's incomplete adjuvant. Antibody responses were evaluated by immunofluorescence with zona-encased oocytes and by inhibition of sperm binding to canine oocytes in vitro. The two bitches receiving canine ZP developed only low titers of antibody (1:100-1:1000). In contrast, all three bitches immunized with porcine ZP proteins developed high (1:10000-1:100000) titers of antibody that crossreacted strongly with canine ZP and abolished binding of sperm to treated oocytes. A breeding trial was conducted as part of this research, but suffered considerably from the small number of animals and the fact that two did not cycle during the study period.

However, none of the three bitches immunized with porcine ZP became pregnant following natural service. The authors indicated that the animals with high titers of anti-ZP antibodies had abnormal cycles, but in reading their description, a majority of the abnormalities appeared to be within the normal range of physiology observed in dogs. The abnormal ovarian cycles observed in dogs immunized against porcine ZP were more completely investigated in subsequent studies by Mahi-Brown and associates (1985), designed to determine whether the type of antigen or adjuvant had an effect on development of abnormal cycles. Dogs were immunized with solubilized pig ZP or a partially purified preparation of porcine ZP proteins produced by gel filtration and ion exchange chromatography. These antigens were administered either without adjuvant or in combination with Freund's, alum, or CP-20961 (a synthetic lipid amine) adjuvants. In the one animal in which no adjuvant was used, low titers of antibody resulted, and that animal cycled normally and was fertile. In combination with any of the adjuvants, both antigen preparations elicited an immune response sufficient in magnitude to prevent pregnancy. Bitches immunized with the purified ZP proteins developed lower titers (1:1000-1:4000) than those immunized with solubilized whole ZP (1:1000-1:20000). The highest titers resulted from the use of either Freund's or CP-20961 adjuvants, whereas moderate titers were elicited by proteins administered with alum. Animals with high titers (1:4000-

1:20,000) of antibodies against pig ZP displayed abnormal estrous cycles in which estradiol rose during proestrus, but did not fall in early estrus, and progesterone failed to become elevated. Moderate titers (1:2000) were apparently sufficient to cause infertility, and only one animal out of six became pregnant. Histopathologic evaluation of ovaries from these dogs was reported recently (Mahi-Brown et al. 1988). Immunization with either preparation of antigen was associated with development of follicular cysts; in those immunized with solubilized ZP, the cysts were lined with a thin layer of granulosa cells, while those in bitches immunized with the purified ZP fraction were lined by a basement membrane and clumps of luteinized cells. In animals developing high titers of antibodies, oocytes were present only in primordial follicles. Large follicles were present, but lacked oocytes. This study established that abnormal estrous cycles resulted from follicular dysgenesis or cyst formation, but the exact etiology of these conditions is not as yet completely resolved.

The contraceptive potential of immunization with porcine ZP proteins has been demonstrated in several species of primates, including squirrel monkeys (Sacco et al. 1983), bonnet monkeys (Upadhyay et al. 1989) and baboons (Dunbar et al. 1989). Sacco et al. (1983) immunized squirrel monkeys with purified ZP3 in Freund's adjuvant, which resulted in production of high antibody titers as determined by formation of precipitates on porcine ZP. The high titers

elicited in these animals persisted for up to one year. Breeding trials were not conducted in this study, but significantly fewer oocytes were obtained from immunized monkeys (2 oocytes from 18 animals) in comparison to controls (33 oocytes from 18 animals). These findings were extended with another group of animals immunized with porcine ZP3 proteins (Sacco et al. 1987a). Development of high antibody titers to ZP3 was associated with a deficiency in secondary and large antral follicles. By 10-15 months after immunization, follicle numbers began returning to normal, demonstrating that the contraceptive effect of ZP vaccines could be reversible. An important observation from this study was that administration of Freund's adjuvant by itself resulted in disruption in fertility and abnormalities in endocrine patterns. Control animals receiving Freund's adjuvant without ZP proteins manifest an initial reduction in estradiol secretion detected 114 days after immunization and none of 20 controls monkeys became pregnant during the first breeding cycle.

Sacco et al. (1989) conducted a dose-response study to define in squirrel monkeys the amount of porcine ZP3 required for contraceptive immunization and to investigate the efficacy of different adjuvants. Three groups of six animals were used to evaluate dosage levels of ZP3. Each animal was injected in multiple intradermal sites with 50, 25 and 5 ug of ZP3 in Freund's complete adjuvant and boosted two times one week apart with the same dose of ZP3 in

Freund's incomplete adjuvant. Additional animals were utilized to compare three adjuvants, Freund's, alum, and a muramyl dipeptide analog (MDP), each of which was administered with 200 ug of ZP3. Antibody responses were monitored by radioimmunoassay (Sacco et al. 1981; Subramanian et al. 1981). Comparable antibody titers were achieved following vaccination with ZP3 in either Freund's or MDP adjuvant, while the use of alum resulted in significantly lower titers. Similar antibody titers and response kinetics were observed among the different doses of ZP3 in Freund's adjuvant. For breeding experiments 20 animals from the dosage experiment were used. Females were housed continuously with males (1 male to 5 females) during the breeding season (mid-February to mid-June). Pregnancy was monitored by palpation and by assay of serum concentrations of estradiol and progesterone. One pregnancy resulted in each of the groups immunized with 25 or 5 ug of ZP3. No pregnancies resulted in the group immunized with 50 ug. Diluted serum from animals in each of the groups blocked attachment of boar spermatozoa to porcine oocytes (Sacco et al. 1984), demonstrating that even at a dilution of 1:100 there was a significant inhibition of sperm attachment and suggesting immunization with even small amounts of ZP3 is capable of producing antibody responses that interfere with fertilization.

The effect of adjuvants in ZP immunizations was also investigated by Upadhyay et al. (1989) in a morphological

study utilizing bonnet monkeys. Freund's adjuvant and sodium phthalylatial lipopolysaccharide (SPLPS) were used as adjuvants for porcine ZP3. Both adjutants were equally effective in producing high titers of antibodies, but maintenance of high titers was greater with Freund's. Use of Freund's adjuvant resulted in ovarian atrophy with only primordial follicles surviving, while use of SPLPS was not associated with detectable changes in ovarian morphology. The ability of Freund's adjuvant to produce abnormal ovarian function is not fully understood, but recently it has been speculated that mycobacterial infections may trigger autoimmune reactions (Shoenfeld and Isenberg 1988) and that mycobacterial components of this adjuvant may trigger such a response within the ovary. In the bonnet monkeys, follicular atrophy was reversible and return to normalcy occurred as antibody titers fell and primordial follicles unaffected by immunization began to develop normally.

Immunization of baboons has recently been used to investigate the utility of peptide adjuvants with porcine ZP vaccines, and to compare native to deglycosylated antigens (Dunbar et al. 1989). Ten adult female baboons were immunized in this study. Five received 0.5 mg of porcine ZP3 (group I) and five were immunized with 0.1 mg of deglycosylated ZP1 (group II); both antigens were delivered with the adjuvant MDP. An additional three animals were used as controls to determine normal cycle length, sex skin changes and progesterone concentrations. Animals in group I

developed significantly higher titers of antibodies to ZP compared to those in group II. Two of the five animals in group I exhibited normal cycle length and ovulation, while the other three had at least one cycle of extended duration or a period of anovulation lasting for several months. In each of the animals in group I, immunization was associated with a reduction in serum concentration of estradiol to approximately 60 pg/ml on day 13 of the cycle compared to 180-250 pg/ml observed at that time prior to immunization. Progesterone levels were not significantly affected by this vaccine. Histologic analysis of ovarian sections revealed that the number of antral follicles was significantly reduced in group I animals compared to controls. Animals in group II did not show significant alterations in cycle length, but the number of antral follicles were reduced in comparison to control baboons, and two of these animals had abnormal follicular cell clusters similar to those previously described in rabbits after immunization with ZP proteins (Skinner et al. 1984).

Immunization of humans with ZP proteins has not been reported. However, investigations of unexplained infertility have provided some evidence suggesting that women can develop antibodies to their own ZP proteins (Caudle et al. 1987). Incubation of serum from such women with porcine oocytes has been shown to result in the deposition of human immunoglobulins on the ZP, and the titer of such antibodies over time correlated with periods of

infertility and return to fertility. In these women there was no indication of abnormal ovarian function. The reasons for the appearance of these antibodies and their subsequent drop are not understood.

Horses represent an additional species in which a ZP vaccine has been evaluated (Liu et al. 1989). Fourteen fertile domestic mares were immunized with heat-solubilized porcine ZP. Each mare received four injections at 2-4 week intervals, each equivalent to 2000-5000 ZP, with a final boost of 20,000 ZP 6-10 months after the last of the initial immunizations. Animals developed high titers of antibodies to pig ZP and 12 of 14 immunized mares failed to become pregnant during the first breeding season following immunization. Disruption of ovarian function was not observed in any of these animals. Even though fertility was only inhibited for one mating season, this immunization scheme showed considerable promise as a means of controlling wild horse populations.

Summary of Zona Pellucida Vaccines and Future Directions - It has been clearly demonstrated that immunization with ZP proteins is a promising strategy for population control in diverse species of animals. However, evaluation of a ZP vaccine in large numbers of animals has not been reported for any species. Part of this problem is undoubtedly related to the difficulty and expense of obtaining sufficient amounts of ZP proteins from their natural source to conduct such a trial. An obvious route

around this obstacle is to clone the genes encoding ZP proteins and prepare a vaccine using recombinant proteins. Such is the ultimate goal of the studies presented in this dissertation.

CHAPTER II

CLONING AND EXPRESSION OF A PORCINE ZONA PELLUCIDA GENE

INTRODUCTION

Cloning and expression of genes for zona pellucida (ZP) proteins would greatly simplify isolation of material required for development of contraceptive vaccines. The majority of research using ZP proteins as antigens to immunologically inhibit fertilization have utilized porcine ZP proteins. Additionally, the immune response elicited by immunization with porcine ZP proteins is broadly crossreactive with ZP from several other species, notably dogs. For these reasons I chose to focus on cloning and expression of a porcine ZP gene. At the start of this project, nucleic acid sequence data was available only for mouse ZP3, the sperm receptor of the mouse ZP, and several lines of evidence indicated that rodent ZP proteins would be ineffective as vaccines to prevent pregnancy in non-rodent animals. Antibodies formed against solubilized porcine ZP, which have been shown to inhibit fertilization in various species, did not crossreact appreciably with mouse ZP and antibodies formed against a synthetic peptide equivalent to the sperm-binding epitope of mouse ZP3 did not react with ZP proteins isolated from hamster, guinea pig, cat or dog

ovaries. Despite the apparent lack of immunologic similarity between porcine and mouse ZP proteins, it was thought possible that the mouse ZP3 cDNA might be useful for identifying porcine ZP sequences. This approach was investigated initially, but shown to be unproductive. I therefore adopted the strategy of immunologic identification of phage harboring porcine ZP gene sequences.

MATERIALS AND METHODS

Overview and Cloning Strategy

To identify genes encoding porcine ZP proteins, a polyclonal antiserum was developed against solubilized porcine ZP and used to screen a porcine ovarian expression library. It was anticipated that ZP mRNAs would constitute a very small fraction of total ovarian mRNA, and therefore lambda gt11 phage, rather than a plasmid vector, was selected for construction of the library. The library was screened with rabbit antiserum to porcine ZP proteins and immunoreactive phage were plaque purified. The cDNA inserts from these phage were subcloned into a plasmid vector for basal characterization and sequencing. To confirm the identity of the sequence isolated as representing a ZP gene, the cDNA was cloned into another vector for expression of protein in E. coli. The resulting recombinant protein was used to generate an antiserum that reacted with native porcine ZP protein.

Production of a Rabbit Antiserum to Porcine ZP Proteins

Isolation of Porcine ZP - Porcine ZP were isolated from pig ovaries obtained from a slaughterhouse, using techniques similar to those described by Dunbar and coworkers (1980). Frozen ovaries were thawed on ice and finely minced with razor blades in PBS. Successively smaller pieces of tissue debris were removed by straining the minced ovaries through gauze and then 140 um mesh teflon screens. Oocytes were collected on 74 um mesh teflon screens and washed free by inverting the screens and flushing with PBS. With the aid of a dissecting microscope, oocytes were individually collected from the resulting fluid by aspiration into a micropipet, insuring minimal contamination of the final product by follicular cells. Oocytes were cracked out of their ZP in PBS with 5 strokes of a teflon homogenizer, and this homogenate was passed over a 40 um mesh screen to separate ZP from residual oocytes. The ZP were collected by backflushing the screens with PBS and centrifuging the collected fluid at 10,000 x g for a few seconds. Finally, ZP were solubilized in 0.1 mM bicarbonate buffer (pH 9.0) by heating to 68°C for 20 min.

Generation of a Rabbit Antiserum to Porcine ZP - One hundred ul of solubilized porcine ZP representing approximately 1000 ZP (35 ug of protein) were mixed with 100 ul of Freund's complete adjuvant and injected into multiple subcutaneous and intramuscular sites of a rabbit. Four, eight and twelve weeks later, the rabbit was boosted with

the same dose of solubilized ZP in Freund's incomplete adjuvant. Serum was collected one week after the final immunization.

Characterization of Rabbit Antiserum to Porcine ZP Proteins - Characteristics of the antiserum to porcine ZP were initially assessed by immunocytochemistry and immunoblotting. For immunofluorescence assay of intact ZP, oocytes were collected from pigs, dogs and cats by mincing ovaries as described above, and from cattle, sheep and mice by flushing oviducts of superovulated females to recover unfertilized ova. Washed oocytes were incubated 60 min at 37°C in a 1:100 dilution of immune or normal rabbit serum in PBS containing 1% bovine serum. The oocytes were then washed three times in PBS, incubated an additional 30 min in a FITC conjugate of goat anti-rabbit IgG (H+L) and washed again as before. The oocytes were pipeted into mounting medium (9 parts glycerol, 1 part PBS), placed under a 5 x 5 mm glass coverslip and examined by epifluroescence microscopy.

Ovarian sections were stained with the rabbit anti-solubilized ZP serum to assess specificity of the antiserum. Paraffin sections of Bouin's-fixed porcine and canine ovary were deparaffinized in two changes of xylene (2-3 min each). The sections were then hydrated by passage through 100%, 95% and 60% ethanol (5 min each), with a final hydration in water for 30 min. Nonspecific-binding was blocked by incubation for 30 min at 37°C in Tris-buffered saline (TBS;

10 mM Tris, 150 mM NaCl, pH 8.0) containing 1% normal horse serum (NHS) and 2% bovine serum albumin (BSA). Normal and immune rabbit sera were diluted 1:100 in TBS and incubated with sections for 2 hr at ambient temperature. After washing in TBS three times (5 min each), sections were incubated with a 1:10,000 dilution of a biotin conjugate of anti-rabbit IgG (H+L; Sigma B-7389) for 1 hr at 37°C and washed as before. Sections were then incubated with a 1:250 dilution of avidin-peroxidase (Sigma A-3151) for 1 hr at 37°C, washed as before and immersed in substrate. The substrate solution was prepared by dissolving 7 mg of 3,3'-diaminobenzidine (Sigma D-5637) in 50 ml of 10 mM Tris (pH 8.0), and adding 4 ul of 30% hydrogen peroxide just before use. Sections were incubated with substrate until a brownish color began to appear (15-30 min), then washed briefly with water to stop the reaction, dried and mounted with Permount.

Antibodies to be used for screening expression libraries must necessarily recognize denatured protein, and it was therefore necessary to determine if antibodies in the rabbit anti-ZP serum would recognize denatured forms of ZP proteins. This was evaluated by immunoblot analysis of ZP proteins electrophoresed on denaturing polyacrylamide gels. Components of the separating and stacking portions of the gels were prepared as described below.

Component	Separating gel	Stacking Gel
	10% T 2.7% C	4%T 2.7%C
30%T 2.7%C	20 ml	2.66 ml
1.5 M Tris-HCl pH=8.8	15 ml	
0.5 M Tris-HCl pH=6.8		5 ml
10% SDS	0.6 ml	0.2 ml
H_2O	24.1 ml	12.2 ml
10% Ammonium persulfate	300 ul	100 ul
TEMED	20 ul	10 ul

Components of the separating gel, except for ammonium persulfate and TEMED, were mixed and degassed for 10 min. Ammonium persulfate and TEMED were then added, and the mixture was poured between prepared glass plates, overlayed with water and allow to polymerize for several hours. The stacking gel portion was prepared in the same manner as the separating gel and, after removal of the water overlay, poured onto the separating gel. Combs were inserted and the gels were allowed to polymerize for 1 hr. After polymerization was complete, the combs were removed and wells rinsed with running buffer (0.025 M Tris, pH 8.3, 0.192 M glycine, 0.1% SDS). Samples of ZP proteins were mixed with 2X sample buffer (0.125 Tris pH 8.0, 4% SDS, 20% glycerol, 10% beta-mercaptoethanol), heated at 95°C for 2 min and applied to gels. Gels were electrophoresed in running buffer overnight at 30 V constant voltage. Following electrophoresis, proteins were transferred to nitrocellulose membranes by electroblotting at 200 V for 1 hr in transfer buffer (0.192 M glycine, 0.025 M Tris, pH

8.3, 20% methanol). The membrane was rinsed with TBS and placed in blocking buffer (TBS containing 1% BSA and 1.25% nonfat dry milk) overnight at 4°C. Filters were washed three times in TBS containing 0.05% Tween 20 (TBST) then incubated at ambient temperture for 4-5 hr in normal or immune rabbit serum diluted in TBST supplemented with 1% bovine serum. Normal and immune sera were tested at dilutions of 100, 500 and 1000. After incubation with test sera, the membranes were washed three times (10 min each) in TBST, and transferred to TBST containing an alkaline phosphatase conjugate of anti-rabbit IgG (H+L; Sigma A-8025) diluted according to manufacturers directions. The membranes were incubated for 45 min at ambient temperature, washed two times in TBST and once in TBS (10 min each) and then immersed in substrate solution until a colored reaction product appeared (30-45 min). The substrate was prepared just before use in a buffer consisting of 100 mM Tris, pH 9.5, 100 mM NaCl and 5 mM $MgCl_2$. To 30 ml of this buffer were added 990 ul of nitroblue tetrazolium (NBT; Sigma N-5514; stock solution: 25 mg/ml in water) and 99 ul of 5-bromo-4-chloro-3-indolyl phosphate (BCIP; Sigma B-0274; stock solution: 20 mg/ml in 2,4-dimethylformamide). Filters were washed with water and air dried.

Production of a Porcine Ovarian cDNA Library

Messenger RNA Isolation - Total ovarian RNA was isolated by a guanidinium/cesium chloride method (Glisin et al. 1974, Ullrich et al. 1977). A 4 M solution of

guanidinium thiocyanate (Sigma G-6639) was prepared by dissolving 50 g of guanidinium thiocyanate and 0.5 g n-lauryl sarcosine in 30 ml of water and heating to 60°C. After dissolution, 2.5 ml of 1 M sodium citrate (pH 7.0) were added, the pH was adjusted to 7.0 with 10 M NaOH, 0.7 ml of beta-mercaptoethanol was added and the final volume was brought up 100 ml. Porcine ovaries, freshly collected at a local slaughterhouse, were homogenized in five parts guanidinium solution using a Polytron. One gram of cesium chloride (Pharmacia 17 0846 06) was added to each 2.5 ml of homogenate, and 3.8 ml of this mixture was layered onto a 1.2 ml cushion of 5.7 M cesium chloride/0.1 M EDTA (pH 7.5) in Beckman SW60 ultracentrifuge tubes. These samples were centrifuged at 35,000 rpm for 12 hr at 20°C. Supernatants were discarded and the RNA pellet resuspended in a 1 ml of 10 mM Tris (pH 7.4), 5 mM EDTA, 0.1% SDS. They were then extracted once with phenol-chloroform-isoamyl alcohol (50:48:2) and once with chloroform-isoamyl alcohol (24:1). The water used in all subsequent steps was treated with diethyl pyrocarbonate to inhibit RNase activity. One-tenth volume of 3 M sodium acetate (pH 5.2) and 2.5 volumes of absolute ethanol were added and the RNA precipitated at -20°C for 2 hr. RNA was recovered by centrifugation at 10,000 x g for 30 min, dissolved in water, and concentration was estimated spectrophotometrically (1 OD_{260} = 40 ug/ml). The samples of RNA were then reprecipitated in ethanol and stored at -70°C.

Polyadenylated RNA was isolated from total RNA by chromatography on oligo (dT)-cellulose (Edmonds et al. 1971; Aviv and Leder 1972). One half gram of oligo (dT)-cellulose (Type 3 from Collaborative Research) was mixed with buffer A (20 mM Tris, pH 7.4, 0.1 M NaCl, 1 mM EDTA, 0.1% SDS) to form a slurry which was then poured into a sterile, disposable plastic chromatography column. The column was washed with three volumes 0.1 M NaOH/0.5 mM EDTA, then with 10 volumes of water until the pH was less than 8.0. The column was prepared for binding by washing with five volumes of buffer B (40 mM Tris, pH 7.4, 1 M NaCl, 1 mM EDTA, 0.1% SDS). Total RNA (1-10 mg) was dissolved in 500 ul of water and heated to 65°C for 5 min. Five hundred ul of buffer B, preheated to 65°C, were added and the solution was cooled to room temperature. This was then applied to the column, eluate was collected, heated to 65°C, cooled and reapplied to the column. The column was washed with 5 volumes of buffer A and polyadenylated RNA collected by elution with a solution of 10 mM Tris (pH 7.4), 1 mM EDTA and 0.05% SDS. One-tenth volume of 3 M sodium acetate and 2.5 volumes of ethanol were added and RNA was precipitated overnight at -20°C. The RNA was collected by centrifugation at 10,000 x g for 30 min, dissolved in water and concentration determined as before. Polyadenylated RNA was stored at -70°C as an ethanol precipitate.

Complementary DNA synthesis - Double stranded complementary DNA (cDNA) was produced with a kit purchased

from Bethesda Research Labs (Cat. No. 8267SA) which was based on modifications of the technique of Gubler and Hoffman (1983). Ten ug of polyadenylated RNA isolated from porcine ovaries were reverse transcribed into first strand cDNA by the following reaction. Into a sterile DEPC-treated microcentrifuge tube was mixed 10 ul of 5X first strand buffer, 2.5 ul 10 mM dNTP mix, 5 ul oligo $(dT)_{12-18}$, 10 ul mRNA (10 ug), 20 ul water and 2.5 ul cloned murine leukemia virus reverse transcriptase (MLV-RT). The final buffer concentration of the first strand synthesis mixture was 50 mM Tris pH 8.3, 75 mM KCl, 3 mM $MgCl_2$, 10 mM dithiothreitol, 500 uM each dATP, dGTP, dCTP, dTTP, 50 ug/ml oligo $(dT)_{12-18}$, 200 ug/ml polyadenylated RNA and 10,000 U MLV-RT/ml. The reaction was incubated for 1 hr at 37°C then placed on ice.

To the 50 ul reaction the following reagants were added to produce second strand cDNA: 288 ul water, 7.5 ul 10 mM dNTP mix, 10 ul second strand buffer, 1.25 ul alpha ^{32}P-dCTP (10 uCi/ul), 10 ul E. coli DNA polymerase I, 1.75 ul RNase H and 1.25 ul T4 DNA ligase. The final concentration of constituents in the second strand reaction mixture was 25 mM Tris, pH 8.3, 100 mM KCl, 10 mM $(NH_4)_2SO_4$, 5 mM $MgCl_2$, 250 um each dATP, dGTP, dCTP, dTTP, 0.15 mM dithiothreitol, 250 U/ml DNA polymerase I, 8.5 U/ml RNase H and 30 U/ml DNA ligase. The reaction was mixed and incubated for 2 hr at 16°C. To terminate the reaction, 25 ul of 0.25 M EDTA were added and the reaction tube was cooled on ice. Double stranded cDNA was purified from the reaction mixture by

adsorption to glass particles (Vogelstein and Gillespie 1979) and eluted in 25 ul of water. Glass particles were used instead of phenol and ethanol precipitation for several reasons. The cDNA can be recovered much more quickly, the risk of phenol contamination is eliminated and small cDNAs (<75 bp) will irreversibly bind to the glass particles eliminating the need to size select the final library.

Glass particles were prepared by first suspending 250 g of 325-mesh powdered flint glass in 200 ml of water. This slurry was stirred for 1 hr and allowed to settle for 1 hr. The supernatant was transferred to 50 ml tubes and centrifuged 5000 x g for 10 min. Pellets were resuspended in 150 ml of water in a glass beaker, one-half volume of concentrated nitric acid was added and the mixture was heated almost to boiling. The acid-treated glass was collected by centrifugation at 5000 x g for 10 min and washed with water until the pH of the mixture was 7.0. The fines were stored as a 50% slurry in water. DNA was purified from reaction mixtures by adding 2 volumes of sodium iodide solution (90.8 g sodium iodide, 1.5 g sodium sulfite per 100 ml of water) and 1 ul of glass particle solution per ug of DNA. This was vortexed and incubated for 10 min at room temperature. The glass particles were collected by brief centrifugation in a microcentrifuge and washed three times with 750 ul of an ethanol wash solution (50 ml ethanol, 50 ml 20 mM Tris, pH 7.4, 1 mM EDTA, 0.1 M

NaCl). DNA was eluted into water by incubation for 10 min at 50°C.

Assembly of a cDNA Expression Library - Double stranded cDNA was modified for ligation into the lambda gt11 by methylation of internal Eco RI restriction sites, addition of Eco RI linkers and ligation into lambda gt11 arms (Young and Davis 1983). Double stranded cDNA in 25 ul was methylated by adding 5 ul 1M Tris (pH 8.0), 5 ul 50 mM EDTA, 5 ul BSA (4 mg/ml), 5 ul 150 uM S-adenosyl methionine and 5 ul Eco RI methylase (20 U/ul). This reaction was incubated for 1 hr at 37°C, then terminated by incubation at 65°C for 10 min. After methylation, the ends of the double stranded cDNA were blunted by adding 6 ul of 1 mM dGTP, dATP, dCTP, dTTP, 6 ul of 0.15 M $MgCl_2$, 3 ul (5 U/ul) T4 DNA polymerase and incubating for 15 min at 37°C. The cDNA was purified by adsorption to glass particles and eluted into 22 ul of water. Eco RI linkers were ligated to the cDNA by adding 8 ul of phosphorylated linkers (GGAATTCC; OD260 = 10), 4 ul 10X T4 ligase buffer (250 mM Tris, pH 7.5, 100 mM $MgCl_2$, 100 mM DTT, 4 mM ATP) and 4 ul T4 ligase (400 New England Biolabs units/ul) and incubating overnight at 15°C. To the resulting 38 ul reaction, 10 ul water, 6 ul 10X digestion buffer (500 mM tris 8, 100 mM $MgCl_2$, 500 mM NaCl) and 200 U Eco RI were added and the mixture incubated at 37°C for 2 hr. Excess unligated linkers were removed by passing the reaction over a column of Sephadex G-50-80.

Radioactive peaks representing cDNAs were pooled and concentrated into a volume of 5 ul using glass particle adsorption. One ul containing 1 ug of dephosphorylated lambda gt11 arms, 5 ul cDNA, 1 ul 10X T4 DNA ligase buffer, 3 ul water and 1 ul T4 DNA ligase were mixed and incubated at room temperature for 4 hr. Ligated DNA was packaged in Gigapack II Plus packaging extract (Stratagene Cat. No. 200213). Four ul of the ligation reaction were added to phage protein extracts supplied in the kit. The packaging reaction was incubated at room temperature for 2 hr, then 500 ul of SM (5.8 g NaCl, 2 g $MgSO_4$, 50 ml 1 M Tris, pH 7.5, 5 ml 2% gelatin) were added. Twenty ul of chloroform were gently mixed in and the library was stored until titering.

The cDNA library was titered by plaque assay on lawns of E. coli. Agar plates (1.5% agarose in LB medium) were poured in 100 x 15 mm petri dishes and allowed to partially dry overnight. Ten-fold dilutions of phage ranging from 10^{-2} to 10^{-7} were prepared in SM, and 100 ul of each dilution were added to 100 ul of plating bacteria (E. coli Y1090) and incubated for 20 min at 37°C. Plating bacteria were produced by inoculating 50 ml of LB medium containing 10 mM $MgSO_4$ and 50 ug ampicillin/ml with an isolated colony of Y1090; this culture was incubated overnight at 37°C with vigorous shaking, centrifuged at 2000 x g for 15 min and resuspended in 0.5 volume of 0.01 M $MgSO_4$. After allowing 20 min for absorption of phage to E. coli, 2.5 ml of LB medium containing 0.7% agar and 0.01 M $MgCl_2$ were added to

the bacteria/phage mixture, and the tube was vortexed briefly and poured onto plates. Plates were inverted and incubated overnight at 37°C. The titer of the library was expressed in plaque forming units (pfu) per ml. The library was stored with chloroform at 4°C.

Isolation of immunologically reactive clones

Antibody Screening of Expression Library - Primary screening of the cDNA library was inititated by incubating approximately 50,000 pfu of phage with plating cells (E. coli Y1090) on a 150 x 10 mm petri dish. Plates were incubated at 42°C for 3.5 hours, then transferred to a 37°C incubator for 4.5 hours. The agar surfaces were overlaid with nylon filters (Zeta-Probe, Bio Rad Cat. No. 162-0159) that had been soaked in 10 mM isopropyl-beta-D-thiogalactopyranoside (IPTG) and partially dried, and growth was continued overnight (Young and Davis 1983).

Filters were marked with a 30 gauge needle at asymmetric points around its periphery, removed from the agar, washed briefly in TBST and then twice more for 30 min each in TBST. Washed filters were incubated in blocking buffer (TBST containing 10% bovine serum and 1.25% nonfat dry milk) at 37°C for 3-4 hr, washed as before and incubated with absorbed and diluted primary antibody for 3-4 hours at room temperature. The rabbit antiserum to porcine ZP was absorbed in TBST containing 1% bovine serum and 1:1000 dilution of Y1090 extract for 3 hours at 4°C, then diluted to a final concentration of 1:500. Extracts were produced

from overnight cultures of Y1090 that had been centrifuged, sonicated, boiled 10 min, recentrifuged and stored at 4°C until needed. Before use, the absorbed antibody was centrifuged at 10,000 x g for 15 min. After blocking, the filters were washed three times (10 min each) with TBST and incubated for 45 min at room temperature with secondary antibody (alkaline phosphatase conjugate of anti-rabbit IgG; Sigma A-8025). Filters were then washed twice more with TBST, once with TBS and immersed in substrate. The NBT-BCIP substrate was prepared as described above.

Filters showing positive signals on primary screening were aligned with their matching plates and the area around the positive signal was removed using the large end of a pasteur pipet. The plug of agar was placed into 1 ml of SM and phage were allowed to diffuse out for 2-3 hr at room temperature. From a plug of this size on a primary screen of 50,000 pfu, approximately 5 x 10^7 puf's/ml were obtained. The titer of the phage solution was determined and 2000 pfu plated out on a 100 x 10 mm plate and rescreened using the same techniques. Screening was continued until a single isolated plaque produced a filter in which all plaques gave positive signals. The single plaque was then amplified to obtain a sample with high titer.

Polymerase Chain Reaction (PCR) Analysis of Positive Clones - In some cases, cDNA inserts from immunologically reactive lambda clones were analyzed initially by PCR to

determine size of insert and to eliminate duplication of clones (Herrmann et al. 1990). Phage clones to be analysed were amplified to a final titer of greater that 10^8 by standard techniques (Maniatis et al. 1982). Five microliters of phage suspension were mixed with 100 pmol of extention primer 1 (5'-ACGACTCCTGGAGCCCGTCAGTA-3'), 100 pmol of extension primer 2 (5'-GGTAATGGTAGCGACCGGCGCTC-3'), 8 ul of dNTP mix (0.5 mM each of dATP, aCTP, dGTP, and dTTP) and 10 ul of 10X reaction buffer (Perkin-Elmer Cetus). The reaction was then brought to a final volume of 100 ul with water. PCR was carried out in a Perkin-Elmer Cetus DNA Thermal Cycler. Phage heads were disrupted at 94°C for 2 min and, after cooling to 80°C to allow primers to anneal, 2.5 U of Taq polymerase were added and 30 cycles of PCR initiated. Denaturing, annealing and extention steps were performed at 94°C for 1.5 min, 60°C for 2 min and 72°C for 4 min, respectively. Five to 10 ul of the final reaction were analyzed by agarose gel electrophoresis to determine size of inserts.

Mini-preparation of lambda clone DNA - Each immunoreactive phage clone was plated out on 100 x 15 mm plates and incubated at 37°C to produce a plate with confluent lysis. Five ml of SM were poured onto the plate and phage were allowed to diffuse out for 2-4 hr. SM was collected, a few drops of chloroform added and the sample stored until DNA was extracted. For each mini preparation of DNA, 0.6 ml of the lysate was processed, which yielded

enough DNA for 2 or 3 Southern blots. To 0.6 ml of lysate, 0.6 ml of DEAE-cellulose slurry equilibrated with LB media was added. The slurry was prepared by placing 100 g of DEAE-cellulose (Whatman DE52) in a beaker and slowly adding several volumes of 0.05 N HCl, checking to ensure that the pH dropped below 4.5. Then, with constant, gentle stirring, concentrated NaOH was added until the pH approached 7.5. The resin was allowed to settle and was washed three times with two volumes of LB medium. Finally, the DE52 was resuspended in LB to make a final slurry of approximately 75% resin and 25% LB. Sodium azide was added to 0.1% and the slurry was stored at 4°C. The phage lysate-slurry mixture was mixed gently 20-30 times and centrifuged 12,000 x g for 5 min. The supernatant was removed and again centrifuged 5 min, then adjusted to contain 10 mM EDTA and 0.5% SDS, extracted with phenol, phenol-chloroform, and chloroform. The resulting solution was made 0.2 M with sodium acetate and two volumes of ethanol were added. The resulting mixture was incubated at -70°C for 15 min, DNA collected by centrifugation (12,000 x g for 15 min) and washed with 70% ethanol, and the DNA pellet was resuspended in 20 ul of water.

Characterization of nucleic acid sequences

Gene subcloning - After a immunoreactive clone had been plaque purified and amplified, DNA was collected to further characterize the clone and ultimately to obtain its DNA

sequence. A fifty ml overnight culture of E. coli Y1090 was produced in medium without maltose. An aliquot containing 10^{10} cells (1 OD_{600} = 8 x 10^8 cell/ml) was centrifuged (2000 x g for 15 min) and bacteria were resuspended in 3 ml of SM. Recombinant phage (5 x 10^7 pfu) were added, mixed rapidly and incubated at 37°C for 20 min. The infected culture was added to 1 liter of LB containing 10 mM $MgCl_2$, and incubation continued overnight at 37°C with vigorous shaking. The following morning, the culture was inspected to assure that lysis had occurred, 10 ml of chloroform were added to the culture and it was vigorously shaken for 30 min. Cultures were centrifuged 15 min at 5000 x g and phage precipitated from the supernatant by the addition of 54 g NaCl and 63 g polyethylene glycol-8000 per liter and incubation overnight at 4°C. Phage were pelleted by centrifugation at 5000 x g for 20 min and gently resuspended in 5 ml SM using a wide bore pipet. Phage suspensions were then extracted with 5 ml chloroform and centrifuged at 5000 x g for 15 min. The opalescent aqueous phase was collected and, after addition of 0.5 g of $CsCl_2$ per ml, layered onto a cesium chloride step gradient (densities of 1.7, 1.5 and 1.45 g/ml). A mark was made at the interface of the 1.45 and 1.5 g/ml layers, which was the predicted location of the light blue band of phage at the end of centrifugation. The gradient was centrifuged in a SW27 rotor at 22,000 rpm for 4 hr at 4°C. Phage were collected by puncturing the side of the tube with a needle and collecting the blue band. Cesium

chloride was removed by dialysis for several hours against 2 changes of buffer (10 mM Tris, pH 7.5, 10 mM $MgSO_4$). After dialysis, 1/20th volume of 0.25 M EDTA was added and phage were extracted 3-4 times with phenol-chloroform and once with chloroform.

Purified lambda DNA was digested with Eco RI for 1 hr at 37°C and electrophoresed in 0.8% agarose. The gel was stained with ethidium bromide and DNA bands were visualized with ultraviolet light. Bands corresponding to the insert were cut out and DNA was isolated with glass particles by melting agarose in two volumes of sodium iodide solution at 55°C, adding 5 ul of glass particles, and binding, washing and eluting as described previously. Fragments were subcloned into Bluescript KS- (Stratagene) that had been digested with Eco RI and treated with 2 units calf intestinal phosphatase for 1 hr in buffer consisting of 0.05 M Tris, pH 9, 1 mM $MgCl_2$ and 0.1 mM zinc. Ligation of the fragment to the vector (1:1 molar ratio) was conducted at 16°C overnight and a fraction of the mixture used to transform E. coli DH5 alpha. The transformed bacteria were plated onto LB agar containing 40 ug 5-bromo-4-chloro-3-indolyl-beta-D-galactoside, 120 ug IPTG and 100 ug ampicillin per ml to allow color selection of bacterial colonies containing plasmid with insert.

The presence of the fragment was confirmed by restriction enzyme digestion of DNA from "minipreps" of 12-18 white colonies. One and one-half ml of a 5 ml overnight

culture from an isolated colony were centrifuged briefly to pellet bacteria. The pellet was resuspended in 100 ul STET (8% sucrose, 50 mM Tris, pH 8.0, 50 mM EDTA, 5% Triton X-100), 10 ul of lysozyme (10 mg/ml) were added and the tubes incubated on ice for 10 min. Tubes were then boiled for 2 min and centrifuged at 10,000 x g for 15 min. Sixty-five ul of supernatant were removed and plasmid was isolated with glass particles as described previously. Isolated plasmid DNA was digested with Eco RI and electrophoresed in 0.8% agarose. Bacteria confirmed as having the desired plasmid construct were frozen at -70°C in 30% glycerol.

Bacteria containing the desired plasmid were plated out on LB plates and incubated overnight at 37°C. An isolated colony was inoculated into 50 ml LB broth containing 50 ug ampicillin/ml and incubated overnight with shaking. A 1:10 dilution of the overnight culture was made into 500 ml LB broth containing ampicillin and grown with shaking to an OD_{600} of approximately 0.7. Chloramphenicol was then added to a final concentration of 10 ug/ml and the cultures incubated overnight with shaking at 37°C. Amplified bacteria were collected by centrifugation (5000 x g for 10 min) and resuspended in 3 ml of a solution containing 50 mM Tris (pH 7.5), 50 mM EDTA, 25% sucrose and 10 mg lysozyme. This mixture was incubated on ice for 5 min, then mixed with 6 ml of "Triton mix" (prepared by mixing 2 ml 10% Triton X-100, 50 ml 0.25 M EDTA, 10 ml 1 M Tris, pH 8.0 in a final volume 200 ml), incubated again on ice for 20 min and

centrifuged at 43,000 x g for 45-60 min. To 10 ml of the resulting supernatant was added 9.5 g CsCl and 1 ml ethidium bromide (10 mg/ml); after dissolution, this solution was centrifuged at 43,000 x g (19,000 rpm in Beckman J-20 rotor) for 10 min and the supernatant carefully separated from protein aggregates formed at the top of the tube. The clarified solution was added to polyallomer centrifuge tubes (Seton Scientific no. 9041), topped with mineral oil and centrifuged in a Beckman Ti70.1 rotor at 55K for 16 hr, 40K for 1 hr and then decelerated at profile 2 (full braking to 350 rpm then no braking). Plasmid bands were identified with ultraviolet light and collected with a 20 gauge needle through the side of the centrifuge tube, dialyzed 2 hr against TE (10 mM Tris, pH 8.0, 1 mM EDTA), extracted with phenol and chloroform and, after addition of 1/20 volume of 7.5 M ammonium acetate, precipitated with ethanol. Plasmid DNA was collected by centrifugation (10,000 x g for 30 min), washed with 70% ethanol, air dried and resuspended in TE (pH 8.0). DNA concentration was determined spectrophotometrically.

Sequencing - Sequencing was performed using Sequenase kits purchased from US Biochemical Corporation. Cesium chloride gradient-purified plasmid DNA (1-2 ug) containing fragments to be sequenced was dissolved in 40 ul denaturing buffer (0.2 M NaOH, 0.2 mM EDTA) and incubated at room temperature for 5 min. Four ul of neutralization buffer (2 M ammonium acetate, pH 4.5) were added and immediately

afterward 100 ul ethanol were added. The mixture was incubated at -70°C for 5 min, then centrifuged at 10,000 x g for 15 min. The DNA pellet was washed in 70% ethanol and dried under vacuum. Primers complementary to plasmid sequences were annealed to denatured plasmid by adding 1 ul (2.5 pmol) of primer DNA, 1.5 ul 10X reaction buffer, 1 ul (alpha-^{35}S)dATP (10 uCi/ul) in a final volume of 15 ul. After incubation for 15 min at 37°C, 1 ul (2 units) of Klenow DNA polymerase was added to the reaction tube, mixed, and 3 ul of that mixture added to each of four tubes labeled A (100 uM dGTP, dTTP, dCTP, ddATP), C (100 uM dGTP, dTTP, 10 uM dCTP, 100 uM ddCTP), G (5uM dGTP, 100 uM dTTP, dCTP, 120 uM ddGTP) and T (100 uM dGTP, dCTP, 5 uM dTTP, 500 uM ddTTP). These reaction tubes were incubated at 37°C for 15 min, followed by addition of 1.5 ul deoxynucleotide mixture (0.125 mM of each dATP, dCTP, dGTP, dTTP). Incubation at 37°C was continued for 15 min and the reaction was terminated by adding 4 ul of formamide buffer.

Sequencing reactions were electrophoresed on urea-polyacrylamide gels. One hundred ml 7M urea-6% polyacrylamide (424.2 g urea, 150 ml 38:2 acrylamide:bis acrylamide, 200 ml 5X TBE per liter) were degassed for 15 min. One ml of 10% ammonium persulfate and 20 ul of TEMED were mixed in, the solution poured between two glass plates and allowed to polymerize at least 45 min. Gels were pre-run in a Model S2 sequencing apparatus (Bethesda Research Laboratory) for 45 min at 60 Watts. Sample wells were

thoroughly rinsed with TBE running buffer (1X TBE = 0.089 M Tris-borate, 0.025 M EDTA, pH 8.3) and heat denatured samples were added and electrophoresed at 60 Watts for 2-6 hours. After electrophoresis, the plates were separated and the gel transferred onto filter paper, dried under vacuum at 80°C for 1-2 hr and exposed on X-Omat AR film overnight.

Computer analysis of nucleic acid and protein sequence data - The PC/GENE program (Intelligenetics, Inc.) was used to align data from DNA sequencing runs, generate restriction maps, translate DNA sequencing into protein sequence, and search for glycosylation signatures.

Southern Analysis - Samples of DNA were electrophoresed in 0.8% agarose prepared in TAE buffer (16 mM Tris acetate, 0.02 mM EDTA, pH 8) at 90 V for 4-5 hours or 35 V overnight until the bromophenol blue dye marker was at least 2/3 of the way down the gel. The gel was incubated in 0.25 N HCl for 10 min, rinsed briefly in water, denatured in two changes of 0.5 M NaOH/1.5 M NaCl (1 hour each) and then rinsed in water. The gels were then processed through two one-hour changes of 0.5 M Tris (pH 7.5)/1.5 M NaCl, rinsed in water and DNA fragments were transferred overnight onto nylon filters in 10X SSC (1X SSC is 150 mM NaCl and 15 mM sodium citrate; pH 7.0). Filters were then dried under a heat lamp for several hours.

Dried filters were soaked briefly in 4X SSPE (1X SSPE contains 180 mM NaCl, 10 mM NaH_2PO_4 and 1 mM EDTA; pH 7.4) in a Seal-a-Meal bag and prehybridized for 1 hr at 43°C in a

solution of 50% deionized formamide, 0.5% Denhardt's solution, 4X SSPE, 1% SDS, 0.15 mg heat denatured, sonicated salmon sperm DNA/ml and 0.35% w/v non-fat dry milk.

Pre-hybridization solution was poured off and hybridization solution was added. Hybridization solution was the same in composition as pre-hybridization solution with the addition of heat denatured, ^{32}P-labeled probe. Hybridization progressed overnight at 43°C with slow shaking.

The radioactive cDNA probes were prepared by the random priming method (Feinberg, 1984). Fifty ng of DNA in 32.5 ul were boiled 10 min and cooled rapidly on ice. To this was added 10 ul of oligo-labeling buffer (containing the hexanucleotides), 2 ul of BSA (10 mg/ml), 5 ul of alpha ^{32}P-dCTP (50 uCi) and 2 units of Klenow fragment DNA polyerase. The reaction mixture was incubated 2 hours at room temperature, followed by 0.5 hr at 37°C. Radiolabeled probe was purified by passage over a column of Sephadex G-50-80 prepared in a siliconized pasture pipet plugged with glass wool (Maniatis et al. 1982). Dextran blue was added to the reaction mixture to follow its progression down the column. The blue band collected represented the labeled cDNA and was used in the hybridization mixture. The specific activity of probes generated with this method was typically greater than 10^9 cpm/ug.

After hybridization was complete, hybridization solution was removed, filters were successively washed in 2X

SSC/0.1% SDS at room temperature for 1 hr, 1X SSC/0.1% SDS at 65°C for 30 min and 0.2X SSC/0.1% SDS at 65°C for 20 min. They were then wrapped in plasticwrap and exposed to X-OMAT AR film with intensifying screens at -80°C for up to 2-3 days.

Northern Analysis - Total and polyadenylated RNA was prepared as described previously. Ten ug of total RNA or 3 ug of polyadenylated RNA were dissolved in 4 ul of water and mixed with 16 ul of RNA sample buffer (0.75 ml deionized formamide, 0.15 ml 10X MOPS buffer, 0.24 ml 37% formaldehyde, 0.1 ml water, 0.1 ml glycerol and 0.08 ml 10% bromophenol blue). The sample was then heated at 55°C for 15 min and electrophoresed with recirculation of buffer at 90 V in 1X 3-(n-morpholino) propanesulfonic acid (MOPS) buffer (10X MOPS contains 0.2 M MOPS, 50 mM sodium acetate, 10 mM EDTA, pH 8.0) for 3-4 hours. The denaturing agarose gel was composed of 1% agarose in 1X MOPS buffer containing 2.2 M formaldehyde (Lehrach et al. 1977; Goldberg 1980).

Following electrophoresis, the gel was processed through two 1-hour incubations with 10X SSC at room temperature, and RNA was transferred to nylon membranes as described for Southerns blots. Probe preparation, pre-hybridization and hybridization were also performed as described for Southern hybridizations except that washings were conducted at 55°C.

Characterization of the zona pellucida gene

Protein expression - The vector selected for production of recombinant protein was pGEX-2T (Smith and Johnson 1988) which expresses inserted cDNA in E. coli as a fusion with glutathione-S-transferase (GST); expression is under control of the IPTG-inducible tac promotor. After growth and lysis of bacteria containing plasmid, fusion protein is isolated by glutathione-agarose chromatography. The GST portion of the fusion protein binds to glutathione, which is covalently attached to agarose, and other proteins are washed away. Fusion protein is then competitively eluted from the solid phase by incubation with free glutathione.

After sequencing the PZP.3 fragment of porcine ZP cDNA, it was determined that a 412 bp HincII-Eco RI fragment could be ligated in frame into pGEX-2T digested with SmaI and Eco RI. An isolated bacterial colony containing this plasmid was grown overnight in 50 ml LB at 37°C, diluted 1:10 into 500 ml fresh LB at 37°C and grown to mid-log phase (OD_{600} = 0.6-1.0). IPTG was added to 1 mM and incubation continued for an additional 5 hr. Bacteria were collected by centrifugation (4000 x g for 10 min) and resuspended in 1/50th volume of MTPBS (150 mM NaCl, 16 mM Na_2HPO_4,4 mM NaH_2PO_4, pH 7.3) and sonicated briefly. Triton X-100 was added to a concentration of 1% and the lysate centrifuged for 5 min at 10,000 x g at 4°C. The resulting supernatant was mixed with 1-2 ml 50% glutathione-agarose (40 mg/ml, swollen in and washed 3 times with MTPBS) and incubated for

2 min at room temperature. The agarose beads were pelleted at 500 x g for 10 sec, then washed three times with MTPBS. Fusion protein was collected by elution in a solution of 5 mM glutathione in 50 mM Tris, pH 8.0. Protein concentration was determined by dye-binding assay (BioRad) and protein size and purity was assessed by SDS-PAGE.

A rabbit was immunized with the fusion protein to develop an antiserum for confirmation that the clone represented a ZP DNA sequence. One mg of fusion protein in a volume of 500 ul was mixed with an equal volume of Freund's complete adjuvant and injected subcutaneously and intramuscularly. Booster injections of the same dose in Freund's incomplete adjuvant were given every 7 days for 6 weeks.

Zona pellucida staining - Normal rabbit serum and immune serum from the rabbit immunized with fusion protein were used to fluorescently stain the ZP of porcine oocytes as described previously. Sera were diluted 1:50 and incubated at 4°C overnight.

Immunoprecipitations - Immunoprecipitations were conducted using normal rabbit serum, rabbit antiserum against solubilized ZP proteins and serum from the rabbit immunized with GST fusion protein. Solubilized porcine ZP proteins were enzymatically labeled with ^{125}I using lactoperoxidase and separated from free iodine by sephadex chromatography (kindly performed by personnel in the laboratory of Dr. G. D. Niswender). Fifty microliters of

serum were incubated on ice with 1 x 10^6 cpm of ^{125}I-labeled ZP protein for 1 hour in a total volume of 400 ul. To this was added 100 ul of protein A-Sepharose slurry (10% v/v in RIPA buffer, which was composed of 150 mM NaCl, 1% NP-40, 0.5% sodium deoxycholate, 0.1% SDS, 50 mM Tris, pH 7.6). The mixture was incubated for 1 hour at 4°C, then washed three times with cold RIPA buffer. After washing, 25 ul of 1X PAGE sample buffer were added, the mixture was boiled for 10 min and briefly centrifuged. The supernatant was removed and analyzed by one-dimensional or two-dimensional PAGE.

RESULTS

Production of Antiserum to Porcine Zona Pellucida Proteins

At the outset of this project, the only ZP gene that had been cloned and sequenced was mouse ZP3. It therefore seemed worth investigating whether this sequence could be used to identify a homologous ZP protein from pigs. Toward that end, I first performed Northern analysis to determine whether a radiolabeled mouse ZP3 probe would hybridize to swine ovarian RNA. The complete mouse ZP3 cDNA was kindly provided by Dr. Jurrian Dean at the NIH (Ringuette, et al., 1986). Total ovarian RNA was prepared using tissue from five different swine. One of these samples consistently displayed hybridization to mouse ZP3 (Fig. 2.1, lane c), but repeated blots using RNA from the other four animals failed to show any evidence of hybridization. Polyadenylated RNA purified from the sample of total RNA showing hybridization

to mouse ZP3 also failed to reveal any evidence of a hybridizing sequence, as did polyadenylated RNA purified from two of the other samples of total RNA. I concluded that the hybridization observed with total RNA from that one animal was spurious and that the mouse ZP3 clone would not be effective in identifying porcine ZP genes. It was therefore decided to develop antibodies against porcine ZP proteins for use in immunoscreening an expression library.

An antiserum was prepared against solubilized porcine ZP proteins and its reactivity characterized in several ways. In comparison to normal rabbit serum, the antiserum bound avidly to ZP surrounding porcine oocytes, as demonstrated by bright immunofluorescent staining (Fig. 2.2). The specificity of the antiserum for the ZP was initially assessed by immunocytochemical staining of porcine ovarian sections. Zonae pellucidae were the only structures observed to show staining above background (Fig. 2.3), indicating that the antigen preparation used for immunization did not contain significant quantities of antigens derived from follicular or other cells.

It was also of interest to determine the extent to which the antiserum crossreacted with ZP from other species. Immunofluorescence was again utilized to assess binding of

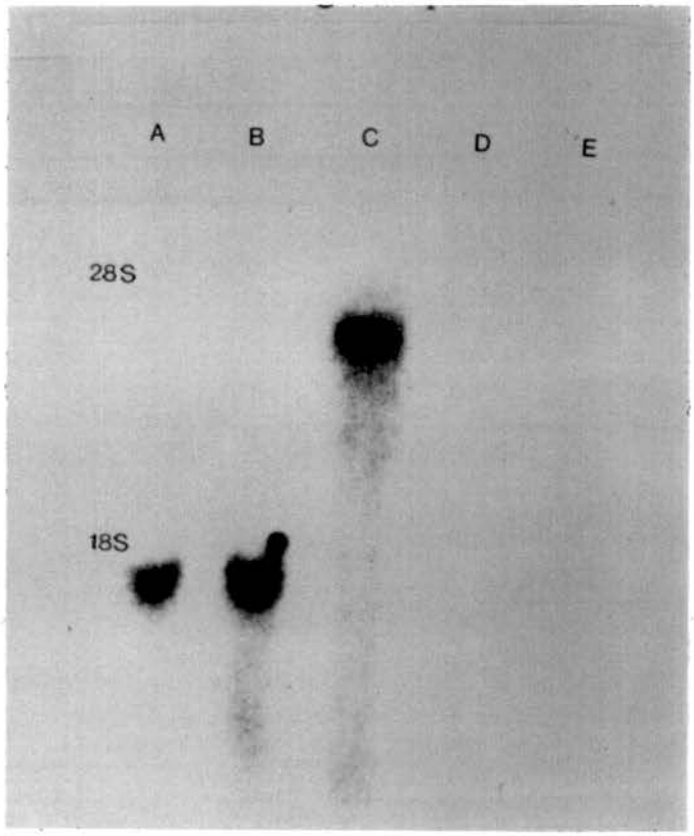

Fig. 2.1 Northern hybridization analysis of mouse and porcine ovarian RNA probed with mouse ZP3 sequences. Lanes A and B: mouse ovarian RNA; C-E: porcine ovarian RNA isolated from three different animals.

antibodies to ZP-encased oocytes from mice, cats, dogs, sheep and cattle. As expected from work of other investigators, the antiserum against porcine ZP failed to bind detectably to mouse ZP (Fig 2.4). It did however stain ZP from each of the other species (Fig. 2.5 - 2.8) Canine ovarian sections were also stained with the immune serum. In this case, antibodies were shown to not only bind to the ZP, but also to a subpopulation of follicular cells (Fig 2.9).

The antiserum against porcine ZP proteins was produced by immunization with native proteins, and there was a need to determine whether the antibodies would recognize non-glycosylated and effectively denatured ZP proteins, as would be produced by recombinant phage. Immunoblotting after separation of proteins by SDS-PAGE under reducing conditions was therefore performed. Immunoglobulins in the antiserum clearly bound to ZP proteins subjected to those conditions (Fig. 2.10). Dilution of the antiserum 500-fold consistently gave a distinct signal with little background staining; this dilution was therefore used for immunoscreening the lambda library; higher or lower dilutions of antiserum resulted in greatly reduced intensity of the bands or problems with non-specific and background staining. To evaluate the antiserum under conditions simulating plaque screening, dilutions of solubilized ZP proteins were adsorbed to a nylon membrane and detected by immunologic dot blot with the anti-ZP antiserum (Fig 2.10).

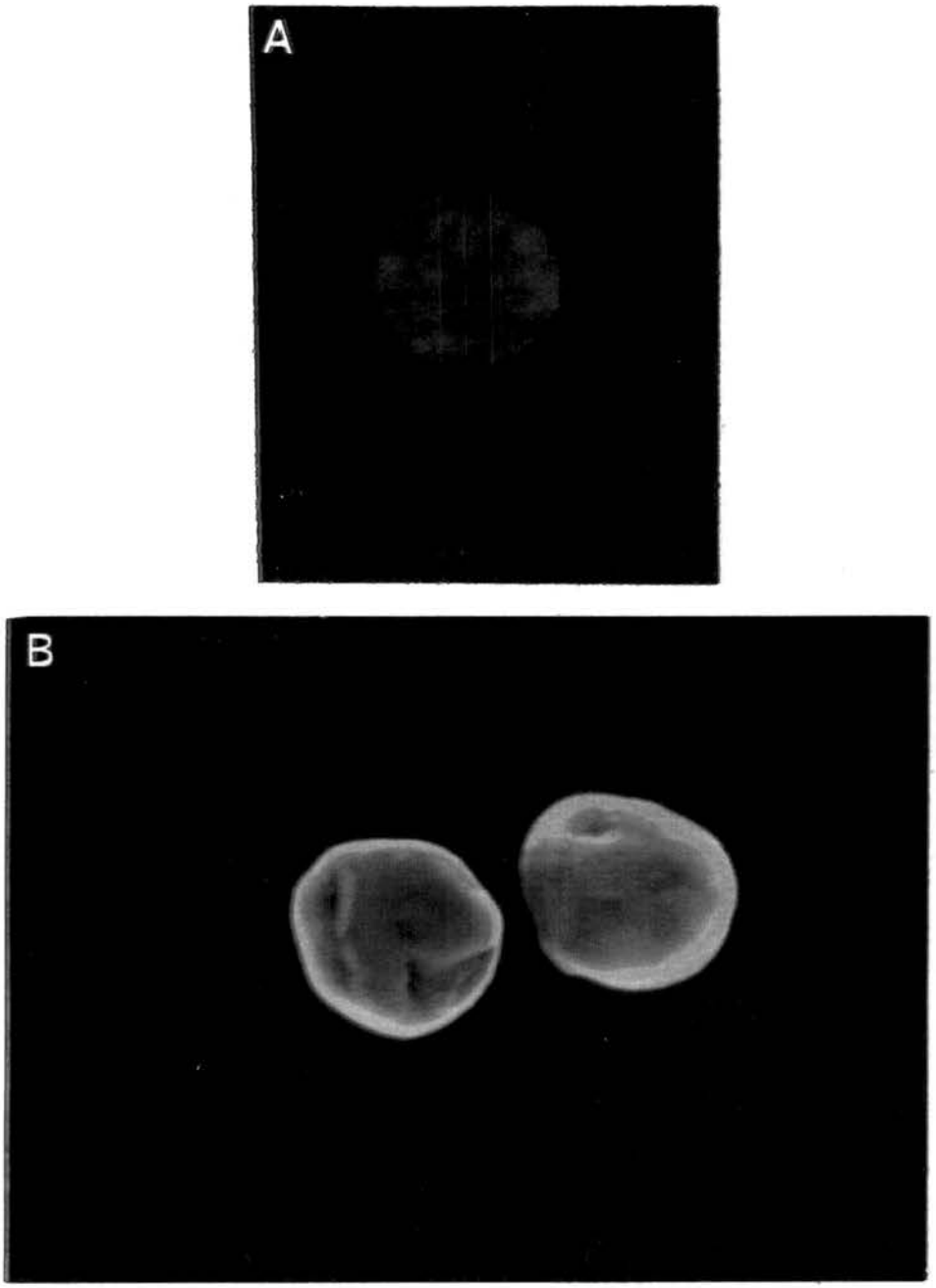

Fig. 2.2 Immunofluorescence staining of ZP-encased porcine oocytes. A) Normal rabbit serum; B) Rabbit antiserum to solubilized porcine ZP (both sera diluted 1:100).

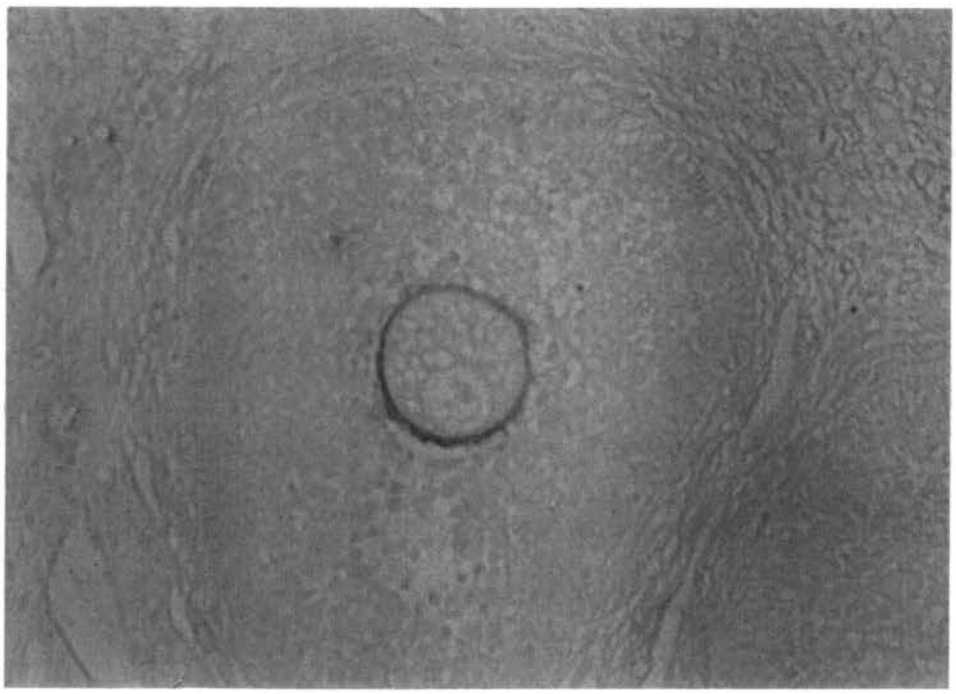

Fig. 2.3 Immunohistochemical staining of porcine ovarian section with antiserum to porcine solubilized ZP. Dark ring of reaction product localizes the ZP surrounding an oocyte (100X magnification).

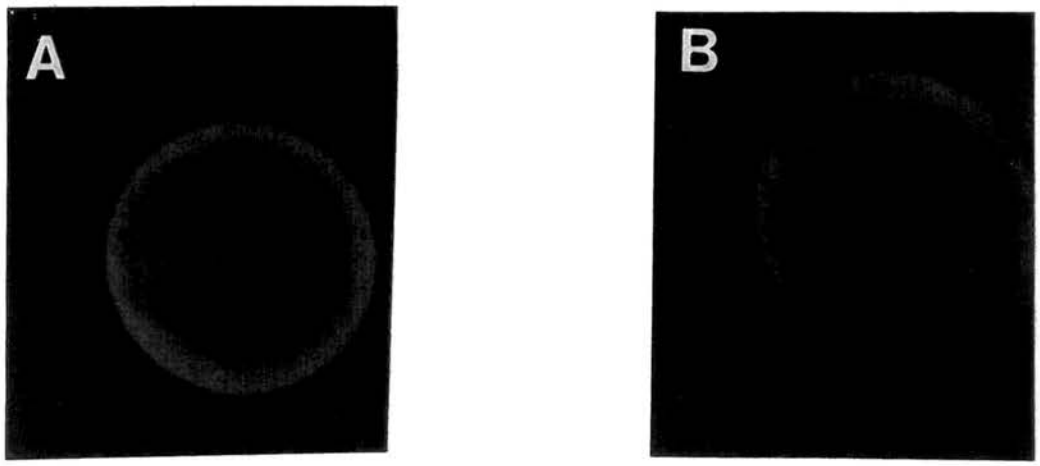

Fig. 2.4 Immunofluorescence staining of ZP-encased mouse oocytes. A) Normal rabbit serum; B) Rabbit antiserum to solubilized porcine ZP (both sera diluted 1:100).

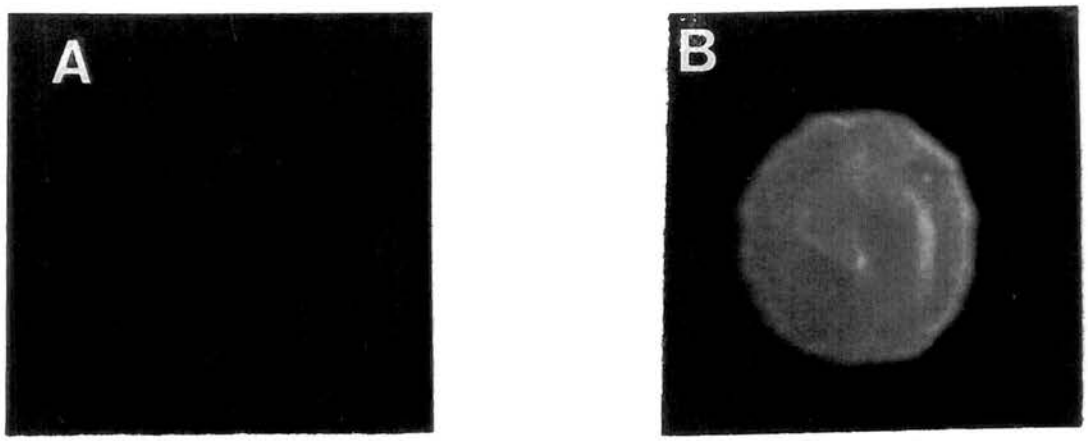

Fig. 2.5 Immunofluorescence staining of ZP-encased feline oocytes. A) Normal rabbit serum; B) Rabbit antiserum to solubilized porcine ZP (both sera diluted 1:100).

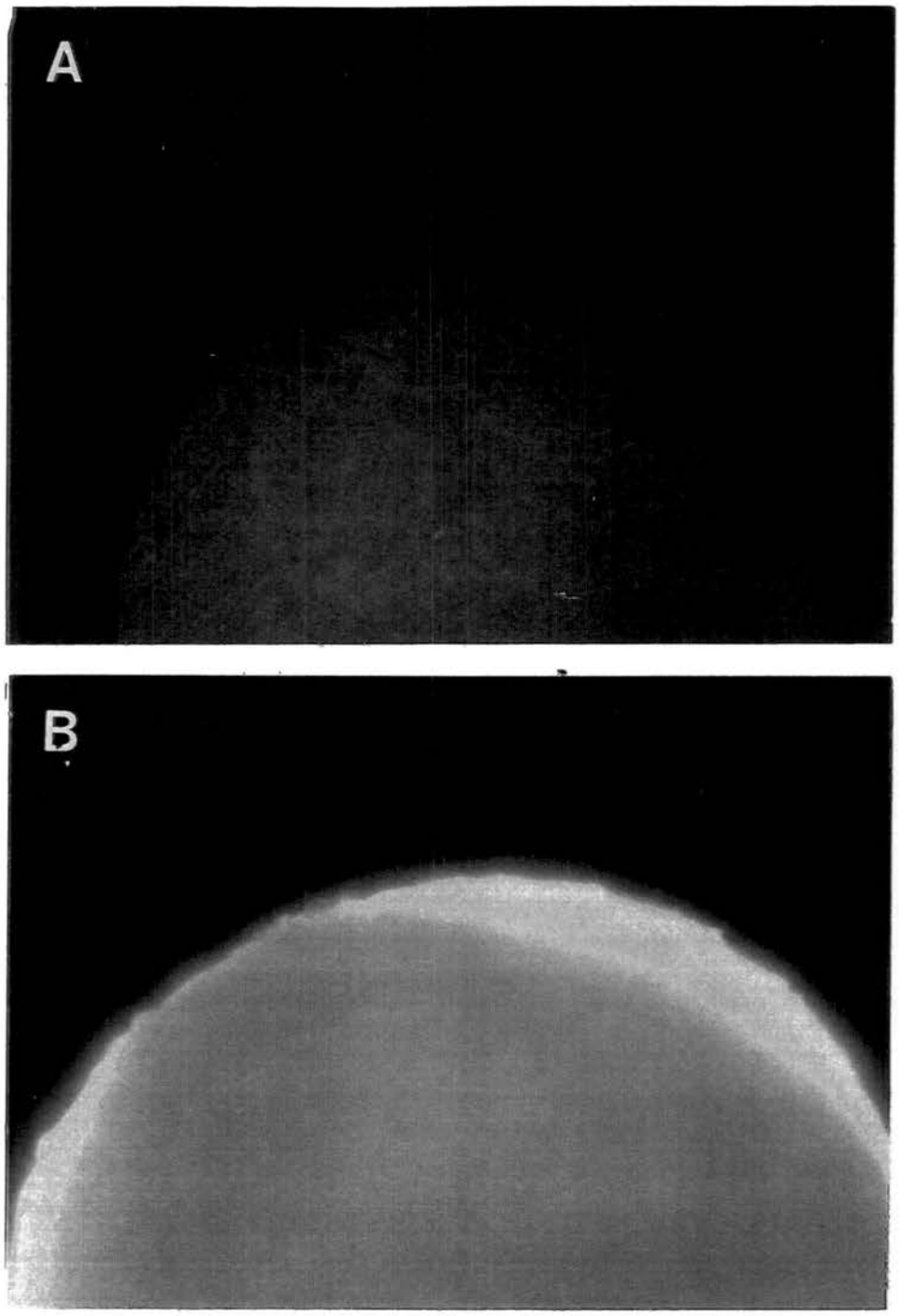

Fig. 2.6 Immunofluorescence staining of ZP-encased canine oocytes. A) Normal rabbit serum; B) Rabbit antiserum to solubilized porcine ZP (both sera diluted 1:100).

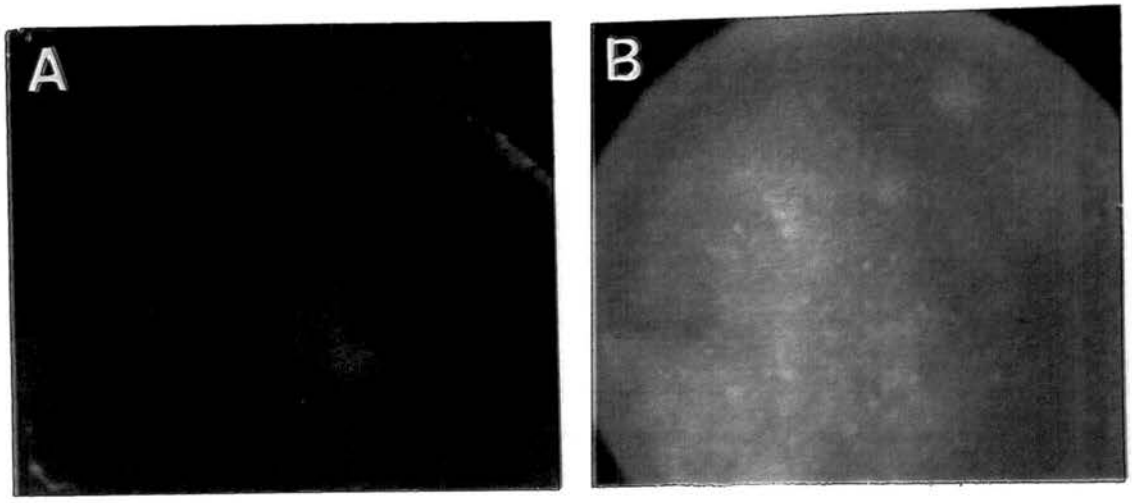

Fig. 2.7 Immunofluorescence staining of ZP-encased ovine oocytes. A) Normal rabbit serum; B) Rabbit antiserum to solubilized porcine ZP (both sera diluted 1:100).

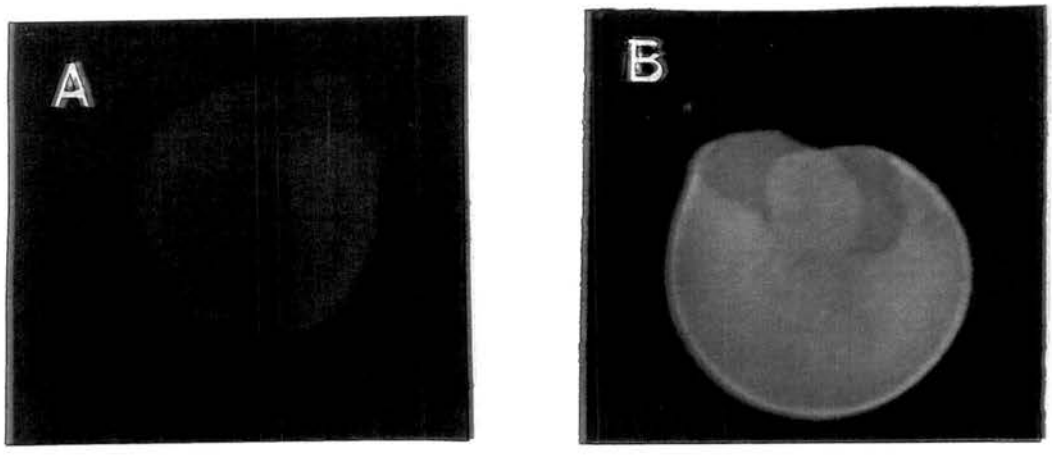

Fig. 2.8 Immunofluorescence staining of ZP-encased bovine oocytes. A) Normal rabbit serum; B) Rabbit antiserum to solubilized porcine ZP (both sera diluted 1:100).

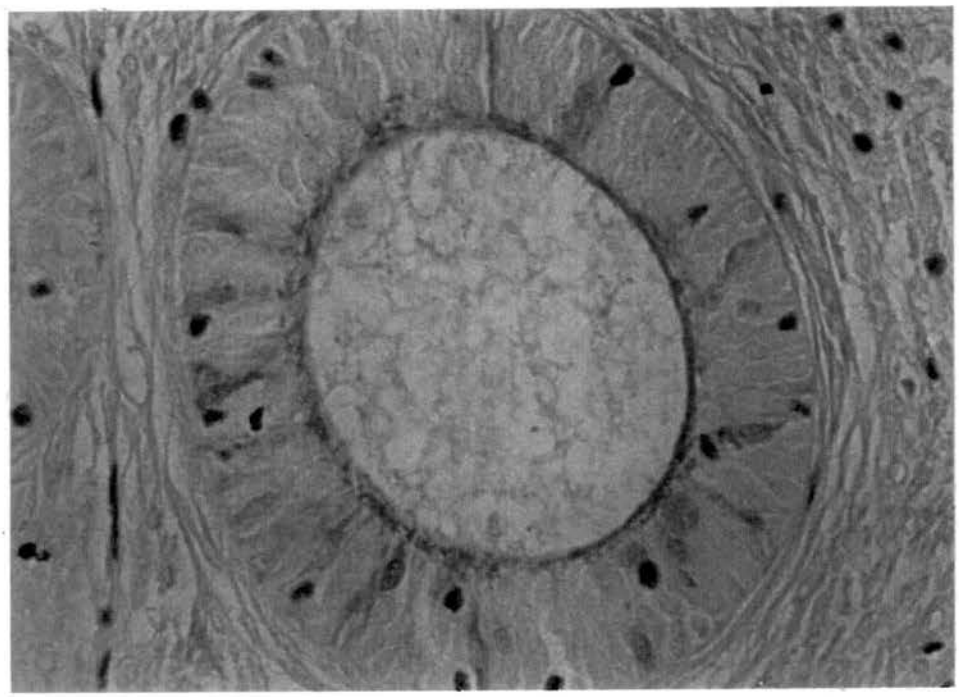

Fig. 2.9 Immunohistochemical staining of canine ovarian section with antiserum to porcine solubilized ZP. Reaction product is seen as a dark precipitate in the ZP surrounding the oocyte and in a subpopulation of follicular cells (400X magnification).

Fig. 2.10 Immunoblot analysis of solubilized porcine ZP proteins. ZP proteins were electroblotted from a SDS-polyacrylamide gel onto a nitrocellulose membrane, which was then cut into strips and exposed to different dilutions of either rabbit antiserum to solubilized porcine ZP (strips 1, 3 and 5) or normal rabbit serum (strips 2, 4 and 6). These sera were used at dilutions of 1:100 (A), 1:500 (B) and 1:1000 (C). The lower immunoblot consists of decreasing quantities of solubilized porcine ZP spotted onto nitrocellulose and detected after exposure to a 1:500 dilution of anti-ZP immune serum. (a. 50 ug, b. 25 ng, c. 10 ng, d. 5 ng, e. 2 ng, f. 1 ng, g. 0.5 ng, h. 0.2 ng, i. 0.1 ng, j. 0.05 ng, k. 0.02 ng, l. 0.01 ng).

With this assay, spots containing 100-1000 pg of ZP protein were clearly detectable, affording confidence that the antiserum would allow detection of the approximately 1 ng of recombinant protein contained in a phage plaque.

Construction and Screening of an Ovarian cDNA Expression Library

Polyadenylated RNA purified from total swine ovarian RNA was used to generate double stranded cDNA. After addition of Eco RI linkers to the cDNA, it was ligated to lambda gt11 arms and packaged into phage particles. Plaque titration revealed that the library contained approximately 1.5×10^6 pfu and that approximately 95% of the phage in the library were recombinants, as revealed by staining for beta-galactosidase activity. Electrophoretic analysis of an aliquot of the first strand cDNA showed that the average length of cDNA was 0.5 - 1.5 kb. This original phage library was purposefully not amplified and during the course of these studies was consumed in screening. In the hopes of obtaining a full length cDNA for that isolated from this library, a second lambda gt11 library was produced by Clonetech Inc., using polyadenylated swine ovarian RNA that I provided.

Immunoscreening of approximately 300,000 phage plaques from the original library resulted in detection of one reactive clone, referred to hereafter as PZP, which was plaque-purified to homogeneity (Fig. 2.11). Restriction analysis of Eco RI-digested lambda DNA isolated from this

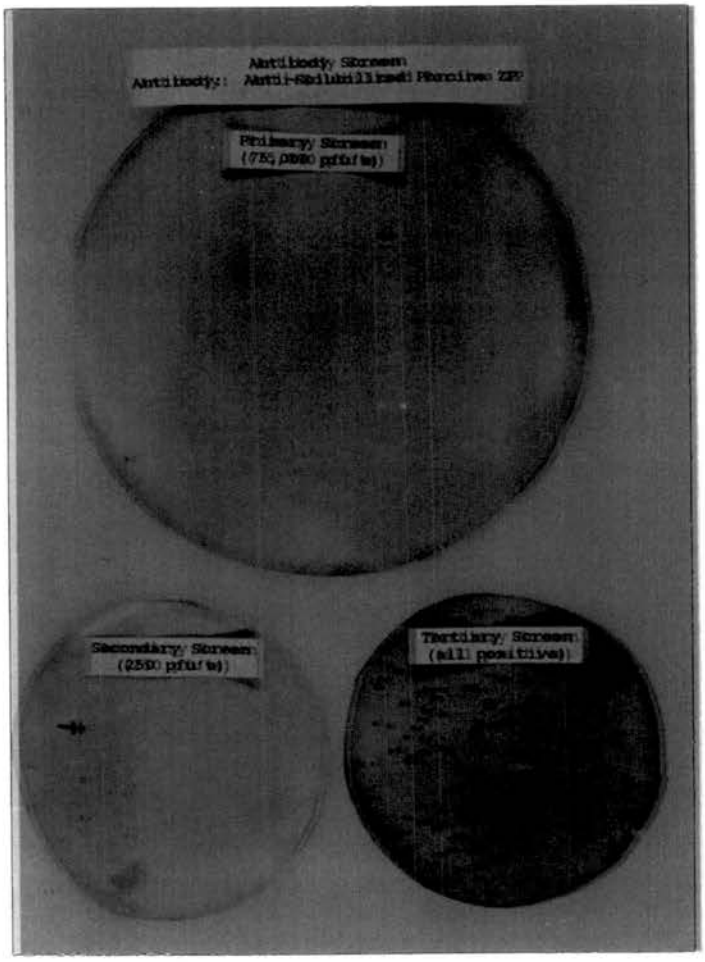

Fig. 2.11 Immunoscreening of recombinant lambda gt11 phage plaques demonstrating identification of the original PZP-containing phage plaque and its subsequent purification to homogeneity.

clone showed the cDNA to be composed of three Eco RI fragments with sizes of approximately 1.2, 0.8 and 0.5 kb; these were designated fragments 2.1, 2.2 and 2.3, respectively. Each of these fragments was isolated from an agarose gel and subcloned into a plasmid vector (Bluescript KS$^-$). In addition, each of the fragments was individually subcloned back into lambda gt11 to allow immunoscreening in that environment. Only one of the three derivative lambda clones, that constructed with the 0.5 kb fragment, was reactive with the anti-ZP antiserum, indicating that it was first in the sequence of the three fragments and in-frame with the lacZ gene of lambda gt11 in the original phage clone.

An attempt was made to identify a full length or longer clone of PZP by screening the original library with radiolabeled PZP.3 fragment. Because this fragment had been shown to represent the 5'-most end of the cDNA and was only 600 bp in length, phage harboring cDNAs much shorter than PZP would not have been detected. Two hybridizing plaques were detected in the approximately 300,000 pfu remaining in the library. Neither of these two plaques, when partially sequenced, contained additional 5'-sequences. One positive plaque in 500,000 pfs's was identified from the Clone Tech pig ovarian library, plaque purified, and shown to harbor an insert approximately 1100 bp in length. Restriction digests and partial sequence analysis of the insert in this phage revealed it to overlap the PZP.3 and PZP.1 fragments of PZP,

but failed to contain additional 5'-sequences. Isolation of this clone thus provided confirmation that PZP.3 and PZP.1 were contiguous and derived from the same mRNA, but due to its being entirely contained within PZP, this clone was not pursued further.

I was also interested in continuing the search for additional porcine ZP genes and toward that end screened a fraction of the Clontech amplified cDNA library with the antiserum to porcine ZP proteins. A total of six immunoreactive plaques were identified and purified (designated as clones ZPA, ZPB, ZPC, ZPD, ZPE and ZPF). In order to rapidly determine whether the inserts in these phage represented mRNAs different from that of PZP and which were duplicates, the inserts plus small flanking regions from the phage were amplified from crude phage preparations by PCR using standard lambda gt11 sequencing primers (Fig. 2.12). These PCR products were then radiolabeled by random priming and used to conduct cross-hybridization studies. DNA from minipreparations of the six new clones, as well as DNA from PZP, were digested with Eco RI and subjected to Southern hybridization with PZP sequences and probes derived from the other clones (Fig. 2.13). Probe prepared from PZP (fragments PZP.3 and PZP.1 combined) failed to hybridize with any of the six new clones and vica versa. Among the six new clones, probe made from ZPA hybridized to itself and ZPC, while probe made from ZPB failed to hybridized to sequences besides itself. None of the probes used in this

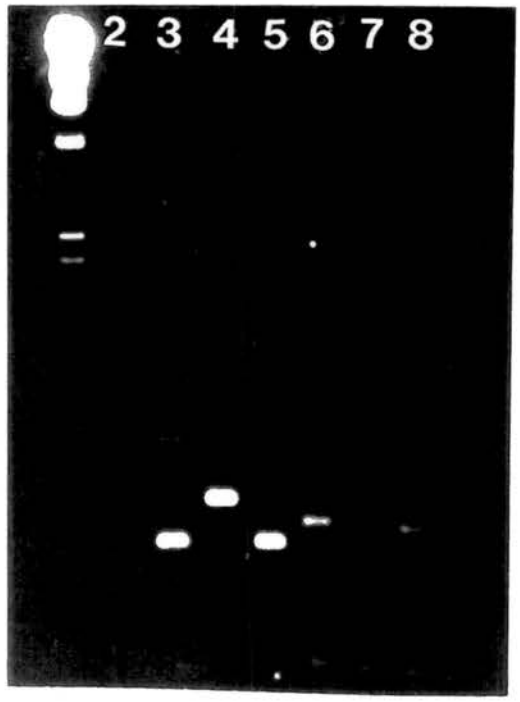

Fig. 2.12 PCR analysis of cDNA inserts in phage detected by immunoscreening. Lambda gt11 sequencing primers were used to amplify the insert from DNA in phage lysates. Lanes (1): Molecular weight markers (Hind III-digested wild-type lambda); (2) PZP (this insert was approximately 2.3 kb in length and could not be amplifed; (3) - (8) additional uncharacterized but immunoreactive clones (designated ZP A-F).

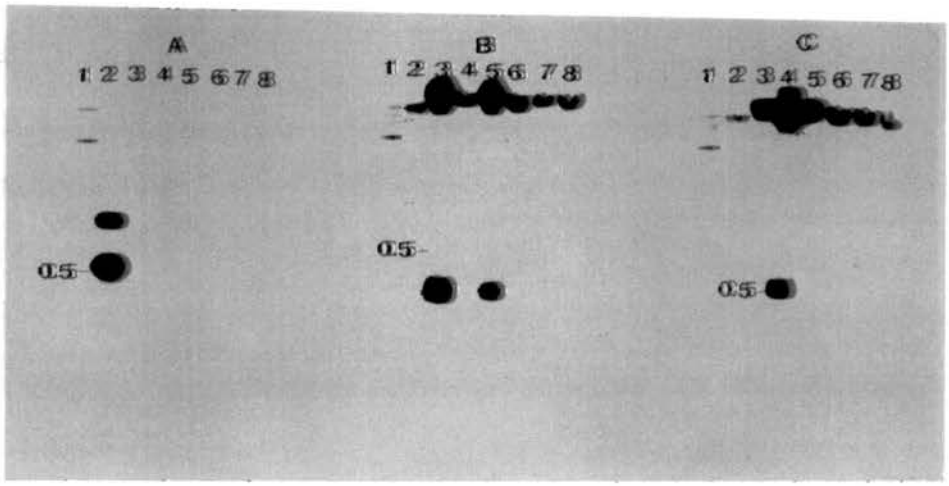

Fig. 2.13 Southern hybridization of immunoreactive phage clones. Lane numbers correspond exactly to those in Figure 2.12 and consist of phage DNA isolated from minipreps and digested with Eco RI. Radiolabeled probes were generated from (A) PZP (fragments PZP.3 and PZP.1); (B) Phage ZPA; (C) Phage ZPB.

experiment hybridized to ZPD, ZPE or ZPF. Although a considerable amount of work remains to be done with these clones to establish their identity and confirm that they indeed represent porcine ZP genes, it seems likely that portions of at least three additional porcine ZP genes have been isolated. The remainder of this dissertation deals with further characterization of the PZP clone.

Characterization of a Porcine Zona Pellucida Gene

Expression of PZP protein in E. coli - It was necessary to confirm that the mRNA represented in the PZP clone encoded a ZP protein. If such were the case, immunization of an animal with recombinant protein expressed from the cDNA should result in an antiserum that recognized a native ZP protein. Also, because proteins in the porcine ZP appear to be encoded by three genes, it was desireable to identify which of the genes had been isolated. I therefore inserted a portion of the PZP cDNA into a procaryotic expression system to obtain recombinant protein. The PZP.3 fragment was selected for use in this effort because it was the first to be well characterized and appeared to encode a relatively hydrophilic portion of the protein. This fragment was inserted in-frame into pGEX-2T and the resulting plasmid transformed into E. coli. Preliminary experiments were performed to determine the optimal time for harvest of bacteria after induction of protein expression with IPTG; on the basis of these studies, an induction time of 5 hours was selected (Fig 2.14). It was ultimately determined that

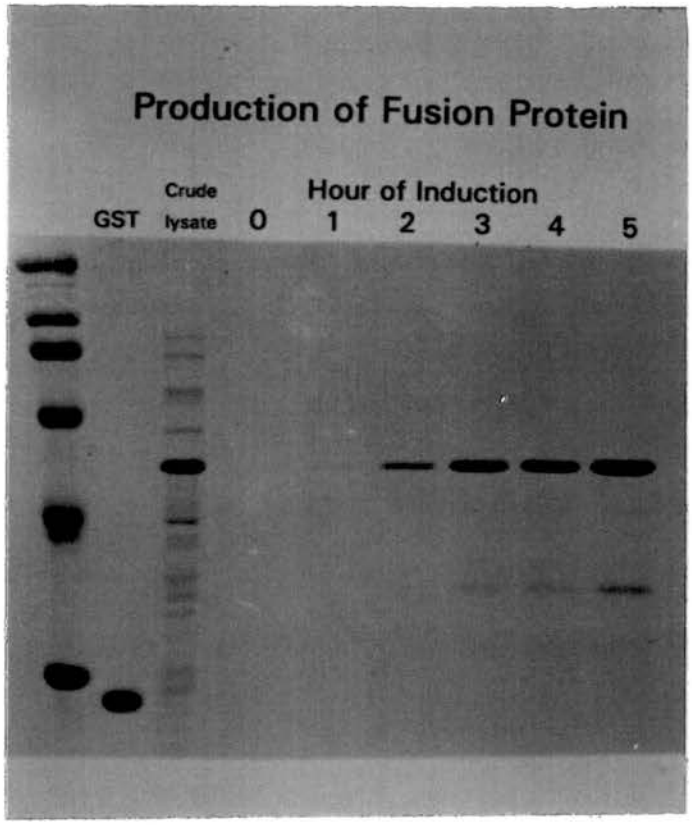

Fig. 2.14 Time course of induction of GST-PZP protein synthesis in E. coli. Time marks indicate hours of induction after addition of IPTG.

at this time we could obtain approximately 40 mg of fusion protein per liter of bacterial culture, and that affinity chromatography on glutathione-agarose allowed very efficient purification of the fusion protein (designated GST-PZP). A rabbit was immunized by multiple injections of affinity-purified GST-PZP in Freund's adjuvant, and the resulting antiserum used to further characterize the PZP cDNA clone.

Porcine zona pellucida staining - Immune serum raised to GST-PZP protein was initially used in immunofluorescent staining of intact porcine ZP (Fig. 2.15). The fluorscence observed was not of the magnitude seen with the antiserum to native ZP protein, but clearly above the background obtained with non-immune serum. Sections of porcine ovary sections stained with the antiserum to GST-PZP also localized the reactive antigen to ZP (Fig. 2.16), whereas non-immune rabbit serum at the same dilution failed to generate specific staining.

Immunoprecipitation experiments - Immunoprecipiation of radiolabeled ZP protein with the antiserum to GST-PZP was used as a second method to establish that PZP represented a ZP mRNA sequence. Solubilized porcine ZP proteins were labeled with ^{125}I, incubated with a battery of positive and negative sera, and the resulting antibody-protein complexes isolated by binding to protein A-sepharose. The sera tested were non-immune rabbit serum, antiserum to a lysate of E. coli administered in Freund's adjuvant, immune serum to

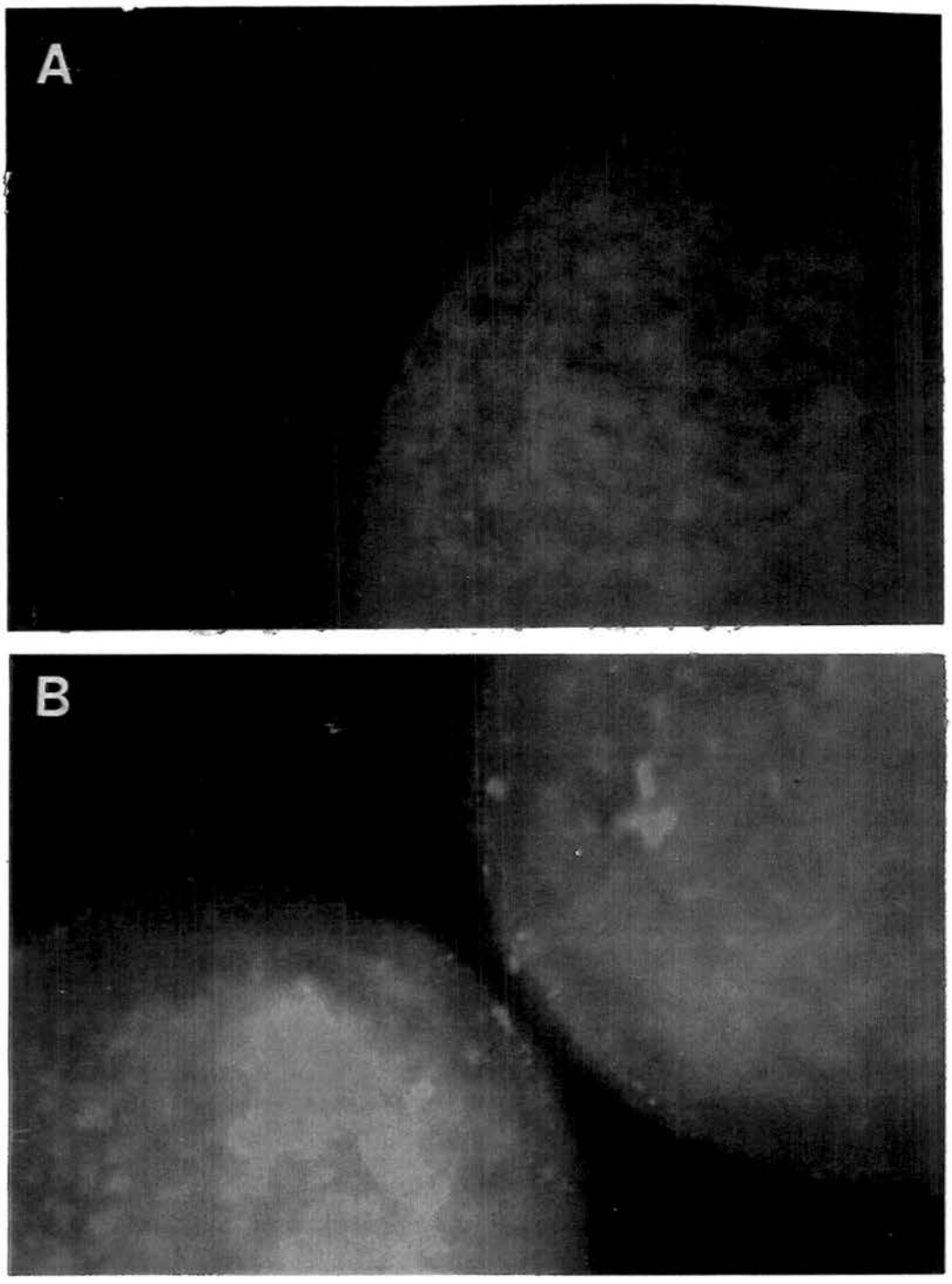

Fig. 2.15 Immunofluorescence staining of ZP-encased porcine oocytes. A) Normal rabbit serum; B) Rabbit antiserum to GST-PZP (both sera diluted 1:50).

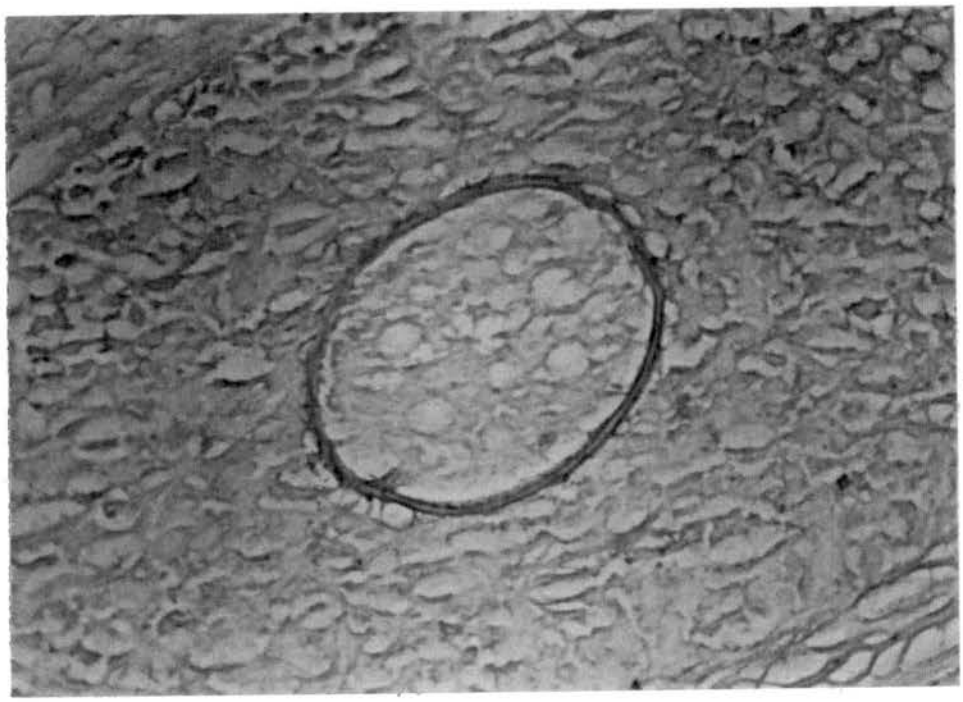

Fig. 2.16 Immunohistochemical staining of porcine ovarian section with antiserum to GST-PZP (400X magnification).

solubilized porcine ZP (the antiserum used to screen the library) and immune serum to GST-PZP. Washed immunoprecipitates were first analysed by one-dimensional PAGE (Fig. 2.17). An autoradiographic signal was not observed in the samples incubated with either normal rabbit serum or antiserum to bacterial lysate. The precipitate from antiserum to solubilized ZP resolved as a broad band of radioactivity, similar to that observed with immunoblotting and typical of the overlapping ZP glycoproteins observed by other investigators. A distinct, but much more narrow band was obtained from the sample precipitated with antiserum to GST-PZP.

Due to the inability to resolve individual porcine ZP proteins by electrophoresis in one dimension, immunoprecipitates obtained with antisera to solubilized ZP and GST-ZP were subjected to two-dimensional PAGE (Fig. 2.18). As expected, a series of charge isomer strings representing individual ZP proteins were observed from the sample precipitated with antserum to solubilized ZP. Immune serum raised against GST-PZP precipitated a single species of protein that appeared most likely to be ZP2.

DNA and Protein Sequence analyses - The nucleotide sequence was first obtained from the PZP.3 fragment. This clone was digested and subcloned as several smaller fragments and each subclone sequenced using primers that annealed to flanking plasmid sequences. Restriction digests of the 2.1 fragment revealed a distressing lack of sites for

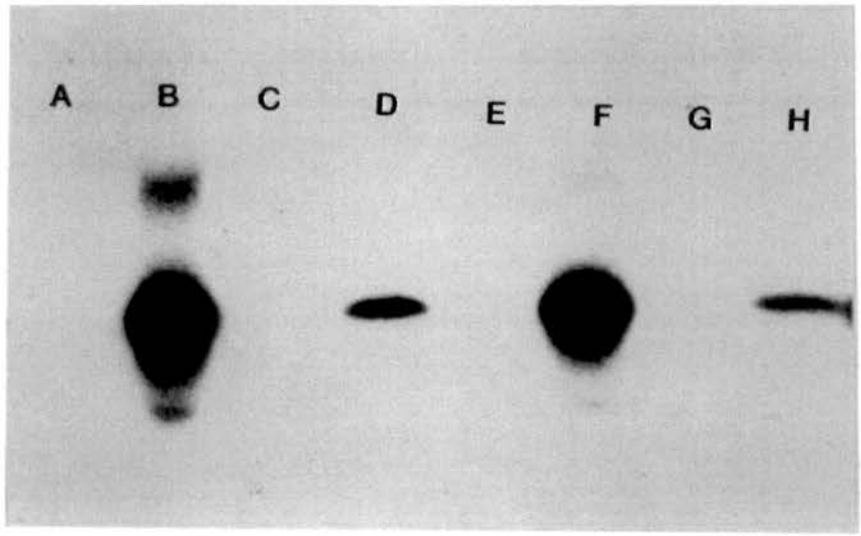

Fig. 2.17 One-dimensional SDS-PAGE analysis of immunoprecipitates of ^{125}I-labeled porcine solubilized ZP proteins. Lanes A and E: normal rabbit serum; lanes B and F: rabbit antiserum to solubilized porcine ZP; lanes C and G: rabbit antiserum to E. coli lysate; lanes D and H: antiserum to GST-PZP.

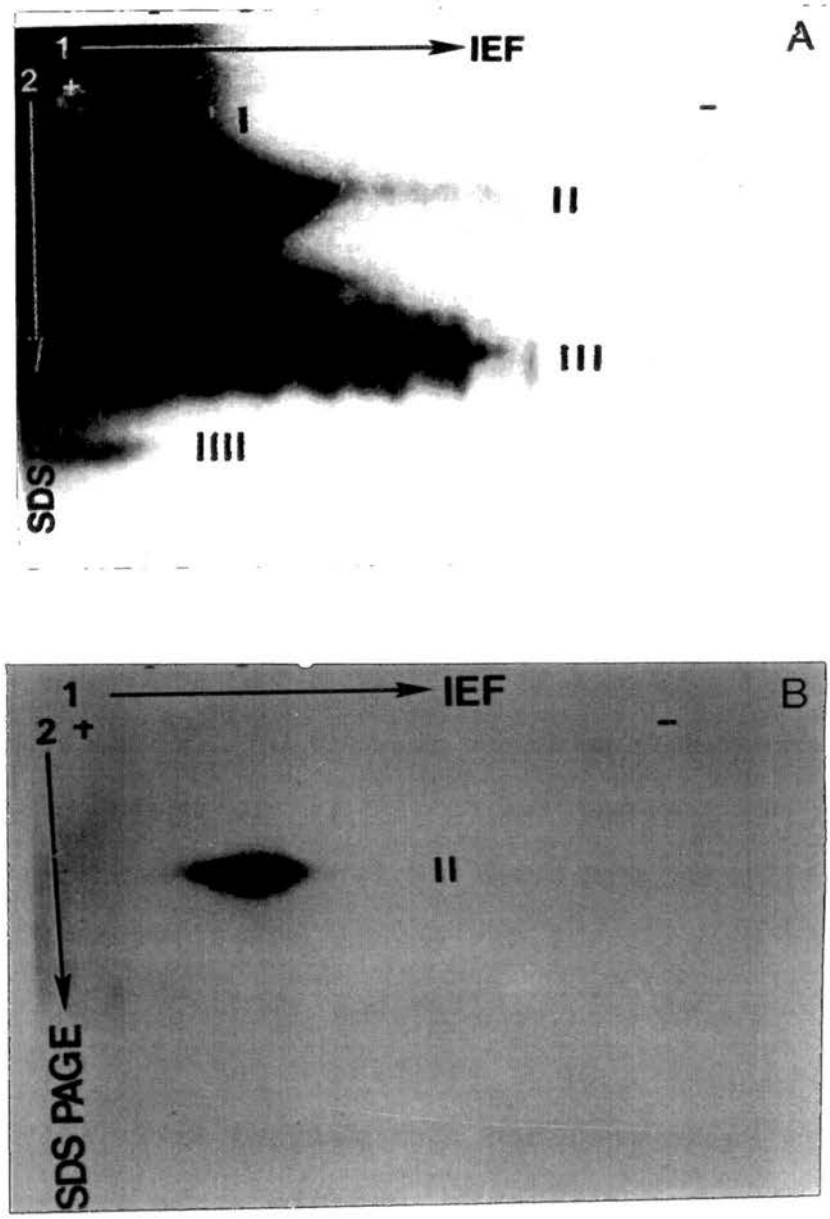

Fig. 2.18 Two-dimensional analysis of immunoprecipitates of ^{125}I-labeled porcine solubilized ZP proteins. A) Antiserum to solubilized porcine ZP. B) antiserum to GST-PZP.

common enzymes, and its sequence was obtained by priming with a series of oligonucleotides synthesized to match the ends of known sequences within the fragment (the "look and leap" approach). These oligonucletide primers were designed to hybridize to sequences approximately 50 bp 5-prime to the end of the last sequence determined, to be 18 to 22 nucleotides in length and to have a G+C content as close to 50% as possible.

The complete sequence of PZP.3 and PZP.1 was determined by sequencing both strands of those fragments, using the strategy presented in Figure 2.19. The end sequences of fragment PZP.2 were also determined, and demonstrated that this fragment contained a portion of a polyadenylated tail at its 3′ terminus. To provide additional assurance that all three fragments of the PZP clone represented the same mRNA, I performed additional Northern hybridization analyses. The PZP.1, PZP.2 and PZP.3 fragments were excised from their host plasmids and radiolabeled by random priming. These probes were then hybridized to polyadenylated RNA prepared from swine ovaries that had been transferred to nylon membranes after electrophoresis in agarose. The autoradiographic signals obtained with each of the three probes were in identical locations relative to the origin of electrophoresis, as would be expected if each was derived from the same mRNA molecule.

The complete nucleotide sequence and deduced amino acid sequence of fragments PZP.3 and PZP.1 is presented in

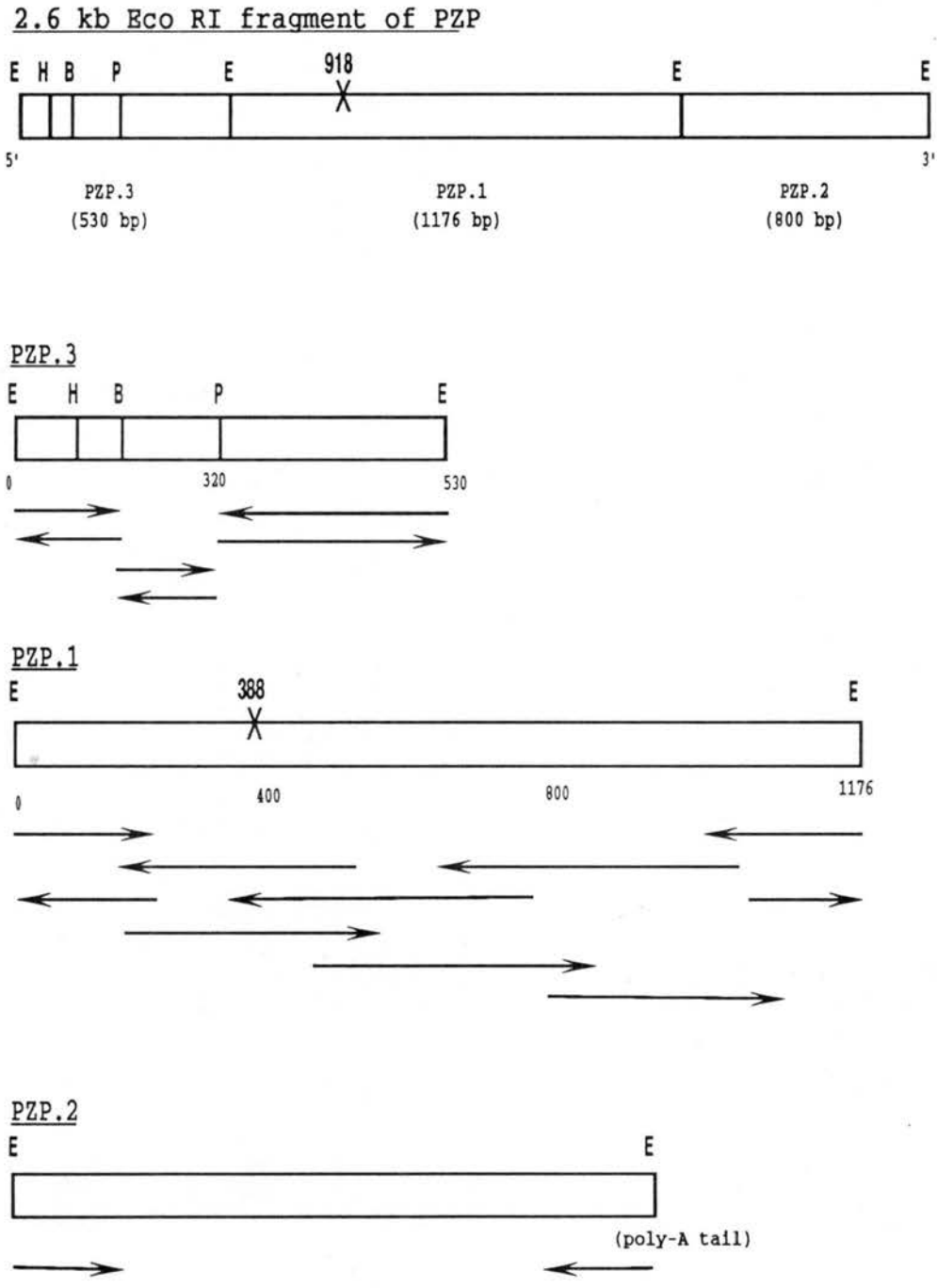

Fig 2.19. Restriction map and sequencing strategies of antibody reactive clone PZP. Position of stop codon is indicated by X. Restriction abbreviations: E, Eco RI; H, Hinc II; B, Bam HI; P, Pst I.

Figure 2.20. An open reading frame of 917 nucleotides is present starting at the 5′ end of the cDNA, with a TAG stop codon beginning at nucleotide 918 (nucleotide 1 being the first nucleotide 3′ to the Eco RI linker in fragment 2.3). Multiple stop codons are present throughout the sequence in both of the other reading frames. The open reading frame spanning fragments 2.3 and 2.1 consisted of 215 A's, 245 T's, 215 C's, and 238 G's (G/C content of 49.4%). The untranslated portion of fragment 2.1 was shown to contain 263 A's, 292 T's, 109 C's and 129 G's (G/C content of 30%). The nucleotide sequence of fragment 2.2 was not determined, other than confirming that a stretch of 50 A's was present defining the 3′ terminus. The 3′ untranslated region was therefore estimated to be approximately 1500 bp in length and very AT-rich.

The presence of a stop codon roughly in the middle of PZP suggested that the clone was not full length. This suspicion was confirmed by Northern analysis. Polyadenylated RNA purified from the ovaries of 3 different swine and probed with radiolabeled PZP.3 revealed that the entire transcript for this gene was approximately 3 kb in length (Fig 2.21). PZP.1 and PZP.2 were also used as probes for Northern analysis on porcine ovarian polyadenylated RNA. Each of these fragments recognized the same size transcript as PZP.3, demonstrating that the three fragments cDNA's as a whole encoded PZP.

Figure 2.20. Nucleotide and deduced amino acid sequence of PZP.3 - PZP.1 cDNA

```
        10         20         30         40         50         60
         |          |          |          |          |          |
GAATTCCCTGCGGCCGACTCGGAGGCTTCCGCGGCTTCTGCGGCACGGACGGGAAGTGAG
GluPheProAlaAlaAspSerGluAlaSerAlaAlaSerAlaAlaArgThrGlySerGlu

        70         80         90        100        110        120
         |          |          |          |          |          |
GCGGCCGGGCCGGGCGGCTCGCGGCAGAAACAGAGGCGGAAGAGTCGCTGCGGCACAGCG
AlaAlaGlyProGlyGlySerArgGlnLysGlnArgArgLysSerArgCysGlyThrAla

       130        140        150        160        170        180
         |          |          |          |          |          |
TCGTCGACCCGGGTGACCGAGAGTGCTGAGCGCCGAGGGGACTCGCCCGCTCGGAAGCCG
SerSerThrArgValThrGluSerAlaGluArgArgGlyAspSerProAlaArgLysPro

       190        200        210        220        230        240
         |          |          |          |          |          |
GATCCCGAGCCGGGCAGGATGGATCATCACCAGCCTGGGACTGGTCGCTACCAGGTGCTT
AspProGluProGlyArgMETAspHisHisGlnProGlyThrGlyArgTyrGlnValLeu

       250        260        270        280        290        300
         |          |          |          |          |          |
CATAATGAAGAGGATAATTCAGAATCATCTGCAGTAGAGCAGCATCAACTTCAGACCCAG
HisAsnGluGluAspAsnSerGluSerSerAlaValGluGlnHisGlnLeuGlnThrGln

       310        320        330        340        350        360
         |          |          |          |          |          |
TACCACAGACCATGCAGGCTGCTTCTTCAGCATCAGGACTTGAGGCTGACTCTTCTCCTC
TyrHisArgProCysArgLeuLeuLeuGlnHisGlnAspLeuArgLeuThrLeuLeuLeu

       370        380        390        400        410        420
         |          |          |          |          |          |
CACCTTATAGTAGTATTACCGTGGAAGTACCTACAACTTCAGATACAGAAGTTTACAGTG
HisLeuIleValValLeuProTrpLysTyrLeuGlnLeuGlnIleGlnLysPheThrVal

       430        440        450        460        470        480
         |          |          |          |          |          |
ACTTTTATCCTGTGCCACCTCCCTACAGTGTTGCTACCTCCCATTCCTACGTATGATGAA
ThrPheIleLeuCysHisLeuProThrValLeuLeuProProIleProThrTyrAspGlu

       490        500        510        520        530        540
         |          |          |          |          |          |
GCTGAGAAGGCCAAAGCTCGTGCCATAGCAGCCGCAGCAGCAGAAACATCTCAAAGAATT
AlaGluLysAlaLysAlaArgAlaIleAlaAlaAlaAlaAlaGluThrSerGlnArgIle

       550        560        570        580        590        600
         |          |          |          |          |          |
CAGGAGGAAGAATGTCCACCAAGAGATGACTTCAGTGATGCAGACCAGCTCAGAGTGGGT
GlnGluGluGluCysProProArgAspAspPheSerAspAlaAspGlnLeuArgValGly

       610        620        630        640        650        660
         |          |          |          |          |          |
AATGATGGGATTTTCATGCTGGCATTTTTCATGGCATTTATTTTCAACTGGCTTGGATTT
AsnAspGlyIlePheMETLeuAlaPhePheMETAlaPheIlePheAsnTrpLeuGlyPhe

       670        680        690        700        710        720
         |          |          |          |          |          |
TGTTTATCTTTCTGTATCACCAATACCATAGCTGGAAGGTATGGTGCCATCTGTGGATTT
CysLeuSerPheCysIleThrAsnThrIleAlaGlyArgTyrGlyAlaIleCysGlyPhe
```

```
       730       740       750       760       770       780
        |         |         |         |         |         |
GGCCTTTCATTGATCAAGTGGATTCTTATTGTCAGGTTTTCTGATTATTTTACTGGATAT
GlyLeuSerLeuIleLysTrpIleLeuIleValArgPheSerAspTyrPheThrGlyTyr

       790       800       810       820       830       840
        |         |         |         |         |         |
TTCAATGGACAGTATTGGCTTTGGTGGATATTTCTTGTACTTGGCCTGCTTCTTTTCTTC
PheAsnGlyGlnTyrTrpLeuTrpTrpIlePheLeuValLeuGlyLeuLeuLeuPhePhe

       850       860       870       880       890       900
        |         |         |         |         |         |
AGAGGATTTGTTAATTACCTGAAAGTCAGAAACATGTCTGAAAGTATGGCAGCTGCTCAT
ArgGlyPheValAsnTyrLeuLysValArgAsnMETSerGluSerMETAlaAlaAlaHis

       910       920       930       940       950       960
        |         |         |         |         |         |
AGAACAAGATATTTCTTCTTATTATAGAGACTGCATCGACCAGACATTCCTTTCTTATAC
ArgThrArgTyrPhePheLeuLeu---

CAATGTGAAATTTCCAGATCATCTGTAAATCTGCAACTTTAATAGGATAGAAGACTACTA

AAAACGGAAGACAAATTAGTGAAGAAAAGATAGAGCTTCAAAATTGAATGGCAGAGTGGT

TTATGCTTAAAAGCCATTTCTGTTCATTCTCTTAATATTTCATTTGCTTTGCGCATTTGC

ATATGTGCCCATTTAAAAGATTTGCATATTCTTGAAAGAAACCAGAAACCGTAAAGTTGT

AGCAGTAAAGTTTCGTCATATTTGATTAGTGTTTGATATAATTGAAAGAGTTGAGTGATA

AACAGTCTTCCAGCATGTAAATGTGATTGACTTCTGACCTGACATTTAGTATAATAAAAA

TGAAATGATTAACCATGTCAAATGCTTTAGTTTCTGGCTCTTAGATTTATCTAGTAACTC

TGCACATCCTTTGTTATATATAAAATTTTTTGAAACTTGCAGTGCTGATTATTAGAAAGG

CTTTGTCAGATTTTTGAACATGATATTTACATCATTATTTAGCAAAACGTTATGTAAATA

ACCATGCATAAATTACTTTCTGCATTGTTTTCTTAGAAATTGTGTCTAGATATCTTTTCA

TTAATTTTAAATTAAGTGGACTTAATATATATAGAAAATTTTGCCGTCAAAGATAGTTTG

TTTTAGGACAAATTTTAAGAAAAATGTGGGTATTCCTCATGTCCTTTGTAAGAAAATCCT

TTTTTTTTTTAAAGGGACATGCCAGTTTTAGGAATTC
                                      |
                                    1717
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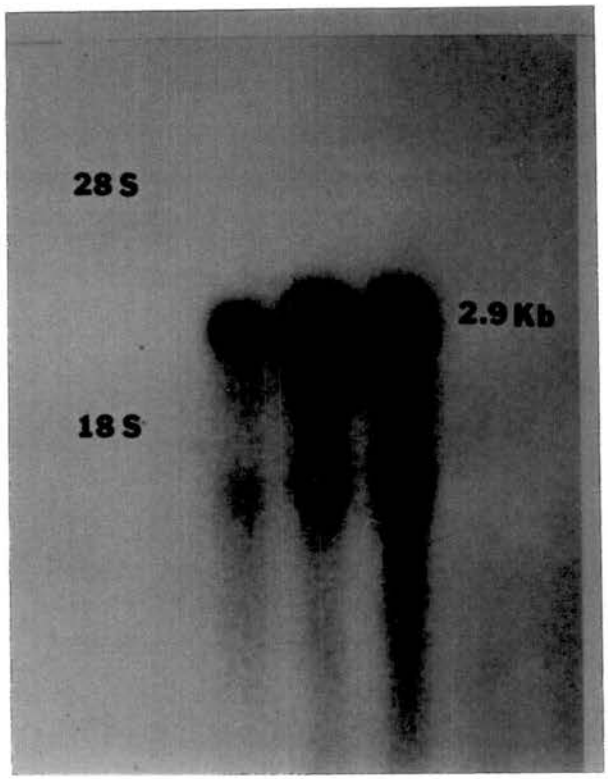

Fig. 2.21 Northern analysis demonstrating hybridization of radiolabeled PZP.3 fragment to samples of polyadenylated RNA purified from the ovaries of three different pigs.

The amino acid sequence deduced from the nucleotide sequence of PZP is considered to represent 305 residues from the carboxy-terminal end of the ZP protein. The first methionine is present at residue 67 of that polypeptide, again indicating that PZP is not a full length clone of the mRNA. Hydrophobicity analysis showed the amino-terminal end of the polypeptide to be relatively hydrophilic, while the carboxy-terminal two-thirds were distinctly hydrophobic (Fig. 2.22). Computer analysis of the deduced amino acid sequence revealed a single N-glycosylation site at residue 288.

The PALIGN program was used to perform protein homology comparisons between the deduced amino acid sequence of PZP and deduced protein sequences from all of the other ZP proteins published to date (Table 2.1). The PZP sequence did not possess apparent similarity to mouse ZP2, mouse ZP3, hamster ZP3, human ZP3 or a rabbit ZP protein.

DISCUSSION AND CONCLUSIONS

Review of the literature that existed at the beginning of this project suggested that rodent ZP proteins would not be effective as contraceptive vaccines in non-rodent species, but that immunization of several of these species with porcine ZP proteins elicited an immune response associated with infertility. It was equally clear that while porcine ZP vaccines had considerable potential to aid

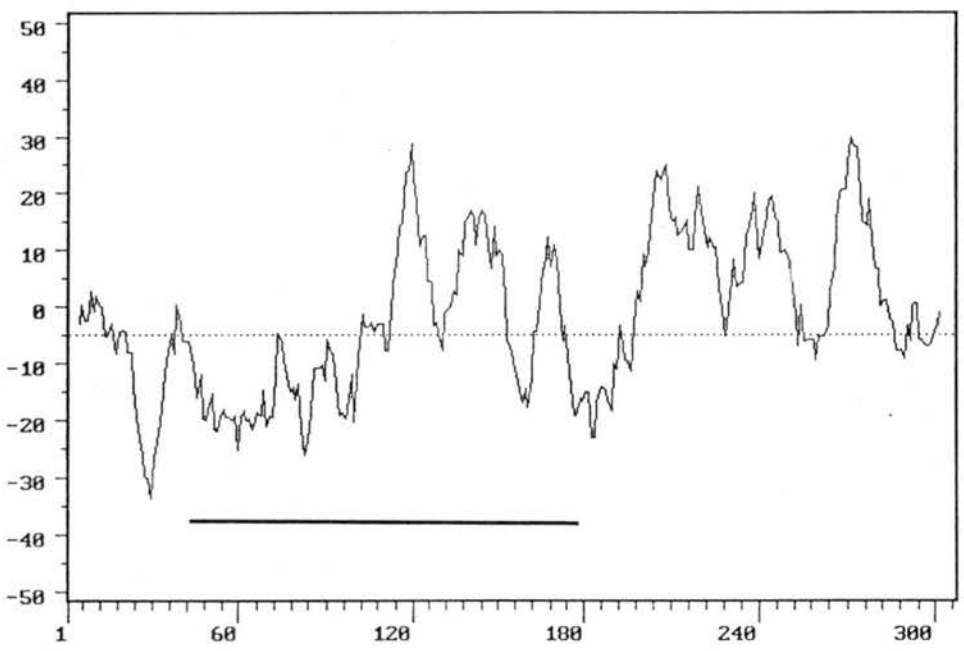

Fig. 2.22 Hydrophobicity profile of deduced polypeptide sequence of PZP. Bar indicates the sequence of protein expressed as part of GST-PZP.

Table 2.1 Sequence similarities among ZP proteins.

	PZP	mZP3	mZP2	haZP3	huZP3	rabbit
PZP	100*	2	5	5	3	6
mZP3	1	100	11	79	68	8
mZP2	5	13	100	10	10	20
haZP3	7	79	10	100	68	5
huZP3	3	67	11	68	100	8
rabbit	7	7	19	4	9	100

* Percentage amino acid identity between ZP proteins as determined by PALIGN of the PC/GENE program.

PZP: porcine
mZP3: mouse (Ringuette et al. 1986)
mZP2: mouse (Liang et al. 1990)
haZP3: hamster (Kinloch et al. 1990)
huZP3: human (Chamberlin and Dean 1990)
rabbit: rabbit (Schwoebel et al. 1991)

in controlling pet and other populations, a vaccine based on natural ZP proteins was not likely to be economically feasible. Production of recombinant porcine ZP proteins was an attractive solution to this problem and formed the basis for the studies presented here.

Immunoscreening of an expression library has become a standard technique for isolating a gene when nucleic acid and protein sequence data are not available, and was the stategy adopted for my studies. To provide an antiserum for screening an ovarian expression library, I immunized a rabbit with solubilized porcine ZP. Aside from use as a tool for isolating a porcine ZP gene, this antiserum provided new information on immunologic crossreactivities of ZP proteins, allowing demonstration that antibodies to porcine ZP proteins reacted with ZP from cats, cattle and sheep.

A cDNA library was constructed in the phage lambda gt11, using cDNA synthesized from porcine ovarian RNA. The frequency of ZP clones in this library was expected to be very low, because oocytes, the known source of ZP mRNA's represent only a small fraction of the cell types in the ovary. Also, ZP mRNAs almost certainly represent a much smaller fraction of total mRNA in swine ovaries than they do in rodent ovaries, where the ratio of oocytes to non-oocyte cells is higher. An additional concern was that expression of ZP protein fragements in phage might be toxic or at least slow their growth. I therefore elected not to amplify the

original library, which would further depress the frequence of ZP clones if such were the case. A single immunoreactive clone was isolated from the original library and formed the basis for a majority of the work described in this dissertation. Several additional putative ZP clones have been identified in another library but have not been characterized further at this time.

The PZP clone isolated from the lambda library reacted with the antiserum to porcine ZP, but further evidence was required that it indeed represented a ZP cDNA. A fragment of the PZP cDNA was inserted into a bacterial expression vector and expressed as a fusion protein in E. coli. Antiserum raised against this fusion protein bound to intact porcine and canine ZP and immunoprecipitated what appears to be a single species of porcine ZP protein from a mixture of total, solubilized ZP proteins.

The nucleotide sequence of the 5′ two Eco RI fragments of the PZP cDNA were determined, as well as a small sequence of the extreme 3′ end of the cDNA, which contained a portion of a polyadenylated tail. Analysis of this DNA sequence showed that a translational stop codon was present at nucleotide 918, implying that the 3′ untranslated region (UTR) of the corresponding mRNA is approximately 1500 bases in length. In comparison to most mRNAs, this is a very long 3′ UTR, but not uniquely large. For instance, the corresponding region of the bovine FSH-beta gene has been shown to be 1295 nucleotides in length (Mauer and Beck

1986). This length does stand in marked contrast to that of rodent ZP mRNAs, which have very short 3′ UTRs (Ringuette et al. 1989). The sequence I derived also shows the porcine 3′ UTR to be very AT-rich, a situation that in several mRNAs has been as associated with transcript instability and short halflife (Jackson and Standart, 1990; Vakalopoulou et al. 1991).

Several factors lead me to believe that the PZP cDNA is not a full length cDNA. First, the open reading frame at the 5′ end of the cDNA (starting with the methionine at nucleotide 192) encodes a protein of only 28,195 Da, which is too small to match the molecular weight of any known porcine ZP protein. Secondly, the 191 nucleotides 5′ to the first methionine in PZP are in translational frame with the sequence after that methionine, which argues against that region being a 5′ UTR. Finally, the first methionine codon in the sequence is not in a context known to be conducive to efficient initiation (Kozak et al. 1984). Obtaining the 5′ end of the gene represented by PZP may require isolating genomic sequences, as was found necessary to obtain full length genes for mouse ZP3 and ZP2 proteins (Ringuette et al. 1989; Liang et al. 1990).

The amino acid sequence of the open reading frame of PZP, deduced from nucleotide sequence data, was compared to deduced protein sequences from the ZP genes of other species described to date. Table 2.1 shows that signficant overall homology does not exist between the PZP sequence and those

of mouse ZP2 and ZP3, hamster ZP3, human ZP3 and a rabbit ZP protein. This lack of homology was not unexpected. As can also be observed in Table 2.1, ZP3 proteins from mouse, hamster and human are similar, but mouse ZP2 and ZP3 are very dissimilar proteins and the rabbit ZP protein shows regions of homology only with mouse ZP2.

A fragment of the PZP cDNA was inserted into a vector for expression of protein, and this system worked very well. The principle impetus for this work was to generate an antiserum to confirm the identity of PZP, but it was also decided to conduct an initial vaccine trial with this bacterially-synthesized ZP polypeptide in rabbits. That study is described in Chapter 3.

CHAPTER III

IMMUNIZATION WITH RECOMBINANT PORCINE ZONA PELLUCIDA PROTEIN

INTRODUCTION

Rabbits immunized with solubilized porcine ZP proteins have been shown to develop histologic abnormalities in ovarian follicles (Skinner et al. 1984) and histologic examination of the ovaries of the rabbit hyperimmunized with a GST-PZP revealed what were initially considered to be similar structures ("abnormal follicular cell clusters"). It was recognized that this was far from a reliable observation, as this was a single animal of unknown age. Therefore, it was decided that a small immunization trial with additional animals was warranted to investigate this phenomenon and determine whether immunization with the small PZP-encoded polypeptide, produced as a fusion protein in E. coli, was capable of having a contraceptive effect in adult rabbits.

MATERIALS AND METHODS

Production and Purification of Recombinant Protein

Subcloning of a 412 bp fragment of PZP.3 into the vector pGEX-2T, transformation of E. coli DH5 alpha and confirmation of the resulting transformant was described in

Chapter II, as are the techniques used for growing those bacteria, inducing expression of fusion protein with IPTG and affinity purification of protein on glutathione-agarose. The purified fusion protein was stored frozen at -80°C until use.

Animals and experimental design

Twelve adult female New Zealand white rabbits, 8 to 14 months of age, were randomly assigned to three treatment groups. The two principle groups consisted of animals immunized with affinity-purified GST-PZP. One group received the protein in Freund's adjuvant (complete for the first injection and incomplete for boosts), while the other group received protein emulsified in TiterMax adjuvant (a proprietary polymer adjuvant purchased from CytRx Corp.). The third group of rabbits served as controls, with two each receiving GST protein (glutathione-S-transferase produced by the vector pGEX-2T in the absence of a recombinant sequence) emulsified in either Freund's or TiterMax adjuvant. Each inoculation consisted of 250 ug of protein (GST-PZP or GST) in adjuvant administered intramuscularly in the hind legs. Animals assigned to the Freund's adjuvant groups were immunized at 0, 2 and 6 weeks, while animals in the TiterMax groups received inoculations at 0 and 6 weeks. To obtain samples for assay of antibody response, each animal was bled for collection of serum prior to initial immunization and at 2, 4, 6, 8 and 16 weeks.

The experiment was designed to evaluate effects of vaccination on reproductive function utilizing four basic parameters: number of ova ovulated, number of ova fertilized, progesterone concentration in serum and subjective evaluation of ovarian histopathology. Fifty-six days (8 weeks) after primary immunization, each rabbit was bled, received an intramuscular injection of 7.5 ug gonadotropin-releasing hormone (GnRH) and was artificially inseminated with 20 x 10^6 spermatozoa. Semen was collected and pooled from two male rabbits of proven fertility. The males were teased with a female for several minutes, then allowed to ejaculate into an artificial vagina prewarmed to 45°C. Semen was diluted in warm egg yolk-TEST extender (Howard et al. 1982) to a final concentration of 40 x 10^6 per ml; motility was checked before and after the addition of extender to ensure that sperm were active and motile. Two days after insemination, each rabbit was subjected to laparotomy for embryo collection and hemiovariectomy; blood was also collected at this time for obtaining serum. Embryo recovery was performed on day 2 following GnRH to insure high recovery rates; we anticipated that the effect of anesthesia and surgical trauma on progesterone output from corpora lutea on the remaining ovary would be minimal eight days later and proportional to what progesterone concentrations would have been had one ovary not been removed. The rabbits were anesthetized with a combination of ketamine and xylazine (approximately 35 mg and 5 mg/kg

respectively), their ventral abdomen clipped and prepared for aseptic surgery. The ovary and uterine horn on each side was exteriorized through a midline incision. An attempt was made to count corpora lutea and the oviducts were then flushed to recover embryos or oocytes. For embryo collection, a plastic cannula was introduced approximately 1.5 cm into the fimbrial end of the oviduct and held in place by a skilled embryo technologist. The oviduct was then flushed retrograde with PBS through a blunt 21 gauge needle inserted from the uterus through the utero-tubal junction. This process was repeated twice for each oviduct. Embryos and oocytes were collected from the recovered flushing medium with the aid of a dissecting microscope, classified (unfertilized or a specific cleavage stage) and fixed in 10% buffered formalin to allow counting of attached spermatozoa if deemed desirable. After ligation of vessels, the right ovary was excised, trimmed of connective tissue, weighed and fixed in Bouin's fluid. The incision was closed and the rabbit allowed to recover. Eight days after surgery (10 days after GnRH), blood for serum was again collected.

Animals were boosted again at week 12 with the same preparation as before. On week 16, serum was collected and GnRH administered as before. Ten days later the rabbits were bled for serum, then killed by barbiturate overdose and their remaining ovary collected and processed as before. Concentrations of progesterone in serum was determined by double antibody radioimmunoassay (Niswender 1973; kindly

performed by personnel in the Endocrinology Laboratory, Colorado State University). Five serum samples from each animal were assayed: three collected in association with the eight week evaluation (at the time of GnRH, two and 10 days later), and two in association with the 16 week evaluation (at the time of GnRH and 10 days later).

Ovaries were fixed intact in Bouin's fluid for 30 minutes then hemisected longitudinally and fixed for an additional day. They were then washed extensively in water and submitted to the Histopathology Laboratory (Department of Pathology) in 70% ethanol. Five micron paraffin sections were cut and stained with periodic acid-Schiff reagent.

Antibody titration

An enzyme-linked immunosorbant assay (ELISA) was developed to quantitate antibody titers in animals immunized with ZP protein preparations. Wells of a microtitration plate were coated with solubilized porcine ZP and incubated with sera to be tested for the presence of anti-ZP antibody. Antibody bound to the coating antigen was detected using a biotinylated second antibody which bound streptavidin-peroxidase. Optimal quantities of coating antigen, biotin-labeled second antibody and streptavidin-peroxidase conjugate were determined by standard checkerboard titration (Harlow and Lane 1988).

Fifty ul of solubilized porcine ZP diluted to 2 ug/ml in PBS (10 mM sodium phosphate, 145 mM NaCl, pH 7.6) were added to wells of a 96-well plate (Falcon 3915) and

incubated overnight at 4°C. Plates were then washed once with PBS and non-specific antibody binding sites blocked by addition of 100 ul of 3% BSA in PBS. After a further incubation of 1-2 hours at room temperature, the blocking buffer was discarded and plates washed three times with PBS containing 0.05% Triton X-100 (PBST). Serum samples were serially diluted in PBST and 50 ul of each sample was added to duplicate wells. The plates were incubated at 37°C for 1 hr and washed three times with PBST. Fifty ul of biotin-labeled anti-rabbit IgG (H+L; Kirkegaard and Perry, Inc; diluted 1:2000 in PBST) was added, incubated for 30 min at 37°C, decanted, and plates were washed as before. Fifty ul of streptavidin-horseradish peroxidase (Kirkegaard and Perry, Inc.; diluted 1:1000 in PBST) was next added and incubated for 30 min at 37°C. After washing three times with PBST and once with PBS, 100 ul of 3,3',5,5'-tetramethyl-benzidine substrate (TMB Microwell 1-Component Peroxidase Substrate, Kirkegard & Perry) was added and incubation continued for 15-30 min. The reaction was stopped by adding 100 ul of 1 M phosphoric acid and absorbance was read at OD 600 nm using a TiterTek microplate reader.

Statistical analyses

Differences among groups in serum progesterone concentration (within days), ovarian weight, number of embryos recovered and numbers of corpora lutea counted were evaluated by one-way analysis of variance (ANOVA program of

the SAS statistical package). For each of these analyses, data were pooled from the two control subgroups. When the F statistic indicated a significant difference ($p < 0.05$), differences among the means of treatment groups were compared using the Student-Newman-Keuls test.

RESULTS

Effect of Immunization on Reproductive Function

Reproductive function in rabbits immunized with GST-PZP was evaluated using several criteria and at two intervals after immunization (Tables 3.1 and 3.2). Based on progesterone concentrations and either the number of ova recovered or number of corpora lutea observed, three of the animals failed to ovulate in response to the first administration of GnRH and two of those same animals to the second GnRH injection; progesterone data from these animals were excluded from statistical analysis. Furthermore, two rabbits had anatomic abnormalities that prevented collection of embryos. Rabbit FTE had bilateral fimbrial adhesions and rabbit KS12 had hemimetria, which allowed embryos to be collected from only one oviduct. Embryo data from each of these animals were eliminated from statistical analysis, but progesterone concentrations and numbers of corpora lutea from these animals were analyzed. A summary of the statistical analyses performed is presented in Table 3.3.

There was no difference among groups in the number of embryos recovered at eight weeks following primary

Table 3.1. Evaluation of reproductive parameters eight weeks after primary immunization.

Treatment Group	Animal	Serum Progesterone (ng/ml on day after GnRH) day 0	day 2	day 10	Ovarian Weight (mg)	Embryos Recovered
GST in Freund's	A2LC	0.41	1.13	4.03	550	10
	MB2L	0.36	0.75	4.35	455	6
GST in TiterMax	T15*	0.07	0.11	0.16	280	0
	ALBC	0.42	1.02	3.57	520	9
GST-PZP Freund's	2LOC	0.39	0.86	3.33	400	8
	H9*	0.17	0.09	0.14	200	0
	H13*	0.26	0.12	0.05	340	0
	9O	0.16	0.63	3.27	410	9
GST-PZP TiterMax	ABCC	0.20	0.61	2.04	360	5
	FTE#	0.22	0.81	3.46	310	0
	KS12#	0.13	0.56	2.24	330	4&
	KS16	0.24	1.01	1.69	320	4

Ovarian weight is for the single ovary removed surgically at this time. Number of embryos refers to the total number of embryos (morulae) recovered from both oviducts.

* All data from these animals excluded from statistical analysis due to failure to respond to GnRH.

Number of embryos recovered from these animals was excluded from statistical analysis due to anatomic abnormalities (see text).

& Embryos from oviduct only.

Table 3.2. Evaluation of reproductive parameters sixteen weeks after primary immunization.

Treatment Group	Animal	Serum Progesterone (ng/ml on day after GnRH) 0	10	Ovarian Weight (mg)	Corpora Lutea
GST in Freund's	A2LC	0.50	11.28	930	10
	MB2L	0.70	6.38	670	10
GST in TiterMax	T15*	--	0.19	170	0
	ALBC	0.02	6.15	710	11
GST-PZP Freund's	2LOC	0.04	7.99	490	6
	H9*	--	0.19	200	0
	H13	0.04	4.84	280	4
	9O	0.17	9.15	530	8
GST-PZP TiterMax	ABCC	0.15	7.49	630	8
	FTE	0.11	11.20	430	5
	KS12	0.29	9.87	540	10
	KS16	0.03	6.94	600	9

Ovarian weight and number of corpora lutea is for the single ovary remaining at this time.

* All data from these animals excluded from statistical analysis due to failure to respond to GnRH.

-- below the sensitivity of the assay (30 pg/ml), but set to that value for statistical analysis

Table 3.3. Statistical analysis of evaluations of reproductive parameters.

	P > F	Control#	ZP-Freund#	ZP-TM#
Eight weeks evaluation				
Progesterone* (day 0)	0.04	0.40	0.28	0.20
Progesterone (day 2)	0.35	0.97	0.74	0.75
Progesterone (day 10)	0.03	3.98^{A}	3.30^{AB}	2.36^{B}
Ovarian weight (mg)	0.01	541^{A}	405^{B}	330^{B}
Eggs recovered	0.09	8.3	8.5	4.5
Sixteen week evaluation				
Progesterone (day 0)	0.18	0.41	0.08	0.15
Progesterone (day 10)	0.69	7.93	7.33	8.88
Ovarian weight (mg)	0.03	770^{A}	433^{B}	550^{B}
Number of corpora lutea	0.06	10.3^{A}	6.0^{B}	8.0^{AB}

#Values represent mean values within treatment groups

*Progesterone concentrations in ng/ml

P > F is the probability of a greater F statistic

^{A,B}Values sharing a common superscript are not different ($P \geq 0.5$)

immunization; all of the ova recovered had been fertilized and had developed to the stage of early morula. At the sixteen week evaluation, the number of corpora lutea on the remaining ovary was lower ($p < .05$) in the rabbits immunized with GST-PZP in Freund's adjuvant than in either of the other two groups. Ovarian weight was significantly lower ($p < .05$) in both groups of rabbits immunized with GST-PZP and at both evaluation times, in comparison to that observed in GST control rabbits.

There was no difference among treatment groups in progesterone concentration in serum at the time of GnRH or at day 2 (8 week evaluation) or day 10 (8 and 16 week evaluations) following GnRH administration. Serum progesterone concentrations were lower ($p < .05$) on day 10 of the eight-week evaluation in animals immunized with GST-PZP in Titermax, in comparison to animals in the other two groups.

Examination of ovarian sections from tissues collected at both 8 and 16 weeks failed to reveal any hint of difference between the GST control and GST-PZP-immunized animals; morphometric analysis was therefore not attempted.

Serologic analyses

In developing the ELISA to detect antibody to ZP proteins, it was found that a dilution of 1:200 was the lowest dilution of normal rabbit serum that did not result in substrate conversion above background. At this dilution, none of the sera from any of the rabbits in this trial were

positive when tested using plates coated with solubilized porcine ZP proteins. These assays included sera from each of the 12 rabbits collected prior to immunization and at 4, 8 and 16 weeks after primary immunization. In contrast, serum collected 13 weeks after the start of immunization of the single rabbit previously immunized with GST-PZP (described in Chapter II) was shown to have a titer of 1:200 in this assay; this titer represented her response after 8 injections of 1 mg of GST-PZP in adjuvant.

The antiserum generated by immunization of a rabbit with solubilized porcine ZP and used to screen the lambda gt11 library had a titer of 1:50,000 in the same assay. This serum, when tested in a similar ELISA against GST protein alone and GST-PZP fusion protein showed binding to the recombinant protein at a dilution of 1:200 and, as expected, no binding to GST protein.

DISCUSSION AND CONCLUSIONS

The trial described here was designed as a first replicate of a study to test the efficacy of a recombinant porcine ZP vaccine produced in bacteria. An additional design constraint was the desire to test the GST-PZP vaccine using an immunization schedule that approached practicality, and for this reason, the rabbits did not receive more than three initial injections of vaccine.

It is difficult to interpret the statistical differences observed in ovarian weight and progesterone

concentrations. One interpretation is that the vaccine did have a biological effect, conceivably due to cell-mediated immune response which we did not attempt to measure. However, due to the small number of animals involved in the study and the fact that data from several animals had to be eliminated from the analysis I prefer to interpret these data conservatively and conclude that the GST-PZP vaccine failed to induce a significant biologic effect on reproductive function.

It is not difficult to predict several factors that may have contributed to the lack of efficacy of the GST-PZP vaccine. The portion of PZP expressed as a fusion protein was small (138 amino acids) and did not include regions of marked hydrophilicity which are associated with highly immunogenic domains (Hopp and Woods 1981) (see Fig 2.22). Had the complete sequence of PZP been determined when this project was initiated, the more hydrophilic domain of the protein (sequence 5′ to that fragment in GEX-PZP) would have been included. Secondly, it is likely that the immune response to native ZP proteins is partially generated to conformational epitopes that would likely not be present in a bacterially-expressed protein. Finally, native ZP proteins are heavily glycosylated, which may contribute substantially to their immunogenicity. The polypeptide used in these studies does not contain a glycosylation signal, but were one present, glycosylation would not have occurred with expression in E. coli.

Personal communications received from two other laboratories indicate that lack of immunogenicity in bacterially-expressed ZP polypeptides is a common problem and suggest that the weak immune response seen in this study was not atypical. Future work in this area should include production of recombinant proteins in systems that are capable of folding and glycosylating proteins in a manner that allows production of recombinant protein more similar to native ZP proteins than was obtained in this study.

CHAPTER IV

CONCLUDING REMARKS

Previous studies from other laboratories have clearly demonstrated the potential for immunization with porcine ZP proteins to disrupt reproductive function in females from diverse species. The work presented in this dissertation represents first steps in the development and testing of a recombinant porcine ZP vaccine, a process that must surely be accomplished if immunization against ZP proteins is to become a practical approach to contraception. Molecular biology now provides the technology to isolate virtually any gene and to express it as protein in substantial quantities, with any of a number of modifications. Just as individual proteins comprising the native porcine ZP had to be characterized for their immunogenic potential, so too do those proteins produced with the aid of molecular technology. Immunocontraceptive epitopes of recombinant proteins need to be identified and characterized to produce the most effective material for the development of anti-fertility vaccines. Deciding upon and optimizing the expression systems for producing recombinant ZP proteins will also play an important role in the development of an efficacious vaccine. The type and degree of protein

modifications required for an optimal immunologic response will affect not only the effectiveness of the vaccine in preventing pregnancy, but also the vaccine's economic feasibility.

The biological, economical and environmental significance of the development of a practical immunocontraceptive is immense. Overpopulation of humans and their pets, and well as of certain wild populations, is a problem that is a major concern in the modern world. I feel that development of immunocontraceptives that block pregnancy at the level of the zona pellucida will play a significant role in solving this problem in the future.

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