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Appearance of Hyaluronan Binding Protein 1 Proprotein in Pachytene Spermatocytes and Round Spermatids Correlates With Spermatogenesis

SONU CHAND THAKUR, VINOD KUMAR, ILORA GHOSH, ARCHANA BHARADWAJ, AND KASTURI DATTA

From the Biochemistry Laboratory, School of Environmental Sciences, Jawaharlal Nehru University, New Delhi, India.

ABSTRACT: The proprotein form of hyaluronan binding protein 1 (HABP1) has been reported to be present in the pachytene spermatocytes and the round spermatids of the adult testis. To explore the role of HABP1 proprotein in spermatogenesis, its expression in the testes of adult rats was compared with that in the testes of developing rats and that in the testes of adult rats that received estriadiol to halt spermatogenesis. Immunoblotting revealed that the mature form of HABP1 was consistently present in the testis, but its precursor form was not found in the testis of animals aged 7, 14, 21, and 28 days. However, immunohistochemical analysis revealed the presence of the proprotein form in the pachytene spermatocytes and the round spermatids of testes from rats aged 21 and the 28 days, the appearance of which correlated well with the appearance of these cells during spermatogenesis. Reverse-transcriptase polymerase chain reaction revealed transcriptional upregulation of HABP1 in the testes of adult rats, compared with the testes of developing rats. Finally, loss of HABP1 proprotein expression from the pachytene spermatocytes and round spermatids was observed in the testes from rats in which spermatogenesis was arrested. Collectively, these findings demonstrate the appearance of HABP1 proprotein in the pachytene spermatocytes and the round spermatids during the initial stages of postnatal testis development and suggest that this expression may be crucial for spermatogenesis.

Key words: Pachytene spermatids, round spermatids, developing rat testis, spermatogenic arrest.

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Introduction

The postnatal phase of spermatogenesis can primarily be divided into the following 4 main stages: the mitotic proliferation of spermatogonial stem cells, the premeiotic differentiation of spermatogonia cells to diploid primary spermatocytes, the meiotic differentiation of primary spermatocytes to haploid early round spermatids and spermiogenesis, and the process of cellular and nuclear reorganization that turns spermatids into spermatozoa. By day 7 of postnatal development, the rat testis contains somatic cells and spermatogonia, and by day 13 or 14, leptotene spermatocytes appear. Early and late pachytene spermatocytes start appearing between days 20 and 22, whereas the haploid round spermatids form around the 24th day (Malkov et al, 1998). In spermatogenesis, the migration of developing germ cells from the basal to the adluminal compartments of seminiferous tubules requires extensive alterations of structural components that may be associated with the extracellular matrix (ECM). Hyaluronan (HA), an important ECM component, is reported to be linked with cell migration (Toole et al, 2000; Turley et al, 2002).We previously reported the presence of a hyaluranan binding protein called hyaluronan-binding protein 1 (HABP1; sequence analysis has shown HABP1 to be identical to P32/gC1qR Ac No AF 275902) on the sperm surface of numerous species, including humans, and has been shown to be involved in sperm oocyte interaction (Ranganathan et al, 1995), HA-mediated signaling (Gupta and Datta, 1991; Rao et al, 1996), and cellular migration (Ghosh et al, 2003).

HABP1 is synthesized as a proprotein of 282 amino acids, which, after translation, is processed by proteolytic cleavage of initial 73 amino acids to a mature form of 209 amino acids. This proprotein form of HABP1 is extremely labile and has only been detected in pachytene spermatocytes and round spermatids in the germ cells in testes of adult rats (Bharadwaj et al, 2002). During spermatogenesis in rats, different subpopulations of germ cells are acquired, depending on the

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Correspondence to: Prof Kasturi Datta, Biochemistry Laboratory, School of Environmental Sciences, Jawaharlal Nehru University, New Delhi-110067, India (e-mail: kdatta@mail.jnu.ac.in).

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developmental stage. We report results of study to determine the expression of HABP1 proprotein in different cell types in the testes of developing rats. Additionally, to understand the role of the proprotein form of HABP1 in spermatogenic differentiation, we evaluated testes from rats in which spermatogenic arrest was induced.

Materials and Methods

Chemicals

All chemicals used in the study were acquired from Sigma Chemical Company (St Louis, Mo) or Sigma Chemie (Deisenhofen, Germany), unless otherwise indicated.

Purification of HABP1 and Generation of Antibodies

HABP1 was purified to homogeneity with an ion exchange column coupled to a Pharmacia FPLC system, as reported elsewhere (Jha et al, 2002). Polyclonal anti-rHABPl antibodies were raised against the purified recombinant HABP1 (rHABPl) in rabbit, as previously described (Deb and Datta, 1996).

Arrest of Spermatogenesis

Adult male rats weighing 250–300 g received intramuscular injections of 75 μg of β-estradiol-17-benzoate suspended in 0.25 mL of olive oil (vehicle) daily for 30 days, as reported earlier (Chinmoy et al, 1984). The spermatogenic status was examined microscopically after hematoxylin-eosin staining of testis sections.

After β-estradiol-17-benzoate treatment, the seminiferous tubules showed an arrest in the development of spermatozoa, as revealed by a complete absence of elongated spermatids (or testicular spermatozoa). However, Sertoli cells, spermatogonia, spermatocytes, and a few round spermatids were seen.

Tissues and Lysate Preparation

Testes from rats aged 7, 14, 21, and 28 days and from adult rats were collected from approximately 10 Sprague Dawley rats per age group (Animal House, Jawaharlal Nehru University, New Delhi, India). The animals were killed in accordance with the protocol approved by the animal ethics committee at Jawaharlal Nehru University. For sodium dodecyl sulfate polyacrylamide gel electrophoresis (SDS-PAGE) and Western blotting, the testes lysate was prepared by removing tunica albuginea, followed by immediate boiling of the seminiferous tubules in Laemmli buffer (Tris-glycerol with 5% β-mercaptoethanol, 2% SDS, and 100 mmol/L PMSF [phenyl methyl sulfonyl fluride]) for 15 minutes (Laemmli, 1970). Subsequently, the lysate was centrifuged at 14 000 × *g* for 15 minutes at room temperature, and the supernatant containing the total lysate protein was stored at −20°C until use. The protein concentration was estimated using the bicinchoninic acid protein assay system with bovine serum albumin (BSA) as a standard (Smith et al, 1985).

SDS-PAGE and Western Blot Analysis

Testicular lysate protein (70 μg) was resolved on a 12.5% PAGE under denaturing conditions (Laemmli, 1970). Resolved proteins were electroblotted onto a nitrocellulose membrane and were probed with polyclonal anti-rHABP1 antibody (titer, 1:2500). After incubation with goat anti-rabbit IgG conjugated to alkaline phosphatase (AP), the immunoreactive bands were detected with the nitroblue tetrazolium/5-bromo-4-chloro-3-indolyl phosphate (NBT/BCIP) system (USB Corporation, Cleveland, Ohio).

Immunohistochemistry

For immunohistochemical study, the organs fixed by immersion in Bouin's fixative were dehydrated through graded alcohol and xylene and were embedded in paraffin wax. The sections of rat testis (5-μm thick) were deparaffinized, rehydrated, and blocked with 3% wt/vol BSA in Tris-buffered saline (TBS, 0.05 mol/L Tris-Cl, pH 7.6; 0.15 mol/L NaCl). After specimens were washed with 0.05% Tween 20–supplemented TBS (TBST), they were incubated with polyclonal anti-rHABPl antibody (titer, 1:200). After incubation with goat anti-rabbit IgG conjugated to AP (titer, 1:500), Fast Red substrate (DAKO, A/S, Glostrup, Denmark) was used to develop color, in accordance with the manufacturer's protocol. The counterstaining was done using hematoxylin, and sections were mounted in glycerol.

RNA Isolation and Reverse-Transcriptase Polymerase Chain Reaction (RT-PCR) Analysis

Total RNA was isolated from the testes of developing rats by use of TRIZOL reagent (Invitrogen, Life Technologies, Carlsbad, Calif), according to the manufacturer's protocol (Chomezynski and Sacchi, 1987). Single step RT-PCR was performed on 2 μg of total RNA from different tissues with the rTth enzyme (PE Applied Biosystems, Branchburg, NJ). We used the RAB3 forward HABP1 primer 5′-CCGGCCC-TTCGGTTTGCTCAGCGT-3′ and the RAB4 reverse HABP1 primer 5′ GGCCCAGTCCAGGGAGTCTGTGTT 3′, which designed on the basis of the exons of the gene encoding HABP1. The amplification was performed for 25 cycles to ensure linear amplification. β-Actin amplification was performed to obtain an internal control, using the same reaction mixture. RT-PCR for β-actin was done using the following primers: forward primer, 5′-CGTGCGCCGCCC-TAGGCACCA-3′; and reverse primer, 5′-TTGGCCTTA-GGGTTCAGGGGGG-3′.

Results

Expression of HABP1 Proprotein in the Testes of Developing Rats

Immunoblot analysis of testes from developing rats (7, 14, 21, and 28 days old) and adult rats was performed using polyclonal anti-rHABPl antibody. As shown in Figure 1, the mature 34-kDa form of HABP1 was detected in testes lysates for all age groups. However,

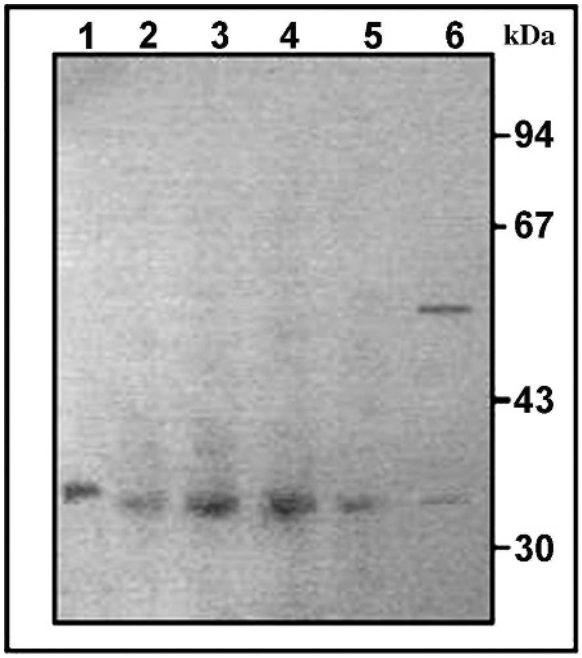

Figure 1. Presence of HABP1 proprotein in developing rat testis. Lysates from rats aged 7 (lane 2), 14 (lane 3), 21 (lane 4), and 28 (lane 5) days and from adult rats (lane 6, control) were prepared in Laemmli buffer, and 70 μg of each lysate was resolved on a 12.5% SDS-PAGE and probed with anti-rHABP1 antibody. Purified rHABP1 is in lane 1.

the 55-kDa HABP1 proprotein was present only in the testes lysates for adult rats, whereas no band could be seen in the testis lysates of developing rats (Figure 1).

To study the differential expression of the proprotein form of HABP1 in different cell types during testis development, testis sections from developing rats (7, 14, 21, and 28 days old) and adult rats were immunostained with anti-rHABP1 antibody. Testes from the 7-day-old rats, which contained only gonocytes and spermatogonia (Figure 2A), and testes from the 14-day-old rats, which had spermatogonia and leptotene spermatocytes, did not show any expression of the proprotein form of HABP1 (Figure 2C).

HABP1 proprotein expression was first seen at 21 days, which correlated with the first appearance of the pachytene spermatocytes during testis development (Figure 2E). It may be noted that, although some tubules with few early spermatocytes (leptotene and zygotene) were present, they did not stain positive with anti-rHABP1 antibody. This suggests that HABP1 proprotein is present only in those spermatocytes, which have reached a certain degree of maturity.

By day 28, haploid round spermatids were detected in testes of the developing rats, and the proprotein form were observed in these cells in testes from the 28-day-old rats (Figure 2G). Intense cytoplasmic staining was also seen in the cytoplasm of pachytene spermatocytes. However, no staining was detected in the spermatogonia and the leptotene and zygotene spermatocytes.

As expected, in testes from adult rats (where all germ cells are present), cytoplasmic staining was seen in the pachytene spermatocytes and round spermatids, but no staining was detected in the spermatogonia, early spermatocytes, and elongated spermatids (Figure 2I). It is evident from the data that, when the germ cells in developing testes differentiate into either pachytene spermatocytes or round spermatids, HABP1 proprotein expression occurs. Thus, expression was detected in the testis only after day 20 and continued until adulthood, which suggests that the proprotein form of HABP1 plays a role during spermatogenesis.

HABPl mRNA Expression in Developing Rat Testis

To determine the relative level of HABP1 transcript in testes of developing and adult rats, total RNA isolated from testes from rats in each age groups were analyzed by RT-PCR under semiquantitative conditions. A 583-bp amplification product was obtained from RNA samples of all testes, using RAB3 and RAB4 primers of the gene encoding HABP1 (Figure 3A). Control RT-PCR was performed with β-actin primers. Comparable amounts of amplified β-actin transcripts obtained from equal quantities of RNA from 5 different testes confirmed the integrity and the quantitative nature of the RT-PCR reaction (Figure 3B). The same level of HABP1 transcript was also detected in the testes from rats aged 7, 14, and 21 days. In testes from rats aged 28 days, the level of transcript was higher than that in testes from the other developing rats. The most significant increase in RNA transcripts was observed in adult rats. This observation was consistent across at least 10 independent experiments. Higher levels of HABP1 mRNA transcript in testes from rats aged 28 days and from adult rats implies a higher expression of the gene encoding HABP1 in these age groups (Figure 3C). This observation correlates well with the fact that rats in these age groups contain higher number of pachytene spermatocytes and round spermatids, which also demonstrates higher expression of the translated product.

Absence of HABP1 Proprotein in Spermatogenic-Arrested Testis Induced by β-Estradiol-17-Benzoate

Spermatogenic arrest was induced in adult rats by means of β-estradiol-17-benzoate administration. HABP1 expression was then studied in testes from these rats, to understand the role of HABP1 proprotein in spermatogenesis. Interestingly, as seen by immunoblot analysis, the HABP1 proprotein was absent in the testes lysates of adult rats with spermatogenic arrest, but there was no

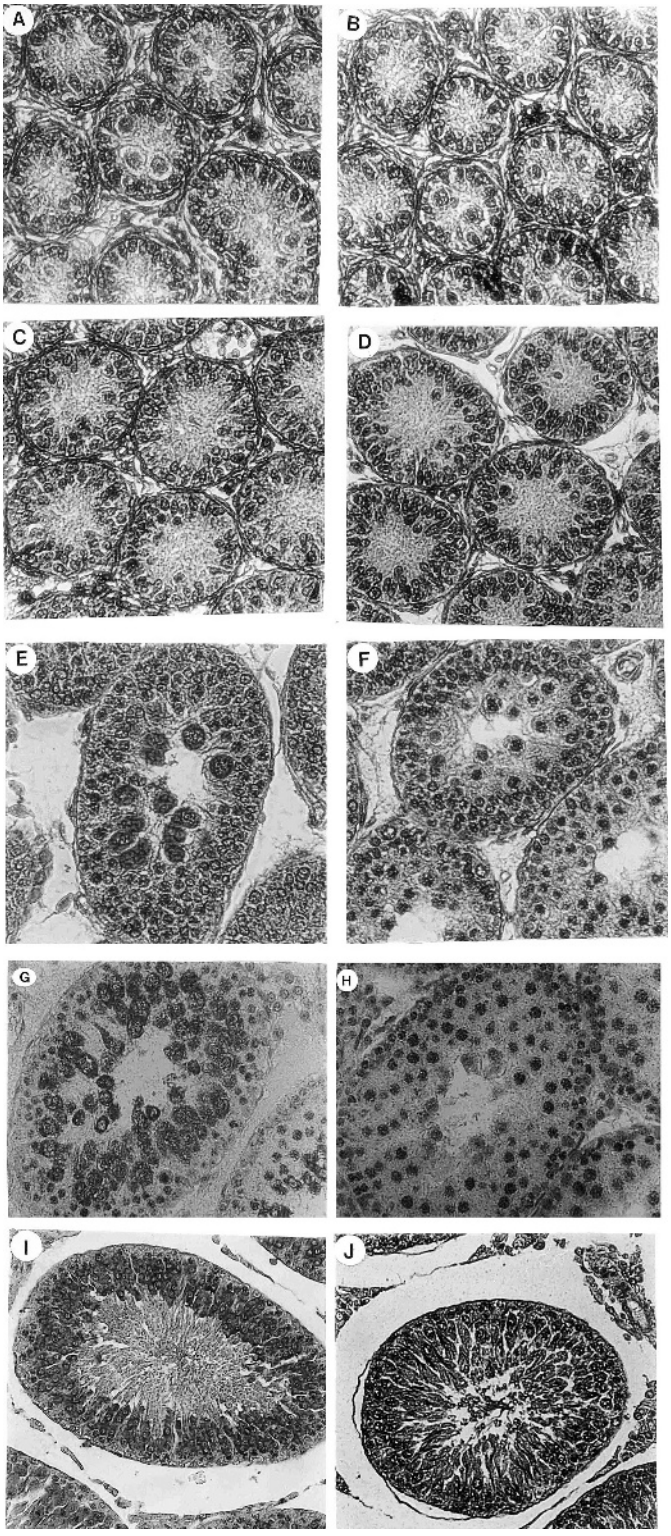

Figure 2. Localization of HABP1 proprotein in testes from rats aged 7, 14, 21, and 28 days and from adult rats. Histochemical staining for HABP1 proprotein by means of anti-rHABP1 antibodies and Fast-Red in testes from rats aged 7 **(A)**, 14 **(C)**, 21 **(E)**, and 28 days **(G)** and from adult rats **(I)**. Histochemical staining with normal rabbit serum control in testes from rats aged 7 days **(B)**, 14 days **(D)**, 21 days **(F)**, and 28 days **(H)** and from adult rats **(J)**.

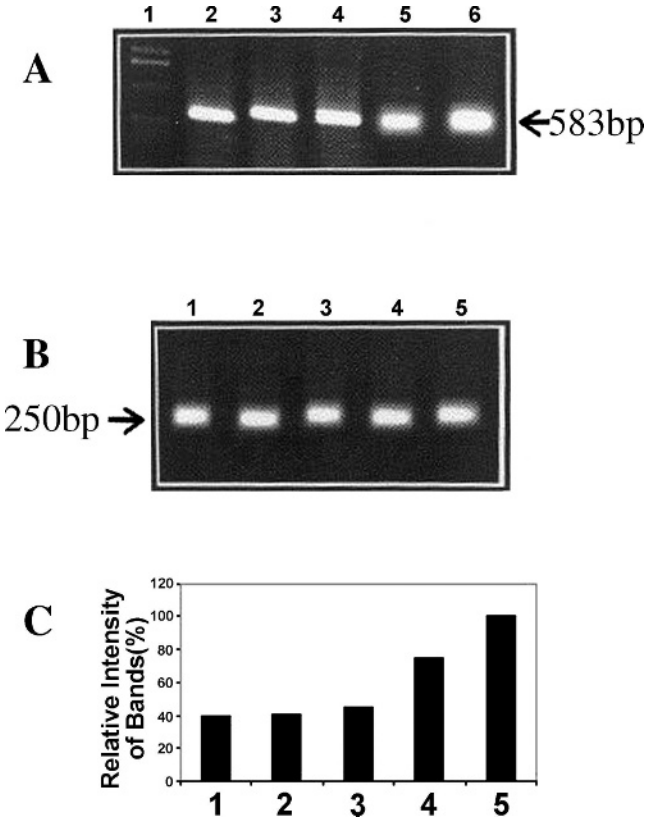

Figure 3. Evidence of enhanced mRNA expression of HABP1. **(A)** RT-PCR analysis using HABP1 specific primers RAB3-RAB-4 with equal amounts of total RNA from testes of rats aged 7 (lane 2), 14 (lane 3), 21 (lane 4), and 28 (lane 5) days and from adult rats (lane 6). Lane 1 shows the 1 kb DNA ladder. **(B)** β-Actin mRNA expression in testes from rats aged 7 (lane 5), 14 (lane 4), 21 (lane 3), and 28 (lane 2) days old; expression in testes from adult rats (lane 1) served as an internal control for the integrity and quantity of RNA used. **(C)** Quantitation and comparison of relative HABP1 mRNA expression in testes from rats aged 7 (1), 14 (2), 21 (3), and 28 (4) days and from adult rats (5). The expression of HABP1 mRNA in testes from adult rats was considered to be 100%.

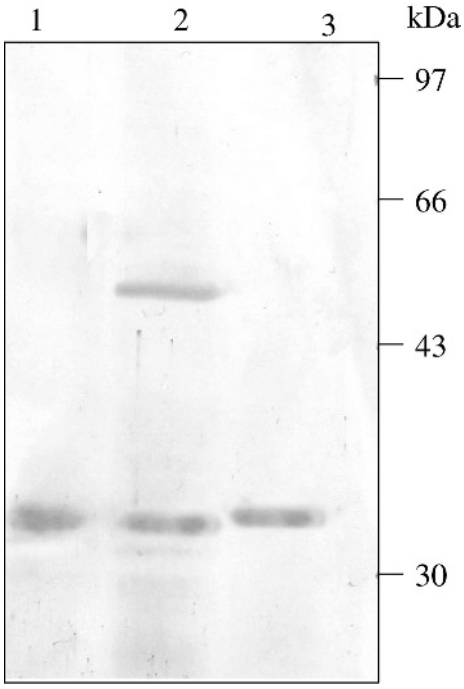

Figure 4. Absence of HABP1 proprotein in testes from spermatogenic-arrested rats. Lysates of testes from spermatogenic-arrested rats (lane 1) and adult rats (lane 2) were prepared in Laemmli buffer, and 70 μg of each lysate was resolved on a 12.5% SDS-PAGE and probed with anti-rHABP1 antibody. Purified rHABP1 is in lane 3.

difference in the level of the mature form of HABP1 (Figure 4).

Immunohistochemical analysis was performed to determine the expression of HABP1 proprotein in spermatogenic-arrested testis. Complete arrest of spermatogenesis in sections of rat testes from treated adult rats was indicated by the absence of sperm in the seminiferous tubules (data not shown). A significant reduction in HABP1 proprotein expression was seen in pachytene spermatocytes from the spermatogenic-arrested testicular tubules, compared with that in testes from healthy, untreated rats. Even the round spermatids that were present in few numbers were devoid of HABP1 proprotein (Figure 5A and B). In contrast, immunostaining of testes from untreated adult rats showed the usual intense staining in the pachytene spermatocytes and round spermatids (Figure 5D and E).

Discussion

Remodeling of the ECM is expected to play a vital role in testicular development. The present article reveals that the proprotein form of HABP1—a novel ECM molecule—likely plays a role during spermatogenesis. This was concluded from the observation that the expression of HABP1 protein occurred only in meiotically differentiated cells (ie, pachytene spermatocytes and round spermatids) and correlated well with the first appearance of these cells during postnatal testis development. This hypothesis is also supported by the fact that, although HABP1 is present in the testes of healthy adult rats, it was not detected in adult rats with spermatogenic arrest.

During spermatogenesis, certain testis-specific genes and testis-specific variants of somatic genes are differentially expressed (Wolgermuth and Watrin, 1991; Penttila et al, 1995). Similar the expression of other cell-specific genes, HABP1 proprotein expression solely in pachytene spermatocytes and round spermatids

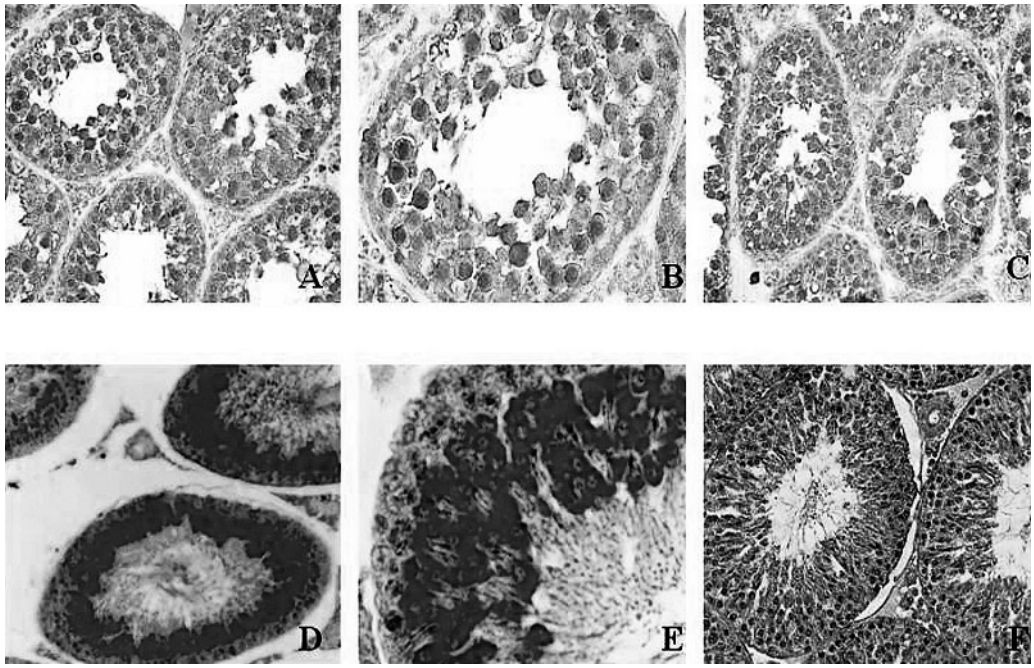

Figure 5. Localization of HABP1 proprotein in testes from spermatogenic-arrested rats. Histochemical staining for HABP1 proprotein with anti-rHABP1 antibodies and Fast-Red in testes from spermatogenic-arrested rats **(A, B)** and adult rats **(D, E)**. Histochemical staining with a normal rabbit serum control of testes from spermatogenic-arrested rats **(C)** and adult rats **(F)**.

suggests that it plays a role in these cells types during spermatogenesis.

Immunohistochemical analysis of testes from rats aged 7 and 14 days showed the absence of the proprotein form of HABP1 in all cell types, whereas after the onset of pachytene spermatocyte formation in rats aged 21 days and round spermatid formation in rats aged 28 days, the proprotein form could be detected by the antibody against HABP1. Because the proprotein form is present only in a small number of cells in testes from rats aged 21 and 28 days, it could not be detected by immunoblot analysis of the total testicular extract. However, because the adult testis contains more pachytene spermatocytes and round spermatids (as evident from the immunohistochemical analysis), the proprotein form could be detected by immunoblotting.

The level of expression of HABP1 transcripts in adults rats was also significantly higher than that in rats aged 7 days. In testes from adult rats, where continuous meiosis takes place, the HABP1 mRNA expression increased several fold, and this finding correlates well with HABP1 proprotein accumulation. The higher level of HABP1 transcripts might be due to transcriptional upregulation or to mRNA storage and delayed post-translational cleavage in meiotic and postmeiotic cells. mRNA storage is common in pachytene spermatocytes, where the mRNA is saved for use during spermatogenesis (Soderstrom, 1976). Therefore, the accumulation of HABP1 proprotein and the storage of mRNA transcripts may imply that the proprotein form is developmentally regulated, suggesting its role in testicular development.

In the testis, the early spermatocytes gradually move from the basal compartment towards the adluminal compartment as they mature. During spermatogenesis, this passage is mediated by the morphological intimacy between Sertoli cell tight junctions and the ECM (Cheng and Mruk, 2002). It is also known that the homeostasis of ECM proteins is regulated by the coordinated activity between proteases and protease inhibitors (Werb, 1997; Sternlicht, 2001). Several matrix metalloproteinases are synthesized as active zymogens (pro-MMPs), which undergo an initial proteolytic cleavage, generating an active protein (Araceli et al, 2005). This activation process is largely regulated by tissue inhibitor of metalloproteinase (TIMPS). The interplay between these molecules regulates the remodeling of the ECM (Siu et al, 2003). Several reports indicate that MMPs and TIMPs produced in testicular cells are under the regulation of hormones, such as follicle-stimulating hormone (Ulisse et al, 1994), and cytokines (Walsh et al, 2000) during development. It is important to mention that MT-1 MMP regulates the proteolysis of multifunctional HABP1 (Rozanov et al, 2002). Thus, the role of HABP1 proprotein accumulation either due to higher rates of transcription or lower rates of protein proces-

sing in the presence of TIMPs can be correlated with ECM remodeling. We must also note that ECM remodeling is known to play a crucial role in spermatogenesis by regulating the Sertoli cell junction dynamics (Mruk et al, 2004).

Taken together, these observations reveal that HABP1 proprotein accumulation during spermatogenesis occurs specifically in the pachytene spermatocytes and round spermatids, which suggests that HABP1 might play a role in the meiotic and the postmeiotic stages of spermatogenesis and that this accumulation might be further regulated by the important phenomenon of proteolysis.

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