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Sequence of Complementary Deoxyribonucleic Acid Encoding Bonnet Monkey (*Macaca radiata*) Zona Pellucida Glycoprotein-ZP1 and Its High-Level Expression in *Escherichia coli*[1]

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ABSTRACT

Zona pellucida (ZP) glycoproteins have been proposed as candidate antigens for immunocontraception. Studies on this potential use can be facilitated by the availability of recombinant proteins. A cDNA λgt11 library was constructed using poly(A)$^+$ mRNA isolated from bonnet monkey (*Macaca radiata*) ovaries and was screened for bonnet monkey ZP1 using a 404-basepair (bp) human ZP1 fragment (nucleotides 818–1221) as probe. Bonnet monkey ZP1 cDNA comprises 1617 nucleotides and encodes a polypeptide of 539 amino acid residues that share 92.0% identity with human ZP1. The major difference between bonnet monkey ZP1 and human ZP1 is the deletion of a 28-amino acid domain (amino acid residues 100–127 corresponding to human ZP1). An internal fragment (1317 bp) of bonnet monkey ZP1, excluding the N-terminus signal sequence and the C-terminus transmembrane-like domain, was amplified by polymerase chain reaction. The amplified *Sac* I and *Kpn* I restricted fragment was cloned in a frame downstream of the T5 promoter under the *lac* operator control for expression in the pQE-30 vector. Recombinant ZP1 (r-ZP1) was expressed as a polyhistidine fusion protein in *Escherichia coli* strains SG13009[pREP4] and *ompT* and *lon* protease-deficient BL21(plysS). SDS-PAGE analysis and immunoblotting with a murine monoclonal antibody, MA-410 (raised against porcine ZP3α—a homologue of bonnet monkey ZP1—and cross-reactive with bonnet monkey zona pellucida), revealed major bands of 51 and 40 kDa besides truncated fragments. Optimum expression of r-ZP1 was observed at 0.5 mM isopropyl β-D-thiogalactopyranoside (IPTG). Immunization of male rabbits with r-ZP1 purified on nickel-nitrilotriacetic acid (NTA) resin under denaturing conditions and of female rabbits with r-ZP1 conjugated with diphtheria toxoid-generated antibodies reactive with r-ZP1 in ELISA. Moreover, immune sera, when tested by indirect immunofluorescence on bonnet monkey ovarian sections, showed positive fluorescence with zona pellucida. The information on the sequence of bonnet monkey ZP1 and the availability of the recombinant protein will help toward better understanding and evaluation of the contraceptive potential of homologous immunization in a nonhuman primate model.

INTRODUCTION

The recognition and binding of sperm by the extracellular glycoprotein coat of the mammalian egg, the zona pellucida (ZP), initiates the fertilization process. Gamete adhesion is mediated by complementary molecules on the respective surfaces of sperm and ZP [1]. This critical role during fertilization has made the ZP glycoproteins potential candidate antigens for immunocontraception. ZP in most species comprises three biochemically and immunologically distinct glycoproteins—ZP1, ZP2, and ZP3. On the basis of the size of their mRNAs, these have been also classified as ZPA (ZP2), ZPB (ZP1), and ZPC (ZP3); ZPA is the longest and ZPC the shortest [2]. In the mouse, the three zona transcripts have been detected only in the ovary, in which their expression is restricted to oocytes [3–5]. However, in cynomolgus monkeys, ZP2 and ZP3 transcripts are present not only in oocytes at all stages of folliculogenesis but also in granulosa cells [6]. In contrast, the ZP1 transcript is present in oocytes in secondary follicles and to a lesser extent in tertiary follicles but is absent in primordial, primary, or antral follicles and granulosa cells [6]. ZP1 has been cloned and sequenced from humans [2], mice [7], rabbits [8], pigs [9], and cats [2]. To avoid or minimize ovarian dysfunction from a ZP glycoprotein-based immunocontraceptive vaccine for human application, it is desirable that the candidate antigen not be present on the primordial/primary follicles and granulosa cells. Moreover, it will be desirable to use either human zona proteins or those with a high sequence homology with human zona proteins and available in pure form without contamination of other ovarian-associated antigens. Keeping this in view, in this communication we describe cloning and sequencing of bonnet monkey (*Macaca radiata*) ZP1 and strategies for its expression in a prokaryotic system. The ability of the recombinant ZP1 (r-ZP1) to generate an antibody reactive to the native protein is also investigated.

MATERIALS AND METHODS

Bonnet Monkey Ovarian cDNA Library, and Screening and Sequencing of ZP1 Clones

Ovaries were collected from a 2-yr-old female bonnet monkey, snap-frozen in liquid nitrogen, and stored at −70°C until used. Total RNA was isolated from frozen ovaries, and the poly(A)$^+$ fraction was purified by using the polyATtract mRNA isolation system (Promega, Madison, WI). Complementary DNA was synthesized from the purified poly(A)$^+$ RNA by using a cDNA synthesis system (Riboclone; Promega) and was cloned in λgt11 to construct a library (Packagene Lambda DNA Packaging System; Promega). The λgt11 ovarian cDNA library was screened using a human 404-base pair (bp) ZP1 fragment (nucleotides 818–1221; kindly provided by Dr. Jurien Dean, Laboratory of Cellular and Developmental Biology, National Institutes of Health, Bethesda, MD). An insert from each positive clone was amplified by polymerase chain reaction (PCR) by means of λgt11 forward (5′-GGTGGCGACGACTCCTGGAGCCCG-3′) and reverse (5′-TTGACACCAGACCAACTGGTAATG-3′) primers; this involved an initial melt at 94°C for 10 min and 30 cycles at 94°C for 1 min, 58°C for 2 min, and 72°C for 3 min, followed by a final extension at 72°C for 15 min. The PCR amplification was carried out in 50 μl final reaction volume with *Taq* polymerase (Stratagene, La Jolla, CA). The amplified

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cDNA fragment was resolved by electrophoresis and purified by use of the GenecleanII kit (Bio 101 Inc., La Jolla, CA). The product was cloned in pCRScript SK(+) cloning vector (Stratagene) and sense-strand sequenced using T3 (5′-AATTAACCCTCACTAAAGGG-3′) and T7 (5′-GTAATACGACTCACTATAGGGC-3′) primers by the dideoxy chain termination method [10] using Sequenase Version 2.0 DNA sequencing kit (USB, Amersham Life Sciences Inc., Buckinghamshire, UK). The complete sequence of the insert was determined by following two different strategies: 1) digesting the insert with *Eco*RI and cloning *Eco*RI-digested fragments into pCRScript SK(+) followed by sequencing; and 2) sequencing the full insert by designing and synthesizing internal primers. The amino acid sequence was derived using the DNASIS program (Pharmacia LKB Biotechnology AB, Bromma, Sweden). Various characteristics of the ZP1 sequence were analyzed by PCGene (IntelliGenetics, Inc., Mountain View, CA). The multiple alignment of the ZP1 amino acid sequence of different species with the bonnet monkey ZP1 sequence was carried out using the Clustal V Multiple Alignment Program [11].

Expression of Bonnet Monkey ZP1 cDNA in Escherichia coli

An internal fragment of the cDNA, excluding the signal sequence and the transmembrane-like domain and following the putative furin cleavage site, was amplified by PCR using the forward primer 5′-GGTGAGCTCAAGCCTGAGACACCAGGT-3′, incorporating a *Sac* I site, and the reverse primer 5′-TCTGGTACCGAGATCAGGACAGGT-3′, incorporating a *Kpn* I site. The PCR was done in a 50-μl of final reaction volume using 50 pmol of each primer and Vent polymerase (NEB Inc., Beverly, MA) for extension. The pBluescript-ZP1 (clone 5, 10 ng) was used as the template and was initially denatured at 95°C for 10 min. Amplification was carried out for 30 cycles of denaturation at 94°C for 1 min, primer annealing at 55°C for 2 min, and extension at 72°C for 3 min, followed by a final extension at 72°C for 15 min. The amplified 1.317-kilobase (kb) fragment was digested with *Sac* I and *Kpn* I and cloned in a frame downstream of a 6X histidine tag under the T-5 promoter-lac operator control in the pQE-30 vector (QIAexpress; Qiagen GmbH, Hilden, Germany). The pQE-ZP1 plasmid was transformed in SG13009[pREP4] bacterial strains provided with the kit. In addition, BL21(plysS) cells, deficient in *omp* T and *lon* proteases, were also transformed with the pQE-ZP1 plasmid.

For expression of r-ZP1 cloned in the pQE-30 vector, a single transformed colony was inoculated and grown overnight at 37°C in 1 ml of Luria Broth (Difco Laboratories, Detroit, MI) containing 100 μg/ml of ampicillin and 25 μg/ml of kanamycin. The next morning, cells were subcultured 1:10 and grown until cell density reached an absorbance at 600 nm (A_{600}) of approximately 0.6–0.7. The cells were further grown in the presence of isopropyl β-D-thiogalactopyranoside (IPTG) to induce expression of the fusion protein under the T-5 promoter. Conditions with respect to the time and IPTG concentration used for induction of r-ZP1 were standardized. The cells were collected by centrifugation at 13 000 × *g* for 60 sec, and the resulting pellet was stored at −70°C until used.

SDS-PAGE and Immunoblotting

The cell pellet obtained from 1 ml culture was solubilized by boiling for 5 min in 100 μl of double-strength sample buffer (0.0625 M Tris [pH 6.8], 2% SDS, 10% glycerol, 5% β-mercaptoethanol, and 0.001% bromophenol blue), and the proteins were resolved on a 0.1% SDS-10% polyacrylamide gel [12]. The proteins were electrophoretically blotted to 0.45-μm nitrocellulose membrane (Bio-Rad, Hercules, CA) overnight at a constant voltage of 15 V in Tris glycine buffer with 20% methanol [13]. For immunoblotting, nonspecific sites on the membrane were blocked by incubation with 5% BSA in 50 mM PBS (pH 7.4) for 1 h and then washed 3 times (15 min each) with PBS containing 0.1% Tween-20 (PBST). For detection of bonnet monkey r-ZP1, a murine monoclonal antibody (mAb), MA-410, generated against porcine ZP3α (a homologue of bonnet monkey ZP1) and immunologically cross-reactive with bonnet monkey ZP, was used. The membrane was incubated for 1 h with a 1:5 dilution of MA-410 culture supernatant and washed 3 times in PBST. Horseradish-peroxidase (HRP)-conjugated goat anti-mouse immunoglobulin (Ig; Bio-Rad) were used to reveal bound antibody. Color was developed with 0.6% (w:v) 4-chloro-napthol in 50 mM PBS containing 25% methanol and 0.06% H_2O_2. The reaction was stopped by washing the membrane with PBS.

Purification and Conjugation of r-ZP1 with Diphtheria Toxoid (DT) and Immunization

For purification of r-ZP1 fusion protein, SG13009[pREP4] cells transformed with the PQE-ZP1 r-plasmid were grown at the shaker flask level (250 ml culture/flask; total volume 2 L). Cells were pelleted at 1500 × *g* for 30 min at 4°C and stored at −70°C until used. The cell pellet (1 g) was solubilized in 5 ml of buffer A (6 M guanidine hydrochloride, 0.1 M NaH_2PO_4, 0.01 M Tris, pH 8.0). The suspension was centrifuged at 10 000 × *g* for 5 min at 4°C, and the supernatant containing the recombinant fusion protein was mixed with gentle end-to-end shaking for 1 h at room temperature (RT) with nickel-nitrilotriacetic acid (NTA) resin (Qiagen GmbH). The resin was loaded onto a column and washed with 5 volumes of buffer A. The column was subsequently washed with 5 volumes each of buffers B and C, which contained 8 M urea, 0.1 M NaH_2PO_4, and 0.01 M Tris and had successively reducing pH values of 8 and 6.3, respectively. The recombinant fusion protein was eluted with buffers D and E (of composition identical to buffers B and C), in which the pH was further reduced to 5.9 and 4.5, respectively. The eluted protein was concentrated in an Amicon concentrator using a YM5 membrane (Amicon Corp., Lexington, MA) and then dialyzed against 100 mM phosphate buffer (pH 7.4) containing 4 M urea. The purified protein was quantified with bicinchoninic acid (Sigma Chemical Co., St. Louis, MO). Five milligrams of r-ZP1 was conjugated to 2.5 mg of DT (Serum Institute, Pune, India) using the "one-step" glutaraldehyde coupling procedure [14]. Conjugation was done in 100 mM phosphate buffer, pH 7.4, with 4 M urea using 0.1% glutaraldehyde, overnight at RT with gentle end-to-end mixing. Unreacted sites were blocked with 100 mM lysine for 3 h at RT. The conjugate was dialyzed against 10 mM PBS with 0.3 M urea.

Two 6-mo-old male New Zealand white rabbits (Small Experimental Animal Facility, National Institute of Immunology, New Delhi, India) were immunized intradermally at multiple sites with 200 μg of r-ZP1, emulsified in complete Freund's adjuvant. The animals received i.m. boosters after 4 wk with an equivalent amount of r-ZP1 in incomplete Freund's adjuvant. In addition, two 6-mo-old female New Zealand white rabbits were also immunized with

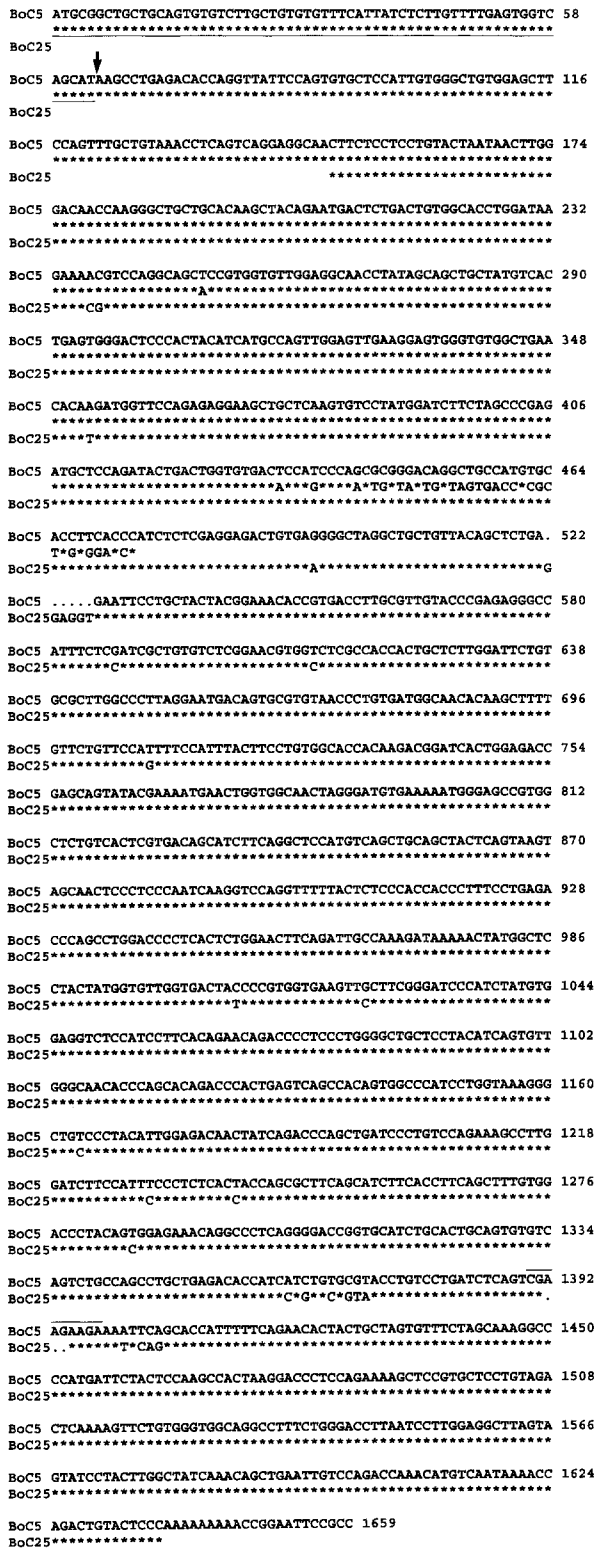

FIG. 1. Nucleotide sequence of bonnet monkey ZP1 cDNA clones 5 (BoC5) and 25 (BoC25). Line 2 under BoC5 represents a 474-nucleotide repeat fragment that precedes the remaining sequence of the BoC5 clone. The BoC25 sequence starts at nucleotide 149 after ATG. The putative nucleotide 63 signal sequence is underlined, and the arrow after 63 nt points to the predicted signal peptidase cleavage site. The predicted furin cleavage site is denoted by overlining. Asterisks represent conserved nucleotides with respect to clone 5, and dots represent the deletion of nucleotides in the respective clones. The nucleotide sequence data reported in this figure will appear in the EMBL, GenBank, and DDBJ nucleotide sequence databases under accession numbers Y10381, Y10382, and Y10383.

r-ZP1-DT conjugate (equivalent to 200 μg r-ZP1/animal) according to the procedure described for the male rabbits. Seven days after the booster, animals were bled to characterize the antibody response.

ELISA

Reactivity of anti-r-ZP1 antibodies was tested by a direct binding ELISA. Microtitration plates were coated with either 200 ng of r-ZP1 (for which 1 mg stock r-ZP1 in 100 mM phosphate buffer, pH 7.4, with 4 M urea was diluted in 50 mM PBS, pH 7.4) or 500 ng of DT per well in 50 mM PBS, pH 7.4, were incubated for 1 h at 37°C and then overnight at 4°C. All subsequent incubations were at 37°C for 1 h. After each incubation, the plates were washed three times in 50 mM PBS containing 0.05% Tween-20. Plates were blocked with 1% BSA. After blocking, 100 μl of doubling dilutions of the preimmune and immune serum samples were added in duplicate. HRP-conjugated goat anti-rabbit Ig (Pierce, Rockford, IL) at a 1:5000 dilution was used as revealing antibodies. Enzyme activity was detected by adding 100 μl of 0.05% orthophenylene diamine and 0.06% H_2O_2 in 50 mM citrate phosphate buffer (pH 5.0) to each well, and the reaction was stopped by adding 50 μl/well of 5 N H_2SO_4. A_{492} was read, and antibody titers were calculated by regression analysis. Antibody titer is expressed as antibody unit, i.e., the dilution of serum giving an absorbance of 1.0.

Immunofluorescence on Bonnet Monkey Ovarian Sections

A normal cycling female monkey (10 yr old) was ovariectomized, and the ovaries were snap-frozen in liquid nitrogen. Sections of 5-μm thickness were cut in a cryostat at −20°C and fixed for 20 min in chilled methanol. Sections passing through a follicle were selected, washed in PBS, and blocked for 30 min in 5% normal goat serum. The sections were incubated at 37°C with a 1:2000 dilution of rabbit preimmune and immune sera for 1 h, washed with PBS, and incubated for 1 h with a 1:1000 dilution of goat anti-rabbit Ig-fluorescein isothiocyanate (FITC) conjugate (Pierce). Slides were washed with PBS, mounted in glycerol:PBS (9:1), and examined under a fluorescent microscope (Optiphot; Nikon, Chiyoda-Ku, Tokyo, Japan).

RESULTS

Nucleotide Sequence of cDNA Encoding Bonnet Monkey ZP1

The constructed bonnet monkey ovarian cDNA λgt11 library had 1.79×10^6 independent plaques with 96% recombinant before amplification. Six positive clones were obtained by plaque hybridization of an amplified library with a 404-bp fragment of human ZP1 cDNA. Amplification of the insert by PCR using λgt11 forward and reverse primers and its analysis by electrophoresis revealed one clone (clone 5, BoC5) to be 2.1 kb, two clones (clones 23—BoC23 and 25—BoC25) 1.6 kb, and three clones (clones 22, 24, and 28) smaller than 1.6 kb (data not shown). The PCR-amplified product from three clones (5, 23, and 25) was cloned into pCRScript SK(+) vector. Sequences from both ends of these three fragments revealed that clone 5 had a full-length cDNA with both the translation initiation codon (ATG) and the termination codon (TAA) along with the poly(A) tail at the 3′ end. Clones 23 and 25 had the

translation termination codon but not the translation initiation codon.

Further mapping of clone 5 with *Eco*RI resulted in 4 fragments of ~750, ~550, ~500, and ~250 bp. Nucleotide sequence analysis revealed that both the ~500-bp (474 nt) and the ~550-bp fragments had a translation initiation codon. The ~500-bp fragment had a sequence identical to that of the ~550-bp fragment (except 42 bp at the 3′-end). The ~550-bp fragment had a *Xho* I restriction site, and one additional site was also present in the pCRScript SK(+) vector. Digestion of clone 5 with *Xho* I generated two fragments of 3.9 and 1.2 kb, indicating that the ~550-bp fragment was 3′ to the ~500-bp fragment. Hence, the order of the various fragments in clone 5 was 5′—~500 bp, ~550 bp, ~750 bp, ~250 bp—3′. Figure 1 shows the nucleotide sequences, within the limit of PCR errors, of clone 5 and 25. The nucleotide sequence of clone 23 was identical to that of clone 25 and hence is not presented in Figure 1. Clone 25 could be read only from 148 bp after ATG and was identical to clone 5 except for 6 extra nucleotides at nucleotide 521 and deletion of three nucleotides after nucleotide 1391. Bonnet monkey ZP1 had a single open reading frame of 1617 nucleotides, resulting in a polypeptide core 539 amino acids in length with a calculated molecular weight of 59 497. A potential signal peptidase site with amino acids at the −1 and −3 positions that are in accordance with the rule proposed by von Heijne [15] was found after 21 amino acids in bonnet monkey ZP1. Comparison of the deduced amino acid sequence of clone 5 with that of human, rabbit, pig, cat, and mouse ZP1 is shown in Figure 2. For this comparison, the sequence of the translation of the extra ~500-bp 5′ fragment of BoC5 was excluded because 11 of 13 amino acids at the C terminus were different from those in human ZP1 (only 2 amino acids differed in the ~550-bp fragment). Furthermore, amino acids E and V at positions 175 and 176, which are present in clones 23 and 25 but deleted in clone 5, were included for this comparison. Comparison of the amino acid sequence revealed that bonnet monkey ZP1 had a 28-amino acid deletion from amino acids 100–127 as compared to human ZP1 (Fig. 2). The bonnet monkey ZP1 amino acid sequence showed 92.0% identity with human ZP1. The mature protein, excluding the N-terminal signal sequence region and the C-terminal transmembrane-like domain, which would presumably be processed in a mature molecule, had an identity of 93.7% with the corresponding human protein. Amino acid sequence identity with rabbit ZP1 was 65.7%, pig 62.3%, cat 65.1%, and mouse 36.9% (Fig. 2). Out of 539 amino acids, 131 were conserved in all 6 species (Fig. 2; marked by dashes in the space below the sequences).

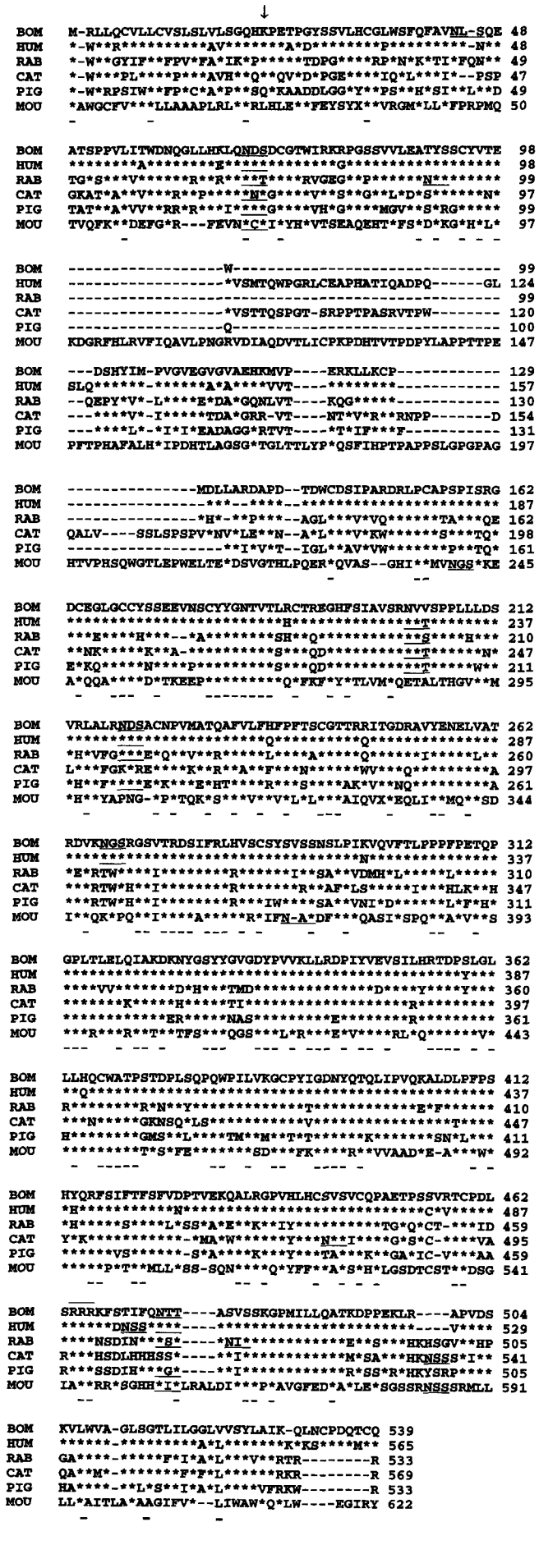

FIG. 2. Comparison of the primary amino acid sequence of bonnet monkey ZP1 with ZP1 of 5 other species. The deduced amino acid sequence (single-letter code) of clone 5 (BoC5) excluding the 5′ ~500-bp (474-nt) repeat fragment is represented. For comparison of amino acid sequence, amino acids E and V at positions 175 and 176, which are present in clones 23 and 25 but are deleted in clone 5, have been included. Asterisks indicate completely conserved amino acids with respect to bonnet monkey ZP1; potential *N*-linked glycosylation sites (Asn-Xaa-Ser/Thr) are underlined. The signal peptidase cleavage site and the tri-basic furin proteolytic processing signal (Arg-Arg-Arg) are indicated by arrows and overlining, respectively. The completely conserved amino acid residues in the six species are marked with dashes in the space beneath the sequences. The amino acid sequence of bonnet monkey ZP1 reported in this figure will appear in the EMBL protein sequence database under accession numbers Y10381 and Y10383.

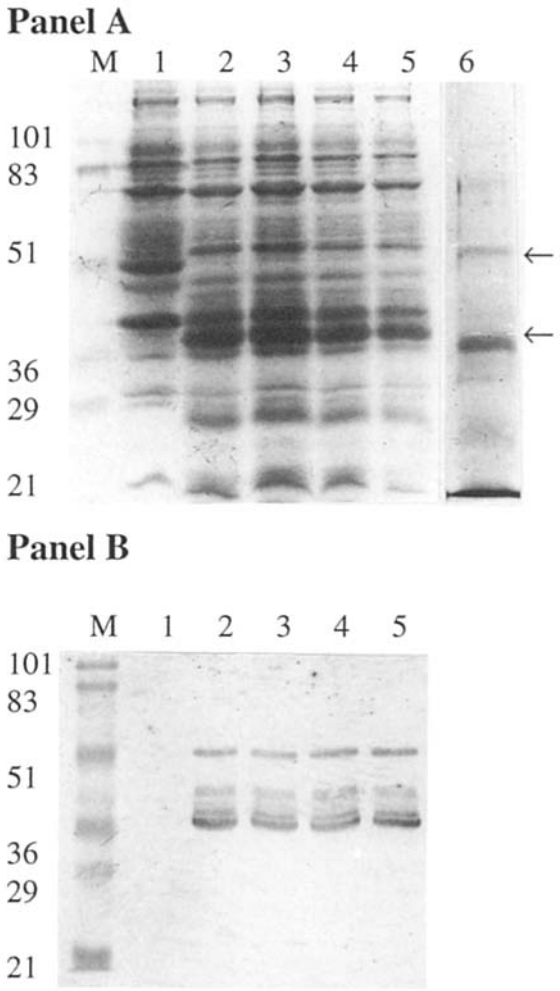

FIG. 3. Electrophoretic analysis and immunoblot of cell lysates prepared from SG13009[pREP4], which harbors the pQE-ZP1 plasmid. SG13009[pREP4] cells transformed with the pQE-ZP1 r-plasmid were grown until A_{600} = 0.7 and were induced with different concentrations of IPTG (lanes 2–5) for 2 h. Cells were lysed by boiling for 5 min in SDS-PAGE buffer and electrophoresed as described in the *Materials and Methods*. **A**) Coomassie-stained gel. Lane M: molecular weight marker ($\times 10^{-3}$); lane 1: uninduced cells; lanes 2, 3, 4, and 5: cells induced with IPTG at a concentrations of 0.5, 1, 2, and 3 mM, respectively; lane 6: r-ZP1 purified on Ni-NTA agarose column (eluted in buffer E). Arrows indicate recombinant 6XHis-ZP1 fusion protein. **B**) Immunoblot of **A**, excluding lane 6, probed with MA-410.

The bonnet monkey ZP1 polypeptide chain contained 5 potential *N*-linked glycosylation sites (Asn-X-Ser/Thr). Two potential cAMP- and cGMP-dependent protein kinase phosphorylation sites at positions 249 and 469 were found in bonnet monkey ZP1 protein. It had 7 potential protein kinase C phosphorylation sites at positions 186, 212, 244, 245, 455, 463, and 480. The protein was also found to have 8 *N*-myristoylation sites at positions 111, 166, 168, 182, 268, 328, 511, and 520. The bonnet monkey ZP1 polypeptide was rich in leucine (11.1%), serine (10.2%), valine (9.0%), and proline (8.3%).

The conformation of bonnet monkey ZP1 protein was 16.5% helical, 28.7% extended, and 54.7% coils as predicted by the Gascuel and Bolmard Basic Statistical Method (PCGene, IntelliGenetics, Inc.). According to the Hopp and Woods [16] analysis, taking an average group of 6 amino acid residues, three domains corresponding to amino acid residues 492–497 (average hydrophilcity (Ah) = 2.0), 461–467 (Ah = 1.75), and 78–83 (Ah = 1.55) were observed to have the highest hydrophilicity. The analysis of the bonnet monkey ZP1 sequence further revealed two predicted transmembrane segments corresponding to LLQCVLLCVSLSLVLSG (amino acid residues 3–19) and VLWVAGLSGTLILGGLV (amino acid residues 506–522) motifs [17].

TABLE 1. Reactivity in ELISA of polyclonal antibodies generated against r-ZP1 and r-ZP1-DT conjugate in rabbits with r-ZP1 and DT.

		Reactivity in ELISA (antibody units)			
		r-ZP1		DT*	
Rabbit	Immunogen	Day 0	Day 35	Day 0	Day 35
Males	r-ZP1				
R-136		507	926672	NA	NA
R-137		726	939283	NA	NA
Females	r-ZP1-DT				
R-29		797	144610	275	484100
R-30		423	172100	200	200930

* NA, Not applicable.

Expression of ZP1 in E. coli

The 1.317-kb fragment from clone 5 corresponding to bonnet monkey ZP1, excluding the signal sequence and the putative transmembrane-like domain, was PCR-amplified and cloned into pQE-30 vector. The recombinant plasmid pQE-ZP1 was used to transform the SG13009[pREP4] bacterial strain. Typical results obtained by 0.1% SDS-10% PAGE and immunoblotting from one of the transformed clones is shown in Figure 3. The fusion protein had an apparent molecular mass of 51 kDa. In addition, a dominant band of 40 kDa along with intermediary products was observed. The optimum expression was obtained at 0.5 mM IPTG. The expression of ZP1 was tightly regulated as this was not observed in the uninduced transformed bacterial cells (Fig. 3). Higher IPTG levels seemed to inhibit growth of transformed bacterial cells without any further increase in expression levels of r-ZP1. Cellular localization revealed that the fusion protein was present in the insoluble intracellular fraction and was not secreted in the periplasm (data not shown). Purification of r-ZP1 on the Ni-NTA agarose affinity matrix revealed major protein bands of 51 and 40 kDa (Fig. 3). The r-ZP1 was also expressed in BL21(plysS) cells deficient in the *ompT* and *lon* proteases. The pattern obtained in the Western blot was comparable to that obtained with SG13009(pREP4). Moreover, r-ZP1 was observed in absence of IPTG, suggesting that its expression is not highly regulated.

Immunogenicity of r-ZP1

From a 2-L batch culture, approximately 15 mg of purified r-ZP1 was obtained and used to immunize male rabbits. Female rabbits were also immunized with r-ZP1 coupled to DT. Immunization of male rabbits R-136 and R-137 resulted in the generation of high antibody titers (926672 and 939283 antibody units, respectively) against r-ZP1 (Table 1). High antibody titers against r-ZP1 and DT were also observed in the immunized female rabbits. However, in contrast to the male rabbits, 5- to 7-fold lower anti-r-ZP1 antibody titers were observed in female animals.

To prove the authenticity of recombinant protein, it is imperative to show that antiserum generated against the r-ZP1 recognizes the native protein. Rabbit antiserum

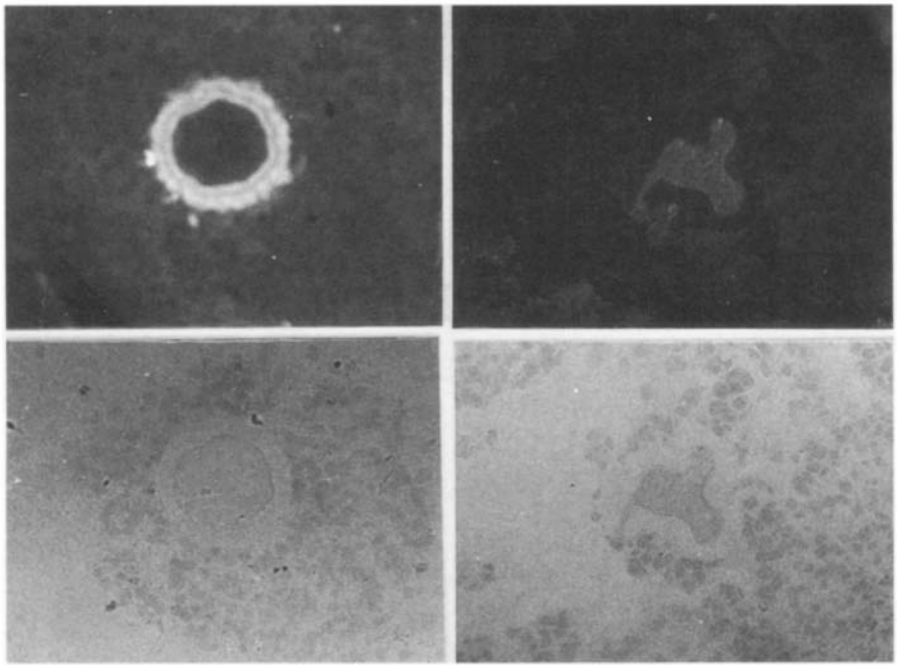

FIG. 4. Reactivity of rabbit anti-r-ZP1 serum with bonnet monkey zona pellucida by indirect immunofluorescence. Representative immunofluorescence patterns are shown. Top left, immune serum; top right, preimmune serum (×100). Microphotographs at bottom left and bottom right represent bright field optics corresponding to top left and top right, respectively.

against r-ZP1 showed intense fluorescence with the zona pellucida on sections from the bonnet monkey ovary (Fig. 4). The preimmune serum failed to show any immunofluorescence (Fig. 4). Moreover, the fluorescence was specific to the zona matrix, and the antibodies did not react with other ovarian cell types.

DISCUSSION

Active immunization of female subjects with native porcine ZP glycoproteins as well as the deglycosylated form leads to a block of fertility in several animal models [18–22]. However, to design a zona glycoprotein/protein-based immunocontraceptive vaccine for human use, it will be desirable to use either human zona proteins or those with a high sequence homology with human zona protein. Recent observations in cynomolgus monkeys reported the presence of a ZP1 transcript in only the oocytes of secondary follicles and its absence in the primordial/primary follicles and granulosa cells [6]. This prompted us to clone and sequence the bonnet monkey ZP1 cDNA. A partial ZP1 sequence has also been reported from the cynomolgus monkey [23]. These investigators failed to isolate full-length ZP1 cDNA clones in spite of exhaustive screening of the two cynomolgus monkey ovarian cDNA libraries with a porcine ZP1 probe. An insert of 866 bp encoding a polypeptide of 270 amino acids corresponding to the C terminus and approximately 50% of the expected full-length sequence has been reported.

Comparison of 270 amino acids corresponding to cynomolgus monkey ZP1 with bonnet monkey ZP1 revealed changes in only 8 amino acids, of which three occur after the predicted furin cleavage site. Bonnet monkey ZP1 has a tri-basic (R-R-R) cleavage site beginning at amino acid 464 that is similar to human ZP1. However, cynomolgus monkey ZP1 has only 2 instead of 3 arginine residues. On the basis of von Heijne's [15] scoring method, bonnet monkey ZP1 has a potential peptidase site after the 21st amino acid, resulting in an N terminal lysine. The polyadenylation signal AAUAAA overlaps with the translation stop codon UAA, thus resulting in a short (17 nucleotide) untranslated region at the 3′ end, a feature common to the mRNA from humans, mice, rabbits, pigs, and cats. Comparison of the deduced amino acid sequence of precursor bonnet monkey ZP1 protein revealed the highest sequence identity with human (92.0%) and the lowest with mouse (36.9%) ZP1. The low sequence homology of bonnet monkey ZP1 with mouse ZP1 is attributed to elongated exons 3 and 12 of mouse ZP1 [24]. There are 4 amino acid residues that are unique in bonnet monkey ZP1 (at positions 2, 121, 455, and 522 of precursor bonnet monkey ZP1 protein) that are otherwise conserved in human, pig, rabbit, cat, and mouse ZP1. There are 5 amino acid residues, identical and unique to both human and bonnet monkey ZP1 (77, 116, 273, 439, and 451 numbered according to the bonnet monkey ZP1 precursor protein) that otherwise are conserved in pig, rabbit, cat, and mouse ZP1. Bonnet monkey ZP1 has 5 potential *N*-glycosylation sites as compared to 6 in human ZP1, of which 3 are conserved (Fig. 2). Interestingly, bonnet monkey ZP1 has 23 cysteine residues that are conserved with respect to human ZP1. However, human ZP1 has 2 additional cysteine residues. A dendrogram (unweighted pair group maximum averages) of the alignment of mouse, cat, pig, rabbit, human, and bonnet monkey ZP1 amino acid sequences revealed that the bonnet monkey is closer than the others to the human in evolution (figure not shown).

At the moment, it is not clear whether repetition of the 500-bp 5′ fragment is an artifact introduced while packaging cDNA into λgt11 or is due to a partial gene duplication. The 3′ nucleotide sequence of the 500-bp fragment does not match the λgt11 arm used for packaging and thus is probably not an artifact of packaging. In chickens, progesterone receptor A and B proteins are synthesized from the same mRNA by alternate initiation sites from two in the frame AUG codons [25]. The chicken progesterone receptor B protein differs from the A protein by an additional 128 amino acids located at the N terminus. The evolutionary and functional significance of the two forms of the steroid hormone receptor is unclear. Whether such a possibility of alternate initiation codon usage exists for expression of ZP1 in nonhuman primates is at best a speculation.

To undertake feasibility studies of an immunocontraceptive vaccine based on bonnet monkey ZP1, it will be desirable to have the antigen in a pure form without contamination of other ovary-associated antigens. Bonnet monkey ZP1 was expressed in *E. coli* as a 6XHis fusion protein, allowing for its convenient purification in a truncated form excluding the signal sequence (21 amino acid residues) and excluding the C-terminal portion (77 amino acid residues including the transmembrane-like domain), which is processed by furin-like proteases. Analyzed by SDS-PAGE, the fusion protein showed dominant bands corresponding to 51 and 40 kDa. Expression was reasonably high (7.5 mg/L in a batch flask culture) although some amounts of intermediary products of the expressed protein were evident in the Western blots. The degradation of r-ZP1 was neither eliminated nor reduced with expression of the protein in a *lon* and *ompT* protease-deficient strain, BL21(plysS), indicating that some other proteases were responsible for the degradation observed. Alternatively, it may represent premature termination during translation of the ZP1 mRNA.

The bonnet monkey r-ZP1 was highly immunogenic in both male and female rabbits, and anti-r-ZP1 antibodies recognized the bonnet monkey zona pellucida. Proteins expressed in a prokaryotic expression system are not glycosylated. Active immunization studies with glycosylated and deglycosylated porcine ZP antigens in the rabbit model system have attributed ovarian pathology to glycosylation [26, 27]. Recently, cynomolgus monkeys were immunized with the rabbit zona protein rc55 (a homologue of bonnet monkey ZP1) expressed in a prokaryotic expression system as cro-β-galactosidase fusion protein and coupled to keyhole-limpet hemocyanin/protein-A. Antibody response against the rc55 inhibited homologous sperm-egg interaction without concomitant disturbances in cyclicity or any associated ovarian pathology [28]. Active immunization of female bonnet monkeys and female baboons of proven fertility with r-ZP1-DT conjugate are in progress to study its efficacy for fertility regulation and effect on ovarian functions.

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