Materials and Methods

REAGENTS

Chemicals: Tris, glycine, acrylamide, N, N′-Methylene-bisacrylamide, sodium dodecyl sulfate (SDS), β-mercaptoethanol, N, N, N′, N′-Tetramethylethylenediamine (TEMED), phenol, ethidium bromide, ethylenediaminetetraacetic acid (EDTA), agarose, Bromophenol blue, Coomassie brilliant blue-R250, calcium chloride, sodium acetate, glucose, lysozyme, glycerol, chloroform, lithium chloride, phenylmethyl sulphonyl fluoride (PMSF), spermidine, saponin, paraformaldehyde were procured from Sigma Chemical Co., St. Louis, MO, USA.

Low melting point (LMP) agarose, isoamyl alcohol, sodium deoxycholate, oxidized glutathione, reduced glutathione, dimethyl sulfoxide (DMSO), 4-chloro-1-naphthol, isopropyl-β-D-thiogalactopyranoside (IPTG) and 5-bromo-4-chloro-3-indolyl-β-D-galactopyranoside (X-gal), were purchased from Amresco, Solon, Ohio, USA. Ammonium persulfate and guanidine hydrochloride were purchased from Amersham, Cleveland, Ohio, USA. Alum was procured from Superfos Biosector, Elsenbakken, Frederikssund, Denmark.

Reagents for Enzyme Immunoassay: Bovine serum albumin (BSA) and orthophenylene diamine (OPD) were purchased from Sigma. Tween-20 (polyoxyethylene-20-sorbitan monolaurate) was obtained from Amresco.

Media and Antibiotics: Bacto tryptone, bacto yeast extract and bacto agar were obtained from Difco laboratories, Detroit, USA, Dulbecco's Modified Eagle's Medium (DMEM) and RPMI-1640 media were purchased from Sigma. Antibiotics such as gentamycin sulfate, ampicillin (sodium salt) and kanamycin were also obtained from Sigma. Fetal calf serum (FCS) was procured from Biological Industries, Hibbutz Beit, Haemek, Israel.

Bacterial strains and plasmids: DH5α and BL21(DE3)pLysS strains of *E. coli* were purchased from Stratagene, La Jolla, CA, USA. SG13009[pREP4] *E. coli* strain was obtained from QIAGEN GmbH, Hilden, Germany. Expression vector, VR1020 was a kind gift from VICAL Incorp., San Diego, CA, USA, pKB3-JE-13 clone was procured from ATCC, Rockville, MD, USA, pQE30 vector was procured from QIAGEN and pRSET-A vector was acquired from Invitrogen Corp., Carlsbad, California, USA.

Kits: PCR-Script™ Amp cloning kit was obtained from Stratagene. Plasmid DNA purification mega kit was purchased from QIAGEN. The pGEM-T Easy cloning kit was purchased from Promega, Madison, WI, USA.

Primers and Enzymes: Various oligonucleotide primers were custom made by Rama Biotechnologies, India Pvt. Ltd., Secundrabad, AP, India, Sigma-Genosys Ltd, New Delhi, India and Microsynth GmbH, Hilden, Germany.

Restriction enzymes were obtained from New England BioLabs (NEB), Beverly, MA, USA and Promega, Taq DNA polymerase and T4 DNA ligase were bought from Promega. Shrimp Alkaline Phosphatase was bought from Amersham.

Molecular weight markers: pGEM DNA markers were procured from Promega, λ DNA-*Hind* III digest and 1 kb DNA ladder were purchased from NEB. Prestained SDS-PAGE standards were obtained from Bio-Rad, Hercules, CA, USA.

Antibodies and Conjugates: Rabbit anti-mouse IgG (whole molecule) conjugated to horseradish peroxidase (HRPO) was procured from Dako A/S, Glostrup, Denmark. Goat anti-mouse IgG-FITC conjugate was procured from Sigma. Mouse monoclonal antibody isotyping kit was also purchased from Sigma. Goat anti-mouse immunoglobulins-HRPO, goat anti-rabbit immunoglobulins-HRPO, rabbit anti-goat IgG-HRPO and goat anti-rabbit

immunoglobulins-FITC conjugates were obtained from Pierce, Rockford, IL, USA. Anti-rabies monoclonal antibody conjugated to FITC was purchased from Centocor Inc, Malvern, PA, USA.

Others: Sodium chloride was purchased from S. D. fine-chem. Ltd., Mumbai, India, Lipofectamine and Trizol were obtained from Gibco-BRL, Rockville, MD, USA. UM-449 cell line was a kind gift by Prof. Nirbhay Kumar (The W. Harry Feinstone Department of Molecular Microbiology and Immunology, John Hopkins University-School of Public Health, N. Wolfe Street, Baltimore, MD 21205, USA), COS-7 cell line was kindly provided by Dr. Chetan Chitnis (Malaria Research Group, International Center for Genetic Engineering and Biotechnology, New Delhi, India), MNA cell line, CVS-11 rabies virus and Standard Rabies Immune Globulin were kindly gifted by Dr. Charles E. Rupprecht (Rabies section, Division of Viral and Rickettsial Diseases, Centres for Disease Control and Prevention, Atlanta, Georgia 30333, USA), Nickel-nitrilotriacetic acid (Ni-NTA) was procured from QIAGEN, ultrafiltration assembly and YM30 membrane from Amicon Corp., Lexington, MA, USA. Bicinchoninic acid (BCA) was purchased from Pierce. Complete and incomplete Freund's adjuvants were procured from Difco laboratories. Nitrocellulose membrane, Tefzel tubing, gold microcarriers and Helios gene gun assembly were obtained from Bio-Rad.

T-25, T-75 tissue culture flasks, 6-well tissue culture plates, 8-well tissue culture chamber slide and 96-well microtitration plates were procured from Nunc a/s, Rosakilde, Denmark. The 24-well tissue culture plates were procured from Corning glass works, Corning, NY, USA. The 96-well tissue culture plates were purchased from Becton Dickinson and Co.,

Franklin Lakes, NJ, USA. Glass micro-slides and coverslips were purchased from Blue Star, Polar Industries and Co., India.

METHODS

I. CLONING OF cDNA ENCODING bmZP1, dZP3, rG AND dZP3-rG GLYCOPROTEINS IN MAMMALIAN EXPRESSION VECTOR VR1020

1. PCR amplification of bmZP1 cDNA

Expression of the recombinant bmZP1 (r-bmZP1, excluding the N-terminal signal sequence [SS] and the C-terminus transmembrane-like domain [TD]) in *E. coli* using pRSET vector (Invitrogen) has been reported previously (Govind et al., 2001). The above pRSET-bmZP1 clone was used as a template for amplification of the bmZP1 cDNA (64-1389 nt; 1326 bp), by polymerase chain reaction (PCR), using 5'-GA**AGATCT**AAGCCTGAGACACCAGGT-3' as the forward primer, and 5' -TCT**AGATCT**ACTGAGATCAGG-3' as the reverse primer, for cloning in mammalian expression vector, VR1020 (VICAL). Both forward and reverse primers were designed with *Bgl* II restriction sites (denoted in bold). The PCR was performed in a 50 µl of final reaction volume (10 mM Tris-HCl, pH 9.0, 50 mM KCl, 1.5 mM $MgCl_2$ and 0.1% Triton X-100) using 50 pmol of each primer and Taq DNA polymerase for extension. The template was denatured at 94°C for 10 min. Amplification was carried out for 35 cycles of denaturation at 94°C for 1 min, primer annealing at 48°C for 2 min and extension at 72°C for 2 min, followed by a final extension at 72°C for 15 min.

2. PCR amplification of dZP3 cDNA

The dog ZP3 (dZP3) cDNA, excluding the SS and the TD, was cloned in prokaryotic expression vector, pQE30 (QIAGEN) as described previously (Santhanam et al., 1998). To clone dZP3 cDNA in mammalian expression vector, VR1020, the pQE30-dZP3 clone was used as a template to PCR amplify dZP3 cDNA (79-1056 nt; 978 bp) using 5'-

GA**AGATCT**CAGACCATCTGGCCAACT-3' as the forward primer, and 5'-GA**AGATCT**TTAAGTGTGGGAAACAGACTT–3' as the reverse primer as described for bmZP1 except that primer annealing was performed at 53°C for 1 min.

3. PCR amplification of rG cDNAs

To obtain the optimum expression of rG in mammalian cells and to study the influence of the SS and the TD on the immune response generated by DNA vaccine, four different constructs of rG cDNA in VR1020 vector were made (Table 1). For cloning rG, BHK21 cells were infected with PM10 strain of rabies virus. Total RNA from the infected cells was prepared at various time period post-infection using TRIZOL reagent. Total RNA was directly used to amplify the cDNA corresponding to rG without the SS and the TD, by RT-PCR, following the manufacturers instruction provided in the kit (Promega). The RT-PCR resulted in amplification of a 1.314 kb fragment. The fragment was cloned in pPCR-Script Amp SK (+) cloning vector and from there into pQE30 expression vector. One of the positive clones (pQE30-rG) expressing rG in *E. coli* was used as a template to PCR amplify rG cDNA, without the SS and the TD, using *BamH* I restriction site in the forward primer and *Bgl* II restriction site in the reverse primer (Table 1). For amplification of rG cDNA to prepare rGVRt (- SS, + TD), rGVRs (+ SS, - TD) and rGVRst (+ SS, + TD) constructs, the pKB3-JE-13 clone (ATCC) encoding the full length rG from the Challenge Virus Standard (CVS) strain of the rabies virus was used as a template. The DH5α strain of *E. coli* was transformed with pKB3-JE-13 plasmid DNA and one of the positive clones was used to PCR amplify different rG cDNA fragments (for rGVRt, rGVRs and rGVRst constructs) using respective forward and reverse primers as shown in Table 1. All the PCR reactions were carried out with Taq DNA polymerase using the same reaction conditions as

Table 1: Salient features of the four constructs designed to express rG in mammalian cells

Construct number	Construct name	Construct design	Primers used*
1	rGVR	- SS, - TD (58-1371 nt)	5'-CGC**GGATCC**AAATTCCCTATTTACACG-3' 5'- GA**AGATCT**TTACCCCCAGTTCGGGAG-3'
2	rGVRt	- SS, + TD (58-1440 nt)	5'-CGC**GGATCC**AAATTCCCTATTTACACG-3' 5'-GA**AGATCT**TTAACAACATGTCATCAG-3'
3	rGVRs	+ SS, - TD (1-1371 nt)	5'- GA**AGATCT**ATGGTTCCTCAGG-3' 5'- GA**AGATCT**TTACCCCCAGTTCGGGAG-3'
4	rGVRst	+ SS, + TD (1-1440 nt)	5'- GA**AGATCT**ATGGTTCCTCAGG-3' 5'-GA**AGATCT**TTAACAACATGTCATCAG-3'

SS, Signal sequence; TD, Transmembrane-like domain

* Restriction sites are denoted in bold

described for bmZP1 except that for rGVR, rGVRt and rGVRst an annealing temperature of 45°C and for rGVRs an annealing temperature of 50°C was used.

4. Assembly of cDNA encoding dZP3- rG fusion protein by PCR

The general strategy to assemble by PCR the cDNA encoding dZP3-rG fusion protein is schematically shown in Fig. 1. Two rounds of PCR were carried out to assemble the dZP3-rG cDNA. In the first round, cDNA corresponding to dZP3 encompassing part of the N-terminal segment of rG and rG cDNA encompassing part of the C-terminal segment of dZP3 were PCR amplified using pQE30-dZP3 and pQE30-rG plasmids respectively as templates. The cDNA corresponding to dZP3 was PCR amplified using forward primer 5'-GA**AGATCT**CAGACCATCTGGCCAACT-3' having *Bgl* II site and reverse primer 5'-CGTGTAAATAGGGAATTTAGTGTGGGAAACAGACTT-3', containing 12 nucleotides from the N-terminal end of rG cDNA at the 3'end of the primer, using an annealing temperature of 49°C. The cDNA corresponding to rG was PCR amplified using forward primer 5'-AAGTCTGTTTCCCACACTAAATTCCCTATTTACACG-3' containing 12 nucleotides from the C-terminal end of dZP3 cDNA at the 5'end of the primer and reverse primer 5'-GA**AGATCT**TTACCCCCAGTTCGGGAG-3' having *Bgl* II site using an annealing temperature of 45°C. The amplified fragments of dZP3 cDNA containing a part of N-terminal end of rG cDNA at its 3'end and rG cDNA containing a part of C-terminal end of dZP3 cDNA at its 5' end were gel purified and used as templates for the next round of PCR employing forward primer of dZP3 cDNA and reverse primer of rG cDNA to obtain amplified fusion product of dZP3 followed by rG cDNA (dZP3-rG). The templates were denatured at 94°C for 10 min. Initial amplification was carried out for 2 cycles of denaturation at 94°C for 2 min, annealing at 51°C for 2 min and extension at 72°C for 2

min followed by the addition of forward and reverse primers and another round of amplification for 35 cycles involving denaturation at 94°C for 1 min, annealing at 55°C for 2 min and extension at 72°C for 2 min followed by a final extension at 72°C for 15 min. Rest of the PCR conditions were same as described for bmZP1.

5. Agarose gel electrophoresis and ligation of PCR amplified fragments in pPCR-Script Amp SK (+) cloning vector

The PCR products obtained by amplification were resolved on a 0.8% low melting point (LMP) agarose gel using 1X TAE buffer (40 mM Tris, 20 mM acetic acid and 1 mM EDTA) and purified from the gel. The purified PCR products were first blunt-ended at 72°C for 30 min using 0.5 units (U) of cloned Pfu polymerase, 10mM dNTPs, 10X polishing buffer (Stratagene). These PCR products were ligated separately to pPCR-Script Amp SK (+) cloning vector, using vector to insert ratio of 1:20 in a 10 µl reaction volume for 3 h at room temperature (RT). The reaction mixture contained 10 ng of pPCR-Script Amp SK (+) cloning vector, 4 U of T4 DNA ligase, 0.5 µl of 10 mM rATP, 1 µl of 10X reaction buffer, 5 U of *Srf* I restriction enzyme. The buffers and enzymes used were supplied along with the PCR-Script™ Amp cloning kit (Stratagene).

For dZP3-rG fusion, the PCR amplified product was ligated with pGEM-T Easy vector (Promega) without blunting. The reaction mixture contained 50 ng pGEM-T Easy vector, 130 ng of fusion PCR product, 3 U of T4 DNA ligase and 5 µl of 2X Rapid Ligation buffer (30 mM Tris-HCl, pH 7.8, 10 mM $MgCl_2$, 10 mM DTT, 2 mM ATP and 10% polyethylene glycol). The reaction was carried out at 16°C for 16 h.

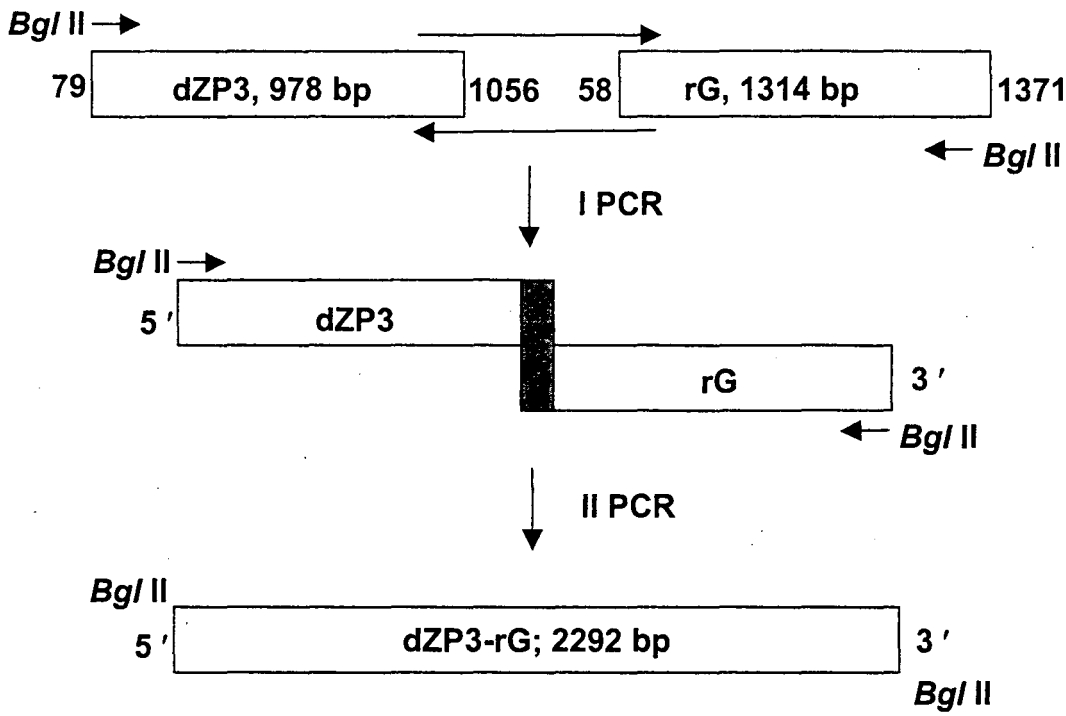

Figure 1: Schematic representation of the assembly of cDNA encoding dZP3-rG fusion protein. Initially, dZP3 cDNA encompassing a part of N-terminal segment of rG cDNA and rG cDNA encompassing a part of C-terminal end of dZP3 cDNA were PCR amplified from pQE30-dZP3 and pQE30-rG plasmid respectively. The cDNA corresponding to dZP3-rG was assembled by second round of PCR using the above PCR-amplified fragments corresponding to dZP3 and rG as templates.

6. Media composition and bacterial culture

The Luria Bertani (LB; pH 7.5) medium was prepared in double distilled water by adding, NaCl 1%, Yeast extract 0.5%, and Tryptone 1% and sterilized by autoclaving under pressure (15 lbs/inch$^{2)}$ for 20 min. Solid growth medium was prepared by adding 1.5% agar to LB prior to autoclaving. Appropriate antibiotics were added after cooling the medium to approximately 50-60°C. Bacterial cultures were grown in LB medium at 37°C in an orbital shaker set at 200 revolutions per minute (rpm).

7. Preparation of competent cells and transformation

The DH5α strain of *E. coli* was grown overnight (O/N) in LB at 37°C and subcultured (1:100) in 100 ml of fresh LB. The culture was grown until absorbance at 600 nm (A_{600}) reached 0.4. The culture was centrifuged at 2500 X g for 15 min at 4°C. The cell pellet was resuspended in 10 ml of freshly prepared sterile ice cold $CaCl_2$ (100 mM) solution and incubated for 30 min on ice. Cells were centrifuged at 1800 X g and the pellet was very gently resuspended in 2 ml of chilled $CaCl_2$ (100 mM) containing 15% glycerol. Aliquots of 100 µl were dispensed into sterile, chilled 1.5 ml eppendorf tubes and stored at -70°C until further use. For transformation, the ligation products from the above reactions were added separately to a vial each of DH5α competent cells thawed on ice. The contents were gently mixed and incubated on ice for 30 min. The cells were then exposed to heat shock at 42°C for 90 sec and incubated on ice for another 2 min. The transformed cells were grown in 1 ml of LB medium for 1h at 37°C with shaking for the expression of the ampicillin resistance marker gene (β-lactamase). Aliquots from each transformation were plated

separately on LB plates containing 100 μg/ml ampicillin, 80 μg/ml of X-gal and 20 mM of IPTG. The plates were incubated at 37°C for 12 h.

8. Small scale plasmid DNA isolation and restriction

Transformants picked following blue-white selection were inoculated in 5 ml LB medium containing 100 μg/ml ampicillin (LB_{amp}) and grown O/N. Following day, 1.5 ml aliquots of O/N culture were harvested by centrifugation at 10,000 X g in a microfuge. The supernatant was discarded and the pellet was resuspended in 100 μl of chilled TEG (25 mM Tris-Cl, pH 8.0, 10 mM EDTA and 50 mM glucose) and incubated for 10 min at RT. After incubation, 200 μl of freshly prepared alkaline-SDS (0.2 N NaOH, 1% SDS; sodium dodecyl sulfate) was added and the contents were mixed gently by inversion. This was followed by incubation on ice for 10 min. Post-incubation, 150 μl of ice-cold sodium acetate solution (3 M, pH 5.2) was added to the mixture and incubated on ice for 15 min. After incubation, the contents were centrifuged at 12,000 X g for 15 min at 4°C and the supernatant was carefully transferred to a fresh tube. DNA was precipitated by adding 0.6 volumes of isopropanol and incubating at RT for 10 min. The DNA pellet was obtained by centrifugation at 12,000 X g at RT for 15 min, air-dried and dissolved in 200 μl of TE. To remove RNA contamination, 50 μg of DNase free RNase was added and incubated for 1 h at 37°C. Plasmid DNA was then extracted once with an equal volume of phenol equilibrated with TE (10 mM Tris, pH 8.0 and 1 mM EDTA) followed by extraction with phenol : chloroform : isoamyl alcohol (25 : 24 : 1) and then with chloroform : isoamyl alcohol (24 : 1). DNA was precipitated by addition of 2 volumes of chilled 100% ethanol to the aqueous phase and incubating the contents at -70°C for 30 min. The DNA pellet was

collected by centrifugation at 12,000 X g for 15 min, and washed with 70% ethanol. The pellet was air-dried and resuspended in 20 µl TE. The clones were checked for the presence of the insert by restriction analysis. The digestion products were checked on 1% agarose gel for the release of the insert. One positive clone was selected from each set of transformations and the plasmid DNA was purified in large amount for the insert preparation.

9. Large scale plasmid DNA isolation

Selected transformants were grown in 250 ml of LB_{amp} O/N. The cells from the O/N cultures were harvested by centrifugation (4°C) at 4000 X g for 30 min. The cell pellet was resuspended in 5 ml of TEG solution containing lysozyme (2.0 mg/ml in 10 mM Tris-HCl, pH 8.0) and incubated at RT for 15 min. Alkaline-SDS (10 ml) was added to the mixture and again incubated at RT for 10 min after mixing the contents gently by inverting the tube. Post-incubation, chilled sodium acetate solution (7.5 ml) was added and the contents were incubated on ice for 15 min. After incubation, the mixture was centrifuged at 10,000 X g at 4°C and processed in the similar fashion as described above upto addition of isopropanol. The DNA pellet was resuspended in 500 µl TE containing 20 µg/ml RNase and incubated for 1 h at 37°C. Plasmid DNA was then extracted as described above. The DNA pellet was air-dried and finally dissolved in 200 µl of TE.

10. Cloning in VR1020 mammalian expression vector

The insert corresponding to bmZP1 was released from the pPCR-Script-bmZP1 clone by *Bgl* II restriction and purified on the agarose gel. VR1020 vector was similarly digested and gel purified. To prevent self-ligation, the digested vector was treated with Shrimp Alkaline Phosphatase (SAP), which removes 5'-phosphate from the termini of double

stranded DNA. The reaction was carried out at 37°C for 1 h. The reaction mixture contained 100 ng *Bgl* II digested VR1020 vector, SAP (0.5 U) and 1 µl 10X SAP buffer (20 mM Tris-HCl, pH 8.0, 10 mM $MgCl_2$) in 10 µl of total reaction volume. The reaction was stopped by inactivating the enzyme at 65°C for 15 min. The digested bmZP1 cDNA was ligated with SAP treated VR1020 at vector : insert ratio of 1:10 in a 10 µl reaction volume for 16 h at 16°C. The reaction mixture contained 10 ng VR1020 vector, 26 ng bmZP1 insert, 1 µl 10X ligase buffer (30 mM Tris-HCl, pH 7.8, 10 mM $MgCl_2$, 10 mM DTT and 1 mM ATP), 1µl T4 DNA ligase (20 U) in a total reaction volume of 10 µl. The ligation product was used for transformation of DH5α competent cells as described previously. Transformants were selected on LB plates containing 50 µg/ml Kanamycin (Kan).

Similarly, the inserts corresponding to dZP3, rG and dZP3-rG fusion were digested with *Bgl* II restriction enzyme, gel purified and cloned in VR1020 vector, except that the ligation product of dZP3-rG fusion with VR1020 was transformed into JM109 competent cells.

II. *IN-VITRO* TRANSFECTION OF MAMMALIAN CELLS WITH VARIOUS PLASMID DNA CONSTRUCTS AND CHARACTERIZATION OF EXPRESSED RECOMBINANT PROTEINS

1. Purification of plasmid DNA in large amount

A single colony of the respective clones was picked up from a freshly streaked LB + Kan (50 µg/ml) plate, inoculated into 5 ml of LB + Kan medium and incubated for 8 h at 37°C with vigorous shaking (~ 250 rpm). Subsequently, 500 µl of this primary culture was inoculated into 500 ml LB+ Kan and grown at 37°C O/N. The culture was centrifuged at

6000 X g for 15 min at 4°C. Plasmid DNA was purified from the pellet using QIAGEN DNA purification kit according to the manufacturer's instructions. The purified plasmid DNA (1.5 - 2.0 mg/ml) was dissolved in autoclaved double distilled water and stored in aliquots (500 µl each) at -20°C until further use.

2. Detection of the expressed recombinant protein following *in vitro* transfection of mammalian cells with the plasmid DNA.

Initial standardization of transfection conditions was done using VRbmZP1 plasmid DNA and COS-1 mammalian cell line. In brief, cells were cultured in T-25 tissue culture flasks in Dulbecco's Modified Eagle's Medium (DMEM) supplemented with 10% fetal calf serum (FCS) at 37°C with 5% CO_2. For subculturing, cells were trypsinized (0.5% trypsin + 0.2% EDTA in DMEM without FCS), centrifuged at 250 X g for 10 min, resuspended in DMEM supplemented with 10% FCS and aliquoted into T-25 flasks. For transfection, cells were seeded on coverslips in a 24-well tissue culture plate at a density of 5×10^4 cells/well, a day prior to transfection. To standardize *in vitro* transfection conditions for optimum expression of bmZP1, varying amount of plasmid DNA was mixed with lipofectamine in DMEM devoid of FCS (final reaction volume 200 µl) and incubated at RT for 45 min. The cells on the coverslips were washed twice with plain DMEM devoid of FCS. DNA-lipofectamine complex was added dropwise to the cells and the plate incubated for 8 h at 37°C in humidified atmosphere of 5% CO_2. Subsequently, 1 ml of DMEM containing 10% FCS was added per well and cells allowed to grow for 48 h. After incubation, cells were processed for visualization of r-bmZP1 by indirect immunofluorescence assay. Cells were washed twice with phosphate buffer saline (PBS; 50 mM Phosphate and 150 mM NaCl, pH 7.4), fixed in chilled methanol (–20°C) for 3 min and blocked with 3% bovine serum

albumin (BSA) in PBS for 2 h at 4°C. For detection of r-bmZP1, a murine monoclonal antibody (MAb), MA-813, generated against *E. coli* expressed r-bmZP1 (Govind et al., 2000), was used as the primary antibody. The cells were incubated with 1:500 dilution of MA-813 ascites fluid for 2 h at 4°C. Cells were washed 5 times with PBS and incubated for 1 h with a 1:800 dilution of goat anti-mouse Ig-fluorescein isothiocyanate (FITC) conjugate (Sigma) at 4°C. After washing with PBS, coverslips with the cells were mounted in glycerol : PBS (9 : 1), and examined under an Optiphot fluorescent microscope (Nikon, Chiyoda-Ku, Tokyo, Japan). For detecting r-dZP3, MAb, MA-451 (1:500 dilution of ascites fluid), generated against porcine ZP3β (a homologue of dZP3) and immunlogically cross-reactive with dZP3 (Santhanam et al., 1998) was used. For detecting r-rG, rabbit polyclonal antibodies (1:1000 dilution) against *E. coli* expressed r-rG, was used as primary antibody. The polyclonal antibody was provided by Dr. Sangeeta Choudhury, Project Associate, Gamete Antigen Laboratory, National Institute of Immunology, New Delhi. Goat anti-mouse immunoglobulins-FITC conjugate (1:800) and goat anti-rabbit immunoglobulins-FITC conjugate (1:2000; Pierce) were used for detecting anti-dZP3 and anti-rG antibodies respectively.

3. Localization of the expressed recombinant protein in COS-1 cells

To investigate if the expressed protein was membrane bound or cytosolic, cells were fixed in 3.7% paraformaldehyde followed by all washings and incubations with primary and secondary antibodies either in presence or absence of 0.1% Saponin and processed for indirect immunofluorescence as described above.

4. Analysis of mammalian cells, transfected *in vitro* with the plasmid DNA, by flow cytometry

COS-1 cells were seeded at a density of 2.5 $\times 10^5$ cells per well in a 6-well tissue culture plate and transfected with plasmid DNA essentially as described above. After 48 h incubation, cells were trypsinized and counted in a hemocytometer. Cells ($\sim 10^6$) were washed twice with PBS and fixed with 0.4% paraformaldehyde in PBS followed by all washings and incubations with respective primary and secondary antibodies in presence of 0.1% Saponin. Antibody concentrations used were same as in indirect immunofluorescence assay. After the final wash, cells were resuspended in PBS and samples were run on an Elite ESP flow cytometer (Coulter Electronics, Hialeh, FL, USA) and data analyzed using WinMDI (version 2.8) software. Cells stained with just secondary antibody were used to account for the background fluorescence. Cells tranfected with VR1020 vector and probed with primary antibody were used as negative control.

5. Analysis of expressed recombinant protein by immunoblot

The cells (2 - 4 x 10^6) transfected with plasmid DNA were resuspended in minimum volume of 2X sample buffer (0.0625 M Tris, pH 6.8, 2% SDS, 10% glycerol, 5% β-mercaptoethanol, and 0.001% bromophenol blue). The samples were boiled for 10 min and resolved on a 0.1% SDS-10% PAGE (Laemmli, 1970). The expression of recombinant proteins was analyzed by Western Blot. The proteins were electrophoretically transferred to 0.45 μm nitrocellulose membrane O/N at a constant current of 30 mA (milliampere) in Tris-Glycine buffer (25 mM of Tris-HCl and 200 mM glycine) containing 20% methanol (Towbin et al., 1979). Post-transfer, the membranes were washed once with PBS and non-specific sites were blocked with 3% BSA in PBS for 90 min at RT. All the subsequent

incubations were carried out for 1 h at RT and each incubation was followed by three washings with PBS containing 0.1% Tween-20 (PBST). Post-blocking, the membranes were incubated with 1:1000 dilution of MA-813 ascites (for detection of r-bmZP1), MA-451 ascites (for detection of r-dZP3) or rabbit polyclonal anti-r-rG antibodies (for detection of r-rG), followed by an incubation with 1:5000 dilution of goat anti-mouse or goat anti-rabbit immunoglobulins conjugated to horseradish peroxidase (HRPO) (Pierce) respectively. The blots were developed with 0.6% (w/v) 4-chloro-1-naphthol in 50 mM PBS containing 25% methanol and 0.06% H_2O_2. The reaction was stopped by extensive washing with double distilled water.

III. PURIFICATION OF r-bmZP1, r-dZP3 AND r-rG EXPRESSED IN *E. coli*

For use in enzyme linked immunosorbant assay (ELISA), r-bmZP1, r-dZP3 and r-rG, expressed in *E. coli* were purified under denaturing conditions. For purification of r-bmZP1, as described previously, the pRSET-bmZP1 clone, encoding bmZP1, excluding the SS and the TD (22-462 aa) was used (Govind et al., 2001). Similarly, the pQE30-dZP3 and pQE30-rG clones encoding dZP3 and rG, excluding the SS and the TD were used for purification of r-dZP3 (26-352 aa) (Santhanam et al., 1998) and r-rG (20-457 aa) (unpublished observations) respectively. For T cell proliferation assays, the above recombinant proteins were purified from inclusion bodies in the absence of chaotropic agents in refolded form as described below.

The above clones were inoculated in 10 ml of LB containing appropriate antibiotics and grown O/N at 37°C. Following day, the cells were subcultured (1:100 dilution) in 1 liter of LB (250 ml/flask) containing appropriate antibiotics and grown at 37°C until the A_{600}

reached a value of 0.5-0.6. The cultures were then induced with 1 mM IPTG for 2 h at 37°C. Cells were pelleted at 4000 X g for 30 min at 4°C and stored at -70°C until used.

1. Purification in denatured form

The proteins were purified by nickel affinity chromatography. The cell pellet (~1g) of each clone 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 8000 X g for 15 min at 4°C and the supernatant containing the recombinant protein was mixed with Ni-NTA resin (Nickel-Nitrilotriacetic acid equilibrated with buffer A) and kept for gentle end-to-end shaking for 1 h at RT. The resin was loaded on a column and washed with 10 bed-volumes of buffer A. The column was subsequently washed with 5 bed-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 8.0 and 6.3 respectively. The protein was eluted with buffers D and E (composition same as buffer B) in which the pH was further reduced to 5.9 and 4.5 respectively. Five fractions of 4 ml each were collected during elution with buffer D and buffer E respectively. The eluted proteins were analysed by 0.1% SDS-10% PAGE (gels stained with Coomassie blue) and Western blot. The fractions showing the purified recombinant protein were pooled and concentrated in an Amicon concentrator using a YM30 membrane and dialyzed against 100 mM phosphate buffer, pH 7.4, containing 4 M urea. The concentration of each purified protein was estimated by bicinchoninic acid (BCA).

2. Purification in refolded form

a) Purification of inclusion bodies

For the purification of inclusion bodies, the bacterial cell pellet from 1 liter culture was resuspended in 10 ml of Tris-HCl buffer (50 mM; pH 8.5) containing 5 mM EDTA and sonicated using Branson sonifier-450 for 8 cycles of 90 sec each (30 watt output; Branson Ultrasonic Corp., Danbury, CT, USA) on ice. The inclusion bodies were collected by centrifugation of the sonicate at 8000 X g for 30 min at 4°C. The pellet was washed twice with 15 ml of 50 mM Tris-HCl buffer with 5 mM EDTA containing 2% sodium deoxycholate in order to remove loosely bound *E. coli* proteins from the inclusion bodies. Subsequently, the inclusion body pellet was washed with 50 mM Tris-HCl buffer (pH 8.5), followed by a washing with the double distilled water. All the buffers used for the purification contained 20 mM of phenylmethyl sulphonyl fluoride (PMSF).

b) Solubilization and renaturation

The purified inclusion bodies were solubilized in 100 mM Tris-HCl (pH 12.0) containing 2 M urea at RT for 30 min, and centrifuged at 8000 X g for 30 min at 4°C. The pH of the supernatant was brought down immediately to 8.5 with 1 N HCl and then extensively dialyzed against renaturation buffer (50 mM Tris-HCl buffer; pH 8.5, 1 mM EDTA, 0.1 mM reduced glutathione, 0.01 mM oxidized glutathione and 10% sucrose). The protein was finally dialyzed against 20 mM Tris-HCl, pH 8.5 and its concentration estimated using BCA.

IV. *IN-VIVO* IMMUNIZATION STUDIES

These experiments were carried out with the approval of Institutional Animal Ethics Committee.

Three different modes of administration were used:

1. Plasmid DNA administered in saline

Inbred male BALB/cJ mice (6-8 week, Small Experimental Animal Facility, National Institute of Immunology, New Delhi, India) were immunized intramuscularly (i.m.) with 100 μg of respective plasmid DNA or VR1020 vector in 100 μl saline (0.9% NaCl) in the anterior tibialis muscle in the hind limbs (each receiving 50 μl). Two booster injections of 100 μg DNA in saline were given on day 21 and 35. On day 45, mice in each group received i.m. injection of *E. coli* expressed recombinant protein (20 μg/mouse in saline). Mice were anesthetized and bled retro-orbitally on days 0, 45 and 52 for analysis of respective antibody responses.

2. Plasmid DNA administered by electroporation

A day prior to immunization, hair were removed from both the hind limbs of the mice using a commercial depilatory agent (Anne French cream, Geoffrey Manners & Co. Ltd, Mumbai, India). Mice were immunized in a similar way as in the saline group but in addition, ten very short electric pulses were given at the site of injection immediately after DNA administration using a gas igniter (Upadhyay, 2001). Voltage delivered in each trigger was 18kV for 10^{-7}s.

3. Plasmid DNA adsorbed onto gold microcarriers

Suspension of DNA adsorbed onto gold microcarriers at 0.5 Microcarrier Loading Quantity (MLQ; 50 μg DNA/25 mg gold microcarriers) was prepared and coated inside Tefzel

tubing, which was cut into 0.5 inch pieces (cartridges). These cartridges were used to deliver DNA into epidermis of male/female mice.

a) Preparation of DNA-gold microcarrier suspension

Twenty five mg of gold microcarriers were weighed in a 1.5 ml eppendorf tube to which 100 µl of 0.05 M spermidine was added and vortexed for 10 sec. To the above mixture 100 µl of DNA (0.5 mg/ml) was added and vortexed for another 10 sec. While vortexing, 100 µl of 1 M $CaCl_2$ was added dropwise to the mixture and left at RT for 10 min to allow precipitation of DNA onto gold microcarriers. The DNA-gold pellet was collected by centrifuging at 12,000 X g for 1 min at RT. The pellet was washed thrice with 100% ethanol (freshly opened bottle), resuspended in 3 ml of 0.1mg/ml polyvinylpyrollidone (PVP) in ethanol and stored at -20°C till further use.

b) Loading the DNA/microcarrier suspension into gold-coat tubing using the tubing prep station

A 25 inch length of tubing was cut and fixed on tubing prep station, air dried by passing nitrogen gas through it for 15 min. The DNA/microcarrier suspension was vortexed and injected into the tubing using a 5 ml syringe and the microcarriers allowed to settle in the tubing for 3 min. Ethanol from the tubing was removed by slowly sucking into the syringe. The tubing was rotated, while passing the nitrogen gas, using the tubing prep station, for 20-30 sec to allow the microcarriers to evenly coat the inside of the tubing.

c) Preparation of cartridges using the tubing cutter

The tubing was cut into 0.5 inch long pieces (cartridges) by using the tubing cutter and cartridges stored at 4°C in vials containing desiccant pellets till further use.

d) Particle delivery using the Helios gene gun

A day prior to immunization, hair were removed from the abdominal region of mice using a commercial depilatory agent (Anne French cream). Two cartridges/mouse (~ 2 μg DNA) were shot under pressurized helium gas (400 psi) intradermally at the shaven area of the abdomen of mice using the Helios gene gun. Two boosters comprising of two cartridges each were given on days 21 and 35. On day 45, mice in each group received i.m. injection of *E. coli* expressed recombinant protein (20 μg/mouse in saline). Mice were bled retro-orbitally on days 0, 45 and 52 for analysis of antibody response.

V. CHARACTERIZATION OF THE ANTIBODIES GENERATED IN RESPONSE TO PLASMID DNA IMMUNIZATION

1. Enzyme Linked Immunosorbant Assay (ELISA)

Microtitration plates were coated with optimized concentration of r-bmZP1 (250 ng/well), r-dZP3 (400 ng/well) or r-rG (500 ng/well) in 50 mM PBS, pH 7.4, at 37°C for 1 h and then at 4°C, O/N. The plates were washed once with PBS and incubated with 1% BSA, (200 μl/ well) in PBS for 2 h at 37°C for blocking the non-specific sites. All subsequent incubations were carried out for 1 h at 37°C and each incubation was followed by three washings with PBS containing 0.05% Tween-20 (PBST). Post-blocking, the plates were incubated with 1:50 dilution of either the preimmune or the immune serum samples obtained from mice immunized with the respective plasmid DNA. Antibodies bound to r-bmZP1, r-dZP3 and r-rG were revealed with 1:2000 dilution of goat anti-mouse IgG (whole molecule) HRPO (Dako). Estimation of the enzymatic activity was carried out with 0.05% OPD in 50 mM citrate phosphate buffer, pH 5.0, containing 0.06% H_2O_2 as the substrate. The reaction was stopped with 50 μl of 5 N H_2SO_4 and the absorbance read at

492 nm with 620 nm as the reference filter. The antibody response generated was represented as the geometric mean of the absorbance of individual mice sera in a group of immunized animals.

In addition, the antibody titer against r-dZP3 was also determined by ELISA. The assay was carried out as described above except that 100 µl of doubling dilutions of the serum samples (dilutions made in PBST supplemented with 0.1% BSA) were added per well in duplicate. For each serum sample tested, a reciprocal of dilution giving an absorbance of 1.0 was calculated by regression analysis and represented as antibody units (AU).

2. Antibody isotyping

The antibody isotypes in the immune sera were determined, by indirect ELISA, using mouse MAb isotyping reagents (Sigma). The microtitration plates coated with r-dZP3 (400 ng/well) and blocked with 1% BSA, were incubated with doubling dilution of pooled serum samples of a group of immunized animals. All the incubations were carried out at 37°C and were followed by three washings with PBST. The incubation was followed by addition of goat anti-mouse isotype specific antibodies at 1:1000 dilution. The binding was revealed by rabbit anti-goat IgG-HRPO conjugate (Pierce) at an optimized dilution of 1:10,000 and processed for enzymatic activity estimation as described earlier.

VI. REACTIVITY WITH NATIVE ZP OF THE IMMUNE SERUM SAMPLES OBTAINED FROM MICE IMMUNIZED WITH VRbmZP1 AND VRdZP3 PLASMID DNA

Ability of mouse polyclonal antibodies, generated subsequent to immunization with VRbmZP1 and VRdZP3 plasmid DNA, to recognize native ZP was evaluated by indirect immunofluorescence assay. A normal cycling female bonnet monkey and a female dog were ovariectomized after administration of ketamine hydrochloride (5 mg/kg body

weight) as a general anesthetic and ovaries were snap frozen in liquid nitrogen. Ovarian sections of 5 μm thickness were cut in a cryostat at -20°C and fixed in chilled methanol for 15 min at RT. Sections passing through follicles were selected, washed with 50 mM PBS and blocked with 3% normal goat serum (NGS) in PBS (v/v) at RT for 1 h. Sections were then washed two times with PBS and incubated with 1:10 dilution of immune serum samples. Ovarian sections incubated with 1:10 dilution of mouse preimmune or immune sera from mice immunized with VR1020 vector served as negative controls. After incubation, the sections were washed three times with PBS and incubated with 1:800 dilution of goat anti-mouse IgG conjugated to FITC (Sigma) for 1 h at RT. The slides were washed three times with PBS, mounted in glycerol : PBS (9 : 1), and examined under fluorescence microscope (Optiphot, Nikon, Japan).

VII. T CELL PROLIFERATION ASSAY

Single cell suspensions of splenocytes in RPMI-1640 medium were prepared from plasmid DNA immunized mice, on day 45, by mechanical disruption of the spleen. Red blood cells were lysed by exposing the cell pellet to 10X concentration of 50 mM PBS and immediately bringing the concentration to 1X PBS by addition of water. Cells were diluted to a final concentration of 3×10^6 cells/ml in RPMI-1640 medium supplemented with 10% FCS. A 100 μl aliquot of splenocytes was added to each well of a 96-well microtitration plate containing serial dilutions of refolded recombinant proteins (r-bmZP1, r-dZP3 or r-rG), diluted in the same medium, as a source of antigen. All assays were carried out in triplicates. Three days after the addition of the cells, culture were pulsed with 1 μCi/well of [^{3}H] thymidine (NEN, Life Science Products, Boston, MA) for 16 h. Cells were lysed and harvested onto glass fibre filaments for liquid scintillation counting (Betaplate; Wallac,

Turku, Finland). Data were expressed as mean counts per minute (cpm) ± SE of triplicate cultures.

VIII. RAPID FLUORESCENT FOCUS INHIBITION TEST (RFFIT)

RFFIT is used for determination of rabies virus neutralizing antibody (RVNA) titers. It is an *in vitro* cell culture based technique in which foci of virus-infected cells are observed by fluorescent antibody staining.

In brief, mouse neuroblastoma (MNA) cells were cultured in T-25 tissue culture flasks in DMEM supplemented with 10% FCS at 35°C in humidified atmosphere of 0.5% CO_2. For subculturing, cells were trypsinized (0.5% trypsin + 0.2% EDTA in DMEM without FCS), centrifuged at 150 X g for 10 min, resuspended in DMEM supplemented with 10% FCS and aliquoted into T-25 flasks. Sera from immunized mice were heat inactivated at 56°C for 30 min and the RVNA titers were determined by RFFIT as described previously (Smith et al., 1996). Briefly, 100 µl of various dilutions of the reference (Standard Rabies Immune Globulin, Biological Research and Reviews, FDA, Maryland, US) and the test sera were mixed with 100 µl Challenge Virus Strain-11 of rabies virus (containing 50 FFD_{50}) in 8-well tissue culture chamber slides and incubated at 35°C in presence of 0.5% CO_2 for 90 min. After the incubation period, 0.2 ml of MNA cells (1×10^5) were added to each well and the slides incubated for 40 h following which these were fixed in chilled acetone and stained with FITC conjugated anti-rabies MAb (Centocor Inc, USA) for 45 min. The slides were washed three times with PBS, mounted in glycerol : PBS (9 : 1), and examined under fluorescence microscope (Optiphot, Nikon, Japan). Data was expressed as neutralizing antibody titer that is the reciprocal of the serum dilution resulting in a 50% reduction in the number of the virus infected cells in the presence of the test serum.