CHAPTER-II

Stage specific expression of the proprotein form of HABP1 in testis
5.1 INTRODUCTION

It is well established that HA is present in reproductive tract fluids and we have reported the presence of HABP1 on the spermatozoa of various species and demonstrated its role in fertilization (Ranganathan et al., 1994). We have also shown enhanced phosphorylation of HABP1 in motile sperm (Ranganathan et al., 1994) and confirmed its role in HA-induced signaling in spermatozoa (Ranganathan et al., 1995). In continuation, we have established to some extent the role of HABP1 in a few more sperm functions, as discussed in the previous chapter.

Spermatogenesis, the most complex differentiation process in higher eukaryotes, comprises differentiation of haploid spermatozoa from testicular stem cells. The entire process depends upon the precise and ordered expression of many genes that are unique to spermatogenesis. The transcripts for most sperm proteins are synthesized just before meiosis, since the chromatin is in a decondensed state, while protein synthesis occurs at different time intervals after meiosis (Kleene, 1996). Transcription terminates early during spermatogenesis, and this necessitates an extensive utilization of post-transcriptional processes for gene expression (Hecht, 1998, Lattermann and Muller, 1987). The involvement of certain genes in spermatogenesis and their biosynthesis in cellular differentiation has been reported, though not fully understood. In this regard, presence of HABP1 in testicular lysate and mature spermatozoa has also been shown (Ranganathan et al., 1994), suggested looking at the localization of HABP1 in testicular cells and its role, if any, in germ cell differentiation.

The synthesis of HABP1 as a proprotein and its conversion to the mature form provides a unique opportunity to study the regulation and expression of a protein that is post-translationally modified and thus, study was initiated on HABP1 gene expression in the testis during spermatogenesis.
5.2 RESULTS

5.2.1 Presence of a 55 kDa protein in the testis, immunologically related to the 34 kDa HABP1

The testis lysate used in this experiment was prepared by boiling the seminiferous tubules directly in 1x Laemmli buffer (as mentioned in Material and Methods). Testis lysate (10 µl), resolved on a 10% SDS-PAGE and transferred onto a nitrocellulose membrane was probed with anti-rHABP1 antibody. Anti-rHABP1 antibody detected a strong 55 kDa protein in the testis lysate (Fig. 13 A, lane 2), indicating the existence of a protein of 55 kDa in the testis, which is immunologically related to the mature 34 kDa HABP1.

5.2.2 Possibility that the 55 kDa protein is the proprotein form of HABP1

In order to explore the possibility of the 55 kDa proteins being the proprotein form of HABP1, the same lysate (as used in Fig. 13A) was probed in parallel with anti-proprotein antibody (pAb 610 antibody). This antibody raised against the decapptide is present in that portion of the proprotein, which is cleaved to generate the mature form. Thus, this antibody can detect only the proprotein form, and hence it is being termed as anti-proprotein antibody. Confirmation of the cross-reactivity of anti-human HABP1 proprotein antibody with its rat counterpart was observed after immunoblotting with pAb 610 antibody. The detection of the 55 kDa band in the lysate suggested the identity of the 55 kDa as the proprotein form of HABP1 (Fig. 13B, lane 1). The pAb 610 antibody did not detect the 34 kDa rHABP1 (Fig 13B, lane 2), which corresponds to the mature form of HABP1 formed after cleavage of the first 73 amino acid residues of the proprotein; since the epitope for the pAb 610 antibody lies within the first 73 amino acids. Detection of the 55 kDa protein by the anti-proprotein antibody suggests that the 55 kDa protein - which is also detected by the antibody against rHABP1 - may be the proprotein form of HABP1.

Control blots were developed with pre-immune serum as the primary antibody and also with the secondary antibody directly, to rule out the possibility of the 55kDa band getting detected as a result of non-specific interactions from the rabbit serum and the secondary conjugate, and they were always negative.
Fig. 13 IMMUNODETECTION OF A HIGHER 55 kDa PROTEIN IN THE ADULT RAT TESTIS. A. Using antibody against recombinant HABPI (rHABPI). Lane 2 shows the testis lysate (lane 2), while purified rHABPI is seen in lane 1. B. Using pAb 610 antibody, an antibody specific for a decapeptide in the proprotein region of HABPI. Testis lysate (lane 1) and purified rHABPI (lane 2) were probed with pAb 610 antibody.
5.2.3 Presence of HABP1 proprotein specifically in the testis

In order to determine if the 55 kDa protein was seen only in testis or in other somatic tissues too, a comparative immunoblotting was done with testis, liver and spleen lysates. Lysates used in immunoblotting of different tissues were prepared by boiling seminiferous tubules (from the testis) and a piece each of spleen and liver in 0.5% SDS-PBS. Liver, spleen and testis lysates (90 μg) were resolved on a 10% SDS-PAGE, transferred onto a nitrocellulose membrane and processed for western blotting using anti-rHABP1 antibody. As shown in Fig. 14A (single arrowhead), the 34 kDa HABP1 band was seen in testis (lane 2), spleen (lane 3) and liver (lane 4) lysates. Besides the 34 kDa band, the 55 kDa protein was also detected (lane 2, double arrowhead), but only in the testis lysate. Purified 34 kDa rHABP1, used as a positive control for the blotting, was also detected as seen in lane 1.

The possibility of the 55 kDa protein being the HABP1 proprotein and the presence of this protein only in the testis, necessitated performing immunoblot of the various tissues by probing it with anti-proprotein antibody. Immunoblotting of testis, spleen and liver lysates with pAb 610 antibody confirmed the specific detection of the 55 kDa in the testis (Fig.14B, lane 2, and single arrowhead). No protein of 55 kDa was detected in spleen (lane 3) or liver (lane 4) lysates, a profile similar to the immunoblot of these tissues with anti-rHABP1 antibody (Fig.14A). This antibody did not to detect the purified mature rHABP1 (Fig. 14B, lane 1). Besides the detection of the 55 kDa protein in the testis lysate, a protein of 67 kDa (lane 2, double arrowhead) was occasionally seen. The 67 kDa band was, however, not seen so strongly when testis lysate prepared in 1 x Laemmli buffer was used. The exact identity of this protein still needs to be studied.

These data suggest that both anti-rHABP1 and the pAb 610 antibodies detect the proprotein form of 55 kDa only in the testis through immunoblotting.

5.2.4 Enhanced HABP1 mRNA expression in the testis

Detection of the 55 kDa protein specific to the testis and the immunological identity of this protein with HABP1, encouraged us to study the expression of HABP1 mRNA in these tissues. Equal quantities of total RNA from testis, liver and
Fig 14. PRESENCE OF THE 55 kDa PROTEIN SPECIFICALLY IN TESTIS. A. Immunoblot analysis of the rat testis, spleen and liver lysates with anti-rHABP1 antibody. Samples (lane 1, purified rHABP1; lane 2, testis lysate; lane 3, spleen lysate and lane 4, liver lysate) were resolved on a 10% SDS-PAGE and probed with anti-rHABP1 antibody, goat-anti-rabbit IgG conjugated to AP and NBT/BCIP color system. B. Immunoblotting of rat spleen, liver and testis lysates with pAb 610 antibody. The same samples as in Fig. 14A (lane 1, purified rHABP1; lane 2, testis lysate; lane 3, spleen lysate and lane 4, liver lysate) were resolved on a 10% SDS-PAGE and probed with pAb 610 antibody, goat-anti-rabbit IgG conjugated to AP and NBT/BCIP color system.
spleen were used for RT-PCR with HABP1 cDNA-specific primer set, RAB3-RAB4. RT-PCR yielded the expected band of 583 bp and the data (Fig. 15A, single arrowhead) showed higher HABP1 mRNA levels in testis (lane 3) in comparison to liver (lane 1) and spleen (lane 2). β-Actin gene expression (Fig. 15A, open arrow) was used as an internal control for the quality and the quantity of RNA isolated from liver (lane 4), spleen (lane 5) and testis (lane 6). Full length HABP1 cDNA, used as a positive control, is shown in lane 7. The RT-PCR products after transfer to a nylon membrane were processed for Southern blotting to confirm the authenticity of the amplified product. The blot was probed with [32P]-labelled HABP1 cDNA. As shown in Fig. 15B, the band amplified in the RT-PCR is strongest in the testis (lane 3) compared to the RT-PCR products of spleen (lane 2) and liver (lane 1), thereby confirming the result of higher HABP1 mRNA expression in testis obtained in the RT-PCR. It is important to note here that another band of ~500 bp (Fig 15A, double arrowhead) was seen in the RT-PCR gel and was also detected in Southern hybridization along with the 583 bp fragment from HABP1 cDNA. This band of ~500 bp appears to be a product of genomic DNA amplification and this will be discussed later in details in the next chapter.

Thus, the high level of HABP1 mRNA in the testis, together with the specific detection of the 55 kDa protein by the antibody against the proprotein form in the testis, further supports the identity of this protein as the proprotein form of HABP1, the primary translation product of the HABP1 mRNA.

5.2.5 Immunohistochemical analysis showing the specific expression of HABP1 proprotein in the testis

Absence of the 55 kDa HABP1 proprotein in spleen and liver tissues in comparison to testis, prompted examination of the immunohistochemistry of these tissues with both anti-rHABP1 and pAb 610 antibodies. Immunohistochemistry of all the three tissues was done simultaneously. While immunohistochemistry with anti-rHABP1 antibody gave no staining in either liver (Fig. 16A) or spleen (Fig. 16B), an intense staining was observed in the testis section (Fig. 16C). Testis sections treated with pre-immune serum were negative (Fig. 16D). The pAb 610 antibody too gave
Fig 15. EVIDENCE FOR ENHANCED mRNA EXPRESSION OF HABPI BY RT-PCR. A. Agarose gel analysis of the amplified products of RT-PCR shows that the intensity of the fragments (amplified from HABPI mRNA after RT-PCR) in testis (lane 3), in spleen (lane 2) and liver (lane 1). Equal quantity of RNA used is evident from the β-actin gene expression seen for testis (lane 6), for spleen (lane 5) and liver (lane 4). HABPI cDNA, amplified by PCR is in lane 7. Lane 8 shows the 1 Kb DNA ladder as the marker. Negative control for PCR was run in lane 9. B. Southern Hybridisation data for the RT-PCR products done using α-[ATP]-labelled–HABPI cDNA as the probe. Lane 1, liver HABPI; lane 2, spleen HABPI; lane 3, testis HABPI; lane 4, liver β-actin; lane 5, spleen β-actin; lane 6, testis β-actin and lane 7, HABPI cDNA. The intense signal from HABPI cDNA has masked lane 8 (of 1 Kb ladder).
Fig. 16. IMMUNOHISTOCHEMISTRY OF RAT LIVER, SPLEEN, AND TESTIS SECTIONS WITH ANTI-rHABP1 ANTIBODY. Rat liver (Panel A), spleen (Panel B) and testis (Panel C) were stained with anti-rHABP1 antibody, goat anti-rabbit IgG conjugated to AP and Fast Red™ substrate. A testis section stained with pre-immune serum as the control is seen in D. Magnification used is 100 x.

Fig. 17. CONFIRMATION OF THE PRESENCE OF HABP1 PROPROTEIN IN RAT TESTIS WITH pAb 610 ANTIBODY. Rat liver (Panel A), spleen (Panel B) and testis (Panel C) were stained with pAb 610 antibody, goat anti-rabbit IgG conjugated to AP and Fast Red™ substrate. Testis section stained with normal rabbit serum as the control is seen in D. Magnification used for C is 400 x, while the rest are taken at 100x.
Fig. 18. HABPI PROPRTIEIN LOCALISATION DURING VARIOUS STAGES OF SPERMATOGENESE AS DETECTED WITH ANTIBODIES AGAINST rHABPI (A—C) AND AGAINST THE PROPRTIEIN FORM OF HABPI (D—H). Spermatogenic stages VIII & IV (Panel A), X (Panel B) and XII (Panel C) stained with anti-rHABP1 antibody are shown, along with the spermatogenic stages II & III (Panel D), X (Panel E), XI & XII (Panel F), XIV-I (Panel G) and VII (Panel H), stained with anti-proprotein antibody are shown.
the same staining pattern (Figs. 17A-D) as the anti-rHABP1 (Figs. 16A-D) antibody, with positive staining only in the testis (Fig. 17C). Normal rabbit serum control was negative (Fig. 17D).

Since the results suggested that both anti-rHABP1 and pAb 610 proprotein antibody reacted with the 55 kDa proprotein and both revealed the presence of HABP1 proprotein only in the testis, a detailed analysis of the staining with these two antibodies was mandatory to check if they both give the same staining profile.

5.2.6 Stage-specific expression of HABP1 proprotein in the seminiferous tubules: Comparative analysis of the staining of the testis with anti-rHABP1 and pAb 610 antibody

Close analysis of the stained testis sections was done to check if the staining for HABP1 proprotein follows any specific pattern with reference to the different stages of spermatogenesis.

Immunohistochemical analysis of testis section was performed using antibody against rHABP1 (Fig. 18A-C) and the pAb 610 antibody (Fig. 18D-H). As seen with anti-rHABP1 antibody, only the tubules are stained (Fig. 18A, single black asterisk) and no staining is seen in the interstitium (black double asterisk). The spermatogonia are never stained (Fig. 18A, black double asterisk; Fig. 18B, back double asterisk). The cytoplasm of the round spermatids was stained intensely in stage IV but the HABP1 proprotein staining was not associated with the cytoplasm of sperm about to be released in stage VIII (Fig. 18A, black arrows). The pachytene spermatocytes showed increased intensity of proprotein staining as stage X approached (Fig. 18B). The cytoplasmic blebs of stage X showed very high levels of this protein (Fig. 18B, white single asterisks), but this was lost as it was ultimately pinched off from the elongating spermatid as residual body. In stage XII (Fig. 18C), the proprotein had already left the cytoplasm of the more elongated spermatids (black arrows), while the large diplotene spermatocytes still showed strong expression for the HABP1 proprotein. There was minimal HABP1 proprotein associated with developing acrosome when in spermatogenic stage X (Fig. 18B) or more elongated stage XII (Fig. 18C), as seen with anti-rHABP1 antibody.
The pattern of staining with pAb 610 was similar to that found with anti-rHABP1 antibody. There was cytoplasmic staining of the post-meiotic germ cells (round spermatids) and pachytene spermatocytes (Figs. 18E,F,G), acrosomal granule (Fig. 18D) and cap-phase (Fig. 18H) round spermatids but not spermatogonia (Figs. 18D,E,F,G,H, back double asterisks). There was also little staining of proprotein within the cytoplasm of the elongating spermatids (Figs. 18G,H, black arrows) and at the time of sperm release there was no proprotein in the remaining cytoplasm of the cells or in the residual bodies (Fig. 18H, black arrowheads).

In addition to the cytoplasmic staining of the HABP1 proprotein, stage-specific staining with the pAb 610 antibody was also observed at an additional location, namely, that around the developing acrosome. Intense staining was first observed from its appearance as an acrosomal granule in stages II-III (Fig. 18D) and maintained till stage VII (Fig. 18H). By focusing through the sections, it first became evident that the proprotein was located around the acrosome, rather than within it, in stages VII-VIII (Fig. 18H) and this was more obvious when the acrosomal shape changed to the spade-like forms in stage X, when the proprotein was apparent as a punctate form on the outer edge of the acrosome (Fig. 18E). In later spermatogenic stages (XI-XII), when the elongating spermatids approached the base of the seminiferous epithelium, the proprotein was also aligned along the length of the acrosome appearing as glove-like “fingers” outlining each acrosomal profile (Fig. 18F). Even later (stages XIV-I), it was only associated with the tip of the sperm head (arrows, Fig. 18G). By the time of sperm release, no signal of HABP1 proprotein was visible in the remaining cytoplasm of the spermatids or the residual bodies (Fig. 18H).

Further, another detailed study was further carried out to analyse the expression of proprotein form of HABP1 detected by anti-rHABP1 antibody during all the stages of spermatogenesis (Fig. 19A-H). The results obtained substantiated the observation made from the previous data (Fig. 18) and the complete analysis revealed an extremely defined expression of the HABP1 proprotein during spermatogenesis.
Fig. 19. DIFFERENTIAL IMMUNOHISTOCHEMICAL LOCALIZATION OF rHABPI PROPROTEIN IN VARIOUS STAGES OF THE ADULT RAT TESTIS USING ANTI-rHABPI ANTIBODY. The figure shows the expression of proprotein in various stages in a sequence: Spermatogenic stage III-IV (Panel A), VIII (Panel B), X. (Panel C), XII (Panel D) and XIV-I (Panel E). Panel F shows the differential staining pattern of three tubules with three different stages of spermatogenesis, namely VIII, IX-X and III-IV.
Anti-recombinant HABP Ab, 1 : 250

A III - IV

B VIII

C X

D XII

E XIV - I

F III - IV

Anti-recombinant HABP Ab, 1 : 250
Using the data available from the staining with both anti-rHABP1 antibody and 610 pAb antibody (Fig. 18 & 19), a scheme outlining the temporal and spatial expression of the proprotein form of HABP1 during spermatogenesis, is represented in Fig. 20.

5.2.7 Presence of the HABP1 proprotein in developing rat testis

After detecting the high expression of HABP1 proprotein in the adult rat testis, study on its expression in the developing rat testis was initiated.

Western blot analysis of the testis of developing (7, 14, 21 and 28 days old) and adult rat testis (as a control) was performed on the lysates prepared in 0.5% SDS-PBS using anti-rHABP1 antibody.

As shown in Fig. 21, 55 kDa HABP1 proprotein presence is clearly visible in the testis lysates of all ages (7 day, lane 1; 14 day, lane 2; 21 days, lane 3; 28 days, lane 4 and adult testis, lane 5).

5.2.8 HABP1 mRNA expression in developing rat testis

Total RNA isolated from testes of rats aged 7, 14, 21 and 28 days along with the adult rat testis, was set for an RT-PCR after quantitation. 2 μg of total RNA was used as a template for RT with RAB4 as the reverse primer and PCR was performed with RAB3-RAB4 primer set. β-actin mRNA expression was used as an internal control for the integrity and quantity of RNA used.

Fig. 22A shows the results obtained after RT-PCR analysis. HABP1 mRNA expression is seen getting amplified as a faint band 583 bp after RT-PCR in 7 (lane 2), 14 (lane 3), 21 (lane 4), 28 (lane 5) days and adult (lane 6) rat testes, although not very clear. The size matches with the expected reverse transcription product, but the faint band seen cannot differentiate between the levels of expression. Lane 1 represents the full length HABP1 cDNA used as a template and this served as a positive control. Uniform β-actin expression was seen in all the lanes (Fig. 22B, lanes 2-5, with a lower expression seen in the adult rat testis, suggesting, either a lower quantity of RNA used or a mild degradation of the RNA sample from adult testis. Single arrowhead shows the β-actin product amplified (~350 bp) from the β-
Stage of spermatogenic cycle

Fig. 20. SCHEME SHOWING THE EXPRESSION OF THE PROPROMTEIN FORM OF HABP1 DURING THE 14 STAGES OF SPERMATOGONESIS IN THE RAT TESTIS. Cytoplasmic staining of pachytene spermatocytes and spermatids by anti-rHABP1 and pAb 610 antibodies (red) and additional staining over the acrosome by the anti-proprotein antibody (blue).
Fig. 21. PRESENCE OF HABPI PROPORTEIN IN DEVELOPING RAT TESTIS. Lysates from 7 (lane 2), 14 (lane 1), 21 (lane 3), 28 (lane 4) day old and adult rat (lane 5; as a control) testes were prepared in 0.5% SDS-PBS and 90 µg of each was resolved on a 10% SDS-PAGE (non reducing), electroblotted on an NC membrane and probed with anti-rHABP1 antibody, goat anti-rabbit IgG -AP conjugate and NBT/BCIP color system.
Fig. 22. HABP1 mRNA EXPRESSION IN DEVELOPING RAT TESTIS. A. Agarose gel analysis of the amplified products of RT-PCR from RNA of 7 (lane 2), 14 (lane 3), 21 (lane 4), 28 (lane 5) day old and rat adult (lane 6) testes. PCR of HABP1 cDNA served as a positive control for PCR (lane 1). B. β-actin mRNA expression of 7 (lane 2), 14 (lane 3), 21 (lane 4), 28 (lane 5) day old and adult (lane 6) testes served as a internal control for the integrity and quantity of RNA used. Lane 1 is the 1 Kb DNA ladder used as a reference. C. Southern hybridisation of samples (in Panel A) using [32P] labelled HABP1 cDNA. RNA from 7 (lane 1), 14 (lane 2), 21 (lane 3), 28 (lane 4) day old and adult (lane 5) testes after RT-PCR were resolved on a agarose gel (Panel A) transferred onto a nylon membrane and processed for southern blotting.
actin gene. while double arrowhead is the RT-PCR product coming from actin mRNA (~250 bp). Lane 1 shows the 1 kb DNA ladder as a reference.

The RT-PCR amplified products were processed for Southern hybridization using [³²P]-labeled HABP1 cDNA probe to confirm the authenticity of the products. Fig. 22C shows the autoradiogram after Southern hybridization of the RT-PCR products (shown in Fig. 22A). As is clearly shown, lanes 2 and 5 show a higher hybridization signal as compared to the other lanes. Lane 2 corresponds to 14 day old testis while lane 5 is the lane for adult rat testis. Signal of other ages is not as high as that seen for 14 day and adult rat testis; 7 day (lane 1), 21 day (lane 3) and 28 day (lane 4). The lane corresponding to the HABP1 full length cDNA (Fig. 22A, lane 1) was not transferred, in order to avoid masking of the adjacent lanes (as seen with the masking of the 1 kb ladder in Fig. 15B). This data, thus, suggests a very high expression of HABP1 mRNA at 14 day and in adult rat testis.

5.2.9 Immunohistochemical localization of HABP1 proprotein in developing rat testis

The sections of rat testes from different age groups (7, 14, 21 and 28 days old) were stained with anti-rHABP1 antibody to detect the HABP1 proprotein expression. As shown in Fig. 23C, the gonocytes ('G') and the Leydig cells ('Le') showed positive cytoplasmic staining for the HABP1 proprotein in the 7 day old rat testis. A higher magnification picture of a gonocytes (arrowhead, 'G') stained, is shown in Fig. 23D. Pre-immune serum control of 7 day old testis is shown in Fig. 23E.

Fig. 24C shows the 14 day old testis section stained with anti-rHABP1 antibody. At 14 days, practically no staining was visible in any of the cell types. Few early spermatocytes ‘ES’ (arrowhead) shown had no staining. Fig. 24D is the pre-immune serum control for the 14 day old testis.

A marked change in the expression of HABP1 proprotein was observed in the 21 day old testis. As shown in Fig. 25A, intense positive cytoplasmic staining in the spermatocytes ‘SC’ was seen. Fig. 25B shows a higher magnification of one of the tubules shown in Fig. 25A. One may note that only the spermatocytes are stained.
Fig. 23. IMMUNOHISTOCHEMICAL LOCALIZATION OF HABPI IN THE TESTIS OF 7 DAY OLD RAT. 7 day old testis sections were stained with anti-tHABPI antibody (1:50) [Panel A] and anti-rHABPI antibody (1:250) [Panel C]. Normal rabbit serum control of anti-tHABPI antibody (1:50) [Panel B] and pre-immune serum control for anti-rHABPI antibody (1:250) [Panel E] are also shown. A higher magnification picture of a single gonocyte ('G') after staining anti-rHABPI antibody is seen in Panel D.
Anti-tissue HABP Ab, 1:50

Normal Rabbit Serum, 1:50

Anti-recombinant HABP Ab, 1:250

Preimmune Sera, 1:250
Fig. 24. IMMUNOHISTOCHEMICAL LOCALIZATION OF HABP1 IN THE TESTIS OF 14 DAY OLD RAT. 14 day old testis sections were stained with anti-tHABP1 antibody (1:50) [Panel A] and anti-rHABP1 antibody (1:250) [Panel C]. Normal rabbit serum control of anti-tHABP1 antibodies (1:50) [Panel B] and pre-immune serum control for anti-rHABP1 antibody (1:250) [Panel D] are also shown. The sections were probed with the goat anti-rabbit IgG conjugated to AP and developed with Fast Red™ substrate.
Fig. 25. PRESENCE OF HABPI PROPROTEIN IN THE TESTIS OF 21 DAY OLD RAT. HABPI proprotein expression in 21 day old testis as seen with anti-rHABPI antibody (1:250) in the spermatocytes ('SC') [Panel A]. Higher magnification picture of one of the tubules shown in Panel A with positive staining in spermatocytes is shown in Panel B. Pre-immune serum control (1:250) for anti-rHABPI antibody is shown in Panel C. The sections were probed with the goat anti-rabbit IgG conjugated to AP and developed with Fast Red™ substrate.
positive, though some tubules even with some early spermatocytes (Fig. 25A, asterisk) did not show expression of HABP1 proprotein, suggesting that HABP1 proprotein expression was probably evident only in spermatocytes which had reached a certain degree of maturity. Pre-immune serum control was negative, as shown in Fig. 25C.

Fig. 26A-F present the HABP1 proprotein expression in 28 day old testis. Intense cytoplasmic staining is seen in the cytoplasm of round spermatids ‘RS’ [Fig. 26A, tubules (i) and (ii)] and spermatocytes ‘SC’ [Fig. 26A, tubules (ii) and (iii)]. It is very interesting to note the differences in the pattern of staining in these three tubules [Fig. 26A, tubules (i), (ii) and (iii)]. Tubule (i) shows expression only in round spermatids (more in number) and none in spermatocytes, while tubule (ii) shows stronger staining in rounds spermatids ‘RS’ (few in number) and weaker staining in spermatocytes ‘SC’ (more in number). A part of the tubule (iii) seen shows very intense staining in the spermatocytes [comparable to the staining of round spermatids in tubules (i) and (iii)] (asterisk). Another tubule similar in staining to tubule (iii) is shown in full in Fig. 26B. Higher magnification of round spermatid staining is shown in Figs. 26C & D. It is important to highlight that the spermatocytes showing positive staining may be the pachytenes [Fig. 26A, as seen in tubule (ii)], whereas the early spermatocytes like leptotenes, zygotenes [Fig. 26A, tubule (i)] do not show positive staining for HABP1 proprotein. This data is in accordance with HABP1 expression in adult rat testis, wherein the staining is restricted to pachytene and the more mature spermatocytes (diplotene, Fig. 19C & D) and round spermatids (Fig. 19A & B) only. Pre-immune serum controls of 28 day old testis are shown in Figs. 26E & F and are negative.

5.2.10 Confirmation of localization of HABP1 proprotein using pAb 610 antibody

7, 14, 21 and 28 day old rat testis were stained with pAb 610 antibody in order to establish the localization of HABP1 proprotein in the developing testis as seen with anti-rHABP1 antibody (Figs. 27A-D). In 7 day old testis (A) the cytoplasmic staining in the gonocytes (‘G’) and the leydig cells (‘Le’) was found to be similar to that seen with anti-rHABP1 antibody staining (Fig. 23C). In 14 day old testis (B), no
Fig. 26. PRESENCE OF HABPI PROPROTEIN IN THE TESTIS OF 28 DAY OLD RAT. HABP1 proprotein expression in 28 day old testis is seen with anti-rHABP1 antibody (1:250) in spermatocytes ('SC') [Panel A, tubules (ii)] and round spermatids ('RS') [Panel A, tubules (i) & (ii) and panel B]. Tubule (iii) shown in Panel A is shown in full in Panel B with positive staining in spermatocytes ('SC'). Higher magnification photograph showing positive staining in round spermatids, 'RS' and dividing cells, 'M' is seen in Panels C & D. Pre-immune serum control for anti-rHABP1 antibody (1:250) is shown in Panels E & F.
Anti-recombinant HABP Ab, 1:250

Anti-recombinant HABP Ab, 1:250

Preimmune Sera, 1:250

Preimmune Sera, 1:250
Fig. 27. CONFIRMATION OF LOCALIZATION OF HABPI PROPROTEIN WITH pAb 610 ANTIBODY. 7 day (Panel A), 14 day (Panel B), 21 day (Panel C) and 28 day (Panel D) rat testes sections were probed with pAb 610 antibody and then developed with goat anti-rabbit IgG conjugated to AP and Fast red ™ color system.
cells pick up staining with pAb 610 antibody, showing a pattern similar to the staining with anti-rHABP1 antibody (Fig. 24C). In 21 day old testis (C), staining is seen mostly in the spermatocytes. Spermatocytes ‘SC’ (more mature ones, presumably pachytene) show weak [tubules (i) and (ii)] and strong [(tubules (iii))] expression, depending on the maturity of the cells. Some tubules exist which do not show any expression in the spermatocytes (asterisk). This pattern is similar to anti-rHABP1 staining (Fig. 25 A & B). In 28 day old testis (D), the round spermatids (‘RS’) and spermatocytes (‘SC’) show positive staining depending on the stage of the tubules as exemplified by the staining seen in tubules (i), (ii) and (iii). Note the strong [tubules (iii)], weak [tubules (i)] and no [tubule (ii)] staining in the spermatocytes, which also reflects upon the staining pattern in round spermatids, which too stained well [tubule (i) and (ii)]. When the number of round spermatids [tubule (ii)] is more, the expression of HABP1 proprotein in spermatocytes is not seen, but the expression is low in spermatocytes in those tubules which have fewer number of round spermatids [tubules (i)] and in those tubules which have no round spermatids [tubules (iii)], the staining in spermatocytes is very strongly evident. This expression seen with pAb 610 antibody in 28 day old testis was similar to that seen with anti-rHABP1 antibody (Fig. 26A-D). It is clear that in the developing testis, the proprotein expression is restricted to pachytene spermatocytes and the round spermatids and correlates with the maturity of the spermatocyte population.

5.2.11 Immunoblotting of adult rat testis lysate with anti-tHABP1 antibody

Testis lysate (10 µl as used in Fig. 13A & B) was probed with anti-tHABP1 antibody (1:750). Anti-tHABP1 antibody detected the 34 kDa HABP1 (Fig. 30, lane 1, single arrowhead) in the testis lysate along with another protein (double arrowhead) in ~55-60 kDa range. This protein is clearly not the 68 kDa protein usually seen with anti-tHABP1 antibody, since it well below the 67 kDa marker band. The identity of this protein is, as yet, not clear. Purified rHABP1 is detected in lane 2.
Fig. 30. IMMUNOBLOT ANALYSIS OF ADULT RAT TESTIS LYSATE PROBED WITH ANTI-tHABP1 ANTIBODY. Testis lysate (lane 1) and purified rHABP1 (lane 2) were probed with anti-tHABP1 antibody and developed with anti-rabbit HRP conjugate and ECL detection system.
5.2.12 Immunostaining of developing and adult rat testis using anti-tHABPI antibody

The staining seen with anti-rHABP1 was found to be very specific and was confirmed as the staining for HABP1 proprotein with pAb 610 antibody. It was important to see the staining pattern obtained with anti-tissue HABP1 antibody in the adult rat testis.

Staining of developing rat testis with anti-tHABP1 antibody gave a profile which showed some similarity in staining with that seen with anti-rHABP1 antibody. Fig. 23 shows the staining of 7 day old testis with anti-tHABP1 antibody. Note the intense staining of the interstitium (Int, Fig. 23A). Cytoplasm of the Gonocytes (‘G’) is also stained positive with anti-tHABP1 antibody. Very faint staining in dividing cells (‘M’, Fig. 23A) is also visible. Normal rabbit serum control is seen in Fig. 23B.

Staining of 14 day old testis with anti-tHABP1 antibody is seen in Fig. 24A. Interstitium is stained strongly as seen with 7 day old testis. In contrast to the staining seen with anti-rHABP1 antibody, the anti-tHABP1 antibody does stain few cells in the seminiferous tubules. The staining seen was mostly in early spermatocytes (‘ES’). ES [(1), (2) and (3)] represent three different populations of spermatocytes which stain differently with anti-tHABP1 antibody. ES (1) stain very strongly ES (2) do not stain at all, whereas ES (3) shows a membranic localization of HABP1. Tubules marked with single asterisk and double asterisk do not show any staining in any of the cell types.

21 day old rat testis stained well with anti-tHABP1 antibody (Fig. 28A-D), although no uniformity was seen. As shown in Fig. 28B, interstitium (‘Int’) is stained strongly with anti-tHABP1 antibody. Leptotene (‘L’), Zygotene (‘Z’) and Pachytene (‘P’) spermatocytes are stained in some tubules (‘L’ and ‘Z’ in Fig. 28A & C, ‘P’ in Fig. 28A & C) and not stained in others (double asterisk, Fig. 28C). Membranic staining is also seen in some pachytene spermatocytes (double asterisk, Fig. 28B), spermatogonia are occasionally positive (‘SG’, Fig. 28B). Staining of the Sertoli cell cytoplasm is also visible in some tubules (Fig. 28C). Normal rabbit serum control was negative (Fig. 28D).
Fig. 28. IMMUNOHISTOCHEMICAL LOCALIZATION OF HABP1 IN THE TESTIS OF 21 DAY OLD RATS AS SEEN WITH ANTI-tHABP1 ANTIBODY (1:50). Leptotene ('L'), zygote ('Z') and pachytene ('P') spermatocytes ('SC') show positive staining with anti-tHABP1 antibody (Panels A & C). Pachytene spermatocytes with a membranic localization (arrowhead, double asterisk) along with an intense staining of the few spermatogonia ('SG') and the interstitium ('Int') is seen positively stained in Panel B. Sertoli cell cytoplasm ('s') too stains positively with anti-tHABP1 antibody (Panel C). Negative control with normal rabbit serum (1:50) is shown in Panel D. The sections were probed with the goat anti-rabbit IgG conjugated to AP and developed with Fast Red™ substrate.
28 day old testis, very much like 21 day old testis showed inconsistent staining pattern. While round spermatids and spermatocytes (‘SC’) showed positive cytoplasmic staining in some tubules (Fig. 29A), spermatogonia were stained in some (Fig. 29E). Fig. 29C, shows 4 tubules [(i), (ii), (iii) and (iv)]; each one of which shows a unique staining pattern. Normal rabbit serum control was negative (Fig. 29E). A high magnification photograph of a stained spermatocyte ‘SC’ (Fig. 29B) and probably a round spermatid (Fig. 29D, arrowhead) is presented.

In the adult rat testis, very interestingly as shown in Fig. 31A, anti-tHABP1 antibody gives an altogether different staining profile as compared to the anti-rHABP1 antibody. The staining was not found to be very specific for any cell type as well as for any particular stage of spermatogenesis. Different tubules with the same stage of spermatogenesis showed different staining profiles. Interstitium, however, was consistently stained very strongly. Strong staining was also clearly visible in the crypts in sertoli cells where the sperm heads bind (Fig. 31B & C, arrowhead, ‘sp’). This staining was a very constant feature. Sometimes sertoli cells cytoplasm is also stained (Fig. 31B, arrowhead, ‘S’).

Summarily, anti-tHABP1 antibody did not present any definite staining pattern, which could seem positive in terms of a defined staining pattern with reference to the different stages of rat spermatogenesis.

5.2.13 Expression of HABP1 in human, mouse and hamster testis

After studying the expression of HABP1 in rat testis, its expression in the testis of other species, namely human, mouse and hamster was intended, since immunocross reactivity of anti-HABP1 antibodies is already reported (Deb and Datta, 1996).

The proprotein form of HABP1 was seen by staining the testes sections with anti-rHABP1 antibody. It was interesting to note that HABP1 proprotein expression in human (Figs. 32E & F), mouse (Figs. 33C & E) and hamster (Fig. 34C) testes is seen only in round spermatids and pachytene spermatocytes, a pattern similar to that seen in the rat testis. Preimmune serum controls of human (Fig. 32G), mouse (Figs. 33D & F) and hamster (Fig. 34D) are shown, which are negative.
Fig. 29. IMMUNOHISTOCHEMICAL LOCALIZATION OF HABP1 IN THE TESTIS OF 28 DAY OLD RATS AS SEEN WITH ANTI-tHABP1 ANTIBODY. Positive staining is seen in round spermatids (‘RS’) and spermatocytes (‘SC’) [Panel A]. Different tubules (i-iv) showing different staining patterns; cytoplasmic, membranic and no staining at all are shown in Panel B. Higher magnification picture of a spermatocyte (‘SC’) [Panel C] and a round spermatid (arrowhead, Panel D) are shown along with spermatogonia (‘SG’) [Panel E]. Negative Control with normal rabbit serum control (1:50) is shown in Panel F.
Anti-tissue HABP Ab, 1:50

A

Anti-tissue HABP Ab, 1:50

B

Anti-tissue HABP Ab, 1:50

C

Anti-tissue HABP Ab, 1:50

D

Anti-tissue HABP Ab, 1:50

E

Normal Rabbit Serum, 1:50

F
Fig. 31. IMMUNOHISTOCHEMICAL STAINING OF ADULT TESTIS WITH ANTI-tHABP1 ANTIBODY. Various staining patterns were obtained with anti-tHABP1 antibody as described below: A. Differential staining of adult rat testis with anti-tHABP1 antibody in Sertoli cells cytoplasm (arrowhead, ‘s’) and the crypts where the sperm heads cluster (arrowhead, ‘sp’). B. Higher magnification picture seen in panel A. showing staining in sertoli cell (‘s’) cytoplasm and the region around the sperm head clusters (‘SP’). C. Unique staining pattern in the form of a circular plate (asterisk) is seen along with the strong staining of the crypts (‘SP’). D. Dividing cells (‘M’) are also stained positive. E. Practically no staining is seen in this tubule with a late stage of spermatogenesis except a few peripheral spermatogonia (arrowhead). F. Tubule showing the central whorls and the crypts as positive (as in Panel C) along with a more prevalent spermatogonial staining all along the along the basal membrane as seen in Panel E.
Anti - tissue HABP Ab, 1 : 50

A

Anti - tissue HABP Ab, 1 : 50

B

Anti - tissue HABP Ab, 1 : 50

C

Anti - tissue HABP Ab, 1 : 50

D

Anti - tissue HABP Ab, 1 : 50

E

Anti - tissue HABP Ab, 1 : 50

F
Fig. 32. EXPRESSION OF HABP1 IN HUMAN TESTIS. Human testis sections were probed with anti-tHABP1 antibody, 1:50 (Panels A-C) and anti-rHABP1 antibody, 1:250, (Panels E &F.). Normal rabbit serum, 1:50 (panel D) and pre-immune serum, 1:250 (Panel G) served as negative controls.
Anti-tissue HABP Ab, 1:50

Normal Rabbit Serum, 1:50

Anti-recombinant HABP Ab, 1:250

Preimmune Sera, 1:250
Fig. 33. EXPRESSION OF HABP1 IN MOUSE TESTIS. Mouse testis sections were probed with anti-tHABP1 antibody, 1:50 (Panel A) and anti-rHABP1 antibody, 1:250, (Panels C & E). Normal rabbit serum, 1:50 (Panel B) and pre-immune serum, 1:250 (Panels D & F) served as negative controls.
Anti-tissue HABP Ab, 1:50

Normal Rabbit Serum, 1:50

Anti-recombinant HABP Ab, 1:250

Preimmune Sera, 1:250

Anti-recombinant HABP Ab, 1:250

Preimmune Sera, 1:250
Anti-tissue HABP Ab, 1:50

Normal Rabbit Serum, 1:50

Anti-recombinant HABP Ab, 1:250

Preimmune Sera, 1:250

Fig. 34 EXPRESSION OF HABP1 IN HAMSTER TESTIS. Hamster testis sections were probed with anti-tHABP1 antibody, 1:50 (Panel A) and anti-rHABP1 antibody, 1:250, (Panel C). Normal rabbit serum, 1:50 (Panel B) and pre-immune serum, 1:250 (Panel D) served as negative controls. The sections were probed with the goat anti-rabbit IgG conjugated to AP and developed with Fast Red™ substrate.
Anti-tHABP1 antibody too stained the interstitium and the crypts in the sertoli cells, into which the sperm heads anchor and show the presence of HABP1, presumably the mature form of HABP1 [human (Fig. 32A-C), mouse (Fig. 33A) and hamster (Fig. 34A) and normal rabbit serum controls are negative. HABP1 [human (Fig. 32D), mouse (Fig. 33B) and hamster (Fig. 34B) and this data implicates that the expression of HABP1 followed the same pattern in different species.

5.2.14 Partial characterization and purification of the 55 kDa HABP1 proprotein

5.2.14.1 Labile nature of the 55 kDa HABP1 proprotein and its processing to the 34 kDa mature HABP1

The fact that the 55 kDa HABP1 proprotein was detected only by immediate boiling of the seminiferous tubules in 0.5% SDS-PBS or Laemmli buffer, suggested the labile nature of the proprotein. Therefore, a confirmation was sought by carrying out a comparative study of lysates prepared in conventional RIPA buffer with and without the addition of protease inhibitors; PMSF, EDTA and DTT and in Laemmli buffer with immediate boiling.

Equal amount of seminiferous tubules (approximately 100 mg) were taken and separately processed in the above mentioned different buffers for lysate preparation. While the tubules in RIPA buffer (with or without protease inhibitors) were incubated in ice for 30 min, the tubules in Laemmli buffer were kept in boiling water bath and processed as described earlier in Material and Methods. During incubation, the tubules in all three tubes were minced. After processing, the lysate with the remaining tubular debris was centrifuged and the supernatant from the lysate in RIPA buffer was boiled in Laemmli buffer before loading. All the lysates prepared in different buffers were separated on a 10% SDS-PAGE and probed with anti-rHABP1 antibody.

As clearly seen in Fig. 35A, a very prominent 55 kDa band was seen in the lysate prepared in Laemmli buffer (lane 1, single arrowhead) along with a very faint 34 kDa band (lane 1, double arrowhead). In the lysate prepared in RIPA lysis buffer with protease inhibitors, the 55 kDa protein band was faint (lane 2, single arrowhead), while the mature 34 kDa protein (double arrowhead) was seen strongly
Fig. 35A. PRESENCE OF HABPI PROPROTEIN IN TESTIS LYSATE PREPARED IN LAEMMLI BUFFER. Testis lysate was prepared by immediate boiling in Laemmli buffer (lane 1), in RIPA lysis buffer with (lane 2) and without (lane 3) protease inhibitors; DTT and EDTA and PMSF. The lysates were resolved on a 10% SDS-PAGE (reducing) transferred onto an NC membrane and probed with anti-rHABPI antibody, goat anti-rabbit IgG-AP conjugate and NBT/BCIP color system.

Fig. 35B. EFFECT OF β-ME ON HABPI PROPROTEIN. Testis lysate prepared in 1 x Laemmli buffer with (lane 1) and without (lane 2) β-mercaptoethanol was resolved on 10% SDS-PAGE, electroblotted onto an NC membrane and probed with anti-rHABPI antibody, goat anti-rabbit IgG-AP conjugate and NBT/BCIP color system.
in RIPA lysis buffer with (lane 2) and without (lane 3) protease inhibitors. The 55 kDa protein was not at all detected in RIPA lysis buffer when protease inhibitors were not used (lane 3, single arrowhead). An additional band of ~44 kDa was visible in the lysate prepared in both kinds of RIPA lysis buffer (lane 2 and 3, triple arrowhead), but not in the sample prepared by immediate boiling of tubules in Laemmli buffer.

This data reveals processing of the 55 kDa HABP1 proprotein to the mature 34 kDa HABP1, which is sensitive to proteases.

**5.2.14.2 Subunit characterization of the 55 kDa HABP1 proprotein**

Testis lysate was prepared by immediate boiling of tubules in Laemmli buffer with and without β-mercaptoethanol (β-ME). Lysate was resolved on a 10% SDS-PAGE, electroblotted onto an NC membrane and probed with anti-rHABP1 antibody. Fig. 35B shows that the 55 kDa protein migrated at the same position in presence (lane 1, arrowhead) and absence (lane 2, arrowhead) of β-ME, suggesting that 55 kDa HABP1 proprotein may exist as a monomer without any higher oligomeric forms, involving inter-chain disulphide linkages.

**5.2.14.3 Relative migration of 55 kDa HABP1 proprotein with Rabbit IgG**

Since the main probe for the 55 kDa detection was the anti-rHABP1 antibody, it was intended to characterize the 55 kDa HABP1 proprotein using immobilized anti-rHABP1 IgG, purified using protein A beads. Since the IgG is also known to have a Mr ~55 kDa (however, Harfenist and Murray, 1990 have clearly mentioned the Mr of heavy chain of IgG as ~50 kDa), a reducing SDS-PAGE with both rat testis lysate and purified anti-rHABP1 IgG (1 μg) was electrophoresed, electroblotted onto an NC membrane and probed with anti-rHABP1 antibody, goat anti-Rabbit IgG-AP conjugate and NBT/BCIP color system. Fig. 35C shows clearly that the 55 kDa protein (lane 1, single arrowhead) in the testis lysate migrated more slowly than the IgG heavy chain (lane 2, double arrowhead) suggesting a clear difference in the molecular weights of 55 kDa HABP1 proprotein and Rabbit IgG. 34 kDa purified rHABP1 is detected in lane 3 after immunoblotting.
Fig. 35C. RELATIVE MIGRATION OF HABPI PROPROTEIN WITH RABBIT IgG. Rabbit IgG (lane 1) and testis lysate (lane 2) prepared in 1 x Laemmli buffer (with β-ME) were resolved on a 10% SDS-PAGE, transferred and probed with anti-rHABPI antibody, goat anti-rabbit IgG AP conjugate and NBT/BCIP color system. Purified 34 kDa rHABPI is seen in lane 3.

Fig. 35D. PARTIAL PURIFICATION OF HABPI PROPROTEIN USING ANTI-rHABPI IgG. Proteins purified using immobilised anti-rHABPI IgG were boiled in 1x Laemmli buffer, resolved on a 10% SDS-PAGE and processed for silver staining (lane 2). Purified rHABPI is seen in lane 1.
5.2.14.4 Partial purification of the 55 kDa HABP1 proprotein

After incubating the testis lysate (prepared in 0.5% SDS-PBS) with anti-rHABP1 IgG coated blot, the bound proteins were eluted using Glycine-HCl, pH 2.2 buffer. The eluted proteins were neutralized, boiled in 1 x Laemmlli buffer with β-ME and resolved on a 10% SDS-PAGE. The gel was processed for silver staining. Fig. 35D shows the silver stained SDS-PAGE gel. The 55 kDa HABP1 proprotein is seen in the eluate (lane 2, single arrowhead) along with the 34 kDa HABP1 (lane 2, double arrowhead). The 68 kDa protein is also seen in lane 2 (triple arrowhead). Also seen are the 34 kDa (double arrowhead) and 68 kDa (triple arrowhead) bands of the purified rHABP1 (lane 1). This data substantiates the observation of the 55 kDa HABP1 proprotein being immunologically related to the 34 kDa HABP1 and the 68 kDa protein.

5.2.15 Overexpression of the proprotein form of HABP1 in E. Coli

Labile nature of the 55 kDa HABP1 proprotein was seen in eukaryotic system, as exemplified by rat testis. To explore the possibility of HABP1 proprotein being inherently unstable and to understand the mechanism of cleavage in eukaryotic system, an attempt to overexpress the full length cDNA, corresponding to the proprotein form of HABP1 (282 amino acids, termed as 282HABP1) in the bacterial system was made.

Full length cDNA was excised out of the recombinant pCIHAI plasmid, using EcoR I-Not I digestion (Fig. 36A, lane 2). The size of the insert was 900 bp, which included the full length cDNA along with a His-tag (18 bp) and Haemaglutinin tag (36 bp). Lane 1 shows the 1 kb DNA ladder as a reference.

5.2.15.1 Ligation and Transformation of the recombinant plasmid (pET30c, 282HABP1) in DH5α and BL21 (DE3) strains of E. coli

pET30c was chosen as the host vector for the 900 bp full length HABP1 cDNA insert. pET30c after digestion with EcoR I-Not I was treated with CIAP to remove the terminal phosphate groups which may result in self-ligation of the vector. The CIAP treated vector and the 900 bp insert were purified after resolving on a preparative LMP-agarose gel.
A.

Fig. 36A. ISOLATION OF FULL LENGTH HABPI cDNA CORRESPONDING TO 282 AMINO ACIDS. pCIHAI plasmid (lane 2) containing the full length cDNA insert was digested with EcoR I-Nor I to get out the ~900 bp fragment (lane 3). Lane 1 shows the 1 Kb DNA ladder as a reference.

B.

Fig. 36B. CONFIRMATION OF POSITIVE pET30c.282HABPI CLONES BY MINIPREP DNA ISOLATION. Random clones after transformation in BL21(DE3) and DH5α E.coli strains were selected, grown and processed for miniprep DNA isolation. Lanes 1-8 of BL21(DE3) and 1-5 of DH5α strains show the recombinant pET30c.282HABPI plasmid. (double arrowhead), isolated after miniprep. Lane C is the control pET30c plasmid (single arrowhead).
The purified fragments of the 282HABP1 insert and pET30c vector were set for an overnight ligation and thereafter, the ligated mix was transformed in competent *E. coli* cells [both DH5<sub>x</sub> and BL21 (DE3) strain]. The colonies appearing after plating were screened for recombinant pET30c.282HABP1, by performing a miniprep of the selected colonies. The miniprep DNA of the recombinant plasmid pET30c.282HABP1 along with the control is shown in Fig. 36B. The control was DH5<sub>x</sub> *E. coli* strain, transformed with only the native pET30c plasmid. As seen clearly, there exists a difference in migration of the recombinant pET30c.282HABP1 plasmid [double arrowhead, BL21 (DE3): 1-8 and DH5<sub>x</sub>: 1-5] and pET30c control plasmid (C, single arrowhead). Lane 5 of BL21 (DE3) is doubtful and lane 4 or DH5<sub>x</sub> is clearly a self-ligated plasmid.

Colony no. 4 of BL21 (DE3) [lane 4] and colony no. 2 in DH5<sub>x</sub> (lane 2) strain were chosen for DNA isolation by midiprep and thereafter, confirming the clones by restriction digestion of the midiprep DNA.

### 5.2.15.2 Confirmation of the recombinant clone

Plasmid DNA was isolated from both DH5<sub>x</sub> and BL21 (DE3) strain and the resulting plasmid was digested with BamHI alone. BamHI digestion should excise out a ~900 bp fragment from the recombinant plasmid pET30c.282HABP1 in contrast to just linearising the native plasmid, pET30c (as shown in Scheme IV). Fig. 36C shows the midiprep DNA from DH5<sub>x</sub> strain having recombinant pET30c.282HABP1 (lane 1) and the native pET30c plasmid (lane 2).

Confirmation of cloning was seen with the BamHI digestion, as shown in Fig. 36C. As seen in lane 5, ~900 bp fragment is excised out from the recombinant pET30c.282HABP1 plasmid (seen in lane 6) while the native plasmid (lane 3) digested with BamHI only gets linearised (lane 4).

### 5.2.15.3 Overexpression of pET30c.282HABP1 in BL21 (DE3) : SDS-PAGE and western blot analysis

Recombinant pET30c.282HABP1 plasmid was transformed into BL21(DE3) strain of *E.Coli* and overexpression was induced with 1mM IPTG. E.Coli lysate before and after 3h of induction were taken out and the cells were harvested, washed
**Fig. 36C. MIDIPREP DNA ISOLATION OF RECOMBINANT pET30c.282HABP1 PLASMID AND CONFIRMATION OF THE CLONE BY BamH I DIGESTION.** PET30c.282HABP1 midiprep DNA was isolated from DH5α strain and then digested with BamH I to get out the ~900 bp fragment. Lanes 1 & 3 show the recombinant pET30c.282HABP1 plasmid, while native pET30c plasmid is seen in lanes 2 & 5. BamH I digested native pET30c plasmid is seen in lane 4. BamH I digested recombinant pET30c.282HABP1 plasmid is seen in lane 6. Lane 7 shows the 1 Kb DNA ladder as the reference.

**Fig. 36D. SDS-PAGE PROFILE OF THE OVEREXPRESSED RECOMBINANT PROTEIN ENCODED BY FULL LENGTH HABP1 cDNA IN E.Coli.** Equal amount of lysates prepared from the uninduced (lane 1) and induced (lane 2) BL21(DE3) *E.Coli* cells (having the recombinant pET30c.282HABP1 plasmid), were resolved on a 10% SDS-PAGE and the bands were visualised by CBB staining of the gel.
once with PBS. The pellet was then boiled directly in Laemmli buffer, centrifuged, to get rid of cell debris, and loaded onto a 10% SDS-PAGE. Same volume of aliquots from both the uninduced and the induced E.Coli cell pellet expressing the HABP1 proprotein was used. Bands on SDS-PAGE were visualised by CBB staining. Fig. 36D shows the CBB stained SDS-PAGE gel. The induced band is seen at the ~45 kDa position in the IPTG induced E. coli lysate (lane 2, arrowhead). The uninduced bacterial cell lysate is seen in lane 1. Thus, it was seen that the protein encoded by full length cDNA (corresponding to 282 amino acids) after overexpression migrates at ~45 kDa.

In order to confirm that the ~45 kDa was indeed the protein of interest, a series of western blots were done using a variety of antibodies against both, the mature and proprotein form of HABP1. Fig. 36E shows the western blots done with anti-rHABP1 antibody (I), anti-tHABP1 antibody (II), pAb 610 antibody (III), mAb 60.11 antibody (IV) and anti-His-tag antibody (V). The pre-immune serum control for the anti-rHABP1 antibody was also run in parallel (VI). The detection of the ~45 kDa overexpressed protein by anti-rHABP1, anti-tHABP1 and mAb 60.11 is clearly evident, suggesting that the ~45 kDa band was indeed HABP1. This was further confirmed by using anti-His-tag antibody (polyclonal), since the overexpressed recombinant protein had an N-terminal and a C-terminal His-tag. To further confirm the identity of the ~45 kDa overexpressed protein as the proprotein form of HABP1 encoded by full length cDNA, the same lysate was probed with pAb 610 antibody. Interestingly, the ~45 kDa overexpressed HABP1 protein did not get detected by the pAb 610 antibody, which detects the decapptide in the proprotein region, revealing that the ~45 kDa HABP1 protein lacks the proprotein region and thus, represents the mature form of HABP1 with the His-tag and haemglutinin tag. This data suggested a processing of the proprotein form of HABP1 to the mature form in the E.Coli.

5.3 DISCUSSION

The present study provides evidence on the identification of a novel, specific, testicular protein of relative molecular mass 55kDa, which is possibly the proprotein form of the hyaluronan binding protein, HABP1. The western blot results are
Fig. 36E. IDENTIFICATION OF THE OVEREXPRESSED 45 kDa PROTEIN AS THE PROCESSED FORM OF HABP1 PRORPOTEIN. Lysates of induced (Lane 2) and uninduced (lane 3) BL21(DE3) cells (having the recombinant pET30c.282HABP1 plasmid), were resolved on a 10% SDS-PAGE along with purified rHABP1 (lane 1; as the positive control), transferred onto an NC membrane, and probed with anti-rHABP1 (I), anti-hABP1 (II), pAb 610 (III), mAb 60.11 (IV) and anti-His-tag (V) antibodies. Control blot probed with pre-immune serum was also run in parallel (VI).
supported by immunohistochemical observations with antibodies against the mature and proprotein forms labeling the same cellular targets and revealing a stage specific expression of the proprotein form of HABP1 in different germ cells during spermatogenesis in adult rat. An additional staining on the developing acrosome with the antibody against the proprotein form of HABP1 may be related to sperm movement within the epithelium. In the developing rat testis too, the HABP1 proprotein expression was found in very specific cell types, namely gonocytes, pachytene spermatocytes and round spermatids.

The data comprises of three main observations. Firstly, antibodies against the denatured HABP1 and against the decapeptide present in the proprotein form of HABP1, both detected the 55 kDa protein only in the testis but not in liver and spleen, showing its testis specific expression. Since HABP1 is synthesised as a proprotein, every cell expressing HABP1 should contain the proprotein form. However, very few reports exist on the detection of the proprotein form of HABP1 in the cells and tissues (Ghebrehiwet et al., 1997; Muta et al., 1997; Dedio et al., 1998). Ghebrehiwet et al (1997) showed a protein of 42 kDa in the Raji cell, by using the same pAb 610 antibody used in this study. Muta et al (1997), detected a protein of 38 kDa in PLC cells transfected with the full length cDNA of HABP1, although they also showed that the 34 kDa mature HABP1 is always strong in such cells, indicating that the proprotein form is efficiently processed to generate the mature form in those cells. Dedio et al (1998) through in vitro transcription/translation experiments (using rabbit reticulocyte lysate) estimated the size of the primary translation product as 39 kDa. There does exist a possibility that the 39 kDa protein is an intermediate form in the processing of the HABP1 proprotein form of 55 kDa to the mature form of 34 kDa, since the rabbit reticulocyte lysate has been reported to contain a variety of post-translational processing activities like proteolysis (Mumford et al., 1981). Moreover, the final product of 39 kDa after in vitro transcription / translation was not probed with the antibody specific for the proprotein region of HABP1 to confirm that the 39 kDa protein was indeed the full length protein comprising 282 amino acids. In support of this hypothesis that the proprotein form of 55 kDa protein is processed to
34 kDa, biosynthetic studies carried out by immunoprecipitation with anti-HABP1 antibody in our laboratory in mouse lymphoma EL4 cells using $[^{35}\text{S}]$methionine have shown that it give a band at 55 kDa, corresponding to the primary translation product and the appearance of the 34 kDa HABP1 could be correlated with the disappearance of the 55 kDa protein (Rao, 1997). Even Krainer et al (1990) reported earlier the detection of a 55 kDa protein in $[^{35}\text{S}]$ methionine labelled HeLa cell nuclear extracts immunoprecipitated with anti-SF2 antibodies. It is important to mention here that the identity of HABP1 with SF2p32, the protein that copurifies with SF2 (Deb and Datta, 1996) has already been reported. Thus, the co-immunoprecipitation of the 55 kDa protein by SF2 antibody in HeLa cells during the biosynthesis studies, does not rule out the possibility of this protein of 55 kDa being the proprotein form of p32/HABP1. Moreover, the 55 kDa protein reported by Krainer et al (1990) in HeLa cells and speculated to be the proprotein form of HABP1 by us, also shows slower migration as compared to IgG heavy chain; a data similar to our observation on slower migration of the 55 kDa HABP1 proprotein to the IgG heavy chain.

This discrepancy between the apparent mass of HABP1 proprotein seen in the present study and the reports by the various groups could be explained by the method of lysate preparation. Repeated efforts to capture the 55 kDa protein by preparing the lysate of the isolated germ cells in the conventional lysis buffer failed. Detection of the 55 kDa protein was possible only when the lysate was prepared by disrupting the tubules and lysing the cells within, directly in the lysis buffer (containing EDTA, PMSF and DTT) instead of lysing the previously isolated cells. The 55 kDa protein was, however, faint and the 34 kDa mature form is seen strongly along with an additional protein of 44 kDa, which was also seen as an intermediate product. The 55 kDa protein was clearly seen getting processed to the 34 kDa mature HABP1. The HABP1 proproteins seen by Muta et al. (1997), Ghebrehiwet et al. (1997) and Dedio et al. (1998) could be the same as 44 kDa seen in this study during the processing of 55 kDa proprotein to the mature 34 kDa HABP1. 44 kDa protein was also seen as an intermediate product in our biosynthetic studies using $[^{35}\text{S}]$ methionine in mouse lymphoma EL4 cells (Rao, 1997). This suggests that the full
length cDNA encoding full length encoding HABP1 proprotein, generates a protein of 55 kDa. which undergoes proteolytic cleavage producing the 44 kDa protein and finally the stable 34 kDa mature HABP1.

Instability of the 55 kDa HABP1 proprotein was clearly evident from the fact that for most of the experiments, the safe method of immediate boiling of the tubules directly in Laemmli buffer or in 0.5%SDS-PBS was followed in order to stabilize the 55 kDa proprotein. Thus, the detection of the 55 kDa proprotein requires the preparation of the lysate in a way that is different from conventional protocols. Efforts to overexpress the HABP1 proprotein (pET30c.282HABP1) in bacteria resulted in detecting a 45 kDa overexpressed protein, which reacted positively only to antibodies (namely, anti-tHABP1 antibody, anti-rHABP1 antibody, 60.11 mAb) directed against epitopes present in the mature portion of HABP1. pAb 610 antibody which recognizes an epitope present in the proprotein region of HABP1 failed to detect the 45 kDa bacterially overexpressed protein suggesting that the 45 kDa protein lacked the proprotein region and corresponded to the mature form of HABP1.

The migration at 45 kDa could be possibly due to His- and HA- tags. pAb 610 antibody being a mono specific antibody, it may be possible that the target epitope, i.e. the decapeptide is masked (although the possibility is less because the analysis was done by SDS-PAGE). To negate this possibility, the monoclonal 60.11 antibody was used which detected the 45 kDa overexpressed protein clearly (another monoclonal antibody, 74.5.2, directed against the C-terminal of HABP1, was also used and that too gave a positive signal with the 45 kDa protein). Although, antibody reactivity suggests towards the 45 kDa protein being the processed (or mature) form of HABP1, purification of this protein and its sequencing (both internal and N-terminal), would confirm the processing of the HABP1 proprotein even in the prokaryotic system. The labile nature of the HABP1 proprotein is also obvious from the observation made by Honore et al (1993). They reported that the cells infected with Vaccinia virus construct having the full length cDNA encoding the proprotein form of HABP1, actually synthesized the protein with the N-terminal sequence the same as that of the mature HABP1/p32 (of 34 kDa), suggesting an efficient
processing of the full length proprotein form of HABP1 to the mature 34 kDa form in the eukaryotic system. In the prokaryotic system also, Tange et al (1996) found that the proprotein form of p32/HABP1, when expressed in bacteria as a GST-fusion protein, was highly unstable and co-migrated with the mature form of p32/HABP1, suggesting that the protein is processed in the bacteria near to, or at, the same site as observed in the human cells.

It may seem difficult to comprehend that the HABP1 proprotein has a Mr of 55 kDa when the theoretical Mr of the HABP1 proprotein is 31.4 kDa. This discrepancy can be explained by the amino acid composition of the protein, which imparts to it a unique property of exhibiting anomalous migration in SDS-PAGE. The mature protein also has an anomalous migration at 34 kDa, although its theoretical molecular weight is 23.8 kDa. The mature protein has a pI of 4.1 and the highly acidic character of the contributing amino acids results in an anomalous migration (Deb and Datta, 1996). The proprotein form of HABP1 is less acidic (pI of 4.52) than the mature protein and the mild acidic character of the protein may be responsible for the anomalous migration. However, a more important reason for the anomalous migration at 55 kDa could be the presence of 11 proline residues in rat HABP1 (and 9 in human HABP1) in the proprotein region alone. Yu et al (1995a) also make a mention that HABP1/p32/TAP show anomalous migration, possibly due to a high proline content. Proline-rich domains have been reported to cause anomalous migration of proteins in SDS-PAGE (Ollo and Maniatis, 1987; Carroll and Scott, 1985). Another report by Bunch et al (1998) also mentions the anomalous migration of glyceraldehyde 3-phosphate dehydrogenase-S (GAPD-S) protein of mouse testis at 69.2 kDa, when its predicted molecular weight is 47.4 kDa. Green (1985) also report of a group C human adenovirus proteins of 26-28 kDa, actually migrating at 40-50 kDa in SDS-PAGE due to high proline content.

Secondly, we demonstrate that the 55 kDa HABP1 proprotein has a cytoplasmic localisation. Being the primary translation product, the HABP1 proprotein is anticipated to have a cytoplasmic location.
Thirdly, RT-PCR data revealed higher levels of HABP1 transcript in the testis, compared to liver and spleen. It is well documented that the testis behaves differently from the somatic tissues (Eddy and O'Brien, 1998) because of unique gene expression during meiosis. Thus, higher expression of HABP1 in the testis could possibly be either due to mRNA storage during meiosis or because of upregulation of transcriptional activity during meiosis, a mechanism commonly seen in the testis. The additional 500 bp product obtained in the RT-PCR experiments hybridized well with HABP1 cDNA, suggesting a homology between HABP1 cDNA and the 500 bp DNA fragment. The observation is highly significant and will be discussed further in Chapter III.

Higher levels of HABP1 transcript may result in increased expression of HABP1 proprotein (the primary translation product) and this could be the reason for detectable levels of HABP1 proprotein in the testis. Absence of the 55 kDa protein in spleen and liver could be a result of extremely low levels of this form of protein in these somatic tissues, making it difficult to be detected by the conventional methods employed in this study. 55 kDa HABP1 proprotein seen in other somatic cell lines [EL4 (Rao, 1997) and HeLa (Krainer et al., 1990)] has been detected only on performing the sensitive [³⁵S] labeling experiments, whereas its detection in testis has been possible even with the lesser sensitive immunoblotting experiments. High levels of HABP1 proprotein in the testis could also be a result of a delayed post-translational cleavage during meiotic and post-meiotic stages. There exist reports on the presence of a number of pro-peptides and precursor proteins in the testis (Kilpatrick et al., 1987; Yoshikawa and Aizawa, 1988) which are assumed to be regulated by the action of several testicular proteases (Pineau et al., 1999). Keeping this in mind, it is likely that the HABP1 proprotein too exists for a transient period in the meiotic and the post-meiotic cells, before getting processed to the mature form of 34 kDa. However, the functional aspect of proprotein expression in a particular stage during spermatogenesis is yet to be clarified and is a subject of future study.

The staining pattern seen with antibody against tissue HABP1 in immunohistochemistry is not easy to explain, more so since the expression was not
seen as a uniform one. One explanation for this could be due to the fact that the anti-tHABP1 antibody has been raised against the 'native' tissue HABP1, which is glycosylated. Thus, there exists a possibility that the anti-tHABP1 antibody cross-reacts with the glyco-moieties of the other proteins in the testis, which are apparently the same as those in HABP1. However this hypothesis itself needs to be proved, since this still does not explain as to why anti-tHABP1 antibody gives no consistent staining pattern. Reports exists, which explain the difference in reactivity of a particular antibody to epitopes in solution, in denatured and in native states (Horan Hand et al., 1992; Byrd et al., 1989) and this may explain differences in reactivity of anti-tHABP1 and anti-rHABP1 antibodies under denatured (immunoblotting) and native condition (immunohistochemistry).

Expression of HABP1 proprotein was restricted to pachytene spermatocytes and the round spermatids. This is not a very uncommon profile of protein expression. A number of proteins expressed in round spermatids and pachytene spermatocytes alone have been reported, e.g. phosphoglycerate kinase-2 (PGK-2) and Lactate dehydrogenase (LDH-C) (McCarrey and Thomas, 1987; Weiben, 1981; Gold et al., 1983; Boer et al., 1987); TATA-binding protein (TBP) (Persengiev et al., 1996) and proto-oncogene c-raf-1 (Wadewitz et al., 1993). However, absence of expression in other cell types because of the sensitivity of immunohistochemically analysis which may not be high enough to detect expression lower than threshold level, cannot be ruled out and will have to be investigated by performing experiments with isolated germ cells (Meistrich et al., 1981). It may thus, be possible that the proprotein form of HABP1, has some-as yet unknown- function which is associated with spermatogenesis.

One could further speculate that the location of the proprotein, surrounding the developing acrosome, and its disappearance at the precise time that sperm move towards the lumen but before release from the seminiferous epithelium, could be causally linked. HABP1 is unlikely to be associated with the tubulobulbar processes that anchor step 19 spermatids to the Sertoli cells (Russell and Clermont, 1976), since no staining was observed in these cells, but a role in translocation within the
seminiferous epithelium could be entertained, associated with cytoskeletal changes occurring in the Sertoli cell at that time. Such a functional significance for attachment/detachment of germ cells would be the case; for example, if the elongating spermatids were attached to the Sertoli cells by hyaluronic acid and the proprotein form were able to bind hyaluronic acid. This speculation requires more detailed study of the whole process before any conclusion can be drawn about the functional importance of the HABP1 proprotein expression in germ cells.

Malkov et al (1998) described the developmental schedule in the post natal rat testis as follows: a postnatal days 6-7 testis contains somatic cells and spermatogonia cells only; by days 13-14, leptotene spermatocytes appear; by day 17-18, zygotene spermatocytes are present; by days 19-20 and days 22-23, early and late pachytene spermatocytes, respectively, are seen. Haploid round spermatids first appear at days 24-25 and elongating spermatids by day 30-31; by day 36, elongated spermatid can be found. The HABP1 proprotein staining is seen at days 7 in gonocytes, at day 21 in pachytene spermatocytes and at day 28 in both in pachytene spermatocytes and round spermatids depending on the stage of the tubule. According to the description of stages by Malkov et al (1998), HABP1 proprotein expression is seen in stage I gonocytes, stage IV and V pachytene spermatocytes and stage VI round spermatids. It seems apparent from the pattern of expression seen that HABP1 proprotein appears in those cells which are newly synthesized after differentiation - the “next generation” cells. This is, however, true only for pachytene spermatocytes and round spermatids, which could apparently mean that HABP1 expression is maintained in late spermatocytes in meiotic cells and on round spermatids in post meiotic cells; a profile similar to that seen in the adult rat testis. The expression in gonocytes cannot be clearly explained just considering the fact that expression of HABP1 proprotein is seen in differentiating cells. Presence of 55 kDa HABP1 proprotein was seen in the developing rat testis (7,14,21 and 28 days) by immunoblotting, although immunohistochemical analysis failed to show the expression of HABP1 proprotein in 14 day old testis. This discrepancy can be explained considering the sensitivity of the techniques involved.
Immunohistochemistry, because of its lower sensitivity is unable to detect the presence of HABP1 proprotein in 14 day old testis section, although the protein is present as evidenced by immunoblot analysis. This data thus, suggests that the level of HABP1 proprotein present in 14 day old testis is significantly low with high transcript levels, suggesting mRNA storage. mRNA expression seen in developing rat testis suggests a very high HABP1 transcript level in 14 day old rat along with a high expression in adult rat testis. High mRNA with low protein expression suggests mRNA storage, or in other words it means that HABP1 mRNA is being produced (i.e. post-transcriptional activity is taking place) but it is not translated efficiently until the pachytene spermatocytes stages. mRNA storage is a common phenomena seen in pachytene spermatocytes where the transcripts are "saved" to be utilized later in spermatogenesis (Söderstrom, 1976).

Moreover, the expression of this protein in the testes of various species has been found to be in the same germ cell types. Identical expression of HABP1 proprotein in various mammalian species suggests that the function of this protein is probably conserved in mammalian spermatogenic cells. Recent experiments in our laboratory, with mouse testis lysate have also shown the migration of HABP1 proprotein at the 55 kDa position, suggesting a structure similarity of this protein among various species. Such an observation has been reported for spermatid specific Histone 2B (ssH2B) (Unni et al., 1995).

During meiosis, the genes normally involved in events like synaptonemal complex formation, chromosome pairing, maintenance of prophase arrest and chromosome segregation are expressed. Currently, the proprotein region of HABP1 is considered to act merely as a leader sequence for HABP1 translocation in the cell (Muta et al., 1997). However, the high HABP1 proprotein expression only in meiotic and post-meiotic cells could imply a role of this form of HABP1 in meiosis, thereby giving functional importance to this form of HABP1. Promoter analysis of the HABP1 gene using the submitted sequence of human chromosome 17 (GenBank accession no. AC004148), reveals binding sites for the transcription factors CREB and NF-κB. The cAMP-responsive element binding protein, CREB (Waebber et al.,...
1991; Ruppert et al., 1992), and its closely related cAMP-responsive element modulator. CREM (Foulkes et al., 1992; which binds to the same promoter element as CREB), have been reported to be responsible for cell- and stage-specific expression of cAMP-regulated genes in male germ cells (Sassone-Corsi, 1997). NF-κB has been shown to be transiently expressed in the nuclei of germ cells of the rat testis with peak levels found in pachytene spermatocytes during stage VII-XI and lower levels in stage I-VII spermatids (Delfino and Walker, 1998). The expression of HABP1 proprotein follows a similar profile and so it may be speculated that the expression of this protein in specific cell types is under the influence of the transcription factors, which are expressed in specific germ cells.

Preliminary experiments using immunoaffinity column of anti-rHABP1 antibody have been partially successful in purifying the 55 kDa protein in addition to the 34 kDa HABP1. However, its labile nature has made it difficult to purify it in quantities large enough to be utilized for further experiments. Work on immunoscreening of the rat testis cDNA expression library with the anti-rHABP1 antibody is also planned to approach this problem at the cDNA level. These additional experiments are mandatory to confirm the identity of the 55 kDa protein as the proprotein and also work on the possibility of this protein being a testis-specific isoform of HABP1, since testis is known to exhibit unique transcriptional, post-transcriptional, and post-translational phenomena resulting in gene products which differ completely from their somatic variants (Hecht, 1998).