CHAPTER-IV

Expression of HABP1 mRNA in rat testis
7.1 INTRODUCTION

High levels of HABP1 proprotein have been demonstrated in adult rat testis along with increased transcript levels (Chapter II), supporting the fact that high levels of proprotein indicate towards high HABP1 transcript levels too, since HABP1 proprotein is the primary translation product of the HABP1 mRNA. Enhanced expression of HABP1 proprotein in the pachytene spermatocytes and the round spermatids suggested looking at the expression and localization of HABP1 mRNA transcript as well. Thus, in situ hybridization was carried out in adult rat testis using DIG-labeled sense and antisense HABP1 riboprobes.

7.2 RESULTS

7.2.1 Preparation of pGEM.HABP1 construct

PCR amplified HABP1 cDNA was set up for a ligation with pGEM®-T-easy PCR cloning vector to get the recombinant plasmid, pGEM.HABP1. HABP1 cDNA was PCR amplified using S1-S2 primer set, as described in Material and Methods. The template used was the pT7A.A32 vector which contained the HABP1 cDNA (corresponding to the mature protein), cloned in it for expression purposes. As shown in Fig. 38A (lane 1), a fragment of 1039 bp was amplified, which was purified and then was set up for ligation with pGEM® T-Easy PCR cloning vector.

7.2.2 Selection of positive colonies containing the recombinant pGEM.HABP1 plasmid

The ligated product of the 1039 bp fragment derived from HABP1 cDNA and pGEM vector was transformed into JM109 cells. The transformed mix was plated on X-gal/IPTG coated plates and blue/white colony screening enabled selection of the recombinant white colonies. White colonies, which carried the recombinant plasmid, were chosen, along with few blue colonies. The blue colonies served as a negative control for ligation and transformation. Inoculating these colonies into LB was followed by processing the cells for PCR (as described in 'Multiple clone test' protocol in Material and Methods). PCR results confirmed the white colonies as the positive ones containing the recombinant pGEM.HABP1 plasmid (Fig. 38B). One
Fig. 38A. PCR AMPLIFICATION OF HABPI cDNA FROM pT7.A.32 PLASMID. 1039 bp HABPI cDNA (lane 1) fragment was PCR amplified from pT7.A.32 plasmid using S1-S2 primer set. Lane 2 shows the 1 Kb DNA ladder as the reference marker.

Fig. 38B. CONFIRMATION OF POSITIVE CLONES HAVING RECOMBINANT pGEM.HABPI PLASMID USING PCR. After ligation of ~1 Kb HABPI cDNA with pGEM T-Easy vector, the ligated mix was transformed and plated on X-gal/IPTG coated plates. Few white and blue (as negative controls) colonies were picked, grown in LB medium and processed for PCR as mentioned in Material and Methods. Lanes 2-8 show the PCR amplification of the white colonies while lanes 9-11 shows the PCR profile of the blue colonies picked. Lane 1 shows the PCR product with HABPI cDNA, the positive control. Lane 12 shows the Hae III digested lambda DNA as the reference marker.
confirmed recombinant clone (No. 2, lane 2) was used for riboprobe preparation in
future.

7.2.3 Preparation of linear pGEM.HABP1 template for in vitro transcription and
checking the orientation of cloning

pGEM.HABP1 recombinant plasmid was linearised using Sal I and Nco I,
individually. Complete digestion was ensured by running small aliquots of the mix
in an agarose gel. Fig. 38C shows the digestion profile with the uncut pGEM.HABP1
(lane 3) plasmid shown along with the Nco I (lane 1) and Sal I (lane 2) digested
pGEM.HABP1 plasmids.

Since the same clone was being used for the preparation of both, the sense
and antisense riboprobes, it was important to know the orientation of cloning, as the
cloning using pGEM, a PCR cloning vector, is non-directional.

Orientation of cloning of HABP1 in pGEM was worked out by Sac I
digestion. As shown in Fig. 38C (i), in the recombinant pGEM.HABP1, one Sac I
site exists in the vector, close to the SP6 promoter site and the other one exists in the
insert. The Sac I site in the insert is at the position 565 from the 5' end in the HABP1
cDNA while the Sac I position in the plasmid is at position 51 and thus, Sac I
digestion would generate a fragment of 616 bp, if the cloning is with the 5' end of
HABP1 cDNA towards the SP6 promoter. However, if the cloning is in a way that
the 5' end of HABP1 cDNA is towards the T7 promoter side, then a fragment of 525
bp will be excised out. After Sac I digestion, it was found that insert size was 616 bp
(shown in Fig. 38D, lane 3). The size of the fragment was calculated to be 616 bp
using a Hae III digested Lambda DNA marker (lane 4). The uncut plasmid is shown
in lane 2. These results suggested orientation of a kind, which would result in a
sense probe with SP6 RNA polymerase and an antisense probe with T7 RNA
polymerase [shown schematically in Fig. 38C(i)]. The orientation was further
confirmed by DNA sequencing [see Fig. 38D(ii)] of the linearised pGEM.HABP1.

Nco I linearised pGEM.HABP1 was used as a template for SP6 RNA
polymerase to generate the sense riboprobe, while Sal I digest was a template for T7
RNA polymerase to generate the antisense probe. After in vitro transcription in the
Fig. 38C. LINEARISING THE RECOMBINANT pGEM.HABP1 VECTOR WITH Nco I AND Sal I. pGEM.HABP1 (lane 3) was linearised with Nco I (lane 1) and Sal I (lane 2). Lane 4 shows the Hind III digested Lambda DNA as reference marker.

Fig. 38D. SacI DIGESTION OF pGEM.HABP1 TO CHECK THE DIRECTION OF CLONING. Presence of two Sac I sites (one in vector and other in insert) in the recombinant pGEM.HABP1 plasmid was utilised to know the orientation of the cloning. Sac I digested recombinant pGEM.HABP1 (lane 2) and the excised fragment of 616 bp (lane 3) as seen migrating above the 603 bp marker band of the Hae III digested lambda DNA (lane 4). Lane 1 shows the Hind III digested lambda DNA marker.
Fig. 38C(i) Orientation of cloning of HABP1 cDNA in the pGEM.HABP1 recombinant plasmid. The diagram shows the 616 bp fragment excised out by SacI digestion (one site in insert (shown in green) and the other one in the vector (shown in pink). The orientation thus works out as follows: T7 RNA polymerase synthesizes the antisense (shown in red) riboprobe, while the sense (shown in green) riboprobe is synthesized by SP6 RNA polymerase. S1-S2 primer binding sites and the 5' and the 3' ends of the HABP1 cDNA are indicated along with the T7 and SP6 promoter sites.
Fig. 38D (i) CONFIRMATION OF THE ORIENTATION OF THE CLONING IN pGEM.HABPI BY SEQUENCING. pGEM.HABPI recombinant plasmid was sequenced in both directions to confirm the orientation of cloning. The sequence shown is the one sequenced using a primer specific for the SP6 promoter. The start site of the sense HABPI strand is indicated with an asterisk. The stretch of sequence underlined is the one belonging to the pGEM vector.

Fig. 38E. PREPARATION OF RIBOPROBES BY IN VITRO TRANSCRIPTION. Linearised pGEM.HABPI plasmids were transcribed in vitro in presence of DIG-II-UTP, run on a 1% formaldehyde-agarose gel, transferred onto a nylon membrane and probed with anti-DIG IgG-AP conjugate and the NBT/BCIP color system. Both sense (lane 2) and the antisense (lane 3) probes (arrowhead) are seen getting detected.
presence of DIG-labeled dUTP, purified probes were resolved on a 1% formaldehyde-agarose gel, transferred and probed with anti-DIG IgG-AP conjugate and the NBT/BCIP color system. As shown in Fig. 38E, the sense Neo I/SP6 (Lane 2) and the antisense Sal I/T7 (lane 3) riboprobes, both are detected as intense bands at ~1 Kb positions (single arrowhead).

7.2.4 In situ Hybridization

In situ hybridization was performed on rat testis sections using sense and antisense HABP1 riboprobes.

Using DIG labeled antisense riboprobe, the distribution of HABP1 mRNA transcript in the different regions of the seminiferous epithelium was clearly seen (Figs 39A-G). The presence was evident in the cytoplasm of round spermatids (Fig. 39A, arrowhead, and Fig. 39B) and the late pachytene spermatocytes (Fig. 39F, arrowhead, and 39G, arrowhead, SC). HABP1 mRNA showed stage-dependent differences in the expression (Fig. 39C-E). As is evident, the expression was seen only in round spermatids during the early stages (Fig. 39C, shown here in stage I) followed by a faint appearance of the HABP1 transcript in the pachytene spermatocytes (Fig. 39D, arrowhead, ‘P’) along with some mRNA in the spermatids (Arrowhead, ‘8’) during stage VIII. The amount of HABP1 mRNA decreased in the round spermatids as they begin to elongate with a result that no expression was apparent in the elongated spermatids of stage XIII (Fig. 39E). The pachytene spermatocytes, now termed as ‘diakinetic’ were still rich in HABP1 mRNA, as is represented by the strong signal seen in the diakinetic spermatocytes of stage XIII (Fig. 39E, arrowhead, ‘Di’).

Surprisingly, however, was the positive signal obtained in situ hybridization even in the sections probed with sense HABP1 riboprobe (Figs. 40A-G). The presence of signal was seen in the cytoplasm of round spermatids (Fig. 40A, arrowhead and Fig. 40B, arrowhead, ‘RS’) and the pachytene spermatocytes (Fig. 40F, arrowhead and Fig. 40G, arrow, ‘SC’). Additionally, fainter signal was seen in the round spermatids (Fig. 40F, arrow and Fig. 40G, arrow, ‘RS’). The staining observed with HABP1 sense riboprobe was identical to that observed with the
Fig. 39. LOCALISATION OF HABP1 mRNA BY IN SITU HYBRIDISATION USING ANTISENSE HABP1 RIBOPROBE. Adult rat testis sections were probed with antisense DIG-labelled HABP1 riboprobe and developed with anti-DIG IgG conjugated to AP and the NBT/BCIP color system. Staining in round spermatids (Panel A, arrowhead, 100 x and Panel B, arrowhead, ‘RS’, 400 x) and the diakinetic spermatocytes (Panel F, arrowhead, 100 x; Panel G, arrowhead, ‘SC’, 400 x) are shown. mRNA signal in round spermatids of stage I (Panel D, arrowhead, ‘1’) and stage VIII (Panel E, arrowhead, ‘8’), pachytene spermatocytes of stage VIII (Panel E, arrowhead, ‘P’) and diakinetic spermatocytes of stage XIII (Panel F, arrowhead, ‘Di’) is also presented.
Fig. 40. POSITIVE HYBRIDISATION SIGNAL IN IN SITU HYBRIDISATION USING SENSE HABP1 RIBOPROBE. Adult rat testis sections were probed with sense DIG-labelled HABP1 riboprobe and developed with anti-DIG IgG conjugated to AP and the NBT/BCIP color system. Staining in round spermatids (Panel A, arrowhead, 100 x; Panel B, arrowhead, "RS", 400 x; Panel F, arrowhead, 100 x; Panel G, arrowhead, "RS", 400 x) and the pachytene spermatocytes (Panel F, arrow, 100 x; Panel G, arrow, "SC", 400 x) is shown. mRNA signal in round spermatids of stage I (Panel D, arrowhead, '1'), pachytene spermatocytes of stage X (Panel E, arrowhead, 'P') and diakinetic spermatocytes of stage XIII (Panel F, arrowhead, 'Di') is also presented.
antisense HABP1 riboprobe (Figs. 39A,B, F & G). In order to understand if the signal with the sense HABP1 riboprobe also revealed a stage specific staining, a closer look of the stained sections was made. Very interestingly, the signal was found to be in the same cells (Figs. 40C-E) which showed positive expression for HABP1 mRNA localization (Figs. 39C-E). Round spermatids (Fig. 40C, arrowhead, ‘1’) in stage I; pachytene spermatocytes (Fig. 40D, arrowhead, ‘P’) in stage X and the diakinetic spermatocytes (Fig. 40E, arrowhead, ‘Di’) in stage XIII gave a positive signal with sense HABP1 riboprobe.

This observation with the sense HABP1 riboprobe is interesting to pursue and the various reasons for this positive signal have been discussed.

7.3 DISCUSSION

Localization of HABP1 mRNA was studied by in situ hybridization using sense and antisense HABP1 riboprobes. Sense riboprobe was used as a negative control for in situ hybridization, while the antisense riboprobe was the probe, which gave the positive signal after binding to the HABP1 mRNA.

Antisense (Sal I/T7) probe very interestingly gave a positive signal (dark brown staining) in round spermatids and pachytene spermatocytes, a pattern similar to that seen for HABP1 proprotein in immunohistochemistry with anti-rHABP1 and pAb 610 antibodies. This suggested the expression of both HABP1 proprotein and HABP1 transcript in the late meiotic (pachytene spermatocytes) and the post-meiotic (round spermatids) cells. As discussed earlier in Chapter II, reports on higher mRNA expression in pachytene spermatocytes and round spermatids exist and these support our observation of a positive signal for HABP1 mRNA in these particular cell types.

More surprising, was the observation that the sense riboprobe too gave a signal similar to that observed with the antisense riboprobe. Apparently, the result is difficult to explain, but keeping in mind the presence of HABP1 pseudogene on chromosome 21, this result seems interesting.

Few hypothesis explaining the signal seen with the control sense riboprobe are being presented and argued. One possibility existing is that the sense riboprobe may hybridize with genomic DNA stretch of the HABP1 pseudogene (or possibly
even the HABP1 gene), made easily accessible by HCl treatment or 50% formamide condition. HCl treatment in the in situ hybridization procedure results in nicks in genomic DNA, making the access of probe easier and the high stringency condition of 50% formamide during hybridization results in denaturation of the DNA stretch of chromosome 21, the HABP1 pseudogene, and thus, eventually may result in the sense riboprobe hybridizing with the complementary strand on the chromosome 21 DNA stretch (HABP1 pseudogene). Also, the signal with the sense riboprobe is the strongest in spermatocytes, since it is in the spermatocytes (typically, pachytene spermatocytes) during meiosis that the DNA is in the most decondensed state, making the access of probe more easy and also, making the target genomic DNA sequence (in this case, the HABP1 pseudogene or HABP1 gene) more available. Another hypothesis says that the signal possibly seen in pachytene spermatocytes, since the DNA content of pachytene spermatocyte (4n) is more than that seen in round spermatids and the other somatic cells of testis. This, however, does not explain the signal seen in round spermatids, which have a haploid genome. Third possibility suggests the signal being due to the hybridizing of the sense strand to the HABP1 pseudogene to the complementary strand on HABP1 pseudogene. This, however, will also be true for the binding of the antisense HABP1 riboprobe, thus making this a weak hypothesis. Moreover, all these hypotheses do not seem very convincing, since the positive hybridization signal seen is in the cytoplasm, although if the signal was as a result of binding of the riboprobes to the HABP1 pseudogene (HABP1 gene), the signal should have been nuclear. Thus, all these arguments lead to the possibility of the cytoplasmic “species” to which the HABP1 sense riboprobe binds to be an “RNA” species.

The possibility of the sense riboprobe binding to an “RNA” species suggests that the RNA molecule of such kind could result from the transcription of the non-coding strand of HABP1 gene or the sense strand in the HABP1 pseudogene. Northern blot analysis of the testis RNA would reveal if another species of RNA other than the HABP1 transcript, having homology to HABP1 cDNA exists, though the RT-PCR data done using set of primers designed from the first and last exon of
the HABP1 gene revealed the presence of only one RNA species (RT-PCR results shown in earlier Chapters). Blast search of HABP1 cDNA carried out with the submitted ESTs in the data bank, shows the existence of ESTs (for e.g. BE379605, AI436606, etc.), whose minus strand shows a sequence homology with HABP1 cDNA (i.e. the HABP1 mRNA (equivalent to the sense strand in HABP1 cDNA) shows homology with the minus strand of the EST submitted or in other words, the HABP1 mRNA shows complementarity with the EST itself). This reveals the existence of ESTs, which are thus, complementary to HABP1 mRNA / the sense strand in HABP1 cDNA and thus, serve as templates during hybridization and result in a positive signal seen with the sense riboprobe in \textit{in situ} hybridization. As mentioned earlier, the template for the sense riboprobe could arise from either the non-coding strand of HABP1 gene on chromosome 17 or from the sense strand of HABP1 pseudogene. However, the possibility of these ESTs having an altogether different origin cannot be ruled out. Reports on pseudogenes getting transcribed has been reported for the \( \alpha \)-fertilin pseudogene. Jury et al (1997) report of the \( \alpha \)-fertilin gene being a transcribed, but non-functional pseudogene in the human testis. This, hypothesis however needs more work, since, there exists at present no experimental evidence of any such molecule in case of HABP1, except the preliminary \textit{in situ} hybridization data. Performing Northern blots with HABP1 sense riboprobe as the probe, would experimentally reveal the presence of the RNA species hybridizing with the HABP1 sense strand. Also, RT-PCR analysis with primers specific for the non-coding strand in HABP1 gene and the sense strand in HABP1 pseudogene would also be the likely approach to understand more about these “RNA species”. The RNA transcribing from the non-coding strand of HABP1 gene, may or may not, however, translate into a polypeptide (Non coding RNAs) (Lakhotia et al., 1999; Lakhotia, 1999). One report on the existence of a non-coding RNA is of the poly [A\(^+\)] RNA. \textit{H19}, from a mouse gene, which does not code for a protein (Brannon et al. 1990). This, however, is unlikely for HABP1 pseudogene, because of the premature termination codons.