Results
CLONING AND SEQUENCING OF bZP3

1.1 PCR Amplification and Cloning of bZP3 in pBluescriptII SK(+)

Attempts to amplify the full length bZP3 by PCR using the forward primer H88, and reverse primer H87 were not successful. Subsequently, an alternate strategy (Fig. 1) was devised to amplify the bZP3 cDNA in 2 fragments, having an overlap of 164 bp, corresponding to exons 1-6 (852 bp) and 4-8 (584 bp) of the human homologue of bZP3. In a second PCR reaction, the 2 amplified fragments were used for assembly and amplification of the full length bZP3 cDNA. The amplified full length gene was digested with Kpn I and Sac I, restriction sites incorporated in the forward and reverse primers, to generate a fragment of 1295 bp corresponding to the full length bZP3 ORF (1272 bp). Fig. 2 represents an agarose gel of the amplified fragments of ~0.9, ~0.6 and ~1.3 kb corresponding to exons 1-6, 4-8 and 1-8. The amplified full length cDNA was cloned in the pBluescriptII SK(+) vector at the same sites, generating the pBluescript-bZP3 plasmid represented schematically in Fig. 3A. Three clones i.e. 401, 403 and 404 were verified by small scale DNA isolation and restriction digestion with Kpn I and Sac I for the presence of the full length bZP3 cDNA insert. Digestion of plasmid DNA prepared from these clones resulted in fragments of ~1.3 kb corresponding to the bZP3 insert and ~2.9 kb corresponding to the vector (Fig. 3B). After confirmation of the digestion pattern, all the three clones were completely sequenced to determine the nt sequence of bZP3.

1.2 Sequencing of the bZP3 cDNA and Analysis of the Sequence

The complete nt and the deduced aa sequence of bZP3 is presented in Fig. 4. bZP3 has a single ORF of 1272 nt, resulting in a polypeptide core of 424 aa with a calculated molecular mass of 47037 Da. It does not have a 3' untranslated region, since the translational stop codon TAA coincides with the RNA polyadenylation signal. According to the method of Klein et al., (1985), the protein is classified as an integral protein. Using von Heijne (1986) scoring method, bZP3 has a potential peptidase site after the twenty second aa resulting in an N-terminal Glu. The method of Rao and
Fig. 1: Schematic representation of the PCR amplification of bZP3. The bZP3 cDNA was amplified by PCR in 2 overlapping fragments corresponding to exons 1-6 using forward primer H88 and reverse primer H79 and exons 4-8 using forward primer H4 and reverse primer H87. In a second PCR reaction, the full length gene (exons 1-8) was assembled using the 1-6 and 4-8 fragments, forward primer H88 and reverse primer H87. The conditions of PCR were as described in the Materials and Methods.
Fig. 2: Agarose gel electrophoresis of PCR amplified bZP3. Amplified fragments corresponding to exons 1-6, 4-8 and 1-8 were resolved on a 0.8% agarose gel. Lanes are 1, pGEM markers (kb); 2, bZP3 exons 1-6 (~0.9 kb); 3, bZP3 exons 4-8 (~0.6 kb); and 4, the full length bZP3 exons 1-8 (~1.3 kb).
Fig. 3: Cloning of bZP3 in pBluescriptII SK(+). The PCR amplified bZP3 was digested with Kpn I and Sac I and cloned in a similarly restricted pBluescriptII SK(+) vector to generate the pBluescript-bZP3 plasmid carrying the full length bZP3 cDNA insert at the Kpn I and Sac I sites. Panel A represents a schematic representation of the pBluescript-bZP3 plasmid. Panel B represents a 0.8% agarose gel showing the restriction pattern of the bZP3 clones 401, 403 and 404. Lanes are represented as 1, φX174 markers (kb); lane 2, λ(Hind III) markers (kb); lanes 3, 5, and 7 represent undigested 401, 403 and 404 plasmids respectively and lanes 4, 6 and 8 represent, 401, 403 and 404 plasmids restricted with Kpn I and Sac I.
| ATG GAG CTC AGC TAT AGG CTC TTT CAG GGT GGA GCC |
|----------------|----------------|
| TGCAGGTACC     | TGCAGGTACC     |
| 49              | 182             |

Fig. 4: Nucleotide and deduced aa sequences of the bZP3 cDNA. The nt sequence of the bZP3 clones 401, 403 and 404 was determined as described in the Materials and Methods. The nt sequence of clone 401 is presented. Clones 403 and 404 had an identical sequence as compared to clone 401. Primers used for amplification of the cDNA have been indicated by underlining and restriction sites are shown in bold. A double underline at the N-terminal end represents the signal sequence. The furin cleavage site RNR is shown in bold and double underline. The termination codon (TAA) is indicated by an asterisk. The polyadenylation site AATAAA shown in bold with a line on top overlaps with the stop codon.
Argos (1986) predicts 2 transmembrane domains from aa 2-17, corresponding to the signal sequence and aa 385-410, corresponding to the transmembrane domain. A tetra basic furin cleavage site (RNRR) is present in the bZP3 sequence (aa 349 to 352) upstream of the hydrophobic transmembrane domain (Hosaka et al., 1991).

Analysis of the Hopp-Woods (1981) and the Kyte-Doolittle (1982) hydrophobicity plots of bonnet and hZP3 proteins revealed a close overlap in the hydrophobicity patterns between the 2 species. Two major hydrophobic domains corresponding to the N-terminal signal sequence and the transmembrane domain were present beside minor internal hydrophobic stretches (Fig. 5).

1.3 Sequence Identity of bZP3 with ZP3 from Other Species

An alignment of the primary aa sequence of bZP3 with ZP3 of human, marmoset, mouse, hamster, rabbit and pig is presented in Fig. 6. bZP3 possesses four potential N-linked glycosylation sites (N-X-S) which are conserved in human and marZP3. However, marZP3 possess one additional site for N-linked glycosylation. Rabbit and pig each have 5 potential sites for N-linked glycosylation, while hamster and mZP3 have 4 and 6 N-linked glycosylation sites respectively, of which 2 positions are conserved among all the above species. In contrast to human and marmoset, bZP3 has 14 instead of 15 Cys residues which are conserved in these three species. However, at position 413 instead of Cys, bZP3 has Trp. There are 31 Thr residues both in bonnet and hZP3 out of which 30 are conserved. bZP3 differs from human by having Thr at position 54 (instead of Met in human) and Leu at position 284 instead of Thr in human. bZP3 has 38 Ser residues as compared to 35 in human out of which 34 are conserved. The cynomolgous monkey partial ZP3 sequence (aa 202-424) has also been reported in an international patent and reveals a difference in 2 aa (383 and 422), both of which occur beyond the furin cleavage site. At the nt level the changes are from G in bZP3 to A (nt 1147) and C in bZP3 to T (nt 1265) in cynomolgous monkey, both of which are transition type of mutations. Ala$_{383}$ and Ala$_{422}$ in bZP3 has been
**Fig. 5: Hydrophobicity plot of bZP3.** The hydrophobicity plots of bZP3 and hZP3 were drawn using the Lasergene DNA and Protein analysis software, using a group length of 7 aa residues and is presented in two formats, the Hopp-Woods and the Kyte-Doolittle plots. Major hydrophobic domains in the protein are present at the N-terminus corresponding to the signal sequence and at the C-terminus corresponding to the transmembrane domain.
Fig. 6: Comparison of the primary aa sequence of bZP3 with six other species. The deduced aa sequence of bZP3 was aligned with the ZP3 sequence from other species such as human, marmoset, mouse, hamster, rabbit and pig using the Cluster V Multiple Alignment Programme. Asterisks indicate completely conserved aa and potential N-linked glycosylation sites (N-X-S/T) are underlined. Cys residues in the bZP3 sequence are indicated by a line on top and the furin cleavage site (RNRR) is indicated by a double underline.
Results

changed to Thr383 and Val422 respectively in cynomolgous monkey (Harris et al., 1994b).

The bZP3 was found to exhibit the highest degree of identity (93.9%) with hZP3. It shows a 88.9% identity with marmoset, 71% with pig, 66.5% with both mouse and hamster, and 66.4% with rabbit ZP3. Comparison of the reported ZP3 sequences had revealed most of the heterogeneity to be localized to specific regions within the protein which include, i) the region corresponding to the N-terminus of the protein, including the signal sequence, ii) the region after the furin cleavage site, including the transmembrane domain, and iii) the C-terminal region (aa 318-348) of the mature protein. Comparison of the bZP3 sequence in these domains revealed that disparity of the N-terminal part of the protein was not restricted to the region that would be processed by the signal peptidase, but extending to approximately the first 50 aa residues. The region upstream to the signal peptidase processing site was conserved in the bonnet, human and marmoset sequences. At the C-terminus, the heterogeneity was also localized mostly to the region beyond the furin cleavage site (aa 353-424) where in bonnet Vs human identity falls to 83.3% from an overall identity of 93.9%. A similar pattern is observed with the marmoset, mouse, hamster, rabbit and pig sequences. In the region aa 318-348, immediately prior to the furin cleavage site, human and bZP3 sequences are relatively well conserved, sharing an identity of 93.5%, not significantly lower than the overall identity of 93.9%, while with the marmoset, mouse, hamster, rabbit and pig sequences, this region is poorly conserved (Table 1). This region is of interest as it has also been implicated as a possible sperm receptor site in the murine model (Millar et al., 1989; Rosiere and Wassarman, 1992).

2 EXPRESSION OF bZP3 IN E. coli

2.1 Cloning of bZP3 for Expression in E. coli

Attempts were made to express (i) the His6-bZP3 protein in the pRSET B vector, under the phage T7 promoter, in BL21(DE3) cells and (ii) under the T5 phage
Table 1:  Sequence identity of different regions of the primary amino acid sequence of bZP3 with the human, marmoset, mouse, hamster, rabbit and pig ZP3 homologues.

<table>
<thead>
<tr>
<th>SEQUENCE IDENTITY (% OF bZP3 WITH THE ZP3 OF</th>
<th>HUMAN</th>
<th>MARMOSET</th>
<th>MOUSE</th>
<th>HAMSTER</th>
<th>RABBIT</th>
<th>PIG</th>
</tr>
</thead>
<tbody>
<tr>
<td>1-424</td>
<td>93.9</td>
<td>88.9</td>
<td>66.5</td>
<td>66.5</td>
<td>66.4</td>
<td>71.0</td>
</tr>
<tr>
<td>1-22#</td>
<td>100</td>
<td>100</td>
<td>60.9</td>
<td>50.0</td>
<td>72.2</td>
<td>63.6</td>
</tr>
<tr>
<td>1-50</td>
<td>92.0</td>
<td>82.0</td>
<td>54.0</td>
<td>48.0</td>
<td>60.9</td>
<td>52.0</td>
</tr>
<tr>
<td>318-348</td>
<td>93.5</td>
<td>80.6</td>
<td>48.4</td>
<td>64.5</td>
<td>35.5</td>
<td>51.6</td>
</tr>
<tr>
<td>353-424*</td>
<td>83.3</td>
<td>83.3</td>
<td>50.0</td>
<td>51.4</td>
<td>41.7</td>
<td>43.1</td>
</tr>
</tbody>
</table>

# Corresponds to the bZP3 signal sequence
* Corresponds to the region of the bZP3 protein after the furin cleavage site
promoter, in the pQE30 vector for expression in SG13009[pREP4] and M15[pREP4] cell strains. Though transformants positive for the bZP3 insert in the right reading frame were recovered, no expression could be detected by SDS-PAGE or immunoblots using MA-451 (raised against pZP3β and recognizing a cross reactive epitope between pZP3β and bZP3). Subsequently, the bZP3 expression construct was redesigned to exclude the major hydrophobic domains corresponding to the signal sequence and the putative transmembrane-like domain (represented schematically in Fig. 7). The truncated bZP3 (bZP3 aa 23-348) was amplified by PCR (Lane 3, Fig. 8), digested with BamH I and Sac I and cloned in frame in a similarly restricted pQE30 vector (lane 5, Fig. 8) with an N-terminal His\textsubscript{6} tag. The design of the pQE-bZP3 plasmid is shown in Fig. 7. The ligation mix was used for transformation of DH5α and transformants were screened by small scale DNA isolation and restriction digestion. A digestion pattern of a representative clone (clone 15), with BamH I and Sac I, is shown in Fig. 8, lane 7.

2.2 Expression of bZP3

M15[pREP4] and SG13009[pREP4] bacterial strains provided with the QIAexpress\textsuperscript{TM} expression kit were transformed with pQE-bZP3 plasmid (clone 15) and studied for the expression of r-bZP3 after induction with 1 mM IPTG for 1 h. The typical results obtained by SDS-PAGE and immunoblot, from one of the transformed clones (clone 15) in M15[pREP4] and SG13009[pREP4] are shown in Fig. 9. MA-451, a murine MAb generated against the pZP3β and recognizing a cross reactive epitope within the bZP3 sequence (EEKLVF in the porcine sequence/EEKLTF in the bonnet sequence), was used for the detection of the r-bZP3. Besides the full length protein, smaller fragments were also detected in the immunoblot, which could be a result of degradation or premature termination during translation of the r-bZP3 protein. The full length fusion protein had an apparent molecular weight of \(~50\) kDa using the Gibco-BRL prestained molecular weight markers. The level of expression of the fusion protein in M15[pREP4] and SG13009[pREP4] strains was comparable and for subsequent experiments the transformed SG13009[pREP4] cells were used.
Fig. 7: Schematic representation of the cloning strategy used for expression of bZP3 in E. coli. A truncated version of the bZP3 gene without the major hydrophobic domains, i.e. the signal sequence and the transmembrane domain was PCR amplified and cloned as a His6-bZP3 fusion protein in the pQE30 vector to express the r-bZP3 protein. The BamH I and Sac I digested bZP3 fragment (corresponding to bZP3 nt 67-1044), amplified from the full length bZP3 cDNA clone in pBluescriptII SK(+) was cloned in frame downstream of a His6 tag in pQE30 vector. T5, promoter of phage T5; AmpR, Amp resistance marker; ORI, origin of replication.
Fig. 8: Restriction analysis of the pQE-bZP3 plasmid. Positive clones harboring the pQE-bZP3 plasmid were identified by restriction analysis of DNA isolated from the transformed colonies. The restriction pattern of one of the positive clones (clone 15) is shown along with the pQE30 vector and the purified PCR amplified fragment (nt 67-1044 corresponding to the bZP3 sequence) that was used for cloning. The figure represents a 0.8% agarose gel showing, lane 1, λ (Hind III) markers (kb); lane 2, φX174 markers (kb); lane 3, the PCR amplified truncated bZP3 fragment; lanes 4 and 5, undigested and digested pQE30 vector respectively; lanes 6 and 7, undigested and digested pQE-bZP3 (clone 15) respectively.
Fig. 9: Expression of r-bZP3 in M15[pREP4] and SG13009[pREP4] E. coli strains. M15[pREP4] and SG13009[pREP4] cells, transformed with the pQE-bZP3 plasmid were grown till A600=0.7. Uninduced cells and cells induced for 1 h with 1 mM IPTG were lysed by boiling for 5 min in 2X reducing buffer and resolved by 0.1% SDS-10% PAGE as described in the Materials and Methods. Panel A represents the Coomassie stained gel and panel B an immunoblot of the same probed with MA-451. Lanes are represented as M, molecular weight markers (kDa); 1 and 2, Uninduced and induced M15[pREP4] cells; 3 and 4, uninduced and induced SG13009[pREP4] cells.
Expression of r-bZP3 was not observed in the absence of IPTG when cells were induced for 1 h (Fig. 9). However, when cells were induced for a longer period (3 h) to increase expression levels, a low level of r-bZP3 was detected in the uninduced controls, indicating that expression was regulated to a large extent, though some level of basal leaky expression existed even in the absence of induction. Localization of the r-bZP3 expressed in E. coli cells (Fig. 10) revealed that the fusion protein was present in the insoluble intracellular fraction (lane 4) and neither in the soluble (lane 3), nor the periplasmic compartments (lane 5).

2.3 Optimization of Expression Conditions
Optimum expression of r-bZP3 was obtained at 0.5 mM IPTG (Fig. 11). Higher IPTG levels seemed to inhibit growth without any further increase in expression levels of r-bZP3. Hence, for further experiments and the scale up of expression for purification of the protein, 0.5 mM IPTG was used for induction. The time kinetics of expression revealed that the quantity of r-bZP3 increased with time, as the total cellular proteins increased, in samples collected from 0-5 h (Fig. 11).

2.4 Expression of r-bZP3 in Protease Deficient E. coli Strains
r-bZP3 was expressed in E. coli strains deficient in proteases, to check if the presence of the lower weight fragments could be attributed to degradation by a specific protease. BL21(DE3) and BL21(pLys S) cells deficient in the omp T and lon proteases and DF5 cells carrying a mutation in the ptr gene were transformed with the pQE-bZP3 plasmid. Transformants were grown and induced as described. Unlike the SG13009[pREP4] and M15[pREP4] strains, in these strains the multiple lower weight fragments were not observed, except in BL21(pLysS), where a single lower molecular weight band was observed (Fig. 12). However, mutants for different proteases (omp T and lon or ptr gene) seemed to show the same effect and hence it was not possible to attribute the degradation to a specific protease. The expression of r-bZP3 in these strains was leaky, and high levels of protein could be observed even without induction (Fig. 12). With the Bio-Rad prestained molecular weight marker, the r-bZP3 showed an apparent
**Fig. 10: Cellular localization of r-bZP3.** SG13009[pREP4] cells transformed with the pQE-bZP3 plasmid were induced with IPTG for 3 h and fractionated into the soluble, insoluble and the periplasmic compartments as described in the Materials and Methods. Expression of r-bZP3 was checked by SDS-PAGE (panel A) and immunoblot (panel B). Lanes are M, molecular weight markers, Gibco-BRL (kDa); lanes 1 and 2 uninduced and induced total cell pellets; lanes 3, 4 and 5 represent the soluble, insoluble and the periplasmic cell compartments respectively.
Fig. 11: Standardization of the expression conditions of r-bZP3. SG13009[pREP4] cells transformed with the pQE-bZP3 plasmid were grown till $A_{600}=0.7$ and induced with different concentrations of IPTG (lanes 1-4) for 3 h or in the presence of 0.5 mM IPTG for varying times (lanes 6-9). Cells were lysed by boiling for 5 min in 2X reducing buffer and electrophoresed as described in the Materials and Methods. Panel A represents Coomassie stained gel and panel B an immunoblot of the same probed with MA-451. Lanes are represented as M, molecular weight markers (kDa); lanes 1, 2, 3 and 4 correspond to cells induced with IPTG at concentrations of 4, 2, 1 and 0.5 mM respectively. Lane 5 represents the uninduced control (0 h) and lanes 6, 7, 8 and 9 correspond to cells induced with 0.5 mM IPTG for 1, 2, 3 and 5 h respectively.
Fig. 12: Expression of r-bZP3 in protease mutant E. coli strains. BL21(pLysS) and BL21(DE3) cells deficient in the omp T and lon proteases and DF5 cells carrying a mutation in the ptr gene were transformed with the pQE-bZP3 plasmid and induced with IPTG for 3 h. Cell lysates were resolved by 0.1% SDS-10% PAGE and analyzed by immunoblotting. Lanes are represented as M, molecular weight markers, Bio-Rad (kDa); 1, untransformed E. coli cells, lanes 2 and 3, uninduced and induced SG13009[pREP4] cells; lanes 4 and 5, uninduced and induced BL21(pLysS) cells; lanes 6 and 7, uninduced and induced BL21(DE3) cells and lanes 8 and 9, uninduced and induced DF5 cells.
molecular weight of ~45 kDa which was closer to the calculated molecular weight of 39 kDa.

2.5 Reactivity of r-bZP3 with Abs Against N- and C-Terminal Peptides

To verify the authenticity of the r-protein, Abs were generated in female bonnet monkeys against peptides corresponding to the bZP3 sequence. Animals were immunized with a synthetic bZP3 peptide from the N-terminus (aa 23-45 corresponding to bZP3 sequence, aa numbers corresponding to the precursor bZP3 protein) and two peptides from the C-terminus (300-322 and 324-347 aa residues), conjugated to DT. Abs thus generated against all the three bZP3 peptides not only recognized the respective peptides but also r-bZP3 protein in ELISA (Table 2). The Abs against the two C-terminal peptides recognized r-bZP3 at a higher dilution as compared to the Ab against N-terminus peptide.

3 EXPRESSION OF bZP3 IN INSECT CELLS

3.1 Cloning of bZP3 for Expression in Insect Cells

Analysis of the Hopp-Woods hydrophobicity plot for bZP3 had revealed the presence of 2 major hydrophobic domains corresponding to the signal sequence and the transmembrane domain. Besides these major domains there were several minor hydrophobic regions within the bZP3 sequence (Fig. 13). The full length bZP3 was cloned in the pBacPAK8 vector for expression in BEVS (V1). In order to mimic the natural processing that occurs and to evaluate the role of the transmembrane domain in expression and secretion a deletion construct, V2, was designed excluding this region (Fig. 13A). The same construct would also help in evaluating whether the heterologous mammalian signal sequence would direct secretion in insect cells. Insect secretory sequences have been reported to enhance expression of heterologous proteins in insect cells and hence two more constructs were designed expressing a short polypeptide from bZP3 aa 23-76 (V3) and also aa 23-348 (V4) as GST-bZP3 fusion proteins with a replacement of the native signal sequence with an insect gp67 signal sequence (Fig. 13).
Table 2: Reactivity in ELISA of Abs generated in female bonnet monkeys against synthetic peptides corresponding to bZP3 deduced aa sequence with the respective peptides and r-bZP3.

<table>
<thead>
<tr>
<th>PEPTIDE</th>
<th>DILUTION OF IMMUNE SERA</th>
<th>A490 WITHb PEPTIDE</th>
<th>bZP3</th>
</tr>
</thead>
<tbody>
<tr>
<td>P1</td>
<td>1:100</td>
<td>2.73</td>
<td>1.63</td>
</tr>
<tr>
<td>KQPFWLLQGGASRAETSVQPVLVE</td>
<td>1:1K</td>
<td>1.80</td>
<td>0.18</td>
</tr>
<tr>
<td>(23-45 aa with an extra K at the N-terminus)</td>
<td>1:10K</td>
<td>0.22</td>
<td>0.04</td>
</tr>
<tr>
<td>P2</td>
<td>1:100</td>
<td>2.70</td>
<td>2.58</td>
</tr>
<tr>
<td>CSFSKSSNSWFPVEGADICQCC</td>
<td>1:1K</td>
<td>1.60</td>
<td>2.51</td>
</tr>
<tr>
<td>(300-322 aa)</td>
<td>1:10K</td>
<td>0.09</td>
<td>0.77</td>
</tr>
<tr>
<td>P3</td>
<td>1:100</td>
<td>2.72</td>
<td>2.45</td>
</tr>
<tr>
<td>KGDCGTSSHRRQPHEVSQWSRSA</td>
<td>1:1K</td>
<td>2.79</td>
<td>2.65</td>
</tr>
<tr>
<td>(324-347 aa)</td>
<td>1:10K</td>
<td>1.07</td>
<td>1.39</td>
</tr>
</tbody>
</table>

a Animals were immunized as described in the Materials and Methods. The serum samples obtained 10 days after the second booster were analyzed.

b The values represent the mean absorbance of two readings minus the mean absorbance obtained with the respective preimmune serum samples.
Fig. 13: Cloning of bZP3 for expression in insect cells: bZP3 was cloned for expression in the BEVS under the polyhedrin promoter. The full length gene (V1; 1-424 aa residues) and its truncated version lacking the C-terminal hydrophobic domain (V2; 1-348 aa residues) were cloned in the pBacPAK8 vector (panel A). Two more constructs were also designed to express bZP3 (V3; 23-76 and V4; 23-348 aa residues) as GST fusion proteins with an insect (gp67) derived secretory signal (panel B). The hydrophobicity plots (Hopp-Woods) for the expressed protein are shown. Numbers in parenthesis represent aa residues corresponding to bZP3.
B). V1-V2 and V3-V4 were compared for expression and secretion efficiency. Fig. 14A depicts a schematic representation of the pBacPAKV1 and Fig. 14B, pBacPAKV2 plasmids while Fig. 15 depicts the resolution of the pBacPAKV1 (Lane 5) and the pBacPAKV2 (Lane 7) recombinant plasmids on an agarose gel after digestion with Kpn I and Sac I restriction enzymes. V1 and V2 digests show bands corresponding to the vector (5.5 kb) and inserts of ~1.3 kb (V1) and ~1.0 kb (V2) respectively. Fig. 16A depicts a schematic representation of the pAcSecG2TV3 and Fig. 16B pAcSecG2TV4 plasmids while Fig. 17 depicts the resolution of the pAcSecG2TV3 (lane 5) and the pAcSecG2TV4 (lane 7) recombinant plasmids on an agarose gel after digestion with BamH I and EcoR I restriction enzymes. V3 and V4 digests show bands corresponding to the vector (~8.6 kb) and insert (~1.0 kb). Sequencing of the V3 transfer vector revealed that the clone carries a dinucleotide deletion at nt position 229-230 of the bZP3 sequence resulting in a frame shift mutation and premature termination after aa 77 of the bZP3 protein. The recombinant transfer vectors were screened by colony hybridization, restriction digestion and Southern blotting as shown for the representative V1 construct (Fig. 18). The labeled bZP3 probe binds to the insert (~1.3 kb band) and not to the vector (5.5 kb band) (Fig. 18C). The transfer vector DNA from one of the positive clones was prepared for transfection and recombinant viruses were obtained as described in Section 3.4 of the Materials and Methods. The recombinant viruses were screened by dot blot, restriction analysis and Southern blotting as shown for the representative virus V1 expressing the full length bZP3 protein (Fig. 19). Southern blotting of viral DNA isolated from two recombinant clones and restricted with Hind III showed a specific signal at ~9.6 kb (Fig. 19C), which correlated with the expected size of the Hind III digested viral DNA fragment with the polyhedrin promoter (~8.3 kb) carrying the bZP3 (~1.3 kb) insert. However, no signal was observed with DNA isolated from uninfected cells (lane 1) or wild type virus, AcNPV, infected cells (lane 2).
Fig. 14: Schematic representation of the pBacPAKV1 and pBacPAKV2 plasmids. The full length gene (V1; 1-424 aa residues) and its truncated version lacking the C-terminal hydrophobic domain (V2; 1-348 aa residues) were cloned in the pBacPAK8 vector. Panel A represents a schematic representation of the pBacPAKV1 and panel B, pBacPAKV2 plasmid constructs carrying the bZP3 insert at the Kpn I and Sac I restriction sites.
Fig. 15: Cloning of bZP3 in pBacPAK8. The Kpn I and Sac I restricted bZP3 insert from pBluescript-bZP3 and the truncated version of the cDNA lacking the C-terminal domain after the furin cleavage site were cloned in the pBacPAK8 vector at the Kpn I and Sac I sites. The figure represents an agarose gel showing lane 1, λ(Hind III) markers (kb); lanes 2 and 3, undigested and digested pBacPAK8 vector; lanes 4 and 5, undigested and digested pBacPAKV1 vector; lanes 6 and 7, undigested and digested pBacPAKV2 vector and lane 8, φX174 markers (kb).
Fig. 16: Schematic representation of the pAcSecG2TV3 and pAcSecG2TV4 plasmids. Constructs were designed to express bZP3 (V3; 23-76 and V4; 23-348 aa residues) as GST fusion proteins with an insect (gp67) derived secretory signal. Panel A represents a schematic representation of the pAcSecG2TV3 and panel B, pAcSecG2TV4 plasmid constructs carrying the bZP3 insert at the BamH I and EcoR I restriction sites.
Fig. 17: Cloning of bZP3 in pAcSecG2T. bZP3 lacking the N-terminal signal sequence and the C-terminal domain after the furin cleavage site was cloned in the pAcSecG2T vector at the BamHI and EcoRI sites. The figure represents an agarose gel showing lane 1, λ(Hind III) markers (kb); lanes 2 and 3, undigested and digested pAcSecG2T vector; lanes 4 and 5, undigested and digested pAcSecG2TV3 vector; lanes 6 and 7, undigested and digested pAcSecG2TV4 vector and lane 8, φX174 markers (kb).
Fig. 18: Screening for the pBacPAKV plasmid. The ligation mix was used for transformation of DH5α cells and transformants were screened on LB Amp plates. The presence of the bZP3 cDNA was verified by colony hybridization of the transformants using $^{32}$P labeled bZP3 cDNA (Panel A), restriction digestion, with Kpn I and Sac I, of the purified DNA from the positive clones (Panel B) and by Southern blotting (Panel C). The + and - indicate the positive and negative controls in the colony hybridization, Lane 1 represents $\lambda$(Hind III) markers (kb); lane 2, pGEM markers (kb); lane 3, undigested pBacPAK8 vector; lanes 4, 5, 6 and 7, Kpn I and Sac I digests of the pBacPAKV1 transfer vector clones, and lane 8, linearized pBacPAK8 vector.
Fig. 19: Screening for the VI virus. The transfer vector was used for co-transfection with linearized AcNPV viral DNA and viruses carrying the bZP3 were produced by homologous recombination. The transfection supernatant was used for a plaque assay and viral stocks were generated from isolated plaques. Viruses were used for infection of Sf9 cells and DNA isolated from the infected cells was screened by dot blot hybridization using $^{32}$P labeled bZP3 cDNA as a probe (Panel A), restriction digestion with Hind III (Panel B) and Southern hybridization (Panel C). Numbers represent, uninfected cells, 1; AcNPV infected cells, 2 and VI infected cells, 3 and 4.
3.2 **Optimization of bZP3 Expression in Insect Cells**

Conditions for expression of bZP3 were optimized using the V1 virus for infection in Sf9 cells. A time course study using total RNA probed with the bZP3 probe showed that the bZP3 was transcribed maximally between 60-72 h pi, corresponding to the peak activity of the viral polyhedrin gene promoter (Fig. 20A). The bZP3 protein could be detected in the cell pellet and not in the supernatant using MA-451 (Fig. 20B and 20C). Expectedly protein expression levels were highest at 60-84 h pi, the time at which the late polyhedrin promoter is maximally transcribed. However, at 84 h pi degradation of the protein was evident and all subsequent harvesting of the infected cells was done at 72 h pi. The r-bZP3 was expressed as a doublet band of 50-60 kDa in size. It is interesting to note that despite the presence of the native secretory signal sequence, bZP3 protein was not secreted into the culture supernatant (lane 3). It appears to be unusual as insect cells are known to recognize with varying efficiency mammalian and other eukaryotic signal sequences (Sridhar and Hasnain, 1993; Sridhar et al., 1993).

3.3 **Influence of Transmembrane and other Hydrophobic Domains on Expression Levels of bZP3**

Metabolic labeling using $^{35}$S-Met, 72 h pi, when the late viral polyhedrin promoter would be active in cells infected with virus, and quantitation of the incorporated label in the proteins expressed under this promoter revealed that expression level of V2 which lacked the C-terminal hydrophobic region was much higher than V1. Direct comparison with polyhedrin expression (lane W) showed that V1 was expressed to $\sim$1% of the polyhedrin protein expressed at 72 h pi in cells infected with AcNPV. Deletion of the transmembrane domain in V2 resulted in a 46 fold increase in expression levels. Expression of V4 was $\sim$16% of polyhedrin expression while expression of a shorter gp67-GST-bZP3 polypeptide, V3 (49% of polyhedrin expression), was $\sim$3 fold higher as compared to V4. Polyhedrin was visible as a single sharp band of $\sim$29 kDa, while the V1-V4 proteins were doublets (Fig. 21).
Fig. 20: Expression of bZP3 in insect cells: Expression of bZP3 in insect cells was standardized using the full length bZP3 (V1) virus. Total RNA isolated from V1 infected Sf9 cells and harvested at different time points pi, was analyzed by Northern blotting using the full length bZP3 probe. Panel A shows the presence of the ZP3 transcript at different time points. The numbers on top indicate the time (h) of harvesting after infection. Expression levels were also analyzed by Western blot using MA-451, a murine monoclonal antibody cross-reactive with bZP3. Cells harvested at different time points were lysed, resolved by 0.1% SDS-10% PAGE and transferred to nitrocellulose membrane as described in the Materials and Methods. Panels B and C represent the Coomassie stained gel and immunoblot respectively. Lanes are M, protein molecular weight markers; W, wild type AcNPV infected cells; S, supernatant from V1 infected cells harvested 72 h pi, 12-84, total cell pellet from cells infected with V1 and harvested at 12-84 h pi.
Fig. 21: Metabolic labeling of infected cells using $^{35}$S methionine: Sf9 cells (1.5X10$^6$) infected with AcNPV or recombinant viruses V1 (bZP3 aa 1-424), V2 (bZP3 aa 1-348), V3 (bZP3 aa 23-76) and V4 (bZP3 aa 23-348), were pulsed with 25 μCi of $^{35}$S Met 72 h pi, to assay for the expression of r-bZP3 and its derivatives, as described in the Materials and Methods. The different lanes are: W, AcNPV infected cells; V1-V4 cells infected with V1, V2, V3 and V4 recombinant viruses respectively.
3.4 Analysis of Expression and Secretion of bZP3 in Insect Cells

Antisera generated against the N- and C-terminal peptides, anti-GST Ab and MA-451 were used as probes in immunoblots to monitor the expression of the r-proteins. While V1, V2 and V4 were recognized by anti N- and C-terminal Abs and MA-451 exclusively in the cell pellet, V3 reacted only with the anti N-terminal Ab both in the cell pellet and supernatant. In addition, anti-GST Ab recognized the V3 protein in the supernatant as well as in the pellet and V4 only in the cell pellet. The results from these experiments are summarized in Table 3. N-terminal anti-peptide Ab demonstrated the presence of the r-bZP3 polypeptides as doublet bands V1, 50-60 kDa; V2, 40-50 kDa; V3, 35-45 kDa and V4, 65-75 kDa (Fig 22, left panel). MA-451 showed the same pattern for V1, V2 and V4, however, V3 was not detected as expected, confirming the fact that the V3 protein was being expressed as a truncated molecule (Fig. 22, right panel). Uninfected cells and AcNPV infected cells did not show any signal with any of the Abs. Quantitation of the secreted versus cellular protein detected in Western blot with the anti-GST Ab revealed that 9-37% of the V3 protein was being secreted (Fig. 23). It therefore appears that the presence of minor hydrophobic domains present within bZP3, and not necessarily only the major domains like the secretory signal sequence and transmembrane-like domain may be responsible for blocking the secretion of bZP3 and gp67-GST-bZP3 in insect cells.

3.5 Immunolocalization of r-bZP3 Protein in Infected Sf9 Cells

Indirect immunofluorescence using MA-451 (Fig. 24) on Sf9 cells infected with recombinant virus revealed the presence of specific immunofluorescence in cells infected with V1, V2 and V4. The distribution of the fluorescence was more or less uniform on the surface of the cells infected with V1 while V2 and V4 infected cells showed a more localized cellular distribution pattern. No immunofluorescence could be detected in cells infected with V3. With the anti-N-terminal Ab however, V3 did show distinct fluorescence. The pattern of distribution in this case was also localized, as in the case of V2 and V4 while V1 showed a uniform distribution of bZP3 (Fig. 25). Both
Table 3: Reactivity in Western blot of Abs generated in rabbits against synthetic peptides corresponding to the bZP3 sequence and MA-451, a murine MAb recognizing the bZP3 protein.

<table>
<thead>
<tr>
<th></th>
<th>CELL PELLET</th>
<th>SUPERNATANT</th>
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<tr>
<td></td>
<td>C  W 1 2 3 4</td>
<td>C  W 1 2 3 4</td>
</tr>
<tr>
<td>N-Terminal Ab@ (23-45 aa residues)</td>
<td>- - + + + +</td>
<td>- - - - + -</td>
</tr>
<tr>
<td>(KQPFWLLQGASRAETSVPVLVE)b</td>
<td></td>
<td></td>
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<tr>
<td>MA-451 (167-172 aa residues)</td>
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<td>- - - - - -</td>
</tr>
<tr>
<td>EEKLVF*/EEKLTF#</td>
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<tr>
<td>C-Terminal Ab (300-322 aa residues)</td>
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<td>- - - - - -</td>
</tr>
<tr>
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<tr>
<td>GST Ab</td>
<td>- - - - + +</td>
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C, W, 1, 2, 3 and 4 refer to uninfected Sf9 cells, Sf9 cells infected with AcNPV or with virus constructs V1, V2, V3 and V4 respectively.

@ Antibody, * Porcine, # Bonnet

Synthetic peptide has an extra lysine at the N-terminus
Fig. 22: Reactivity of bZP3 with anti-peptide sera and MA-451: Cells infected with AcNPV, V1, V2, V3 or V4 recombinant viruses were harvested 72 h pi and lysed as described. The proteins were resolved by 0.1% SDS-10% PAGE and analyzed by Western blot using anti-N-terminal peptide Ab and MA-451. Panel A represents the Coomassie stained gel and panel B Western blots probed with anti N-terminal Ab (left panel) and MA-451 (right panel). The different lanes are: C, uninfected cells; W, AcNPV infected cells; 1-4 represent cells infected with V1, V2, V3 and V4 recombinant viruses respectively.
Fig. 23: Secretion of bZP3: Cells as well as supernatants were harvested from Sf9 cells infected with V1, V2, V3 and V4 viruses 72 h pi and analyzed by Western blotting using anti-N-terminus (Panel A) and anti-GST Ab (Panel B). Lanes 1-4, represent cell pellet/culture supernatants from cells infected with V1, V2, V3 and V4 viruses respectively. Arrows indicate the secreted V3 protein.
MA-451 and anti-N-terminal Ab failed to show any specific fluorescence with uninfected Sf9 cells. Deletion of the transmembrane domain thus changes the cellular distribution of bZP3 and gp67-GST-bZP3 in infected Sf9 cells.

4 IMMUNOGENICITY OF r-bZP3 EXPRESSED IN E. coli

4.1 Purification and Conjugation of r-bZP3
r-bZP3 (His<sub>6</sub>) fusion protein was purified by affinity chromatography using the Ni-NTA resin. From a 2 L batch culture, approximately 20 mg of purified r-ZP3 was obtained from SG13009[pREP 4] and 15 mg from BL21(DE3) cells transformed with pQE-bZP3 plasmid. Representative samples of the purified fractions resolved by SDS-PAGE are shown in Figs. 26A and 27A. While the r-bZP3 protein purified from transformed SG13009[pREP 4] cells showed several lower molecular weight bands besides the full length bZP3 protein, r-bZP3 purified from transformed BL21(DE3) cells showed a single band. The r-bZP3 expressed in SG13009[pREP4] was conjugated to DT and that expressed in BL21(DE3) was conjugated to both DT and TT as described in Materials and Methods. Conjugation of r-bZP3 with DT or TT was confirmed with SDS-PAGE analysis (Figs. 26B and 27B).

4.2 Immunogenicity of r-bZP3
A female rabbit (R-188) was immunized with r-bZP3 expressed in SG13009[pREP 4] and conjugated to DT, as described in Materials and Methods to check the immunogenicity of the r-protein. Ab titres, determined by ELISA using the r-bZP3 coated microtitration plates, were <100 Ab units (AU) at Day 0; 11,112 AU at day 40, and 11,000 AU at day 70.

4.3 Reactivity of Anti-r-bZP3 Ab with Heterologous and Homologous ZP
The ZP3 family of proteins is well conserved in evolution, and it may be possible to determine the cross reactivity of Abs generated against bZP3 with heterologous ZP3. Moreover, it also becomes essential to verify that the Abs generated against the
Fig. 24: Immunocytochemical localization of bZP3: Sf9 cells infected with AcNPV, V1, V2, V3 or V4 were harvested 72 h pi, and probed with MA-451 by indirect immunofluorescence. Panel A represents uninfected cells while panels B, C, D and E are cells infected with V1, V2, V3 and V4 viruses respectively.
Fig. 25: Immunocytochemical localization of bZP3: Sf9 cells infected with AcNPV, V1, V2, V3 or V4 were harvested 72 h pi, and probed with anti-N-terminus Ab by indirect immunofluorescence. Panel A represents uninfected cells while panels B, C, D and E are cells infected with V1, V2, V3 and V4 viruses respectively.
Fig. 26: Purification and conjugation of r-bZP3 expressed in SG13009[pREP4] cells. SG13009[pREP4] cells harboring the pQE-bZP3 plasmid were grown in a batch flask culture (250 ml X 8) and induced with 0.5 mM IPTG for 2 h. r-bZP3 was purified by affinity chromatography on Ni-NTA resin as described in the Materials and Methods. Panel A represents a 0.1% SDS-10% PAGE of the fractions purified on the column and stained with Coomassie blue. Lane 1, column wash with buffer C (8 M Urea, 0.1 M NaH₂PO₄ and 0.01 M Tris, pH 6.3) lanes 2-5 elution with buffer D (composition same as buffer C, pH 5.9) and lanes 6-9 elution with buffer E (composition same as buffer C, pH 4.5). The purified protein was pooled, dialyzed against 100 mM phosphate buffer, pH 7.4 containing 4 M urea and conjugated to DT using the "one step" glutaraldehyde coupling procedure. Panel B represents the 0.1% SDS-10% PAGE (left) and immunoblot probed with MA-451 (right). Lanes are 1, purified r-bZP3 protein; lane 2, DT and lane 3, r-bZP3-DT conjugate.
Fig. 27: Purification and conjugation of r-bZP3 expressed in BL21(DE3) cells. BL21(DE3) cells harbouring the pQE-bZP3 plasmid were grown in a batch flask culture (250 ml X 8) and induced with 0.5 mM IPTG for 2 h. r-bZP3 was purified by affinity chromatography on Ni-NTA resin as described in the Materials and Methods. Panel A represents a 0.1% SDS-10% PAGE of the fractions purified on the column and stained with Coomassie blue. Lane 1, unbound fraction, lanes 2-5 elution with buffer D and lanes 6-9 elution with buffer E. The purified protein was pooled, dialyzed against 100 mM phosphate buffer, pH 7.4 having 4 M urea and conjugated to DT or TT using the "one step" glutaraldehyde coupling procedure. Panel B represents the Coomassie blue stained SDS-PAGE of the conjugate. Lanes are 1, purified r-bZP3 protein; lane 2, DT and lane 3, r-bZP3-DT conjugate; lane 4, TT and lane 5, r-bZP3-TT conjugate.
denatured r-bZP3 protein is able to recognize the glycosylated protein in its native conformation on the oocytes.

a) porcine zona proteins

The reactivity in Western blot of rabbit anti-r-bZP3 Abs with pZP3α and pZP3β and r-bZP3 is shown in Fig. 28. Anti r-bZP3 Abs not only reacted with r-bZP3 but also with pZP3β, the porcine homologue of bZP3. However, Abs did not react with pZP3α the homologue of ZP1 thereby showing the specific reactivity of the anti-r-bZP3 Abs with the ZP3 family of proteins.

b) human ZP

Indirect immunofluorescence using rabbit anti-r-bZP3 Abs on human oocytes revealed the presence of specific fluorescence on human oocytes treated with immune and not the preimmune sera. The distribution of the fluorescence was uniform over the entire surface of the oocytes (Fig. 29). No immunofluorescence could be detected in oocytes treated with the preimmune serum.

c) bonnet ZP

For proving the authenticity of the expressed r-protein, it was imperative to show that the Abs generated against the r-bZP3 recognizes the native protein. Rabbit immune serum against r-bZP3-DT conjugate showed intense fluorescence with the zona pellucida of oocytes in sections from the bonnet monkey ovary. The pre-immune serum failed to show any immunofluorescence. Moreover, the fluorescence was specific to the zona matrix and the Abs did not react with other ovarian cell types (Fig. 30).

4.4 Contraceptive Efficacy Studies in Bonnet Monkeys

Bonnet monkeys were selected for inclusion in the efficacy studies after observing 2-3 normal ovulatory cycles (progesterone values >2 ng/ml). Five monkeys (MRA 375, MRA 515, MRA 640, MRA 672 and MRA 770) were immunized with the r-bZP3 (expressed in SG13009[pREP4])-DT conjugate. Immunized animals were monitored for Ab titres in ELISA against r-bZP3 and DT as well as serum progesterone concentration and menstrual cyclicity. While MRA 515 failed to generate a significant
Fig. 28: Reactivity of rabbit anti-r-bZP3 with pZP3α, pZP3β and r-bZP3 in Western blot. The pZP3β, pZP3α and r-bZP3 (500 ng/lane) were resolved on a 0.1% SDS-10% PAGE and electrophoretically transferred to nitrocellulose membrane as described in the Materials and Methods. Panel A represents the Coomassie blue stained gel and Panel B represents an immunoblot of the same probed with 1:500 dilution of rabbit anti-r-bZP3 immune sera (Day 70). Lanes are represented as M, molecular weight markers (kDa); lane 1, pZP3β; lane 2, pZP3α; and lane 3, r-bZP3.
Fig. 29: Reactivity of rabbit anti-r-bZP3 serum with human oocytes by indirect immunofluorescence. Human oocytes were incubated with 1:50 dilution of preimmune (faintly visible oocyte on the left) or immune sera (lower oocyte) of rabbit immunized with r-bZP3-DT conjugate, followed by incubation with 1:250 dilution of goat anti-rabbit-FITC conjugate. Human oocyte treated with 1:50 dilution of rabbit anti-pZP3 Abs (top) was used as a positive control (200X).
Fig. 30: Reactivity of rabbit anti-r-bZP3 serum with bonnet monkey zona pellucida in an indirect immunofluorescence assay. Bonnet monkey ovarian cryosections (5 μm) were incubated with 1:250 dilution of rabbit anti-r-bZP3-DT immune or preimmune sera followed by incubation with 1:2000 dilution of FITC-labeled anti-rabbit Ig as described in Materials and Methods. Representative results with the immune and preimmune sera are shown. Top panel shows specific immunofluorescence with the ZP following treatment with immune serum (left) and not with preimmune serum (right). Lower panel represents the bright field view of the same areas as shown in the upper panel (100X).
Ab response, MRA 375 and MRA 672 generated moderate Ab titres ($<2000$ AU) and MRA 640 and MRA 770 generated high Ab titres ($>5000$ AU). MRA 640 and MRA 770 were mated with males of proven fertility following the primary immunization schedule comprising of 3 injections. Two more boosters were given to MRA 375 and MRA 672 before initiating mating. MRA 515 was excluded from the mating study, as it failed to generate significant titres even after repeated boosting. Ab titres against r-bZP3 and DT, and serum progesterone concentrations of the four immunized animals i.e. MRA 375, MRA 640, MRA 672 and MRA 770, along with the outcome of the mating, are shown in Figs. 31-34. Ab titres generated against the DT carrier were at least 10-20 fold higher than those against the r-bZP3 antigen. Progesterone levels in all the monkeys showed a disturbance in ovulation in the months of May to August which coincided with the summer amenorrhoea in *M. radiata* (Srinath, 1980).

MRA 375 which showed a low to moderate anti-r-bZP3 response became pregnant in the first ovulatory cycle after summer amenorrhoea and delivered a healthy offspring. Ab titres were $<1000$ AU at the time of conception. Of the remaining 3 animals, 2 animals (MRA 640 and MRA 672) continued to show normal ovulation and remained infertile (MRA 640 for 14 ovulatory cycles and MRA 672 for 8 ovulatory cycles). MRA 770 initially showed a disturbance in ovulation, however, it subsequently recovered and became pregnant in the second ovulatory cycles when Ab titres were $~7000$ AU and delivered a healthy offspring. Since 3 of the 4 animals in this group continued to ovulate normally despite the immunization, it would seem that the disturbed cyclicity observed initially in MRA 770 was a result of normal variation in the colony, or was induced by factors other than the immunization.

During the same period, all 3 unimmunized control animals became pregnant, MRA 647 and MRA 768 within the first 2 cycles of mating and MRA 489 in the first cycle after the resumption of normal cyclicity after summer amenorrhoea as described elsewhere (Afzalpurkar, 1997).
Figs. 31-34: Progesterone profiles and Ab titres of bonnet monkeys immunized with r-bZP3-DT conjugate. Female bonnet monkeys showing ≥2 normal progesterone profiles were immunized with 250 µg of r-bZP3 expressed in SG13009[pREP4] cells, conjugated to DT, using Squalene and Arlacel A (4:1) as adjuvants. The primary injection consisted of 1 mg of SPLPS per animal as an additional adjuvant. Boosters were administered at intervals of 4-6 weeks. The upper panel of each figure shows the serum progesterone levels in ng/ml. Arrows indicate initiation of mating (M) and the onset of pregnancy (P). Summer amenorrhoea is denoted by SA and periods for which samples were not analyzed for progesterone as ND. Onset of menstrual cycle is indicated on the X-coordinate of the top panel as (•). The lower panel represents the Ab titres of the monkeys. Microtitration plates coated with r-bZP3 (200 ng/well -•-) or DT (500 ng/well, -▲-) were incubated with doubling dilutions of immune sera and processed as described in Materials and Methods. The results are expressed as AU calculated by regression analysis and represent the dilution of serum giving an absorbance of 1.0 at 490 nm. The immunization schedule is represented as + on the baseline of the lower panel. Fig. 31: MRA 375; Fig. 32: MRA 640; Fig. 33: MRA 672 and Fig. 34: MRA 770.
Fig. 31: MRA 375

![Graph Image]

- **P4 ng/ml**
  - ND
  - SA

- **Antibody Units**

- **r-bZP3**
- **DT**
Fig. 32: MRA 640

![Graph showing P4 ng/ml and Antibody Units over Days]

- P4 ng/ml
- Antibody Units
- Days

Legend:
- r-bZP3
- DT
Fig. 33: MRA 672

![Graph showing P4 ng/ml and Antibody Units over Days]

- **P4 ng/ml**
  - ND
  - SA
  - ND

- **Antibody Units**
  - 100,000
  - 10,000
  - 1,000
  - 100

- Days range from -100 to 800.

- Symbols: • for r-bZP3, --- for DT.
Fig. 34: MRA 770

[Graph showing antibody units and P4 ng/ml over days with r-bZP3 and DT markers]
Results

To solve the problem of variable Ab titres against bZP3 and to minimize the high anti-DT Ab response observed, a second group of 3 monkeys (MRA 384, MRA 502 and MRA 661) was immunized with the full length r-bZP3 protein expressed in BL21(DE3) cells coupled with either DT or TT as carriers. The primary immunization comprised of 125 μg of r-bZP3-DT and 125 μg r-bZP3-TT/animal. Subsequently, animals received boosters of 250 μg of r-bZP3 alternately with either DT or TT as carrier proteins as described in the Materials and Methods. The results are shown in Figs. 35-37. The primary and the first booster injections failed to generate any significant anti-bZP3 Ab response and hence the second booster was administered with 1 mg SPLPS/animal as an additional adjuvant. The anti-carrier response was significantly lower in the new group of monkeys. However, anti-r-bZP3 titres remained low to moderate over the period of the study. In this group one animal, MRA 661, remained infertile while continuing to have normal ovulatory cycles over the period of the study. MRA 384 became pregnant in the first ovulatory cycle following summer amenorrhoea, when its Ab titres were ~2500 AU while MRA 502 became pregnant in the second cycle following summer amenorrhoea at a time when the Ab titer was ~3500 AU. No disturbance in cyclicity was observed in this group as a result of immunization.

4.5 T-Cell Responses to r-bZP3

In order to analyze if immunization with r-bZP3 was resulting in the breakdown of T-cell tolerance to the ZP3 protein, initially attempts were made to standardize T-cell proliferation assays using the r-bZP3 or DT for stimulation. No significant proliferative responses could be detected in our assays. An alternate strategy was then used in which r-bZP3 expressed in BL21(DE3) cells was conjugated to a hapten arsonate (ars). Naive (MRA 446 and MRA 670) and r-bZP3 immunized monkeys (MRA 375, MRA 640 and MRA 672) were immunized with ars-r-bZP3. It was expected that bZP3 being a self molecule should not be able to provide T cell help unless T cell tolerance was broken. Naive monkeys in that case should not develop an anti-hapten (anti-ars) Ab response and monkeys pre-immunized with r-bZP3-DT should develop an anti-ars response only
Figs. 35-37: *Progesterone profiles and Ab titres of bonnet monkeys immunized with r-bZP3-DT/r-bZP3-TT conjugates.* Female bonnet monkeys showing ≥2 normal progesterone profiles were immunized with r-bZP3 expressed in BL21(DE3) cells. The primary immunization consisted of 125 μg of r-bZP3-DT and 125 μg of r-bZP3-TT conjugates administered using Squalene and Arlacel A (4:1) as adjuvants. The primary injection consisted of 1 mg of SPLPS per animal as an additional adjuvant. Boosters were administered at intervals of 4-6 weeks alternately with 250 μg of r-bZP3-DT or r-bZP3-TT conjugates. The upper panel of each figure shows the serum progesterone levels in ng/ml. Arrows indicate initiation of mating (M) and the onset of pregnancy (P). Periods for which serum progesterone level were not analyzed are represented as ND and onset of menstrual cycle is indicated on the X-coordinate as (●). The lower panel shows the Ab titres. Microtitration plates coated with r-bZP3 (200 ng/well -●-), DT (500 ng/well, -▲-) or TT (500 ng/well, -■-) were incubated with doubling dilutions of immune sera and processed as described in Materials and Methods. The results are expressed as AU calculated by regression analysis and represent the dilution of serum giving an absorbance of 1.0 at 490 nm. Symbols on the baseline of the lower panel represent immunization/boosting schedule with DT (●) or TT (▲). Fig. 35: MRA 384; Fig. 36: MRA 502 and Fig. 37: MRA 661.
Fig. 35: MRA 384

![Graph showing the change in P4 ng/ml (top) and Antibody Units (bottom) over days with markers for r-bZP3, DT, and TT.]
Fig. 36: MRA 502

Graph showing the concentration of P₄ (ng/ml) over days, with antibody units plotted on a log scale. The graph includes data points for r-bZP3, DT, and TT.
Results

if T-cell tolerance was broken as a result of immunization. However, both the naive and pre immunized groups developed an anti-ars response in the absence of a significant increase in the anti-r-bZP3 Ab response, with the exception of MRA 375 which also showed some increase in the anti-r-bZP3 Ab response (Table 4). It thus seems possible that the anti-ars response being observed in the animals was being generated because of T-cell help from some E. coli contaminating protein/proteins in the antigen preparation which were getting arsonated and provided T-cell help for developing an anti-ars response.
Table 4: Reactivity in ELISA of serum samples of female bonnet monkeys immunized with ars-bZP3 against r-bZP3 and arsonate

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<tr>
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<td>2.03</td>
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# Absorbance with 1:500 dilution of antisera
@ Absorbance with 1:100 dilution of antisera