8 STRUCTURAL BIOLOGY

Several research projects, aimed at studying structure-function relationship of proteins involved in reproductive processes have been undertaken at the Institute. Molecular mechanisms involved in the interaction of gonadotropins with their receptors have been carried out using multifaceted approaches like synthetic peptides, antipeptide antibodies and bioinformatics. Identification of mutations and polymorphisms in the gonadotropin receptor genes are being undertaken with an aim to delineate the importance of specific amino acid residues necessary for active conformation of the protein. Identification of structural and functional domains in these proteins would help in designing antagonists of hormone action with potential application in contraceptive development. Programmes have also initiated to understand the functional significance of any given protein based on primary sequence analysis by carrying out in-silico analysis.

8.1 Identification of Ligand Binding Regions on the Extracellular Domain of Human Follicle Stimulating Hormone Receptor

Principal Investigator: **Smita Mahale**

Project Associates: Pallavi Kene, Leena Tendulkar and Vaishali Nakhawa

Duration: 1999-2004

Follicle stimulating hormone receptor (FSHR) has a large extracellular domain (ECD), which has been shown to be crucial for hormone-receptor interaction. In our attempt to delineate different regions in FSHR involved in hormone binding and signal transduction, we have used antipeptide antibodies to potential surface oriented regions of the ECD. The ability of antibodies to modulate FSH binding has been reported earlier (Annual Report 2003-04, p 168). To establish the role of different regions of the ECD in governing FSH-induced cAMP generation, antipeptide antibodies were screened for their ability to affect the same. No antipeptide antibody by itself, in the absence of FSH could stimulate cAMP levels, ruling out the occurrence of potential agonists. Of the antipeptide antibodies screened in this assay, those against region 15-31, 216-235, 285-300 and 327-341 of FSHR behaved as antagonists and inhibited production of FSH-induced cAMP levels significantly (Fig. 109). The effect was found to be dose dependent (Fig. 110). Based on the observations made with respect to accessibility to antipeptide antibodies, ability of antibodies to inhibit FSH binding and subsequent cAMP generation and kinetics of antibody binding, regions 285-300 and 327-341 of FSHR appear to be the chief FSH-binding sites, while regions 15-31 and 216-235 of FSHR serve as ancillary FSH-binding sites.

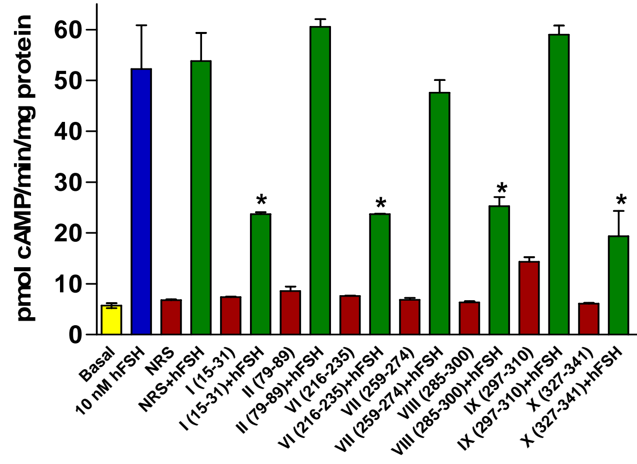

Fig. 109: Effect of NRS or antipeptide antibodies at a concentration of 1μg IgG/tube in the absence or presence of hFSH (10nM) on cAMP production. cAMP levels with and without stimulation with 10nM hFSH are indicated.

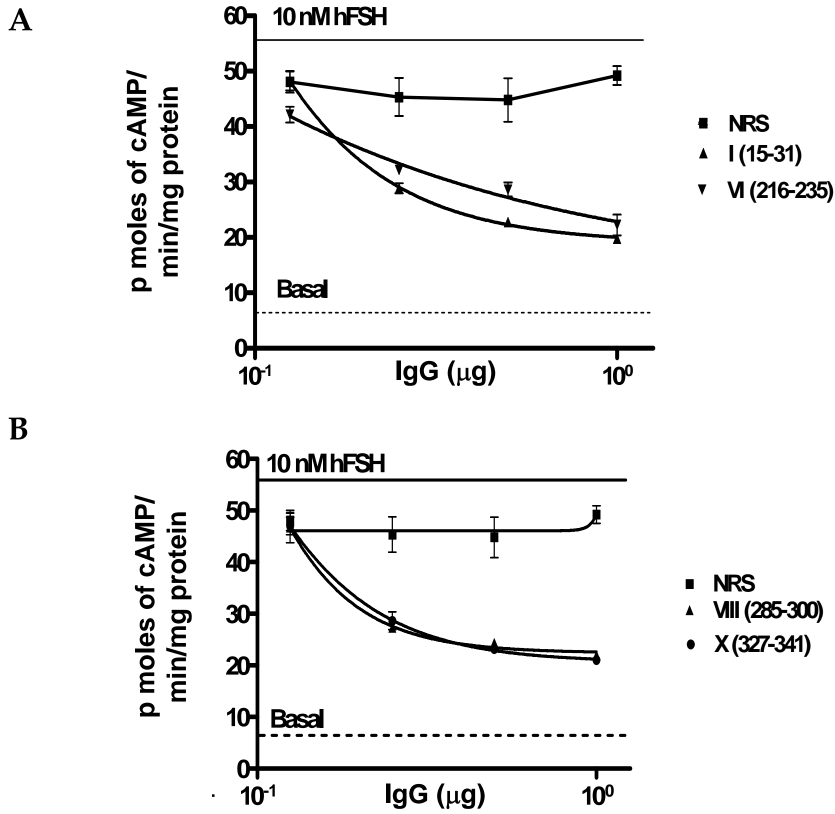

Fig. 110: Dose-dependent inhibition of FSH-induced adenylyl cyclase activation by effective antipeptide antibodies. IgG at different concentrations from 0.125 to 1 μg were studied; (A) with NRS, I (15-31), and VI (216-235) and (B) with NRS, VIII (285-300) and X (327-341). Values for basal levels of cAMP and those for stimulation with 10nM hFSH are indicated by horizontal lines parallel to the X-axis.

8.2 Immunology of the Peptides Corresponding to the Ligand Binding Regions from the Extracellular Domain of Rat Follicle Stimulating Hormone Receptor

Principal Investigator: **Smita Mahale**

Project Associates: J.D. Ghosalkar, Tarala Nandedkar, Dhanashree Jagtap, Leena Tendulkar and Vaishali Nakhawa

Duration: 2002-2006

It is believed that the discrete linear epitopes of the extracellular domain of FSHR are involved in ligand binding. Most of these findings are based on the observation that the specific peptides corresponding to predicted surface oriented regions of the ECD or antibodies to these peptides inhibited hormone receptor interaction under *in vitro* conditions. The present study aims to determine the ability of antibodies raised against hormone binding regions of FSHR to bind and neutralize endogenous receptor activity in a rat model. Peptide 20-30 and 285-309 from FSHR sequence were selected based on the observation that these are the immunodominant epitopes (Annual Report 2002-03, p 127 and Annual Report 2003-04, p 170). Both these peptides also showed FSH antagonistic activity in a FSH radioreceptor assay (Fig. 111) antibodies to both these peptides detected FSHR, as seen by western blot (Fig. 112). *In vivo* studies in rats are in progress.

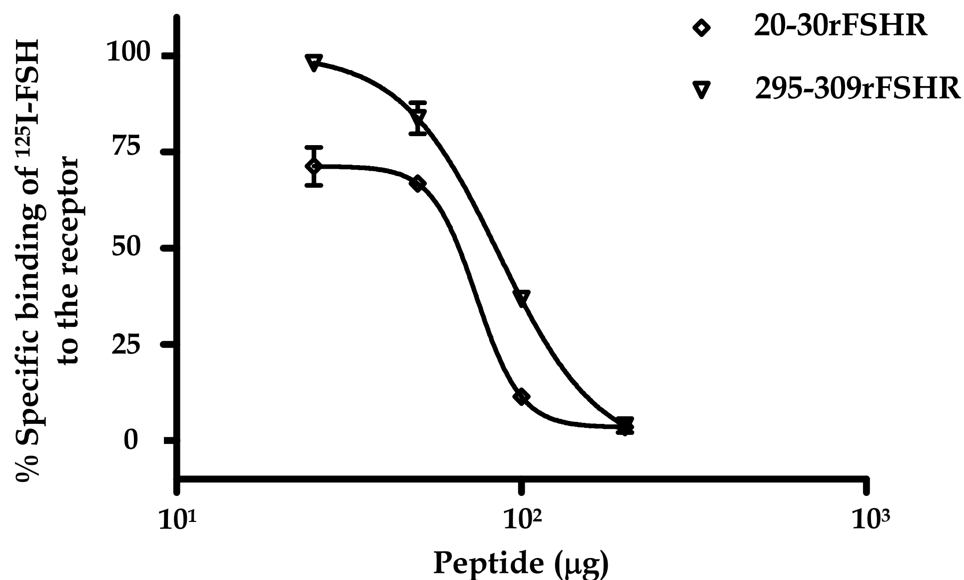

Fig. 111: Inhibition of ^{125}I-FSH binding to FSHR by synthetic peptides corresponding to rat FSHR sequence 20-30 and 295-309.

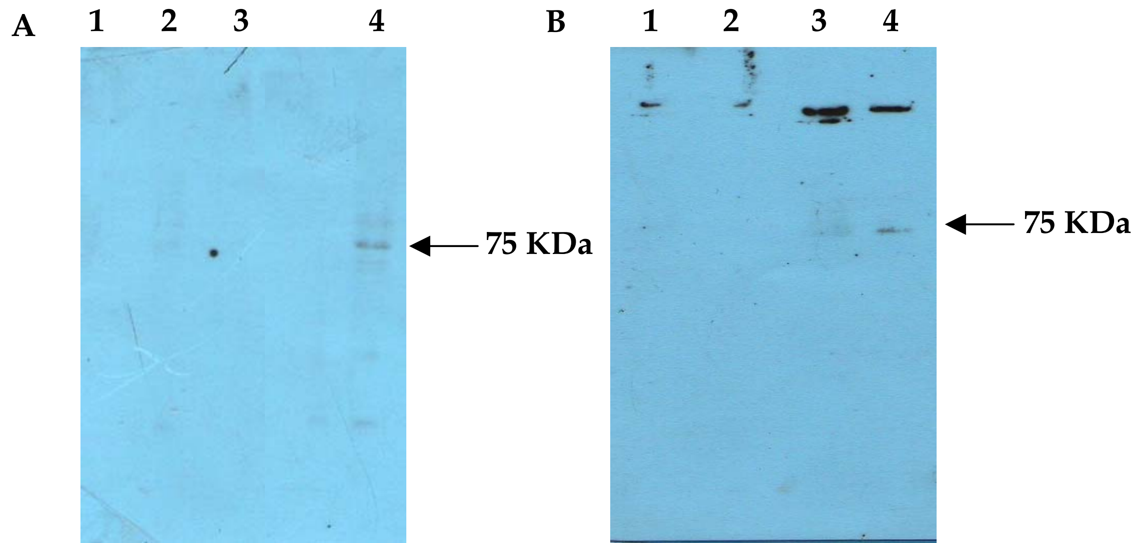

Fig. 112: Western blotting of membrane preparations from untransfected HEK-293 cells (lane 1 and 3) and HEK-293 cells overexpressing rat FSHR (lane 2 and 4). Lane 1 and 2 probed with preimmune sera. Lane 3 and 4 probed with antisera to peptide 20-30 rFSHR (A) and 285-309 rFSHR (B).

8.3 Mutational Analysis of Gonadotropin Receptor Genes and its Implications in Physiology and Pathophysiology of Pituitary Gonadal Function

Principal Investigator: **Smita Mahale**

Project Associates: Swati Achrekar, Pervin Meherji, Vrinda Khole, Zareen Patel and D.N. Modi

Collaborator: Dr Sadhana Desai, Fertility Clinic, Mumbai

Duration: 2002-2007

Inactivating mutations in FSHR gene have been shown in certain cases of infertile women. These mutations result in the formation of a protein with altered structure which makes the receptor inactive. Known mutations in exons 6, 7 and 10 were studied in infertile female subjects who were non-responders to gonadotropin treatment. Using sequence specific primers the exons 6, 7, 10 were amplified by PCR. The PCR products were subjected to restriction fragment length polymorphism (RFLP) for detection of known mutations and single strand conformation polymorphism (SSCP) analysis for detection of novel mutations. Results indicated no mutations in the subjects included in the study. Incidence of FSHR mutations seems to be very rare in our population.

FSHR gene is known to be polymorphic at two sites in exon 10 resulting in change in amino acid at position 307 (T/A) and 680 (N/S). Incidence of presence of S^{680} is found to be high in subjects undergoing IVF treatment who required more ampoules of FSH for ovarian stimulation. These two polymorphisms were studied in group of subjects undergoing ovarian stimulation in an IVF treatment cycle. Distribution of three different variations in study group as well as control subjects at both polymorphic sites is shown in Figs 113 and 114. No significant differences were observed in both the groups. However, the overall frequency of different allelic variants was altered in our study (Fig. 115). Further studies need to be carried out to understand the significance of these polymorphisms.

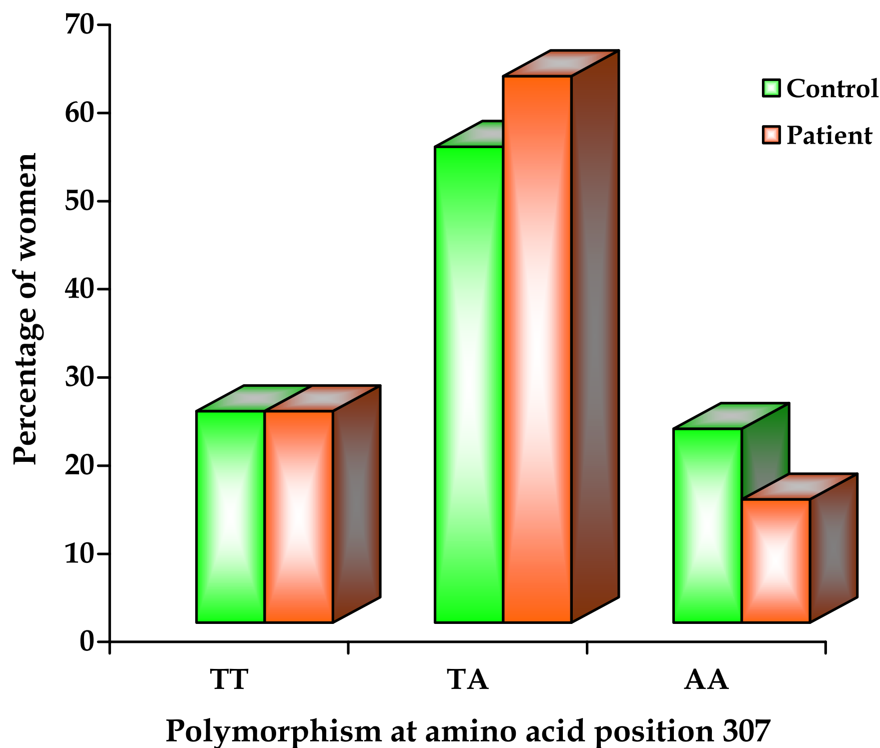

Fig. 113: Distribution of the three possible FSH receptor variants at amino acid position 307 in exon 10 of hFSH receptor gene among normogonadotropic proven fertile controls and subjects undergoing ovulation induction.

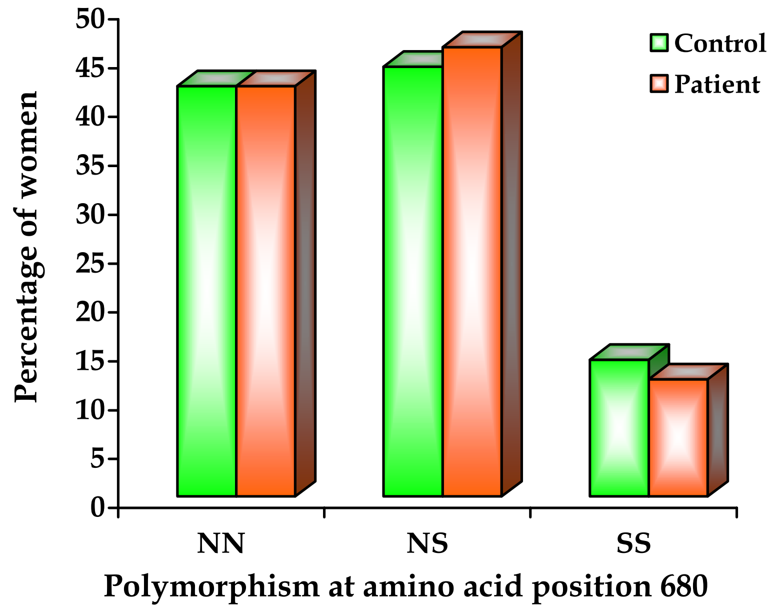

Fig. 114: Distribution of the three possible FSH receptor variants at amino acid position 680 in exon 10 of hFSH receptor gene among normogonadotropic proven fertile controls and subjects undergoing ovulation induction.

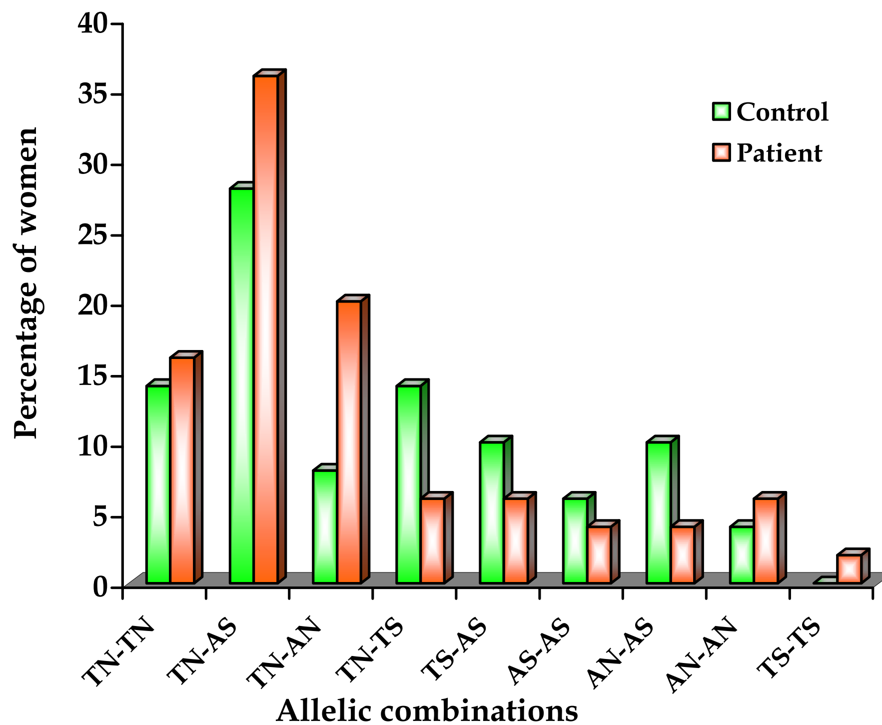

Fig. 115: Distribution of nine different FSH receptor allelic combinations (307 and 680) among proven fertile controls and subjects undergoing ovulation induction.

8.4 Studies on β-Microseminoprotein: Biochemical, Molecular and Bioinformatics Approaches

Principal Investigator: **Smita Mahale**

Project Associates: V.D. Dighe, Dhanashree Jagtap, Mallika Mesharam, Leena Tendulkar, and Vaishali Nakhawa,

Collaborators: Pervin K. Meherji, Jyotsna Gokral, and Geeta Vanage

Duration: 2003-2010

β-Microseminoprotein (β-MSP), also referred as prostate secretory protein or human seminal plasma inhibin (HSPI) is a non-glycosylated protein consisting of 94 amino acids. Originally found in human seminal plasma and prostatic fluids, its presence was later shown in other secretions in humans and its homologs were described in other species. Primary sequence comparisons in different species suggest that the protein has rapidly evolved and is rich in conserved cysteine residues. One of the objectives of this study is to assign disulphide bond pairing in MSP. This information is essential as disulphide bonds are known to play a role in stabilizing a native conformation of most of the proteins which is crucial for its biological actions. Changes occurring in the conformation of the native protein following reduction of disulphide bonds (modified protein) was studied using immunological approach (Annual Report 2003-04, p 174-175). Circular dichroism analysis of native as well as modified protein revealed that the native protein has approximately 80 percent β-sheet content, which decreased significantly upon reduction of disulphide bonds (Fig. 116). These results clearly indicate that the disulphide bonds are crucial for maintaining the native conformation of this protein.

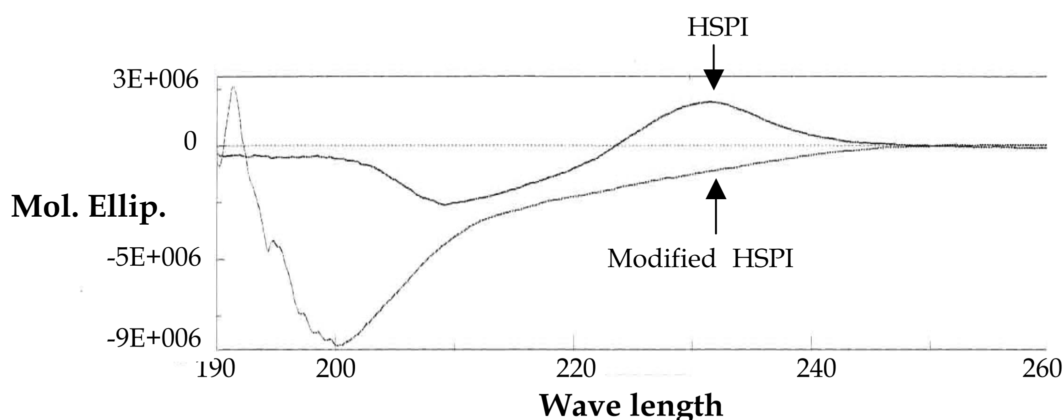

Fig. 116: Comparison of circular dichroism spectra of native and modified HSPI.

In our attempt to identify and characterize binding protein to β-MSP, studies were initiated with rat model. Towards this, specific antibody capable of detecting rat β-MSP was raised using peptide 1-17 of rat β-MSP as an immunogen. The antibody was able to detect the native rat-β-MSP present only in prostate and not in rat testis or epididymis (Fig. 117). RT-PCR using rat sequence specific primers revealed presence of transcript only in the rat prostate (Fig. 118). Efforts are on to identify and characterize the β-MSP proteins present in the rat pituitary, to understand its role in FSH modulation.

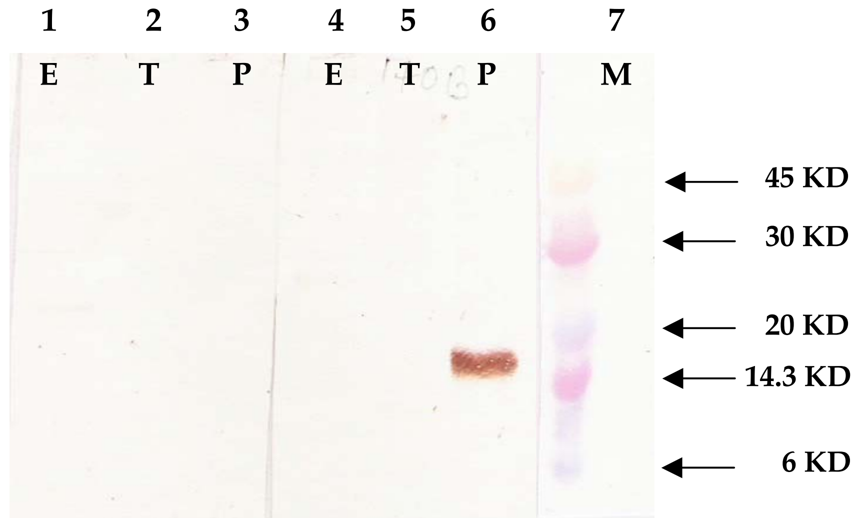

Fig. 117: Detection of β-MSP in rat epididymis (E), testes (T) and prostate (P). Lane 1-3 probed with pre-immune sera and lane 4-6 probed with antiserum to the peptide 1-17 of rat MSP. Lane M represents molecular weight markers.

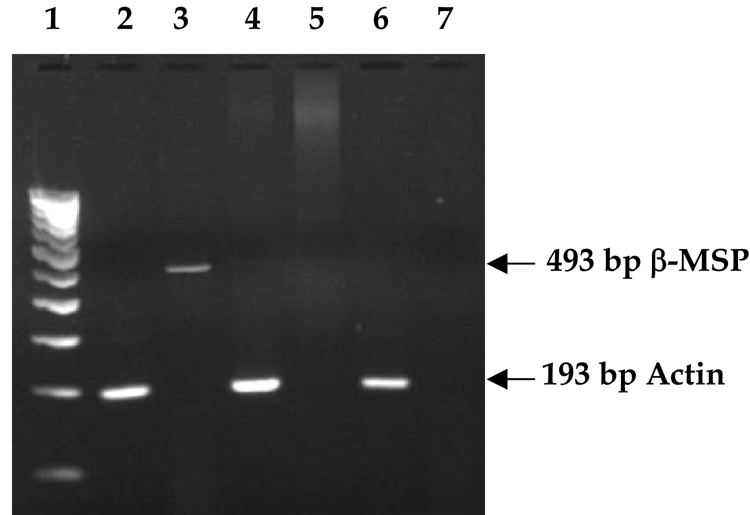

Fig. 118: β-MSP transcripts in rat tissues. Lane 1: 100 bp marker; Lanes 2 and 3: Prostate; Lanes 4 and 5: Testis and Lanes 6 and 7: Epididymis. Actin specific primers were used in Lanes 2, 4 and 6. β-MSP specific primers were used in Lanes 3, 5 and 7.