Materials and Methods

REAGENTS

Chemicals: Tris, glycine, acrylamide, N', N'-methylene bisacrylamide, sodium dodecyl sulfate (SDS), N', N', N', N'-tetramethylethylene diamine (TEMED), ammonium persulfate (APS), β-mercaptoethanol (BME), 4-chloronaphthol, urea, guanidine HCl, guanidine isothiocyanate (GITC), sarcosyl, sodium citrate, phenol, ficoll, polyvinylpyrrolidone (PVP), agarose, bromophenol blue, Coomassie brilliant blue, ethidium bromide, calcium chloride and bicinchoninic acid (BCA) were obtained from Sigma Chemical Co., St. Louis, MO, USA. LMP agarose and isopropyl β-D thiogalactopyranoside (IPTG) were from Amresco, Solon, USA.

Molecular weight standards were obtained from Gibco-BRL, Grand Island, NY, USA, or Bio-Rad Laboratories, Hercules, CA, USA.

Reagents for enzyme immunoassays viz., bovine serum albumin (BSA), orthophenylene diamine (OPD) were procured from Sigma, while Tween-20 was obtained from Amresco.

Reagents for conjugation and immunization, viz. diphtheria toxoid (DT) and tetanus toxoid (TT) were from Serum Institute, Pune, India while glutaraldehyde, L-lysine, 2, 6, 10, 15, 19, 23-hexamethyl-2, 6, 10, 14, 18, 22-tetracosa-hexane (Squalene) and mannide monooleate (Arlacel A) were procured from Sigma; Pergonal® was obtained from Laboratoires Serono S.A., Aubonne, Switzerland; Sodium phthalyl derivative of lipopolysaccharide (SPLPS) was kindly provided by the Immunoendocrinology Laboratory, National Institute of Immunology (NII), New Delhi.

Reagents used in the estimation of progesterone such as gelatin, charcoal, dextran, ^{3}H-progesterone and anti-progesterone Ab were provided by the WHO Matched Reagent Assay Programme while diphenoxazole (PPO), 1-4 bis (5-phenyl-2-oxazolyl) benzene (POPOP), and mercury-[(o-carboxyphenyl)thio]ethyl sodium salt (Thimerosal) were obtained from Sigma.

Media and Antibiotics: Bacto-tryptone, bacto yeast extract and bacto agar were from Difco Laboratories, Detroit, USA; Grace's insect cell medium and antibiotic-antimycotic

from Gibco BRL; ampicillin, kanamycin and neutral red were from Sigma; FCS, was obtained from Biological Industries, Hibbutz Beit, Haemek, Israel.

Bacterial Strains and Plasmids: M15[pREP4] and SG13009[pREP4] from Qiagen GmbH, Hilden, Germany, DH5α, BL21 (DE3) and BL21(pLysS) from Stratagene, La Jolla, USA, DF5 cells were kindly provided by Prof. K. Dharmalingam, Madurai Kamraj University, Madurai, Tamil Nadu, India.

pBluescriptII SK(+) vector from Stratagene, pQE30 from Qiagen, pBacPAK8 vector from Clonetech Laboratories Inc., Palo Alto, CA, USA and pAcSecG2T vector, from Pharmingen, San Diego, CA, USA were obtained.

Kits: PolyAT® tract mRNA isolation system and Riboclone® cDNA synthesis system from Promega Inc.; Geneclean® II kit from Bio 101 Inc., La Jolla, USA; Plasmid Midi kit and QIAexpress™ from Qiagen GmbH, Hilden, Germany; Sequenase version 2.0 DNA sequencing kit and Multiprime DNA labeling system from Amersham, Little Chalfont, Buckinghamshire, UK; BacPAK™ baculovirus expression system from Clonetech and Baculogold™ transfection kit from Pharmingen.

Primers: Various oligonucleotide primers used were custom made by Rama Biotechnologies India Pvt. Ltd., Secunderabad, AP, India.

Enzymes: Various restriction enzymes used and Vent DNA polymerase were procured from New England Biolabs, Beverly, MA, USA. Taq DNA polymerase was obtained from Stratagene.

Antibodies and Conjugates: Goat anti-GST Ab was obtained from Pharmacia Biotech, Uppsala, Sweden. The following secondary revealing Abs were used: i) goat anti-mouse immunoglobulin G (IgG)-horse radish peroxidase (HRPO) from BioRad; ii) goat anti-rabbit IgG-HRPO (Pierce Chemical Co.); iii) goat anti-monkey IgG-HRPO (Sigma); iv) anti-rabbit-FITC and v) anti-goat-HRPO (Reagent Bank, NII, New Delhi) vi) anti-mouse FITC (Dakopatts a/s, Glostrup, Denmark).

Radioisotopes: dCTP[α-^{32}P] (3000 Ci/mM), dATP[α-^{35}S] (1250 Ci/ mM) and ^{35}S Met (1000 Ci/ mM), were procured from NEN Life Sciences Products, Boston, MA, USA

Others: Ni-nitrilo-tri-acetic acid (NTA) affinity resin from Qiagen; Membranes for Western blotting were obtained from BioRad; Hybond N and X-ray films were from Amersham; DNA and protein analysis softwares, PCgene from IntelliGenetics, Inc., Mountain View, CA, USA and Lasergene from DNASTAR Inc., Madison, Wisconsin, USA; Ultrafilteration assembly and YM5 membranes from Amicon Corp., Lexington, MA, USA.

METHODS

I CLONING AND SEQUENCING OF bZP3

1.1 PCR Amplification of bZP3

Total RNA was isolated in the laboratory from frozen bonnet monkey ovaries and the poly (A)+ fraction purified using PolyAT tract® mRNA isolation system and used for cDNA synthesis using Riboclone cDNA synthesis system®. The bonnet monkey ovarian cDNA was used as a template for the amplification by PCR of the region of bZP3, corresponding to hZP3 exons 1-6 using forward primer 5'-TGCA**GGTACC**ATGGAGCTGAGCTATAGGC-3' (corresponding to exon 1 and incorporating a *Kpn* I site shown in bold) and reverse primer 5'-CAGGTGGCAGGTGATGTA-3' (corresponding to exon 6), involving an initial melt at 94°C for 2 min and 35 cycles of 94°C for 1 min, 60°C for 2 min and 72°C for 3 min followed by a final extension at 72°C for 15 min. Similarly, the region corresponding to exons 4-8 of hZP3 was amplified using forward primer 5'- ATCACACCATCGTGGAC-3' (corresponding to exon 4) and reverse primer 5'-AGATCT**GAGCTC**ATTGCTTTCTTCTTTTATTCGGA-3' (corresponding to the exon 8 and incorporating a *Sac* I site) with the same conditions of the PCR as above except using an annealing temperature of 55°C. The PCR amplifications were carried out using *Taq* DNA polymerase in a 50 µl volume using 20 ng of cDNA. The full length bZP3 cDNA was assembled using the above purified fragments by second PCR involving i) one cycle of 94°C for 3 min, 55°C for 2 min, 72°C for 4 min; ii) addition of forward and reverse primers corresponding to exons 1 and 8 respectively; iii) 35 cycles of 94°C for 1 min, 55°C for 2 min, 72°C for 3 min; and iv) final extension at 72°C for 15 min.

1.2 Agarose Gel Electrophoresis, Digestion and Ligation

The PCR amplified cDNA fragment corresponding to bZP3 was resolved on a 0.8% agarose gel run using 1X TAE buffer (0.04 M Tris-acetate, 0.001 M EDTA) and purified using the Geneclean® II kit. The PCR amplified bZP3 was digested with *Kpn* I and *Sac* I and ligated into the pBluescriptII SK(+) vector at the same sites. The digestion and

ligation reactions were carried out using conditions and buffers specified by the manufacturer.

1.3 Media Composition and Bacterial Culture

All bacterial cultures were grown in Luria Bertani (LB) medium (NaCl 1%, Yeast extract 0.5%, and tryptone 1%, pH 7.0) at 37°C with shaking. The medium was sterilized by autoclaving at 15 lbs/inch^2 for 20 min. Solid growth medium was prepared by adding 1.5% agar to LB prior to autoclaving. Antibiotics were added after cooling the medium to 50°C.

1.4 Preparation of Competent Cells and Transformation

E. coli DH5α cells were grown overnight (O/N) in LB at 37°C and subcultured in 100 ml of fresh LB. The culture was maintained at 37°C with shaking till absorbance at 600 nm (A_{600}) reached 0.3. The culture was chilled and centrifuged at 4,500 rpm in a Sorvall SS34 rotor for 15 min. Cells were resuspended in 50 ml of freshly prepared sterile ice cold $CaCl_2$ (100 mM) solution and incubated on ice for 1 h. Cells were pelleted at 2,500 rpm and very gently resuspended in 10 ml of chilled 100 mM $CaCl_2$ having 15% glycerol. 200 µl of competent cells were aliquoted into sterile, chilled 1.5 ml tubes and stored at -70°C. The ligation mix was added to competent cells thawed on ice, tubes were gently mixed and incubated on ice for 1 h. Cells were subjected to a heat shock at 42°C for 90 sec and then revived in 1 ml of LB at 37°C for 1 h with gentle shaking. Aliquots were plated on LB plates containing the appropriate antibiotics and incubated at 37°C O/N.

1.5 Small Scale Plasmid DNA Isolation

Colonies obtained after transformation were inoculated in 5 ml LB and grown O/N in the presence of 100 µg/ml ampicillin (LB_{Amp}). Next morning 1.5 ml of the culture was centrifuged for 1 min at 10,000 rpm in a microfuge. The supernatant was discarded and the pellet was resuspended in 100 µl of chilled GTE (50 mM Glucose, 25 mM Tris HCl and 10 mM EDTA). After an incubation at room temperature (RT) for 5 min, 200 µl of freshly prepared alkaline SDS (0.2 N NaOH, 1% SDS) was added and the contents

mixed by inverting tubes. Following an incubation on ice for 5 min, 150 µl of ice cold potassium acetate solution (prepared by mixing 60 ml of 5 M potassium acetate, 11.5 ml of glacial acetic acid and 28.5 ml of water) was added. The mixture was incubated on ice for 5 min and centrifuged at 12,000 g for 5 min at 4°C. The supernatant was decanted into a fresh tube and extracted once with an equal volume of phenol equilibrated with 10 mM Tris, pH 8 and 1 mM EDTA (TE) followed by extraction with chloroform:isoamyl alcohol (24:1). DNA was precipitated by adding 2 volumes of chilled ethanol, contents mixed and tube incubated on ice for 30 min. The pellet collected after centrifugation at 12,000 g for 15 min was washed once with 70% alcohol, dried and resuspended in 50 µl TE. To remove RNA contamination contents of the tube were treated with 20 µg/ml RNAase for 15 min at RT. DNA was checked and analyzed after restriction digestion by agarose gel electrophoresis.

1.6 Large Scale Plasmid DNA Isolation

A 1000 ml culture of cells harboring the plasmid were grown O/N in LB_{Amp}. Next morning the culture was chilled and cells pelleted at 4,500 rpm in a Sorvall SS34 rotor for 20 min. The supernatant was discarded and cells were washed with 100 ml of STE buffer (0.1 M NaCl, 10 mM Tris HCl and 1 mM EDTA, pH 8.0). The pellet obtained after centrifugation was resuspended in 10 ml of GTE solution containing 1 mg/ml lysozyme and the mixture was incubated at RT for 20 min at 4°C. Alkaline SDS (20 ml) was added and the mixture was incubated at RT for 10 min after mixing gently by inverting the tube. Ice cold potassium acetate solution (15 ml) was added and the tube was chilled on ice for 15 min and then centrifuged at 18,000 rpm at 4°C in a SS34 rotor. The supernatant was carefully transferred to a fresh tube, DNA was precipitated by adding 0.6 volume isopropanol and incubating at RT for 10 min and then recovered by centrifugation at 5000 rpm at RT for 30 min. DNA was rinsed with 70% ethanol, dried and dissolved in 3 ml of TE. To the nucleic acid solution 3 ml of chilled LiCl (5 M) was added, mixed and the precipitate removed after spinning at 10,000 rpm for 10 min at 4° C. DNA was precipitated from the supernatant using an equal volume of isopropanol,

centrifuged at 10,000 rpm for 10 min, washed with 70% ethanol and dried. DNA was resuspended in 500 µl of TE containing 20 µg/ml RNAase, incubated at RT for 30 min and analyzed by agarose gel electrophoresis. DNA for transfection was prepared using the Plasmid midi kit DNA purification system using protocols described in the manual.

1.7 Sequencing of bZP3

Double stranded plasmid pBluescript-bZP3 DNA was sequenced using Sanger's dideoxy chain termination method (Sanger et al., 1977) using the Sequenase version 2.0 kit according to the protocols recommended by the manufacturer. Purified plasmid DNA (5 µg) and 2 pM of the sequencing primer was used in the sequencing reaction. Table 2 gives a list of the primers used for sequencing of the bZP3 cDNA clones. bZP3 sequence was confirmed by sequencing three independent clones 401, 403 and 404.

	PRIMER		SEQUENCE
1	H88	Forward	TGCAGGTACCATGGAGCTGAGCTATAGGC
2	H79	Reverse	CAGGTGGCAGGTGATGTA
3	M4	Forward	ATCACACCATCGTGGAC
4	H87	Reverse	AGATCTGAGCTCATTGCTTTCTTCTTTTATTCGGA
5	BSP1	Forward	TCTGGTCTCCATGGACAC
6	BSP2	Reverse	TGTGGATTTCTGCCTGGAGGT
7	M13(-20)	Forward	GTAAAACGACGGCCAGT
8	M13 R	Reverse	TTCACACAGGAAACAG

1.8 Analysis of Sequence

The bZP3 sequence was analyzed using PCgene and Lasergene DNA and protein analysis softwares. The alignment of the bZP3 aa sequence with the homologous sequences from other species was carried out using the Cluster V Multiple Alignment Programme (Higgins and Sharp, 1989).

2 CLONING AND EXPRESSION IN *E. coli*

2.1 PCR Amplification and Cloning in pQE30 Vector

Our initial attempts to express the full length gene in *E. coli* as a His_6 fusion protein failed. Attempts were initially made to express the His_6-bZP3 protein in the pRSET B

vector, under the phage T7 promoter, in BL21(DE3) cells, and under the T5 phage promoter, in the pQE30 vector for expression in SG13009[pREP4] and M15[pREP4] cell strains. For cloning in pRSET B, the full length bZP3 initially subcloned in the pBacPAK8 vector at the *Kpn* I and *Sac* I sites was released after digestion with *Kpn* I and *Eco*R I and cloned in a similarly restricted pRSETB vector inframe with an N-terminal His_6 tag. For cloning in the pQE30 vector, the pBacPAK8 carrying the full length bZP3 was initially digested with *Not* I, filled in with Klenow and then digested with *Kpn* I. The purified bZP3 fragment was then cloned in the vector digested with *Kpn* I and *Sma* I in frame with an N-terminal His_6 tag. Though transformants positive for the bZP3 insert in the right reading frame were recovered, no expression could be detected by SDS-PAGE or immunoblots in either case.

An alternate strategy was then devised in which an internal fragment of the gene, excluding the signal sequence and the transmembrane-like domain, following the putative furin cleavage site, was amplified by PCR using the forward primer 5'-CG**GGATCC**CAACCCTTCTGGCTCTTG-3' incorporating a *Bam*H I site and the reverse primer 5'-CC**GAGCTC**AGAAGCAGACCTGGACCA-3' incorporating a *Sac* I site. The PCR was done in a 50 µl volume using 50 pM of each primer and Vent polymerase for extension. The pBluescript-bZP3 (10 ng) having a full length bZP3 insert was used as the template and was initially denatured at 95°C for 10 min. Amplification was carried out for 35 cycles of denaturation at 95°C for 2 min, primer annealing at 60°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 bZP3 fragment was digested with *Bam*H I and *Sac* I and cloned in frame downstream of a His_6 tag under the T5 promoter-lac operator control in the pQE30 vector. The authenticity of the construct was confirmed by N-terminal sequencing using an upstream sequencing primer GGCGTATCACGAGGCCCTTTCG.

2.2 Expression in M15[pREP4] and SG13009[pREP4] E. coli Strains

The pQE-bZP3 plasmid was transformed in M15[pREP4] and SG13009[pREP4] bacterial strains provided with the kit. The transformed colonies were analyzed for expression. A single transformed colony was inoculated and grown overnight at 37°C in 1 ml of LB containing 100 µg/ml of ampicillin and 25 µg/ml of kanamycin. Cells were subcultured 1:10, next morning and grown until cell density reached an 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. The cells were collected by centrifugation at 13,000 g for 60 sec and the resulting pellet was stored at -70°C until used.

2.3 SDS-PAGE and Immunoblot

The cell pellet obtained from 1 ml culture was solubilized by boiling for 5 min in 100 µl of 2X sample buffer (0.0625 M Tris, pH 6.8, 2% SDS, 10% glycerol, 5% BME and 0.001% bromophenol blue) and the proteins were resolved on a 0.1% SDS-10% PAGE (Laemmeli, 1970). The gel was stained with Coomassie brilliant blue for staining total cellular proteins. For immunoblotting, the proteins were electrophoretically transferred to 0.45 µm nitrocellulose membrane overnight at a constant voltage of 15 V in Tris glycine buffer with 20% methanol (Towbin et al., 1979). Nonspecific sites on the membrane were blocked by incubation with 5% BSA in 50 mM phosphate buffered saline (PBS), pH 7.4, for 1 h followed by 3 washes (15 min each) with PBS containing 0.1% Tween-20 (PBST). For detection of bZP3, a murine monoclonal antibody (MAb), MA-451, generated against the pZP3β and recognizing a cross reactive epitope (166-171 aa residues) within the bonnet sequence was used (Afzalpurkar and Gupta, 1997). The membrane was incubated for 1 h with a 1:5 dilution of MA-451 culture supernatant, followed by 3 washes in PBST. Horseradish-peroxidase (HRPO) conjugated goat anti-mouse immunoglobulin (Ig) was used to reveal bound Ab. Colour was developed with 0.6% (w/v) 4-chloronaphthol in 50 mM PBS, pH 7.4, containing 25% methanol and 0.06% H_2O_2. The reaction was stopped by washing the membrane with PBS.

2.4 Intracellular Localization

A 100 ml culture was grown and induced according to the procedure mentioned above. The culture was divided into 2 aliquots and cells were pelleted down. For cytosolic localization, one pellet was resuspended in 5 ml of sonication buffer (50 mM Na-phosphate, pH 7.8, 300 mM NaCl). The sample was frozen and then thawed in ice-water and cells lysed by brief sonication. The sample was centrifuged at 10,000 g for 20 min. The soup and the pellet represent the soluble and insoluble components of the cell pellet. In order to check for periplasmic localization, the 2nd aliquot of cells was resuspended in 10 ml of hypertonic solution (30 mM Tris, pH 8, 20% sucrose, 1 mM EDTA) and incubated at RT for 10 min with shaking. Cells were centrifuged at 8,000 g for 10 min. The pellet was subjected to osmotic shock in 5 mM $MgSO_4$. Cells were stirred for 10 min in an ice water bath, centrifuged at 8000 g at 4^oC for 10 min. The soup collected represented the periplasmic fraction. The fractions were analyzed by 0.1% SDS-10% PAGE and Western blotting as described above.

2.5 Standardization of Expression Conditions

Conditions for expression of r-bZP3 in SG13009[pREP4] cells transformed with the pQE-bZP3 plasmid were standardized. Cells were grown till A_{600}=0.7 and induced with different concentrations of IPTG (0.5, 1, 2, or 4 mM) for a constant time period (3h) or induced with a 0.5 mM IPTG for different time periods (0, 1, 2, 3 or 5 h). Cells were harvested and analyzed by SDS-PAGE and immunoblot as described above.

2.6 Expression of r-bZP3 in Different E. coli Strains

E. coli strains deficient in specific proteases were used to study their influence on the expression of r-bZP3. BL21(DE3) and BL21(pLysS) deficient in *ompT* and *lon* proteases and DF5 carrying a targeted mutation of the *ptr* gene, were transformed with the pQE-bZP3 plasmid. Colonies obtained were grown O/N and subcultured next morning and grown till A_{600}=0.7. Cultures were then induced with 0.5 mM IPTG for 3 h. Harvested cells were checked by SDS-PAGE and immunoblotting.

2.8 Reactivity with Anti-peptide Sera

Peptide antisera were generated in the laboratory against peptides P1, 23-45 aa residues with an extra lysine at the N-terminus (KQPFWLLQGGASRAETSVQPVLVE), P2, 300-322 aa residues (CSFSKSSNSWFPVEGPADICQCC) and P3, 324-347 aa residues (KGDCGTPSHSRRQPHVVSQWSRSA) corresponding to bZP3 precursor protein in rabbits and were used to determine their reactivity with the r-bZP3 protein expressed in *E. coli* in an enzyme linked immunosorbent assay (ELISA). Microtitration plates were coated with 200 ng of r-bZP3 or 1 μg/well of the peptide. HRPO conjugated goat anti-rabbit Ig at 1:5000 dilution was used as revealing Ab.

3 EXPRESSION IN BACULOVIRUS

3.1 Plasmid Construction

Constructs were designed to express bZP3 in insect cells under the late polyhedrin promoter. The full length bZP3 1-424 aa residues (construct V1 encoding a 47 kDa polypeptide) including the native eukaryotic N-terminal signal sequence (aa 1-22) and the C-terminal region after the furin cleavage site which includes the transmembrane-like domain (aa 349-424) was digested from pBluescript-bZP3 clone 401 using *Kpn* I and *Sac* I restriction enzymes and subcloned in the pBacPAK8 vector. A second construct V2 was designed containing a truncated version of the gene (aa 1-348), excluding the C-terminal transmembrane domain giving a protein with a calculated mass of 39.8 kDa. The insert was amplified by PCR using the forward primer TGCA**GGTACC**ATGGAGCTGAGGC incorporating a *Kpn* I site (restriction site shown in bold) and the reverse primer CC**GAGCTC**AGAAGCAGACCTGGACCA incorporating a *Sac* I site using 10 ng of 401 template DNA. The amplified fragment was digested with *Kpn* I and *Sac* I, and ligated with a similarly restricted pBacPAK8 vector. Two more constructs were designed to express bZP3 aa 23-76 (V3 encoding a polypeptide 36.6 kDa) and aa 23-348 (V4, encoding a polypeptide 67.3 kDa) inframe as GST fusion proteins with a replacement of the native signal sequence with an insect

(gp67) signal sequence in the pAcSecG2T vector. bZP3 was amplified using the V1 transfer vector as a template in a PCR reaction using forward primer CGGGATCCCAACCCTTCTGGCTCTTG incorporating a *Bam*H I site and reverse primer GCGAATTCCAGAAGCAGACCTGGACCA incorporating an *Eco*R I site. Amplified DNA was digested and ligated with the digested pAcSecG2T vector. A dinucleotide deletion at nt position 239-240 resulted in premature termination of the protein after aa residue 76 and was used for expression of the V3 protein. DNAs from the transfer vector clones were purified using the Plasmid Midi kit DNA purification system.

3.2 Screening of the Recombinant Transfer Vector

The ligation mixture was used for transformation of DH5α cells as described earlier. Transformed bacterial colonies growing on LB_{Amp} plates were screened by colony hybridization. Briefly, colonies were grown for 6-8 h on a Nylon membrane placed on a LB_{Amp} plate. The colonies were lysed by placing the membrane on a Whatman® 3MM paper soaked in 10% SDS for 3 min, followed by treatment with denaturing solution (0.5 N NaOH, 1.5 M NaCl) for 5 min and neutralization solution (0.5 M Tris HCl pH 8, 1.5 M NaCl) for 5 min in the same manner. The membrane was dried, UV cross linked (Ultraviolet crosslinker, Amersham) and processed for prehybridization and hybridization.

Stocks of 20X SSC (174 g/L NaCl, 88.2 g/L sodium citrate, pH 7.0) and 50X Denhardt's (1% ficoll, 1% PVP, 1% BSA) were prepared. The membrane was prehybridized for 4-6 h in the prehybridization solution (5X SSC, 5X Denhardt's, 0.5% SDS, 10 µg/ml sheared and denatured salmon sperm DNA). The bZP3 DNA was labelled using the Multiprime DNA labeling system using 50 ng of purified bZP3 DNA. For hybridization with the probe, 10^6 cpm/ml of the denatured ^{32}P labeled bZP3 probe was added to the prehybridization solution and incubation was further carried out for 14-16 h. For removing the non specifically bound probe, the membrane was washed successively at RT in 2X SSC for 10 min, at 55°C in 0.2X SSC, 0.1% SDS for 10 min and finally at

65°C in 0.2X SSC, 0.1% SDS for 10 min. The membrane was wrapped in Saran wrap and exposed to an X-ray film.

The colonies that were positive by colony hybridization were inoculated in a 3 ml culture and used for preparing DNA for analysis by restriction digestion and Southern blotting. The digested DNA was resolved on a 0.8% agarose gel as described above. The gel was soaked in 4 volumes of denaturing solution (1.5 M NaCl and 0.5 M NaOH) for 1 h at RT with shaking followed by neutralization (1 M Tris HCl, pH 8 and 1.5 M NaCl) for 1 h at RT. The DNA was transferred to a Nylon membrane, UV crosslinked and hybridized with the full length ^{32}P labeled bZP3 probe as described above.

3.3 Cell Culture Techniques

AcNPV and recombinant baculoviruses were isolated, grown and assayed in confluent monolayers of *Spodoptera frugiperda* (Sf9) cell line maintained in TNMFH which is Grace's insect cell culture medium supplemented with 3.33 g/L Lactalbumin hydrolysate, 3.33 g/L Yeast autolysate. Complete medium (CM) was prepared by supplementing TNMFH with 10% heat inactivated FCS and 100X antibiotic-antimycotic.

3.4 *Construction of Recombinant Viruses*

Lipofectin-mediated transfection and *in vivo* homologous recombination was used to introduce foreign DNA into the AcNPV genome at the polyhedrin locus for making the V1, V2, V3 and V4 recombinant virus constructs using the BacPAK™ baculovirus expression system or the Baculogold™ transfection kit (Pharmingen) according to the manufacturer's instructions.

3.5 Plaque Assay for Isolating Viruses

Sf9 cells ($1.8x10^6$) seeded in a 35 mm culture dish were infected in duplicate with 100 µl of the serial dilutions (10^0 to 10^{-2}) of the transfection supernatant for 1 h. The viral inoculum was aspirated and 1.5 ml of the cooled agarose overlay (1.5% LMP agarose, 0.5X CM) was added to each dish and allowed to set. 1 ml of CM was added to each dish and the plates were incubated at 27°C for 5 days. Medium was removed and cells were stained with 2 ml of staining solution (0.03 % neutral red in 10 mM PBS) for 1 h.

The staining solution was aspirated and the plates left at 27°C O/N. Plaques whic appeared as clear zones, were identified, marked and verified under the microscop Plaques were picked up using a sterile 200 µl tip and viruses were allowed to diffuse ou O/N in 200 µl of CM to make the plaque pick stock virus.

3.6 Screening of the Recombinant Viruses

For analysis of viral DNA by dot blot, $1X10^5$ cells were seeded in each well of the 9 well plate and infected in duplicate with 50 µl of the plaque pick for 1 h, followed b addition of 50 µl of CM to each well. Infected cells were incubated for 5 days, afte which the culture supernatant was saved and cells were processed for dot blot analysi Cells were lysed with 200 µl of 0.5 M NaOH. The alkali was neutralized by addition o 50 µl of 4 M ammonium acetate. The nylon membrane was wetted in warm water an washed in dot blot solution (1 M ammonium acetate, 0.02 N NaOH) and the cell lysat was blotted on to the membrane using a dot blot apparatus (Bio-Rad), dried, UV cros linked and processed for prehybridization and hybridization.

For the isolation of total genomic DNA, cells infected in a 35 mm culture dish wer harvested 72 h post infection (pi) and treated with 400 µl of DNA extraction buffer (1 mM Tris HCl, pH 8, 0.6% SDS, 10 mM EDTA) and 50 µl of 20 mg/ml proteinase K a 37°C for 12-16 h. The DNA was extracted twice with phenol:chloroform:isoamy alcohol (25:24:1) and once with chloroform. For each extraction, the suspension wa mixed by inverting the eppendorf and separated by centrifugation at 2,000 rpm for 3 mi in a microfuge. DNA was precipitated with 1 ml of 95% ethanol at -20°C for 4 h an pelleted at 4,500 rpm for 20 min. The pellet was washed with 70% ethanol, dried an resuspended in 50 µl of TE. DNA was digested with *Hind* III, resolved on a 0.8% agarose gel and processed for Southern blotting. Positive clones were amplified b infecting cells at a multiplicity of infection (MOI) of ≤1 for 10 days and the amplifie virus was titrated using a plaque assay. Sf9 cells were infected at ~10 MOI fo expression of the r-proteins.

3.7 Expression of bZP3 in BEVS

Expression conditions for bZP3 under the polyhedrin promoter were standardized using the Northern blot and Western blot analysis of cells infected with the V1 virus. Sf9 cells, seeded at a density of 1.5 million in a 35 mm petridish were allowed to attach for 1 h at 27°C. The medium was removed and the cells were infected with AcNPV (*Autographa californica* nuclear polyhedrosis virus) or V1 at ~10 MOI for 1 h. The infected cells were harvested at different time points from 0-84 h pi. The cells (~2X10^6) were washed with chilled PBS and resuspended in 1 ml of denaturing solution (4 M GITC, 25 mM sodium citrate, pH 7, 0.5% sarcosyl, and 0.1 M BME) followed by addition of 50 µl of 2 M sodium acetate (pH 4) and 500 µl water saturated phenol and 100 µl chloroform:isoamyl alcohol (49:1). The suspension was mixed thoroughly after the addition of each reagent, vortexed for 10 sec and cooled on ice for 15 min. The aqueous and the phenol phases were separated by centrifugation at 12,000 rpm for 20 min in a refrigerated microfuge. The aqueous phase was transferred to a fresh tube and 500 µl isopropanol was added. RNA was precipitated at -20°C for 1 h, and pelleted at 12,000 rpm for 20 min at 4°C. The RNA pellet was dissolved in 300 µl denaturing solution followed by addition of 300 µl of isopropanol. RNA was reprecipitated at -20°C for 1 h, washed with 75% ethanol and the pellet collected by centrifugation at 12,000 rpm in a refrigerated microfuge. RNA was dissolved in 25 µl of 0.5% SDS by heating at 65°C for 10 min and stored at -70°C. RNA was quantitated and 5 µg of RNA corresponding to each time point was resolved on a 1.2% agarose formaldehyde gel, transferred to a nylon membrane and probed with ^{32}P labeled bZP3 probe.

Cells harvested at different time points from ~2X10^6 cells 12-84 h pi were pelleted down, washed with 10 mM PBS, pH 7.4, and lysed in reducing buffer and resolved on a 0.1% SDS-10% PAGE as described earlier. The supernatant was concentrated to 10X for loading on the gel.

3.8 Radiolabeling of Recombinant Proteins in Infected Sf9 Cells

Cells ($1.5X10^6$) infected with AcNPV, V1, V2, V3 or V4 were grown for 72 h pi followed by starvation for methionine (Met) in Met-free medium for 1 h. 25 μCi of ^{35}S Met was added and cells were pulsed for 2 h. Cells were harvested and proteins resolved on a 0.1% SDS-10% PAGE as described above. The gel was dried and exposed to X-ray film. The signal was quantitated and analyzed using the molecular imager (GS 250, BioRad, USA). The intensity of the polyhedrin band was compared with that of the r-bZP3 proteins expressed by virus constructs V1, V2, V3 and V4.

3.9 Analysis of r-bZP3 in V1, V2, V3 and V4 Infected Cells and Supernatant

Cells and supernatant collected 72 h pi were analyzed on a 0.1% SDS-10% PAGE and Western blot using polyclonal Abs generated in rabbit against peptide-DT conjugates using (i) 23-45 aa residue N-terminal peptide with an extra lysine at the N-terminus (KQPFWLLQGGASRAETSVQPVLVE), (ii) 300-322 aa residue C-terminal peptide (CSFSKSSNSWFPVEGPADICQCC) corresponding to the bZP3 sequence (iii) MA-451 and (iv) goat anti-GST Ab. The C-terminal anti-peptide Ab was used for determining whether or not the full length bZP3 was being expressed by the cells infected with the different viruses. Anti-mouse (1:500), anti-rabbit (1:500) or anti-goat (1:500) Abs conjugated to HRPO were used for revealing bound Ab.

3.10 *Immunocytochemical Localization of Recombinant Proteins in Infected Cells*

Sf9 cells infected with AcNPV, V1, V2, V3, or V4 were harvested 72 h pi, washed twice with 10 mM PBS, air dried on a slide and fixed in chilled methanol for 15 min. Cells were incubated with MA-451 culture supernatant and N-terminal anti-peptide serum (1:500) at 37°C for 1 h, washed with PBS and further incubated at 37°C for 1 h with 1:50 dilution of anti-mouse FITC or 1:2000 anti-rabbit FITC. Slides were washed extensively, mounted in 90% glycerol in PBS (50 mM, pH 7.4) and examined under Optiphot fluorescent microscope (Nikon, Tokyo, Japan).

4 IMMUNOGENICITY AND *IN VIVO* CONTRACEPTIVE EFFICACY

4.1 Purification and Conjugation

For purification, the His_6-bZP3 fusion protein was expressed in SG13009[pREP4] and BL21(DE3) strains transformed with the pQE-bZP3 plasmid. Expression was scaled up to a 2000 ml (250 ml X 8) batch flask culture. Cells were pelleted down at 4,000 g for 20 min at 4°C and stored at -70°C till used. The cell pellet (1 g/5 ml) was solubilized in 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 15 min at 4°C and the supernatant containing the r-fusion protein was mixed with gentle end to end shaking for 1 h at RT with the Ni-NTA resin (Qiagen GmbH). The resin was loaded on a column and washed with 10 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. The recombinant fusion protein was eluted with buffers D and E 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 and then dialyzed against 100 mM phosphate buffer pH 7.4 having 4 M urea. The purified protein was quantitated with bicinchoninic acid.

Twenty milligrams of r-bZP3 was conjugated to 13 mg of diphtheria toxoid (DT; Serum Institute, Pune, India) or 19 mg of tetanus toxoid (TT) using a modification of the "one step" glutaraldehyde coupling procedure (Avrameas, 1969). Conjugation was done in 100 mM phosphate buffer, pH 7.4 with 4 M urea using 0.1% glutaraldehyde, O/N 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 having 0.3 M urea.

4.2 Immunization of Rabbit

Female New Zealand White rabbit (Small Animal Facility, National Institute of Immunology, New Delhi, India), 6 months of age was immunized intramuscularly with r-bZP3-DT conjugate equivalent to 125 µg of r-bZP3 (expressed in SG13009[pREP4] cells) in 0.9% saline emulsified with Squalene and Arlacel "A" in a ratio of 4:1 and

administered at two sites. In addition, the primary dose also contained 500 µg of SPLPS as an additional adjuvant. This was followed by 2 booster at 4 weekly intervals with an equal amount of r-bZP3-DT conjugate.

4.3 Titration of Rabbit Anti-bZP3 Sera

Microtitration plates were coated with r-bZP3 at a concentration of 200 ng/well in 50 mM PBS, pH 7.4 for 1 hr at 37°C and then at 4°C overnight. Plates were subsequently washed once with PBS and blocked with 1% BSA for 1 hr at 37°C in PBS to reduce non-specific binding. Blocking was followed by three washes of 5 min each with PBS containing 0.05% Tween-20 (PBST). Plates were incubated with varying dilutions of preimmune and immune sera for 1 h and bound Ab was revealed with the anti rabbit-HRPO conjugate used at an optimized dilution of 1:5000 in PBS. After washing to remove unbound anti-rabbit-HRPO conjugate, the enzyme activity was estimated with 0.1% orthophenylenediamine (OPD) in 50 mM citrate phosphate buffer, pH 5.0 having 0.06% of hydrogen peroxide as the substrate. The reaction was stopped by adding 50 µl/well of 5 N H_2SO_4 and the absorbance read at 490 nm in a microplate reader (Molecular Devices Corporation, California, USA). The Ab titer was calculated by regression analysis and is represented by Ab units (AU) as the reciprocal of the dilution of the Ab giving an A_{490} of 1.0

4.4 Reactivity with Porcine ZP3

Porcine ZP3α and ZP3β prepared as described previously (Yurewicz et al., 1987) and purified r-bZP3 were tested for their reactivity with rabbit anti-r-bZP3 Ab in the immunoblot by the same procedure as described above except that goat anti-rabbit Ig-HRPO conjugate was used.

4.5 Immunofluorescence on Bonnet Monkey Ovarian Sections

A 3 year old monkey was treated daily for 3 days with an intramuscular injection of 25 IU of Pergonal® (Laboratoires Serono S.A., Aubonne, Switzerland). The monkey was ovarectomized on day 6, and the ovary was snap frozen in liquid nitrogen and sections of 5 µm thickness were cut in a cryostat at -20°C and fixed for 20 min in chilled methanol.

In addition, cryosections of an ovary from a normal cycling female (10 years) were also processed. 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 1:250 dilution of rabbit pre-immune and immune sera for 1 h, washed with PBS and incubated for 1 h with 1:2000 dilution of goat anti-rabbit Ig-FITC conjugate. Slides were washed with PBS and mounted in Glycerol:PBS (9:1) and examined under fluorescent microscope.

4.6 Indirect Immunofluorescence on Human Oocytes

Human oocytes were washed twice with PBS containing 0.1% BSA and then incubated with 1:50 dilution of immune or pre-immune serum samples at RT for 30 min. Following washing with PBS (3 changes of 5 min each), the oocytes were treated with goat anti-rabbit Ig-FITC conjugate for 30 min at RT. After washing with PBS, the treated oocytes were mounted in Glycerol:PBS (9:1) and examined under fluorescent microscope.

4.7 Progesterone Radioimmunoassays

Progesterone levels were estimated from sera of bonnet monkeys which were bled biweekly using a radioimmunoassay employing reagents and protocol as prescribed by the W.H.O. Matched Assay Reagent Programme (Sufi et al., 1983). Each sample was run in duplicates. Progesterone was extracted from serum (0.1 ml) by the addition of 2 ml of ice-cold ether in each tube and vortexing for 2 min. The tube was immersed in liquid nitrogen in order to flash freeze the serum phase and the unfrozen ether phase which contained the extracted steroid hormone was decanted into another tube. The ether was allowed to evaporate O/N and 0.5 ml of steroid assay buffer (0.1 M PBS, pH 7.3, 0.1% thiomersal and 0.1% gelatin) was added to the tubes and the tubes were incubated at 40°C for 30 min. Steroid sticking to the walls of the tubes was recovered by vigorous vortexing. 100 µl of anti-progesterone Ab (at a dilution giving ~50% binding of tritiated progesterone in the absence of unlabelled competing progesterone) was then added to the tubes followed by addition of 0.1 ml of ^{3}H-progesterone (~10,000 cpm/tube). The mixture was incubated for atleast 16 hrs at 4°C. Unbound progesterone

was separated by addition of 0.2 ml of ice cold assay buffer containing 0.625% activated charcoal and 0.0625% dextran and incubated for 30 min at 4°C. This was followed by centrifugation at 2500 rpm for 15 min at 4°C. The supernatant was carefully decanted into scintillation vials and 4 ml of scintillation fluid (0.4% 2,5 diphenoxazole; 0.01% POPOP [1-4 bis(5-phenyl-2-oxazolyl)benzene] in sulfur free toluene) was added and counted in a liquid scintillation beta counter (Beckman Instruments, California, USA). The amount of progesterone per ml of serum was calculated from a standard curve with known amounts of progesterone in each assay.

4.8 Immunization of Female Bonnet Monkeys

Female bonnet monkeys (*Macaca radiata*) reared at the Primate Facility (NII, New Delhi) were selected and serum progesterone levels were estimated for atleast three months in samples which were collected biweekly. Animals showing atleast two consecutive normal ovulatory peaks (serum progesterone levels >2 ng/ml) (Bamezai, 1986) were selected for fertility trials.

Five animals (MRA 375, 515, 640, 672, 770) immunized with 250 µg equivalent of r-bZP3, expressed in SG13009[pREP4] cells, conjugated to DT, was emulsified with Squalene and Arlacel A, adjuvants permitted for human use, in a ratio of 4:1 and administered intramuscularly at two sites. In addition, the primary dose also contained 1 mg/animal of SPLPS as an additional adjuvant. Animals were boosted at intervals of 4-6 weeks depending on the Ab titers with 250 µg of r-bZP3-DT using Squalene and Arlacel A as adjuvants. A second group of 3 monkeys (MRA 384, 502, 661) were immunized using a slightly different protocol. The primary immunization consisted of 125 µg of r-bZP3-DT and 125 µg of r-bZP3-TT (expressed in BL21(DE3) cells) using the same adjuvants and immunization protocols mentioned above except that boosters were administered alternately with 250 µg of r-bZP3-DT or -TT conjugates using Squalene and Arlacel A as adjuvants. Following completion of the primary immunization and 2 boosters at monthly intervals, bleeds (1-2 ml) were collected biweekly from the antecubital vein for estimation of progesterone levels and Ab titres. Boosters were

administered as and when required. Animals were put on continuous mating with males of proven fertility after administration of the three primary injections and monitored for menstrual cyclicity and conception. Ab titres were determined as described above except that anti-monkey HRPO conjugate was used as the revealing Ab.

4.9 Arsanilation of r-bZP3

r-bZP3 was arsanilated using a modification of the procedure of Nisnoff (1967). Briefly, arsanilic acid (100 mg) was dissolved in 5 ml of 1 M HCl. A 10 ml stock of $NaNO_2$ (10 mg/ml) was also prepared fresh and added dropwise to the arsanilic acid solution while vortexing. Activation of arsanilic acid was checked on starch-KI paper. The ice cold activated arsanilic acid solution was added dropwise to the protein solution (5 mg of r-bZP3 in 100 mM PB, pH 7.4) stirring constantly in an ice water bath, while pH was maintained between 9.0 and 9.5 with 10 N NaOH. The protein solution was dialyzed extensively against 100 mM PB having 4 M urea. Arsanilation of r-bZP3 was checked by ELISA using ars-r-bZP3 for coating (1 µg/well) and using a 1:100 dilution of murine anti-ars MAb, R16.7 (Durdik et al., 1989). Bound Ab was revealed using anti-mouse HRPO conjugate (1:5000). Three monkeys previously immunized with r-bZP3-DT conjugate (MRA-375, MRA-640 and MRA-672) and 2 naive monkeys (MRA-446 and MRA-670) were immunized at 2 intramuscular sites with 250 µg of ars-bZP3 conjugate using Squalene:Arlacel A (4:1) as an adjuvant. Boosters were administered at intervals of 20 days and bleeds were collected 10 days post immunization. Bleeds were analyzed by ELISA using r-bZP3 and ars-BSA for coating to determine anti-bZP3 and anti-ars Ab titres as described earlier.