MATERIAL AND METHODS
3.1 MATERIAL

Unless otherwise mentioned, chemicals were obtained from Sigma Chemicals Co. (St. Louis, MO, USA). EAH-Sepharose-4B, Resource™-Q column and molecular weight markers were obtained from Pharmacia Biotech Inc. (Uppsala, Sweden). Protoblot Western blot system was purchased from Promega Corp. (Madison, WI, USA). EZ-Link™ Sulfo-NHS-LC-Biotin was purchased from Pierce (Rockford, IL, USA). Chemicals for RT-PCR were purchased from Perkin Elmer, USA. M-MuLV-RT was purchased from Promega Corp. USA. Fast Red™ substrate system was purchased from Dako, Denmark. Actin primer set was a kind gift from Dr. Chandrima Shaha, NII, New Delhi.

Restriction enzymes and other molecular biologicals were purchased from New England Biolabs (NEB, USA), Boehringer Mannheim (Germany) and Amersham Pharmacia Biotech (UK). Oligonucleotide primers were synthesized from MWG – Biotech GmbH (Germany) and Genset SA (France). γ-[32P]-ATP and α-[32P]-ATP were from Board of Radiation and Isotope Technology (BRIT), Bhabha Atomic Research Center (BARC), India. Nylon, nitrocellulose and PVDF membranes were from Amersham (UK).

[All the solutions were prepared in double distilled, Milli-Q water or DEPC H2O (in case of RNA work). Autoclaving was carried out at a pressure of 15 lbs/sq.in for 20 min unless it was for RNA, which was for 30 min].

3.2 METHODS

3.2.1 MAINTENANCE OF ANIMALS

Rats (Wistar and Sprague Dawley) were maintained in the animal house of Jawaharlal Nehru University, New Delhi, India and Charles River, Sulzfeld, Germany. Swiss Albino mice and New Zealand Rabbits were maintained in JNU animal house, New Delhi. The animals were fed ad libitum standard feed.

3.2.2 PROCESSING OF DIALYSIS TUBING

Convenient length of dialysis tubing were boiled in 1 mM EDTA, pH 8.0 for 10 min and thoroughly washed in distilled water and stored at 4°C for subsequent use.
3.2.3 PREPARATION OF HA-SEPHAROSE-4B AFFINITY MATRIX

Hyaluronic acid (HA) from human umbilical cord (grade 1) was coupled to EAH-Sepharose-4B beads by acid catalyzed condensation reaction using N-ethyl-N'- (3-dimethylaminopropyl) carbodiimide (EDC) as the coupling reagent. Here, EDC acts as a homo-bifunctional reactant, which conjugates the carboxy group of the ligand (HA), to amino group of the gel beads. Reaction was performed in distilled water adjusted to pH 4.5-6.0 for 12 h. For 20 ml of EAH-Sepharose-4B (7-10 μmole-NH₂/ml), 310 mg of EDC and 50 mg of HA were added and the pH was checked intermittently and kept between 4.5-6.0. The slurry was washed thoroughly with water and then alternatively with 0.2 M Glycine-HCl, pH 2.2 and 0.1 N NaHCO₃ containing 0.5 M NaCl. The activated Sepharose was blocked by 1M acetic acid, pH 4.0 for 4 h at 4°C under constant stirring condition and again washed as before. The amount of HA bound per ml of gel was estimated by carbazole test (Bitter and Muir, 1962) using glucuronic acid as standard. Column was packed with HA-Sepharose-4B matrix and equilibrated with 0.01 M phosphate buffered saline (PBS), pH 7.2.

3.2.4 PURIFICATION OF HYALURONAN-BINDING PROTEIN (HABP1)

3.2.4.1 Induction and expression of hyaluronan-binding protein (HABP1) in E.coli

Sequence analysis of hyaluronan-binding protein (HABP1) was reported earlier and found to be identical to that of p32, a protein co-purifying with the splicing factor SF2. P32 cDNA cloned in the bacterial expression vector pT7A.A32 was a kind gift from Dr. Adrian Krainer, CSHL, USA. P32 cDNA-plasmid construct expressing the 209 amino acids long mature p32 protein (Krainer et al., 1991; Honore et al., 1993) under the control of T7 promoter was expressed in BL21(DE3) according to Krainer et al (1991). Pre-inneculum of the BL21(DE3) transformed with expression plasmid (pT7A.A32) was grown overnight at 37°C in LB medium containing 50 μg/ml ampicillin, 0.2% glucose. 1% of the pre-inneculum was transferred to fresh LB medium containing 50 μg/ml ampicillin, 0.2% glucose and grown until OD₆₀₀ reached 1.0. Then 0.4 mM IPTG was added to the culture and the culture was grown at 37°C for the next 3h. The cells were pelleted by centrifugation.
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at 5,000 x g for 30 min at 4°C and either stored at -70°C or immediately processed for protein purification. Purification of rHABP1/p32 by HA-affinity column and further, its binding to biotinylated HA in vitro, confirmed the availability of functionally active rHABP1.

3.2.4.2 Purification of recombinant HABP1 using HA-Sepharose affinity

Purification of HABP1 was carried out according to the procedure described by Deb and Datta, 1996. Bacterial pellet obtained from 500 ml culture was washed with PBS and suspended in 15 ml PBS. Triton X-100 (0.01%, v/v) was added and left at room temperature for 45 min. Complete lysis was achieved by sonication (10-15 bursts each of 30 sec) on ice. Cellular debris was pelleted down and the supernatant was dialyzed against PBS and the volume made upto 50 ml with PBS and loaded onto the HA-Sepharose-4B column. After washing with 20 bed-volumes of PBS, pH 7.2 containing 0.15 M NaCl and then, with 20 bed-volumes of 0.01 M phosphate buffer, pH 7.2 containing 0.5 M NaCl; the column was eluted with 0.2 M Glycine-HCl, pH 2.2 at a flow rate of 20 ml/h, and the eluate was collected as 2 ml fractions. The protein-containing fractions were determined by measuring their absorbance at 280 nm. The peak fractions were pooled and concentrated. Whole purification process was carried out essentially at 4°C.

3.2.4.3 Purification of recombinant HABP1 using ion exchange chromatography (Resource™-Q column)

Induction, overexpression of HABP1 and lysis was done as described above. Cellular debris of 50 ml culture were pelleted down and the supernatant was dialyzed against 20 mM Tris-HCl, pH 8.5 (buffer A) and the volume made upto 25 ml with buffer A and loaded onto the Resource™-Q column (Pharmacia) at a rate of 0.5 ml/minute. Column was washed with buffer A containing 0.4 M NaCl until O.D_{280} reached to base line. Bound protein to Resource™-Q column was then eluted with buffer A containing 0.6 M and the eluate was dialyzed overnight against PBS at 4°C and stored.
3.2.4.4 Purification of HABP1 from normal rat kidney

Purification of HABP1 was carried out according to the procedure described by Gupta et al (1991a). Kidney, collected from normal adult rat, were washed in ice cold 0.01 M PBS, pH 7.2 containing 0.15 M NaCl and minced in two volumes of the same buffer. The homogenate was centrifuged at 12,000 x g at 4°C for 30 min. The pellet was homogenised in two volumes of 0.2 M Glycine-HCl, pH 2.2 and centrifuged at 35,500 x g at 4°C for 30 min. The supernatant was neutralised, dialysed against PBS and spun down to get a clear lysate. The clear lysate was loaded onto an HA-Sepharose-4B affinity column pre-equilibrated with PBS. After washing with 20 bed-volumes of 0.01 M phosphate buffer containing 0.15 M NaCl and then, with 20 bed-volumes of 0.01 M phosphate buffer, pH 7.2 containing 0.5 M NaCl; the column was eluted with 0.2 M Glycine-HCl, pH 2.2 at a flow rate of 20 ml/h, and the eluate was collected as 2 ml fractions. The protein-containing fractions were determined by measuring their absorbance at 280 nm. The peak fractions were pooled and concentrated. Whole purification process was carried out essentially at 4°C.

3.2.5 PROTEIN ESTIMATION

Protein content was estimated using either Lowry (Lowry et al., 1951) or Bicinchoninic acid (Smith et al., 1985) method, using bovine serum albumin as standard.

3.2.6 ANTIBODIES

Antibody against the tissue purified HABP1 (from rat kidney) were also raised in the rabbit. Tissue HABP1 (tHABP1) purified from the HA affinity column, was injected into the rabbit after checking its purity by SDS-PAGE. Normal rabbit serum was used as a control for anti-tHABP1 antibody (Gupta et al., 1991a).

Polyclonal anti-rHABP1 antibodies were raised against the purified recombinant HABP1 (rHABP1) in rabbit as mentioned in Deb and Datta (1996). Purified rHABP1 was separated on a preparative 12.5% SDS-PAGE and the denatured rHABP1 band was cut out from the gel after Coomassie Brilliant Blue (CBB) staining and injected into the rabbit after grinding it in PBS, pH 7.2. Pre-
immune serum of the same rabbit was used as the negative control for anti-rHABP1 antibody.

Affinity purified antibody was prepared using tHABP1 and anti-tHABP1 antiserum. Briefly, purified HA-binding protein was electrophoresed on a 12.5% SDS-polyacrylamide gel and transferred onto a nitrocellulose (NC) membrane. The membrane was stained with Ponceau S to visualize the proteins and a strip corresponding to 34 kDa was cut out. The strip was blocked in 2% BSA and incubated for 8 h at 4°C in the antiserum developed against HA-binding protein. The bound antibodies were eluted using 0.2 M Glycine-HCl, pH 2.2, neutralized and stored at -20°C till further use.

Polyclonal antibody against the proprotein form of HABP1 (termed as pAb 610, Ghebrehiwet et al., 1997) was raised in rabbits against a decapeptide 51 G S E R R P G L L R 60, present in that region of the proprotein form of HABP1, which is cleaved off to give rise to the mature protein. pAb 610 is a mono-specific polyclonal antibody and recognizes the above mentioned decapeptide in human HABP1 (above) and has homology with the rat HABP1 decapeptide (51 G S A R R S G L L Q 60). Normal Rabbit serum was used as a control for pAb 610 antibody. In this study, however, another lot of the anti-HABP1 proprotein antibody – ‘611’ (raised in another rabbit) has been used, although ‘610’ is being referred to always in this study since it is pAb ‘610’ antibody which has been reported in the literature (Ghebrehiwet et al., 1997). pAb 610 antibody was a kind gift from Dr. B. Ghebrehiwet (SUNY, Stony Brook, USA ) and Dr. EIB. Peerschke (Cornell University, USA).

Monoclonal antibodies (mAbs), 60.11 and 74.5.2, were directed against epitopes in the N-terminal and C-terminal of human HABP1, respectively. They were a kind gift from Dr. Ghebrehiwet of SUNY, New York. mAb 60.11 is directed against a stretch in mature gC1qR/HABP1 corresponding to amino acid residues 76-93. The 74.5.2 mAb is targeted against residues 204-218 in the same sequence (Ghebrehiwet et al., 1996).


3.2.9 **PURIFICATION OF IgG FROM SERUM USING PROTEIN A-SEPHAROSE BEADS**

IgG was purified from the serum using protein A beads, as described by Harlow and Lane (1988) with a slight modification. Anti-rHABP1 antiserum (1 ml of 1:10 dilution in PBS) was incubated with 40 mg of swelled protein A-sepharose beads, overnight at 4°C. Subsequent to this, the beads were loaded onto a tiny column made in a 1 ml syringe. The antiserum was collected and stored at -20°C for further use. The protein A beads were washed with >40 bed volume (~20 ml) of PBS, pH 7.2 and then the IgG bound to the beads was eluted in fractions of 200 µl each with Glycine-HCl, pH 2.2 for protein estimation was done. The eluted fractions and the protein containing fractions were pooled, dialyzed against PBS and stored at -20°C for further use. A small aliquot was resolved on SDS-PAGE (reducing) to check the purity the IgG eluted. The procedure was repeated for purifying higher amounts of IgG.

3.2.10 **SODIUM DODECYL SULFATE-POLYACRYLAMIDE GEL ELECTROPHORESIS (SDS-PAGE)**

Polyacrylamide gel electrophoresis under denaturing condition (in the presence of 0.1% SDS) was performed according to the method of Laemmli (1970). The protein was stacked at pH 6.8 in a stacking gel containing 4% acrylamide, 0.106% N, N'-methylen bisacrylamide, 0.125 M Tris-HCl, (pH 6.8), 0.01% TEMED and 0.1% APS. The running (separating) gel was made of 10% acrylamide, 0.27% N, N'-methylen bisacrylamide, 0.375 M Tris-HCl (pH 8.8), 0.01% TEMED and 0.1% APS. The protein samples were electrophoresed in running buffer consisting of 0.025 M Tris-base, 0.192 M glycine, pH 8.3 and 0.1% SDS. The protein samples were prepared in sample buffer [0.0625 M Tris-HCl, pH 6.8; 2% SDS, 10% glycerol, and with or without 5% β-mercaptoethanol; Laemmli (1970)] and immersed in a boiling water bath for 5 min. For the lysates prepared by the direct boiling method (in 0.5% SDS-PBS or 1x Laemmli buffer), the samples were loaded as such onto the gel. Standard molecular weight marker (Pharmacia) was also
3.2.7 BIOTINYLLATION OF rHABP1

Biotinylation of rHABP1 was carried out with EZ-link™ Sulfo-NHS-LC-Biotin (Pierce) according to manufacturer's protocol (modified from Suter and Butler, 1986). Briefly, 1 mg of rHABP1 in 0.5 ml of 50 mM sodium bicarbonate buffer, pH 7.5-8.5 in a microfuge tube. Just prior to use, 1 mg of Sulfo-NHS-Biotin was dissolved in 1 ml water and 37.5 µl of Sulfo-NHS-Biotin was added to rHABP1 solution. The microfuge was placed in ice and incubated for 2 hours. Unreacted biotin was removed by dialysis against PBS, pH 7.2. An aliquot of the biotinylated HABP1 was resolved on a 10% SDS-PAGE, transblotted to an NC membrane and probed with streptavidin-AP conjugate and developed with NBT/BCIP color system, to check the integrity of the biotinylated protein. Biotinylated protein was stored at -20°C until use.

3.2.8 SILVER STAINING OF SDS-PAGE GELS

SDS-PAGE gels were silver stained using the protocol described by Merril et al., 1984. Briefly, after the run was over, the gel was fixed for 2 x 30 min or overnight in ethanol (40%) and acetic acid (10%). After fixation, the gel was washed 2 x 10 min in Milli-Q water and then sensitized in sodium thiosulfate pentahydrate solution (0.3g/L) for 1 min. A brief wash of 2 x 1 min in Milli-Q water was given to the gel after sensitization, followed by a 30 min incubation in silver nitrate solution (2g/L silver nitrate and 250µl/L 37% formaldehyde), which was the crucial silver staining step. After staining, the gel was rinsed for about 10-20 sec in Milli-Q water and then the bands were visualized by developing with the developer (30g/L sodium carbonate, 250µl/L 37% formaldehyde and 10 mg/L sodium thiosulfate pentahydrate) till the desired intensity was reached, after which the gel was rinsed several times with Milli-Q water and then photographed. At no point of time, the gel was touched with bare hands or even with gloves. If required, the gel was held only at the corners.
3.2.9 PURIFICATION OF IgG FROM SERUM USING PROTEIN A-SEPHAROSE BEADS

IgG was purified from the serum using protein A beads, as described by Harlow and Lane (1988) with a slight modification. Anti-rHABP1 antiserum (1 ml of 1:10 dilution in PBS) was incubated with 40 mg of swelled protein A-sepharose beads, overnight at 4°C. Subsequent to this, the beads were loaded onto a tiny column made in a 1 ml syringe. The antiserum was collected and stored at -20°C for further use. The protein A beads were washed with >40 bed volume (~20 ml) of PBS, pH 7.2 and then the IgG bound to the beads was eluted in fractions of 200 μl each with Glycine-HCl, pH 2.2 for protein estimation was done. The eluted fractions and the protein containing fractions were pooled, dialyzed against PBS and stored at -20°C for further use. A small aliquot was resolved on SDS-PAGE (reducing) to check the purity the IgG eluted. The procedure was repeated for purifying higher amounts of IgG.

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electrophoresed simultaneously to calculate the subunit molecular size of the proteins.

3.2.11 WESTERN BLOT ANALYSIS

Proteins were separated by SDS-PAGE on 10% gels under reducing conditions and electroblotted onto a Hybond-C™ nitrocellulose membrane (Amersham, UK) in a buffer containing 0.025 M Tris, 0.192 M glycine, 0.037% SDS and 20% methanol, pH 8.3 following the procedure described by Towbin et al (1979). After blocking in 3% (w/v) BSA in Tris buffered saline (TBS - 0.05 M Tris-Cl, pH 7.6 containing 0.15 M NaCl) at room temperature, the blots were probed with primary antibody (depending on the experiment) for 1 h at room temperature. All dilutions were made in 1.5% (w/v) BSA-TBS. Membranes were then washed 5 x 5 min in TBS containing 0.05% (v/v) Tween-20 and further incubated with secondary antibody [depending on the experiment, it was goat-anti-rabbit IgG conjugated to alkaline phosphatase (AP) or goat-anti-mouse IgG conjugated to AP; 1:10,000 in 1.5% BSA-TBS] for 1 h at RT. The bound antibody was detected with the NitroBlue Tetrazolium (NBT) / 5-Bromo-4-Chloro-3- Indolyl Phosphate (BCIP) color system [10 μl of NBT (30 mg/ml) and 10 μl of BCIP (15 mg/ml in 10 ml of AP buffer (0.1 M Tris-HCl, pH 9.5, 0.1 M NaCl and 5 mM MgCl₂)] until the bands developed to desired intensity.

3.2.12 MEDIA FOR SPERM PREPARATION

3.2.12.1 Dulbecco's PBS (D-PBS), pH 2.5

Dulbecco's PBS (D-PBS) used contained per 100 ml: 0.8g NaCl, 0.02g KCl, 0.02 g KH₂PO₄, 0.01 g MgCl₂.6H₂O, 0.01g CaCl₂, 0.216 g Na₂HPO₄ .7H₂O. The pH was set to 2.5 using concentrated HCl.

3.2.12.2 Biggers-Whitten-Whittingham medium (BWW)

Biggers-Whitten-Whittingham medium (BWW) contains per 100 ml: 490.2 mg NaCl, 35.6 mg KCl, 16.2 mg KH₂PO₄, 29.4 mg MgSO₄, 2.8 mg sodium pyruvate, 100 mg dextrose, 300 mg NaHCO₃, 52.7 mg calcium lactate, 0.368 mg sodium lactate, 3 g BSA, 7.5 mg penicillin G, 7.5 mg streptomycin sulfate and 0.02% phenol red (Biggers et al. 1971). The pH was between 7.2 and 7.4.
Incomplete media with NaCl, KCl, KH$_2$PO$_4$ (not used in experiments involving the use of $[^{32}P]$), MgSO$_4$ and Phenol Red was prepared and stored at 4°C after autoclaving. The other components were added as and when it was needed to reconstitute the complete media.

### 3.2.13 ISOLATION OF RAT SPERM

Cauda epididymides were removed from adult male wistar rats after carefully removing both the connective tissue and the blood vessels. Spermatozoa from cauda were obtained by perfusion of the vas deferens with 0.01M PBS, pH 7.2. Tissue fragments were allowed to settle and the supernatant was centrifuged at 200 x g to separate spermatozoa from the fluid. Aliquots of the sperm pellets from the cauda epididymis were washed thrice with in PBS and finally suspended in the BWW medium with 3 mg/ml BSA (BWW-BSA medium) and incubated at 37°C for further experiments.

### 3.2.14 SEMEN COLLECTION PROCESSING AND HUMAN SPERM PREPARATION FOR COMPUTER-AIDED SPERM ANALYSIS (CASA)

Semen samples were collected from patients attending the Institute of Reproductive Medicine, Münster, Germany, for fertility problems. As a part of internal quality control scheme, semen samples reflecting all qualities presented (low concentration, poor morphology and poor motility) were used regularly for assessment by all the technicians in the laboratory to assess inter technician agreement. These samples were assessed according to the WHO (World Health Organization) manual (1992). Sperm kinematic parameter were assessed on an IVOS - Hamilton-Thorne automated semen analyses (Version/0.6, Beverly, MA)

Human semen samples were obtained after atleast 3 days of sexual abstinence by masturbation from patients and healthy donors of proven fertility. 3 ml 70% Percoll in a 15 ml tube was held at 45°C and 3 ml 35% Percoll was gently layered on top. 2 ml semen after liquefaction at 37°C was gently layered onto the Percoll gradient. The solution was centrifuged at 500 x g for 20 min at 25°C. Using a pipette, the remaining seminal plasma and the Percoll was discarded. The sperm pellet was washed with 5 ml BWW medium containing BSA (4 mg/ml) at 400 x g for 5 min at
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25°C. The sperm concentration and percentage motility was counted in a pre-warmed Makler chamber. The sperm concentration was adjusted 20 x 10⁶/ml and the tube was kept at 37°C with 5% CO₂, at an angle, until it was used for CASA.

3.2.14.1 CASA (Computer-Aided Sperm Analysis)

3.2.14.1.1 Treatments for CASA

Different samples for CASA were prepared with the following treatments: HA polysaccharide, HA disaccharide and HA monosaccharides; N-Acetyl Glucosamine and N-Glucuronic acid (all at concentrations 50 and 150 μg/ml) and anti-tissue HABPI IgG (affinity purified) & Rabbit IgG (at dilutions 1:20 and 1:50). Human sperms were incubated with the above mentioned reagents at 37°C, 5% CO₂. After 3, 6 and 22 h, 5 μl sample was drawn out after a gentle mixing (by tapping with a finger), put on a Makler chamber and recorded its motility for 3 min.

3.2.14.1.2 Video recording of sperm preparation

After 0, 3, 6 and 22 h incubation, sperm suspensions (5 μl) were placed in a 10 μm deep Makler Chamber (Sefi Medical Instruments, Israel) and examined in an Olympus BHS microscope equipped with a heated stage set at 37°C and a 10 x negative phase-contrast objective (Olympus AIONH) with 3.3 x photo-ocular, interfaced to a Sony CCD AVC-D7CE video camera (Sony Corp., Tokyo) and a monitor (Compaq Video graphics Monitor, Compaq computer Corp., Houston, TX). At least ten fields were recorded onto VHS videotapes for 10 sec per field. The percentage of motile spermatozoa in a sample was determined using the video pause function, the tape was then allowed to run and the sperm that remained in their original position were counted as immotile or as non-progressing cells, if flagellating. Whenever possible, 200 sperm were counted per sample.

The following set up was used for human sperm analysis: frame rate - 25 Hz; Sort C (track points 13-25, VAP 5-500). Frames acquired - 25; minimum contrast - 50; minimum size - 4; threshold STR-80; medium VAP- 25; low VAP -5; non-motile head-6; non-motile intensity - 80. The optics were calibrated with magnification 1.56 (with Sony camera, 10 x objective, 3.3 x photo-ocular) and illumination intensity Ca 2000.
3.2.14.1.3  **Statistical analysis**

The samples were compared by one way repeated analysis of variance (one way RM ANOVA) in Sigma Stat version 2.03. The graphs were plotted in Sigma Plot version 6.00.

3.2.15  **CAPACITATION OF RAT SPERM AND INDUCTION OF ACROSOME REACTION**

Spermatozoa from cauda epididymis were obtained by perfusion of the vas deferens in BWW-BSA and the contents in the BWW-BSA medium were allowed to stand at room temperature (37°C) for 5 min, for the debris to settle to the bottom of the tube. The supernatant containing sperms was gently aspirated into another clean 10 ml tube and centrifuged at 200 x g for 5 min. After aspirating the supernatant, fresh medium was gently added onto the sperm button and incubated at 37°C for 30 min. Medium gently aspirated by this swim-up method contained motile capacitated spermatozoa.

Acrosome reaction was induced in capacitated spermatozoa using progesterone (Margalioth et al., 1988; Osman et al., 1989). One mg of progesterone was dissolved in 100 μl of DMSO and diluted with 0.9 ml of phosphate buffered saline (PBS). The rat spermatozoa (1 x 10⁶ cells) were incubated with progesterone, at the final concentration of 5 μg/ml at 37°C for 30 min.

3.2.16  **SUPEROVULATION OF MICE, OOCYTE RETREIVAL AND PREPARATION OF SOLUBILISED ZONA PELLUCIDA**

Swiss Albino female mice were injected intraperitoneally with 5 IU of pregnant mare serum gonadotropin (PMSG) and then with 5 IU human chorionic gonadotropin (hCG) 48h later. 16-18 h later, oocytes were collected by washing the oviducts with BWW-BSA medium. Cumuli surrounding the oocytes were dispersed by addition of 0.1% hyaluronidase. After 3 washes in PBS, the oocytes were placed in Dulbecco’s PBS (D-PBS, pH 2.5) till the whole zona pellucida solubilized. After that, the whole suspension was spun down and the supernatant, which contained the solubilized zona proteins was taken, immediately neutralized with dilute NaOH and stored at −20°C until use.
3.2.17 METABOLIC LABELLING OF PROTEINS IN RAT CAUDA SPERMATOZOA AND IMMUNOPRECIPITATION OF HABP1

The contents of cauda epididymides - from mature male wistar rats were released into phosphate free BWW-BSA medium. 1 x 10^6 intact spermatozoa were incubated with 1 mCi of γ-[AT^{32}P] in 1 ml phosphate-free BWW-BSA medium for 20 min at 37°C. After that progesterone (5μg/ml) and hyaluronic acid (250 μg/ml) were added separately and the sperms were further incubated for 15 min. Subsequent to this, sperms were pelleted down and the supernatant was kept aside for immunoprecipitation, while the sperm pellets were rinsed with ice-cold PBS and solubilized with radiimmune precipitation assay (RIPA, 0.15M NaCl, 50 mM Tris-HCl, pH 7.6, 10 mM sodium pyrophosphate, 100 mM sodium fluoride, 100 μM sodium orthovanadate, 1 mM PMSF, 50 μg/ml Aprotinin. 4 mM EDTA, 0.5% Na-deoxycholate, 0.1% SDS and 0.1% Triton X-100) buffer according to the method of Sefton et al (1978) with minor modifications. The cell lysates were centrifuged at 15,000 x g for 3 min at 4°C. The initial supernatant and also the supernatant after cell lysis were incubated with 1.5 mg of swollen protein A sepharose-4B conjugated to anti-tHABP1 antibody (Ranganathan et al., 1994) overnight at 4°C with continuous rocking. Precipitate was washed thoroughly with RIPA buffer and boiled in 1 x Laemmli sample buffer. Electrophoresis was carried out with these samples at 80 V in 10% gel in the presence of SDS. For visualizing the labeled protein, the gels were stained with CBB, dried and exposed to X-ray film for autoradiography at -70°C.

3.2.18 TISSUES AND LYSATE PREPARATION

Testis, spleen and liver were collected from rats, after killing them by CO₂ asphyxiation or anaesthetizing them with chloroform. For immunohistochemical studies, organs were fixed by immersion in Bouin's fixative [75% (v/v) water saturated picric acid, 20% (v/v) formaldehyde (37%) and 5% (v/v) acetic acid] for 16-18 h and then washed immediately in 70% alcohol to get rid of excess fixative. After thorough washing, the tissues were processed further by dehydrating though graded alcohol (absolute, 96%, 80%, 70 % and 50%) and xylene and were finally embedded in paraffin wax. Subsequent to this, using a microtome, 5 μm sections
were prepared for immunohistochemical analysis. For the initial experiments of SDS-polyacrylamide gel electrophoresis (SDS-PAGE) and western blotting, the testis lysate was prepared by immediate boiling of the seminiferous tubules directly in 1 x Laemmli Buffer with and without β-mercaptoethanol (Laemmli, 1970). For comparative analysis of various tissues, lysates were prepared in 0.5% (w/v) SDS-PBS, pH 7.2, as described. To a few testicular tubuli and to a piece each of spleen and liver, 1 ml 0.5% SDS-PBS was added and the tissues were kept in a boiling water bath for 10 min, after which the tissues and the tubuli were crushed manually using the Dounce homogenisor probe and then boiled further for 20 min. Subsequently, the lysates were centrifuged at 12,000 x g for 15 min at room temperature and the supernatant, which contained the lysate proteins, was stored at -20°C until use. For a few experiments, the lysate was also prepared in RIPA buffer with EDTA (1 mM), DTT (1 mM) and PMSF (1 mM). The stocks were diluted 20 fold before protein estimation.

**3.2.19 IMMUNOHISTOCHEMISTRY**

5 μm sections were deparaffinised, rehydrated and the non-specific antibody binding was blocked with 3 % (w/v) BSA in TBS for 1 h at room temperature. After 5 washes of 5 min each in TBS, the slides were incubated with anti-rHABP1 antibody (1:250) or pAb 610 antibody (1:50) for 1 h at room temperature. All dilutions were made in 1.5 % (w/v) BSA-TBS. Sections were washed (5 x 5 min in TBS) and incubated with goat anti-rabbit IgG conjugated to AP (1:200 in 1.5 % BSA-TBS) for 1h at RT. Substrate addition followed 5 x 5 min washes with TBS. Fast Red™ substrate (Dako, Denmark), prepared from ready-made tablets (as per instructions) was used to develop the color. After the development of the color to desired intensity, the sections were washed in TBS, and counter-stained in Haematoxylin, and mounted in Glycerol (E-Merck). At no point during the whole procedure, the sections were allowed to remain dry.

Identification of specific germ cell types was performed according to the criteria of Leblond and Clermont (1952) as described by Hess et al (1990).
3.2.20 SOLUTIONS USED

(1) **TES Buffer for plasmid isolation**: 25 mM Tris-HCl (pH 7.5), 10 mM EDTA (pH 8.0) and 15% Sucrose

(2) **Denaturing solution in midi-scale DNA plasmid preparation**: Freshly prepared solution containing 0.2 N NaOH and 1% SDS

(3) **1 x TAE buffer**: 40 mM Tris-acetate, 1 mM EDTA

(4) **DNA gel loading buffer**: 0.25% (w/v) bromophenol blue, 0.25% (w/v) xylene cyanol FF and 15% (w/v) Ficoll Type 400

(5) **CBS buffer for RNA isolation**: 4 M guanidine thiocyanate (GTC), 0.025 M sodium citrate pH 7.0; 0.5% N-lauryl sarcosine and 0.2 mM β-mercaptoethanol

(6) **Formaldehyde gel running buffer**: MOPS -20 mM 3-(N-morpholino) propane sulfonic acid (pH 7.0), 8 mM sodium acetate and 1 mM EDTA (pH 8.0)

(7) **Formaldehyde gel loading buffer**: 50% (w/v) glycerol, 1 mM EDTA, pH 8.0, 0.25% Xylene Cyanol FF and 0.25% bromophenol blue

(8) **1x STE in in situ hybridisation**: 150 mM NaCl, 2.5 mM Tris, 0.25 mM EDTA

(9) **Pre-hybridization solution for Southern blotting**: 7% SDS, 50 mM Sodium phosphate buffer (pH 7.0), 2% blocking, 50 μg/ml ss DNA, 5 x SSC and 0.1% N-Lauryl Sarcosine

(10) **Digestion/Extraction buffer for genomic DNA isolation**: 100 mM NaCl, 0.01 mm Tris-Cl (pH 8), 0.025 M EDTA (pH 8), 0.5% SDS and 0.1 mg/ml Proteinase K

(11) **T_{10}E_{1**:} 10 mM Tris-HCl (pH 7.5), 1 mm EDTA.

3.2.21 DESIGNING OF PRIMERS TO BE USED IN VARIOUS EXPERIMENTS

Primers were designed in such a way that they generate a fragment of not more than 600 bp for the RT experiments. Also, The GC content was kept in mind and 40-50% was taken as the minimum requirement. After designing, the sequences were fed one by one into a package called “Oligo” to calculate the Tm of the primer and to examine if the primer designed was free from the tendency to form primer dimers, especially those extending up to the 3’ end. ‘Oligo’ also demonstrated if the
Primer had a tendency to have pairing of complementary bases within itself and if so, then was the 3' end free in that case.

Primer sets used in the study for various experiments are as follows:

**HABP1 cDNA specific primer sets:**

1. S1-S2
   - S1 (forward primer): 5' GGAGATATACATATGCACACCGAC 3'
   - S2 (reverse primer): 5' GCAGCCGGATCCTGTAAACTC 3'
   (Amplifies a 1039 bp DNA fragment from pT7A.A32 plasmid)

2. RAB3-RAB4
   - RAB3 (forward primer): 5' CCGGCCCTTCGGTTTGCTCAGCGT 3'
   - RAB4 (reverse primer): 5' GGCCCAGTCCAGGGAGTCTGTGTT 3'
   (Amplifies a 583 bp DNA fragment from rat and human HABP1 mRNA)

3. HABP1-HABP2
   - HABP1 (forward primer): 5' CTCCCTAAGATGTCTGGAGGTTGGG 3'
   - HABP2 (reverse primer): 5' CCAAACCCTGCCATGAAACTATGG 3'
   (Amplifies a 588 bp DNA fragment from human HABP1 mRNA)

**Primer sets used as controls:**

1. HMEx7-HMREx10 [specific for Follicular Stimulating Hormone Receptor (FSH-R) mRNA]
   - HMEx7 (forward primer): 5' CAAGAAATACACAACTGTGC 3'
   - HMREx10 (forward primer): 5' CCCATGATATCTTCACATGG 3'
   (Amplifies a 500 bp DNA fragment from human and mouse FSH-Receptor mRNA)

2. Actin I–Actin II (specific for β-actin mRNA)
   - Actin I (forward primer): 5' CGTGCGCCGCCCTAGGCACCA 3'
   - Actin II (reverse primer): 5' TTGGCCTTACGGGCTAGGGGGG 3'
   (Amplifies a ~250 bp DNA fragment from rat β-actin mRNA)

cDNA corresponding to both the full length (282 amino acids, proprotein form) and the mature (209 amino acids formed after cleavage of 73 amino acid residues from the N-terminal region) HABP1 have been used in this study. The full
length HABPI cDNA contains only the full ORF region of HABPI cDNA (without the UTRs) and has been referred to as the ‘full length HABPI cDNA’ in the whole study. While the HABPI cDNA corresponding to the mature form of HABPI is being referred to as just ‘HABPI cDNA’. ‘HABPI cDNA’ contains the portion of the mature protein (see asterisk in Sequence 1) along with the 3' UTR of the HABPI cDNA also (see Sequence I)].

The position of the various primers have been highlighted in Sequence I and the position of the HABPI cDNA specific primers in the HABPI gene (in different exons) is depicted in Scheme III.

3.2.22 DNA AND RNA ESTIMATION

DNA and RNA were estimated spectrophotometrically as described in Sambrook et al (1989).

3.2.22.1 Analysis of DNA

3.2.22.1.1 Isolation of DNA from mammalian cells

The tissue after isolation was washed twice with ice-cold PBS (sterile) and immediately frozen at −70°C until use. The frozen tissue was then ground to powder form in a sterile pestle-mortar and to the powder 1 ml of Digestion/Extraction buffer was added and the mixture was kept at 50°C for 12-18 h with gentle shaking. After an overnight digestion, the sample was thoroughly extracted with an equal volume of phenol/chloroform/isoamyl alcohol (25/24/1: v/v/v). The sample was then centrifuged for 15 min at 4,000 x g at RT. After transferring the aqueous phase to a sterile microfuge, 20 μg/ml of RNase A was added and kept at 37°C for 30 min (use water-bath). Phenol/chloroform/isoamyl alcohol extraction was repeated once again and then again to the aqueous phase, 0.5 vol. of 7.5 M ammonium acetate and 2 vol. of 100% absolute alcohol (EtOH) were added. The DNA immediately formed a stringy precipitate. The precipitated DNA was recovered by centrifugation at 4,000 x g for min at RT. The DNA pellet was rinsed twice with 70% ethanol. Later, ethanol was decanted the pellet was air dried until the last visible traces of ethanol had evaporated. The pellet was not allowed to dry completely. The DNA was resuspended in 1 ml T10E 1 buffer (pH 8) until dissolved. DNA was occasionally
Sequence I. Homology of rat and human HABP1 cDNA and protein sequences. Primer binding sites are highlighted in both sequences. Similarity of the dicapeptide sequence of human HABP1 against which pAb 610 antibody is directed is highlighted as "GSERRPGLLR" along with the rat homologous sequence "GSARRSGQLQ". The start and stop sites are also highlighted in grey. Asterisk (*) represents the beginning of the mature protein. Human HABP1 protein sequence is written in • whereas the rat sequence is written in •. Homologous bases are written in • and the non-similar ones are shown in •.
Scheme III: Primer position in different exons of the HABP1 gene. Exons are shown in ◆ and marked as E and introns are shown in ○ and marked as I. The grey boxes represent the 3' and 5' UTR regions.
shaken gently at RT or at 65°C for several hours to facilitate solubilization. DNA was then stored at 4°C.

### 3.2.22.1.2 Plasmid DNA isolation

#### 3.2.22.1.2.1 Mini Scale Plasmid DNA isolation

Mini scale plasmid DNA isolation was done according to Sambrook et al (1989) with a modification. Each bacterial colony was transferred into 1 ml LB containing 30 μg/ml kanamycin (or an appropriate antibiotic as per the plasmid used) in 15 ml loosely capped tube and incubated for 4 h at 37°C with vigorous shaking. Each 1 ml culture was poured in a microfuge tube, centrifuged at 10,000 x g for 30 min at 4°C and the medium was removed by aspiration, leaving the bacteria pellet as dry as possible.

500 μl bacterial cell culture containing the desired plasmid was mixed with 500 μl of phenol/chloroform/isoamyl alcohol mixture (25:24:1) and vortexed at full speed for 10 seconds. It was then centrifuged for 10 minutes at 10,000 x g at RT. The supernatant was collected (~450 μl) without disturbing the interface and to it 500 μl isopropanol was added, mixed thoroughly and centrifuged at 12,000 x g for 15 minutes. The pellet, thus, obtained was washed with 80% ethanol, dried and dissolved in 20 μl T_{10}E_{1}.

#### 3.2.22.1.2.2 Midi Scale Plasmid DNA isolation by alkaline lysis method

(Birnboim and Doly, 1979)

Cells were grown overnight in 50 ml LB medium containing ampicillin (50 μg/ml) or kanamycin (30 μg/ml) and collected by centrifugation (5,000 x g at 4°C for 15 min). The cell pellet was resuspended in 1.5 ml of TES Buffer containing 2 mg/ml lysozyme, and kept on ice for 10 min. The lysed cells were subjected to 3 ml of denaturing solution on ice for 10 min. The chromosomal DNA-Protein complex was selectively removed by incubating with 1.6 ml of 3 M sodium acetate (pH 4.6) for 20 min on ice followed by centrifugation at 12,000 x g for 20 min. The RNA was digested by treating with 5 μl of RNase A for 45 min at 37°C. The crude preparation was further purified by extracting twice with an equal volume of phenol/chloroform/isoamyl alcohol [25/24/1, (v/v/v)] and once with equal volume of chloroform/
isoamyl alcohol [24/1, (v/v)]. The crude plasmid DNA was precipitated by the addition of three volumes of pre-chilled ethanol, and the precipitate collected by centrifugation at 12,000 x g for 30 min at 4°C. The pellet was resuspended in 0.4 ml of nuclease-free water and 0.12 ml of 4 M NaCl. DNA was precipitated with 0.5 ml of 13% polyethylene glycol (average Mol. Wt. 8000), and collected by centrifugation at 12,000 x g for 10 min at room temperature. The pellet was washed with 70% (v/v) ethanol, and dissolved in 50 μl of T<sub>10</sub>E<sub>1</sub>.

3.2.22.1.3 Agarose Gel Electrophoresis for DNA

Agarose gel electrophoresis was performed as described by Sambrook et al (1989). For DNA samples, the required amount of agarose was melted by heating in 1 x TAE buffer, cooled to 55°C and ethidium bromide (0.5 μg/ml) was added prior to the casting of the gel on the gel tray. One-sixth volume of DNA gel loading buffer was mixed with samples and loaded in the wells. The electrophoresis was performed at 5 V/cm in 1 x TAE buffer and the DNA fragments were visualized on a UV transilluminator at 302 nm.

3.2.22.1.4 Southern Blot Analysis (Southern, 1975)

The RT-PCR products were resolved on agarose gels as described before. The fragments were transferred from agarose gels onto a nylon membranes (Hybond-N<sup>TM</sup>) as described. The gel was removed from the tray and placed in a dish containing 0.25 N HCl for 15 min with intermittent shaking to depurinate the DNA. After depurination, the DNA was denatured by incubation in 0.4 N NaOH containing 0.6 M NaCl with two changes for 30 min. The gel was then neutralized with 0.5 M Tris-Cl, pH 7.5 containing 1.5 M NaCl for 30 min with 2 changes. The treated gel was placed on four sheets of Whatman 3 MM papers saturated with 10 x SSC [1.5 M NaCl and 0.15 sodium citrate (pH 7.0)]. A sheet of Hybond-N<sup>TM</sup> presoaked in 10 x SSC was placed on the gel taking care that no air bubble(s) got trapped between the gel and the membrane. This was followed by two sheets of Whatman 3 MM paper presoaked in 10 x SSC and a thick stack of dry absorbent paper (all cut to the size of the gel). Finally, a weight was placed over this assembly to keep the papers firmly pressed on the gel. After 16-20 h, the nylon membrane was
carefully dislodged from the dry gel after marking the position of the wells and immersed in a 0.4 N NaOH solution for 45 sec to ensure complete denaturing of immobilized DNA. The membrane was transferred to a neutralizing solution (2 x SSC and 0.2 M Tris-Cl, pH 7.5) for 1 min and then UV cross-linked.

3.2.2.2 ANALYSIS OF RNA

Glassware used for RNA isolation and analysis were baked at 200°C for 16-18 h. Plasticware were treated with 3.0% (w/v) H₂O₂ for 2h and later autoclaved at 15 lb/sq. in for 30 min. All the solutions (except Tris buffer) were prepared in DEPC water. DEPC was dissolved (0.1%, v/v) in Milli-Q water with vigorous stirring for 2h followed by an overnight incubation at 37°C and then autoclaving.

3.2.2.2.1 RNA isolation

Total RNA was isolated from adult rat testis, spleen and liver by the guanidinium thiocyanate-β-mercaptoethanol-phenol-chloroform extraction method (Doyle, 1996).

Freshly isolated tissues (1 g) were suspended in 12 ml CBS buffer and homogenised for 1 min in a glass teflon homogenisor with 2 ml of 2M sodium acetate (pH 4.0), 10 ml DEPC H₂O saturated phenol and 2 ml of isoamyl alcohol (49:1, v/v) followed by mixing the tube for 10 seconds. The phases were separated by centrifugation at 10,000 x g for 20 min at 4°C. The crude RNA was recovered from the aqueous phase by precipitating with 10 ml of isopropanol at 4°C overnight, followed by centrifugation of 10,000 x g for 20 min at 4°C. The pellet, after a wash with 70% (v/v) ethanol in DEPC H₂O was resuspended in CBS buffer with Guanidine thiocyanate (GTC) and the RNA was re-precipitated at -70°C for 1 h with using 1/10 volume 3 M sodium acetate, pH 5.2 and 2 volumes cold absolute ethanol, followed by washing with 70% (v/v) ethanol. The purified RNA preparation was briefly dried and resuspended in DEPC H₂O.

Total RNA was also isolated from the testis of developing animals using a commercial reagent TRIZOL® (Life Technologies, UK) according to the manufacturer's protocol.

RNA from human testis was isolated from frozen tissue using RNAZOL™
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(Cinna/Biotecx), a reagent similar to TRIZOL®. Both RNAZOL™ and TRIZOL® work on the modified protocol as described by Chomczynski and Sacchi (1989).

**3.2.22.2 Fractionation of RNA by Agarose Gel Electrophoresis** (Sambrook et al., 1989)

The agarose gels were prepared in formaldehyde gel running buffer and electrophoresed without sample for 5 min at 5 V/cm. ~20 μg of RNA was denatured (Vr-15 μl) in the presence of 50% (v/v) deionized formamide (pH 4.0), 2.2 M formaldehyde and 1 x formaldehyde running buffer at 65°C for 15 min, chilled on ice and 5 μl of DEPC treated formaldehyde gel loading buffer was added. The RNA samples were loaded onto the gel and electrophoresed in 1x formaldehyde gel running buffer at 3 V/cm for 3 h. The gel while setting itself contained ethidium bromide (0.4 μg/ml).

**3.2.22.3 Northern Blot Analysis**

The formaldehyde gel was rinsed with several changes of DEPC H2O to remove formaldehyde. RNA was then transferred from the gel to the nylon membrane essentially as described for Southern blotting. The blots after the transfer were UV-crosslinked.

**3.2.22.3 HYBRIDIZATION OF RADIOLABELLED PROBES TO IMMOBILIZED NUCLEIC ACIDS**

Preparation of radiolabelled DNA by random priming method (Feinberg and Vogelstein, 1983).

About 50-100 ng of HABP1 cDNA was denatured by heating in a boiling water bath for 10 min and immediately chilled on ice. To the tube containing denatured DNA, 2 μl each of 0.5 mM dGTP, dCTP and dTTP, 5 μl of hexanucleotide labelling mixture (containing random hexamers and reaction buffer at 10 x concentration (2M HEPES, pH 6.6, 2 mM Tris-Cl (pH 7.0), 0.1 mM EDTA and 4 mg/ml (BSA), 30-50 μCi α-[ 32 P] -dATP (3000 Ci/mMol, 10 μCi/μl]) and nuclease free water to make up the volume to 50 μl. The reaction was initiated by adding 5 units of Klenow enzyme. Incubation was carried out for 2-3 h at 37°C before stopping by addition of EDTA (to a final concentration of 20 mM). The
unincorporated dNTPs were removed by ethanol precipitation in the presence of 2.5 M ammonium acetate and absolute alcohol.

Hybridization of radiolabelled probe to DNA immobilized on Hybond-N\textsuperscript{TM} nylon membrane DNA and RNA blots were first incubated in pre-hybridization solution at 65\degree C in hybridization bottles. After 2-3 h heat denatured radiolabelled probe (2-5 x 10\textsuperscript{5} cpmlml) and 100 \mu g/ml denatured Salmon sperm DNA was added to the prehybridization mix. The hybridization was carried out at 65\degree C for 16-18 h. The membrane were washed sequentially to remove the non-specifically bound probe using the following protocol: twice with 2 x SSC containing 0.1% SDS at room temperature for 5 min each and twice with 0.5 x SSC containing 0.1% SDS at 65\degree C for 15 min each.

3.2.22.4 REMOVAL OF BOUND PROBE FROM NYLON MEMBRANE FOR REHYBRIDIZATION

The probes were removed by washing the membranes in a solution containing 0.1 x SSC, 0.1% SDS at 15 min at 100\degree C with several changes, before hybridization.

3.2.22.5 AUTORADIOGRAPHY

After hybridization and washing, the blots were wrapped in a thin plastic film and exposed with intensifying screens in cassettes at -70\degree C for an appropriate amount of time.

3.2.22.6 REVERSE TRANSCRIPTION - POLYMERASE CHAIN REACTION (RT-PCR)

2 \mu g of total RNA from the rat tissues was used as a template for RT-PCR. Single tube RT-PCR was done using rTth enzyme (Perkin Elmer, USA). The reaction volume (25 \mu l) included 2 \mu g of total RNA, 0.2 Units rTth enzyme, 1x EZ\textsuperscript{TM} buffer, 5 Units RNase inhibitor, 2.5 mM manganese acetate solution, 50 pmol of each primer and 300 \mu M of each dNTP. HABP1 cDNA served as a positive control and a negative control for RT-PCR with all components except template DNA was also run in parallel. An RT-PCR using actin primers was also done as a control for the quantity and integrity of the RNA samples used in the reaction. RT-
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PCR was done at 60°C for 30 min followed by the PCR profile as follows: for the first round, initial denaturation at 94°C for 7 min, followed by 39 cycles of 1 min denaturation at 94°C; and 1 min annealing and extension at 60°C. Final annealing and extension was performed at 60°C for 7 min. The PCR amplified product was resolved on a 1.5% agarose gel containing 0.4 µg/ml ethidium bromide with a 1-Kb DNA ladder run as a reference for fragment size. An image of the gel was recorded with a CCD camera and stored in digitized form. The gel after image capture was treated for transfer onto a nylon membrane (Hybond-N™) to be processed further for Southern hybridization. Southern hybridization using α-[32P] labeled HABP1 cDNA probe was done in aqueous medium at 65°C.

Human RNA samples were reverse transcribed by M-MuLV RT. Briefly, 5 µg of total RNA was taken in a microfuge tube and to that was added 200 pmoles of primer to be used as the reverse primer (Oligo dT, HABP2 or HMREx10, as the case may be) for the first strand cDNA synthesis reaction. 2.5 mM dNTP mix containing all the four dNTPs, 2 µl of M-MuLV reverse transcriptase buffer [50 mM Tris-HCl (pH 8.3), 75 mM KCl, 3 mM MgCl₂, 10 mM DTT], 1 µl RNAsin (ribonuclease inhibitor) and 1 µl of M-MuLV RT (50 U/µl). The reaction mix without RNAsin was incubated at 65°C for 5 min, followed by incubation at 37° for 30 min after the addition of RNAsin. After the reaction, the mix was immediately chilled in ice and the mixture was then used for a typical PCR by adding 100 pmoles of the second primer. PCR was performed as described in the next section.

3.2.22.7 POLYMERASE CHAIN REACTION (PCR)

Sense (forward) and antisense (reverse) oligonucleotide primers flanking the desired region to be amplified were used for PCR. DNA was amplified in a total volume of 100 µl consisting of 50 mM KCl, 10 mM Tris-Cl (pH 8.3), 1.6 mM MgCl₂, 200 µM of each dNTPs, 50 pmoles of each primer, 100 pmoles of template DNA and 5 units of Taq DNA polymerase. The PCR cycle comprised of annealing 5-10 degrees below the Tm of the primers used (59°C for HABP1-HABP2 and HMEx7-HMREx10 primer sets; 60°C for RAB3-RAB4 and Actin I-Actin II primer set and 52°C for S1-S2 primer set) for 2 min, extension at 72°C for 1 min and
denaturation at 94°C for 1 min. The last extension was done for additional 10 min. The amplification reaction was carried out in a DNA thermal cycler [MJ Industries, USA] after overlaying the samples with mineral oil to prevent evaporation (used generally for $V_f < 25 \mu l$). The sample containing all reagents except template DNA served as the negative control for PCR. The size and integrity of products was checked by electrophoresing 10 μl of the sample on a 1% agarose gel at 5V/cm for an appropriate time period.

3.2.22.8 OVEREXPRESSION OF FULL LENGTH HABP1 (COMPRISING OF 282 AMINO ACIDS)

3.2.22.8.1 Isolation of the full length cDNA insert

Full length cDNA insert (corresponding to 282 amino acids) was taken out from the vector pCIHA1 (a kind gift from Dr. T. Muta, Kyushu University, Japan; TM Muta et al., 1997) to be cloned into pET30c vector (Novagen, USA) for over expression of full length HABP1.

3.2.22.8.2 Restriction digestion of pCIHA1 and pET30c with EcoRI and NotI

The purified full length cDNA insert was cloned into the digested and purified pET30c vector (Novagen, USA) according to Sambrook et al (1989).

Two separate digestions were put for pET30c and pCIHA1 vectors. Briefly, in a 1.5 ml microfuge tube, 40 μl of digestion mixture containing 15 μg of the vector, 4 μl EcoRI buffer (10 mM Tris-HCl, 10 mM MgCl2, 50 mM NaCl, 1 mM DTT, pH 7.9), 2 μl BSA (1mg/ml) and 0.5 μl each of EcoRI and NotI (each stock -20 U/μl) and 13.8 μl of Milli-Q water were mixed and incubated at 37°C for 3 h. The digestion was monitored by loading 2 μl of digestion mixture on a 1% agarose gel. After complete digestion, the digested plasmid was kept at 4°C and later the required band was excised from a preparative agarose gel, and the DNA was eluted out and purified.

3.2.22.8.3 Purification of DNA from agarose gels by freeze-fracture method

Low melting agarose (LMP-Agarose) was used when the DNA was required to be gel purified. LMP-agarose was prepared the same way as the normal melting agarose (as described earlier), except that the casting was done at 4°C. After
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electrophoresis, DNA was visualized on a UV transilluminator. Agarose blocks containing the desired DNA fragments were cut out and put in a preweighed microfuge tube. The agarose containing the insert was mixed with 2-3 volumes of T₁₀ E₄ and then incubated at 70-80°C for 15 min and vortexed vigorously. The tube was frozen in an isopropanol slush for 5 min and then thawed at room temperature. This step was repeated thrice. The agarose was then pelleted at 12,000 x g, 20 min at 4°C. The supernatant containing the DNA was extracted once with Phenol/chloroform/isoamyl alcohol [25/24/1, (v/v/v)] and once with chloroform/isoamyl alcohol [24/1, (v/v)] and precipitated with alcohol as described earlier.

3.2.22.8.4 CIAP treatment of pET30c

Digested vector pET30c was treated with CIAP (Calf Intestinal Alkaline Phosphatase, NEB) in the same buffer in which the enzymatic digestions with EcoR I-Not I were performed.

3.2.22.8.5 Ligation of cDNA insert with EcoR I-Not I digested pET30c

The EcoR I-Not I digested pET30c was ligated to the full length cDNA insert (got out by EcoR I-Not I digestion from pCIHA1) as follows: In a microfuge tube (V₉ = 10 μl), 100 ng digested pET30c vector, 500 ng cDNA insert, 1 μl 10 x T4 DNA ligase buffer (200 mM Tris-HCl, pH 7.6, 50 mM MgCl₂, 50 mM DTT, 500 μg/ml BSA), and 1 μl of bacteriophage T4 DNA ligase (6 Weiss unit/μl) were mixed and left overnight at 16°C.

3.2.22.8.6 Preparation of competent cells and Transformation (Hanahan, 1985) of ligated mix in E.Coli

E.coli [DH5α and BL21(DE3)] were grown in 50 ml LB until the optical density at 600 nm reached 0.38-0.42. Cells were transferred to a pre-chilled 500 ml flask. After 15 min of vigorous shaking in ice water, the cells were collected by centrifugation at 5,000 x g for 5 min at 4°C and were resuspended in 20 ml of pre-chilled 0.1 M CaCl₂. After 15 min on ice with occasional shaking, the cells were collected again by centrifugation. The cell pellet was finally resuspended in 2 ml of ice cold 1M CaCl₂. The competent cells were stored as 15% glycerol stocks in small
Scheme IV. Cloning strategy for overexpression of protein form of HABP1

Scheme V. Cloning strategy of HABP1 cDNA in pGEM T-Easy for riboprobe preparation for *in situ* hybridisation.
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Aliquots at \(-70^\circ C\) after flash freezing in liquid nitrogen. All the steps were performed in the laminar flow.

Competent cells (100 \(\mu l\)) were incubated with 5-10 ng of the plasmid DNA (after ligation). The cells were then kept in ice for 45 min and were subjected to heat shock at \(42^\circ C\) for 45 sec, diluted in LB medium (0.9 ml) and incubated for 1 h at \(37^\circ C\) with shaking at 225 rpm. The transformants were selected on a LB plate supplemented with kanamycin (60 \(\mu g/ml\)) and then the selected colonies were picked, grown and the plasmid DNA from them was isolated to confirm the positive clones containing the recombinant pET30c.282HABP1 plasmid by mini and midi prep isolation. The strategy of cloning of pET30c.282HABP1 is shown in Scheme IV.

3.2.22.8.7 Confirmation of recombinant pET30c.282HABP1 by restriction enzyme digestion

Purified recombinant pET30c.282HABP1 plasmid was digested with \(BamHI\). Briefly, in a 1.5 ml microfuge tube, 20 \(\mu l\) of digestion mixture containing 1\(\mu g\) of the vector, 2 \(\mu l\) \(BamHI\) buffer (10 mM Tris-HCl, 10 mM MgCl\(_2\), 50 mM NaCl, 1 mM DTT, pH 7.9), 2 \(\mu l\) BSA (1mg/ml) and 0.5 \(\mu l\) each of EcoRI and NotI (each stock -20 U/\(\mu l\)) and 13.8 \(\mu l\) of Milli-Q water were mixed and incubated at \(37^\circ C\) for 3 h. The digestion was monitored by loading 2 \(\mu l\) of digestion mixture on a 1% agarose gel. After digestion, the digested plasmid was kept at 4°C and later the required band was excised from a preparative agarose gel, and the DNA was eluted out and purified.

3.2.22.8.8 Induction and expression of pET30c.282HABP1 in E.coli

pET30c.282HABP1 under T7 promoter was expressed in BL21(DE3). Pre-innoculum of the BL21(DE3) transformed with expression plasmid pET30c.282HABP1 was grown overnight at \(37^\circ C\) in LB medium containing 30 \(\mu g/ml\) kanamycin. 1\% of the pre-innoculum was transferred to fresh LB medium containing 30 \(\mu g/ml\) kanamycin, and grown until \(OD_{600}\) reached 0.6-0.8. Then 1.0 mM IPTG was added to the culture and the culture was grown at \(37^\circ C\) for the next
3h. The cells were pelleted by centrifugation at 5,000 x g for 30 min at 4°C and either stored at −70°C or immediately processed for protein purification.

3.2.8.23 \textbf{IN SITU HYBRIDIZATION}

3.2.8.23.1 \textit{Ligation of the PCR amplified 1 Kb HABP1 cDNA in pGEM\textsuperscript{®}–T-Easy vector}

PCR cloning vector pGEM\textsuperscript{®}–T-Easy (Promega Corp., USA) was used for cloning the PCR amplified 1 Kb HABP1 cDNA fragment, which was amplified using S1-S2 primer set. 17 ng of amplified HABP1 cDNA insert and 50 ng of pGEM\textsuperscript{®}–T-Easy vector were taken and a ligation was set up as described earlier for pET30c and full length HABP1 cDNA. A blank ligation was set up with just 50 ng of pGEM\textsuperscript{®}–T-Easy vector and no insert.

3.2.8.23.2 \textit{Transformation}

The ligated product of pGEM\textsuperscript{®}–T-Easy vector and HABP1 cDNA insert was transformed into JM109 competent cells (provided with the pGEM\textsuperscript{®}–T-Easy vector system II) using the routine transformation protocol described by Hanahan (1985) and plated onto LB-Amp (100 μg/ml) plates coated with X-gal/IPTG for blue-white colony screening (Horwitz et al., 1964).

3.2.8.23.3 \textit{Confirmation of positive colonies by PCR method}

Few white (positive for insert isolation) and some blue (as a negative control) colonies were picked up and grown in LB medium. Multiple clone test was done by PCR method. Briefly, each clone was grown in 50-100 μl LB-Amp medium for 3-4 h after which to 5 μl of the bacterial suspension, 15 μl of sterile distilled water was added and the whole set up was kept at 98°C for 5 min for lysing the bacterial cells. From the lysed suspension, 5 μl was picked up and used as a template for setting up a PCR using S1-S2 primer set. After the PCR, the samples were run in a 1% agarose gel and one confirmed positive colony was selected for the preparation of riboprobes for \textit{in situ} hybridization.

A part of the remaining bacterial suspension (from which 5 μl was used for the multiple clone test) was inoculated into 2 x 20 ml LB-Amp medium for isolating
the recombinant clone (pGEM.HABP1) in larger quantities (as described earlier in Medium scale plasmid DNA preparation) for further studies.

3.2.8.23.4 Linearising circular plasmids and purification of linearised plasmids

To 14 µg of circular recombinant clone (pGEM.HABP1), 20 U of *Nco* I was added and kept at 37°C for 2 h and an aliquot of it was run on 1% agarose gel to check for complete linearisation. To another aliquot of 14 µg of pGEM.HABP1, 20 U of *Sal* I was added and kept overnight at 37°C for complete digestion, as checked by running an aliquot on a 1% agarose gel. After complete digestion with both the enzymes, the linearised pGEM.HABP1 plasmid were purified using high pure PCR product purification kit (Boehringer Mannheim, Germany).

3.2.8.23.5 Checking for the orientation of the HABP1 insert in recombinant pGEM.HABP1 plasmid

2 µg of pGEM-HABP1 plasmid was digested with 10U of *Sac* I for 2h at 37°C and the size of the fragment generated was estimated after running an aliquot of the digested sample in 1% agarose gel and the orientation of the cloning was worked out. The whole strategy for the cloning of pGEM.HABP1 is shown in Scheme V.

3.2.8.23.6 Digoxigenin (DIG)-labelled riboprobe preparation by In vitro transcription

DIG riboprobes were prepared using the *in vitro* transcription kit from Promega, USA. Labelled sense and antisense cRNA (riboprobes) were synthesized by incubating the *Nco* I and *Sal* I linearised template (4 µg) with DIG-labelled UTP (DIG-11-dUTP, 3.5 mmol/litre) (Boehringer Mannheim) in the presence of SP6 or T7 RNA polymerase respectively, for 2 h at 37°C. After synthesis, the amount of DIG-labelled RNA was determined by comparison with a DIG-labelled DNA standards (Boehringer Mannheim) by dot blot analysis and the quality of the riboprobes was checked by running an RNA gel for these riboprobes, transferring them onto a nylon membrane and probing it with the DIG high prime DNA labeling and detection starter kit II (Boehringer Mannheim, Germany).
3.2.8.23.7 \textit{In Situ Hybridization}

The expression of HABP1 mRNA within the rat testis was determined by hybridization with DIG-labelled antisense cRNA probes (riboprobes). For \textit{in situ} hybridization, sections were deparaffinised using xylene and rehydrated though graded alcohols. All the dilutions were made in DEPC H$_2$O. To increase probe accessibility, sections were treated with 0.2 N HCl for 20 min at room temperature and partially digested with proteinase K (at 2 mg/ml in 50 mM Tris/HCl pH 8.0, 5 mM EDTA pH 8.0) for 20 min at 37°C. After incubation with 0.2% glycine (in DEPC H$_2$O) for 10 min at 4°C, sections were treated with 0.1M triethanolamine (pH 8.0) and then acetylated in 0.25% acetic anhydride and prehybridized for a minimum of 2 h at 37°C. Prehybridization solution was composed of 6 x STE, 1 x Denhardt's solution, 50% deionised formamide, 125µg/ml salmon sperm DNA, and 125 µg/ml yeast tRNA. Hybridization was performed overnight at 37°C in hybridization buffer (prehybridization buffer plus 10% dextran sulphate; Pharmacia Biotech) containing 200 ng/ml of DIG-labelled probe. Excess and mismatched probe was removed by treating sections with 20 µg/ml RNase A (ICN) (in 10 mM Tris/HCl pH 8.0, 0.5 M NaCl, 5 mM EDTA) at 37°C for 30 min, followed by sequential 2 x 15-min washes in 2 x SSC solution to a maximum stringency of 0.1 x SSC at 32°C for 15 min. Hybridized probe was localized on tissue sections using an anti-DIG alkaline phosphatase-conjugated antibody (diluted 1:330) (Boehringer Mannheim). Excess antibody was removed by washing in TBS, pH 7.6. Nonspecific antibody binding was minimized by preincubating sections in 1:5 dilution of sheep serum in TBS. The tissue mRNA:DIG-cRNA dimers were visualized by an enzyme catalyzed color reaction using NBT/BCIP ready-made tablets, Boehringer Mannheim. Sections were counter-stained with Haematoxylin and mounted for viewing under the microscope.