

Osteopontin Gene Expression in the Holstein Bull Reproductive Tract

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ABSTRACT: The objective of this study was to localize gene expression of osteopontin in the Holstein bull reproductive tract using Northern blot analysis and in situ hybridization. For Northern blot analysis, a digoxigenin-labeled osteopontin complementary deoxyribonucleic acid (cDNA) was used to probe blots containing total ribonucleic acid (RNA) isolated from the testis, epididymis, vas deferens, ampulla, seminal vesicle, prostate, and bulbourethral glands. The digoxigenin-labeled cDNA for the bovine homologue of osteopontin was hybridized to a single band at approximately 1.6 kb to RNA samples from the ampulla and seminal vesicle. For in situ hybridization studies, antisense and sense riboprobes were synthesized and used to hybridize cryosections that had been obtained from bull reproductive tissues. In situ hybridization of the bull testis

detected osteopontin messenger RNA in the developing germ cells. Osteopontin gene expression was detected only in seminiferous tubules that contained elongated spermatids, which suggests that expression varies with the stage of the seminiferous epithelium. Within the epididymis, silver grains were distributed over the sperm that were located within the lumen of the caput, corpus, and cauda epididymis. Osteopontin expression was primarily observed in the epithelial cells of the ampulla. Antisense riboprobes also hybridized to sperm that were located within the lumen of the ampulla, confirming the presence of osteopontin transcripts in the haploid male gamete.

Key words: Spermatozoa, mRNA, testis, epididymis, ampulla, seminal vesicle.

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Our laboratory recently identified osteopontin (OPN), the 55-kd fertility-associated protein found in bull seminal plasma (Cancel et al, 1997). OPN is an acidic glycoprotein rich in aspartic acid, glutamic acid, and serine (Sørensen and Petersen, 1994). Also known as secreted phosphoprotein-1, 2ar, and bone sialoprotein, OPN is a prominent bone matrix protein that is involved in mineralization and resorption of the bone matrix (Craig and Denhardt, 1991). Immunohistochemical studies in human tissues localized OPN to the luminal surfaces of epithelial cells of the gastrointestinal tract, gall bladder, pancreas, urinary tract, reproductive tract, lung, breast, salivary glands, and sweat glands (Brown et al, 1992). Moreover, OPN is present in biological fluids such as urine and milk (Senger, 1989). In urine, OPN is believed to inhibit precipitation of calcium salts, particularly calcium oxalate (Shiraga et al, 1992). In milk, OPN is present at a concentration of 3–10 µg/mL and has been suggested to bind to calcium (Senger et al, 1989).

Although OPN is present in many tissues, few laboratories have reported its presence in reproductive tissues. In mice, OPN messenger ribonucleic acid (mRNA) was demonstrated in the ovary, but was not detected in the

testis by Northern blot analysis (Craig and Denhardt, 1991). Siiteri and Hamilton (1995) reported OPN mRNA in adult rat Sertoli-cell extracts. Reverse transcription-polymerase chain reaction (RT-PCR) studies identified OPN mRNA in the rat testis and epididymis (Siiteri et al, 1995). Immunofluorescence studies confirmed the presence of OPN in the rat epididymis and also detected OPN associated with the rat sperm tail (Siiteri et al, 1995).

Because OPN is more prevalent in semen of higher-fertility bulls (Cancel et al, 1997), we undertook studies to identify the origin of OPN in the bull reproductive tract to gather information that may provide insight into how this protein influences male fertility. Recently, we established that OPN is present in bull seminal vesicles and ampulla, as well as in the fluids of these tissues (Cancel et al, 1999).

The objective of this study was to localize gene expression of OPN in the Holstein bull reproductive tract using Northern blot analysis and in situ hybridization.

Materials and Methods

Collection of Bull Reproductive Tissues

The reproductive tracts of 4 Holstein bulls previously maintained at the J.O. Almquist Research Center were collected within 30 minutes of slaughter. The testes; caput, corpus, and cauda epididymides; vasa deferentia; ampullae; seminal vesicles; prostate; and bulbourethral glands were isolated and plunged into liquid

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nitrogen. After being transported to the laboratory, the tissues were stored at -80°C until they were used.

Amplification of OPN Complementary Deoxyribonucleic Acid

Kerr et al (1991) sequenced the full-length bovine OPN complementary deoxyribonucleic acid (cDNA). The cDNA for the bovine homologue of OPN (cloned into a pBluescript vector) was kindly provided by Dr Larry Fisher of the National Institute of Dental Health, National Institutes of Health, Bethesda, Maryland. The pBluescript vector containing the OPN cDNA was transformed into *Epicurian coli* TOPP Competent Cells (Stratagene, La Jolla, Calif) according to the manufacturer's instructions. Following the transformation, bacterial colonies were grown and the bacteria were harvested and lysed by the alkali method (Sambrook et al, 1989). The plasmid was then isolated and purified by precipitation with polyethylene glycol (Sambrook et al, 1989). The purified plasmid was stored at -20°C until it was used to synthesize probes for Northern blot analysis and in situ hybridization.

Northern Blot Analysis

Total RNA was isolated from bull reproductive tissues using the TRI REAGENT (Sigma Chemical Company, St Louis, Mo) according to the manufacturer's instructions. Ten- μg RNA samples were loaded in 1% agarose gels containing 2.2 M of formaldehyde and the gels were run at 100 volts (constant voltage) for 3 to 4 hours. Following gel electrophoresis, the RNA was transferred onto a nylon membrane (Nytran; Schleicher & Schuell, Keene, NH) and baked, under vacuum, for 2 hours at 80°C .

The OPN cDNA was released from the pBluescript vector by restriction enzyme digestion with XbaI and XhoI. Following enzyme digestion, the samples were run in a 0.8% agarose gel and the OPN cDNA was isolated and purified using the Gene Clean Kit (Bio 101 Inc, La Jolla, Calif) according to the manufacturer's instructions. The OPN cDNA was stored at -20°C until it was used for Northern blot analysis.

Northern blot analysis was carried out using the digoxigenin (DIG) labeling and detection system (Boehringer Mannheim, Indianapolis, Ind). Briefly, the OPN cDNA was labeled by random priming using the DIG-high prime solution (Boehringer Mannheim) according to the manufacturer's instructions. The membrane was prehybridized in standard hybridization solution (5X SSC, 0.1% N-lauroylsarcosine, 0.02% SDS, 1% blocking reagent) for 30 minutes at 68°C and hybridized overnight at 68°C in 30 mL of standard hybridization solution containing the DIG-labeled OPN cDNA.

Following hybridization, the membrane was washed twice for 15 minutes each time in 2X SSC/0.1% SDS at room temperature followed by 2 15-minute washes in 0.1X SSC, 0.1% SDS at 68°C . The membrane was rinsed in washing buffer (0.3% Tween 20 in 0.1M maleic acid buffer/0.15M sodium chloride, pH 7.5) and incubated for 30 minutes in blocking solution (prepared by diluting 10X blocking solution 1:10 in maleic acid buffer) followed by a 30-minute incubation in anti-DIG-AP conjugate solution (1:10,000 dilution; Boehringer Mannheim). The membrane was washed twice for 15 minutes each time in washing buffer and equilibrated for 5 minutes in 30 mL of detection

buffer. The hybridized DIG-labeled OPN probe was visualized with the chemiluminescence substrate CSPD ready-to-use solution (Boehringer Mannheim). The membrane was exposed to x-ray film (Fuji film, Fisher Scientific, Pittsburgh, Pa) at 37°C to enhance the luminescent reaction. Following Northern blot analysis the membrane was then stripped and hybridized to a DIG-labeled 18S ribosomal RNA to check for even loading of the wells as well as even transfer of RNA samples to the nylon membrane.

In Situ Hybridization

Ten-micron sections of bull reproductive tissues were created using a cryostat and were placed on poly-L-lysine-coated slides (Sigma Chemical Company, St Louis, Mo). Sections were stored at -80°C until they were used for in situ hybridization.

The pBluescript Amp SK vector containing the OPN cDNA was linearized using either the restriction enzyme XbaI (Pharmacia, Piscataway, NJ) or XhoI (Pharmacia) to produce anti-sense and sense riboprobes, respectively. Linearized templates were treated with proteinase K, phenol/chloroform extracted and ethanol precipitated. Template DNAs were resuspended in RNase-free water ($\sim 1 \mu\text{g}/\mu\text{L}$) and stored at -20°C until they were used for riboprobe synthesis.

Riboprobes were synthesized using the T_3/T_7 Riboprobe in vitro transcription system (Promega, Madison, Wis) according to the manufacturer's instructions in the presence of $[\alpha^{35}\text{S}]\text{UTP}$ (NEN, Boston, Mass). For the synthesis of OPN riboprobes, the XbaI digest was transcribed with the T_3 polymerase and the XhoI digest was transcribed with the T_7 polymerase. Following transcription, DNA templates were digested with RQ_1 DNase (Promega) and unincorporated nucleotides were removed using a NucTrap Probe Purification Column (Stratagene) and the Push Column Beta Shield Device (Stratagene) according to the manufacturer's instructions. Riboprobes were recovered by ammonium acetate precipitation and were resuspended in hybridization buffer (50% Formamide, 4X SSC, 5X Denhardt's solution, 1% SDS, 10% dextran sulfate, 250 $\mu\text{g}/\text{mL}$ transcription RNA, 25 $\mu\text{g}/\text{mL}$ poly A and C, 0.1M DTT) to 500,000 CPM per μL .

Frozen sections were brought to room temperature by placing them on a slide warming plate. The sections were fixed for 30 minutes in 4% paraformaldehyde in PBS (pH 7.0) followed by 3 10-minute washes in 1X PBS. Sections were then rinsed in ddH_2O and 0.1 M triethanolamine for 1 minute each before acetylation for 10 minutes in 0.1 M TEA containing 0.25% acetic acid. Slides were rinsed in ddH_2O for 1 minute and dehydrated in 50%, 70%, 85%, 95%, and twice in a 100% ethanol series for 3 minutes each. Slides were dried on a slide warming plate. Riboprobes were denatured at 68°C for 10 minutes. Approximately 5–10 μL of riboprobe/hybridization buffer were added to each section and cover slips were placed over the sections. Tissue sections were hybridized at 50°C for 3 hours in a humidified chamber. Following hybridization, slides were incubated in 4X SSC/0.02 M DTT for 15 minutes and rinsed in 4X SSC for 10 minutes. Slides were then treated with RNase A (20 $\mu\text{g}/\text{mL}$ RNase A in 5X TE) at 37°C for 30 minutes. Slides were subjected to a high criterion wash for 1 hour at 55°C followed by an overnight wash in 0.5X SSC/0.02 M β -mercaptoethanol at room temperature. The following day, the sections were dehydrated, air-

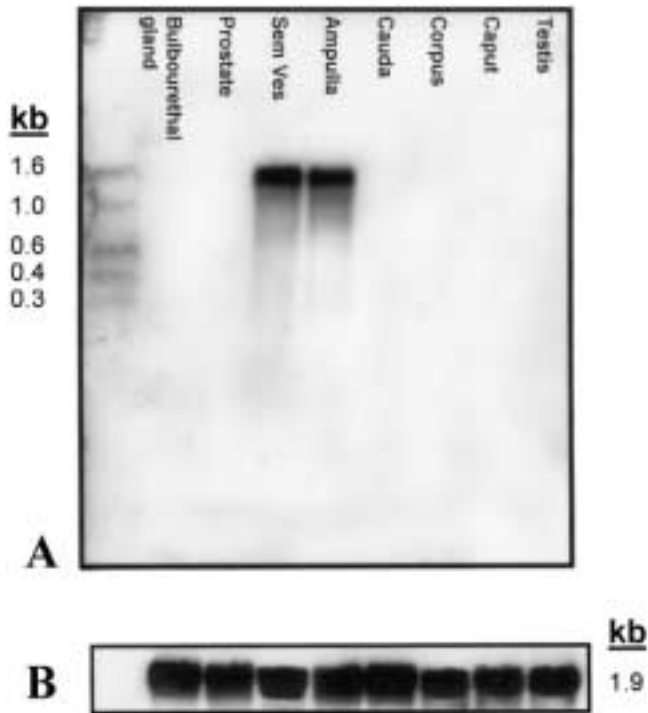


Figure 1. Northern blot analysis of osteopontin (OPN) gene expression in the bull reproductive tract. Total ribonucleic acid (RNA) was isolated, electrophoresed in a 1% agarose formaldehyde gel, and blotted onto a nylon membrane. Following hybridization, the membrane was washed and the signal detected by chemiluminescence. (A) Blot probed with digoxigenin-labeled OPN complementary deoxyribonucleic acid. (B) Blot probed with digoxigenin-labeled ribosomal 18S RNA.

dried, taped to a piece of cardboard, and exposed to x-ray film overnight at room temperature. Following autoradiography, the slides were dipped in emulsion (NTB2, Kodak, VWR Scientific Products, Philadelphia, Pa) and exposed in the dark for 2 to 5 days. The slides were developed with D-19 developer (Kodak) for 5 minutes, fixed (in Kodak fixer) for 10 minutes, and rinsed for at least 1 hour under running deionized water. Finally, the sections were counterstained with hematoxylin/eosin and mounted with Permount (Fisher Scientific). Slides were allowed to air-dry for 2 days before being examined by bright field microscopy.

Results

A digoxigenin-labeled cDNA for the bovine homologue of OPN was used to probe blots containing RNA from

bull testis, epididymis, vas deferens, ampulla, seminal vesicle, prostate, and bulbourethral gland. The DIG-labeled OPN cDNA hybridized to a single band at approximately 1.6 kb to RNA samples from the ampulla and seminal vesicle (Figure 1A). OPN transcripts were not detected in RNA samples that were isolated from the testis, epididymis, prostate, bulbourethral gland (Figure 1A), or vas deferens (not shown). Following Northern blot analysis with the OPN cDNA, the membrane was stripped and hybridized to a DIG-labeled 18S ribosomal RNA to check for even loading of the wells and complete transfer of the RNA (Figure 1B).

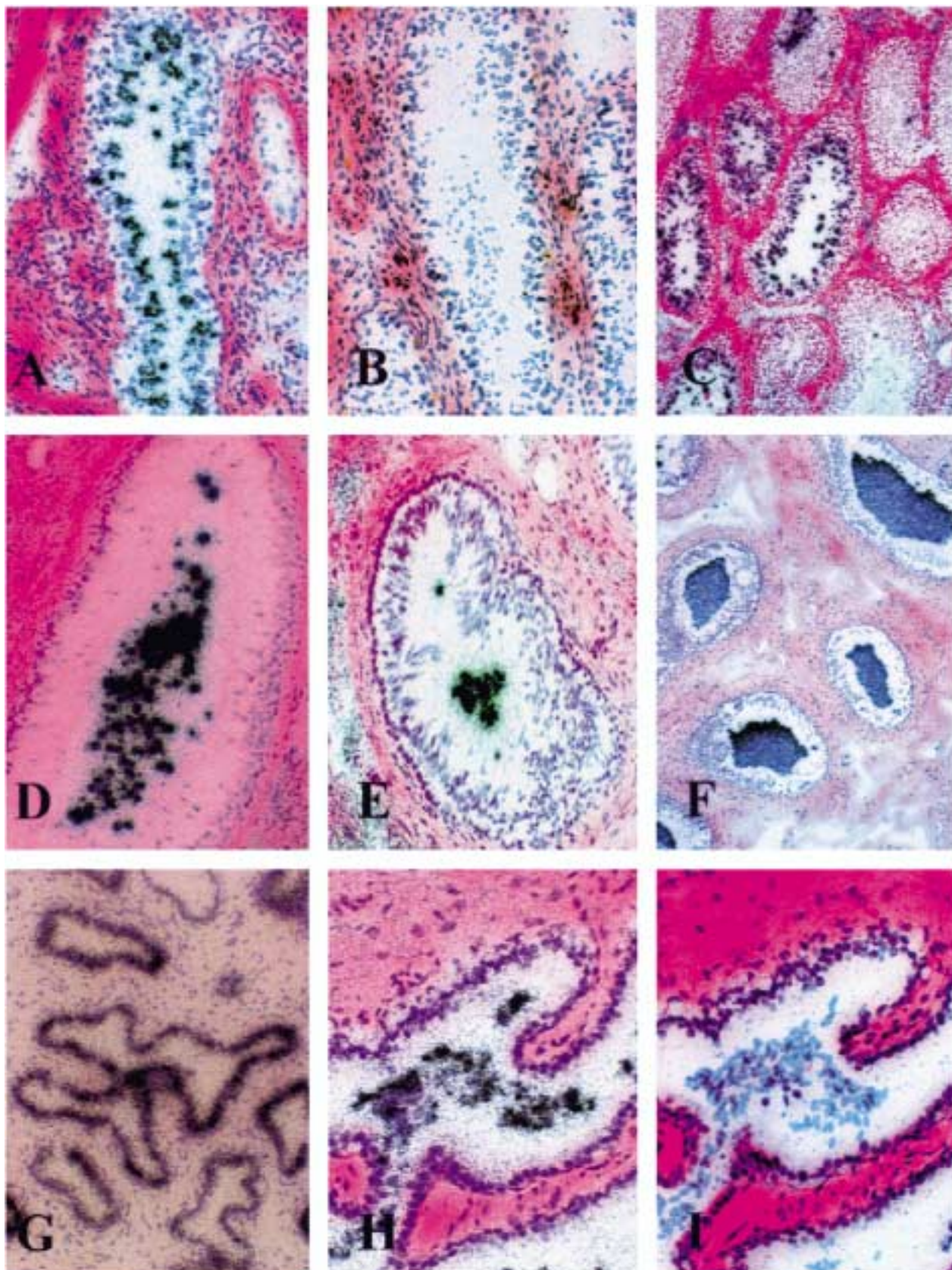
In situ hybridization was used to identify the cell types expressing the OPN transcript. In the testis, OPN expression was observed in the developing germ cells of the adluminal compartment of the seminiferous tubule (Figures 2A and C), but OPN mRNA was not detected in the interstitial tissue (Figure 2C). In addition, OPN transcripts were observed primarily in tubules containing elongated spermatids (Figures 2A and C), which suggests that OPN gene expression may vary with the stage of the seminiferous cycle. However, morphological details in the cryosections were insufficient for definitive staging of the seminiferous tubules.

Within the epididymis, OPN expression was not observed in the epithelial cells of the caput, corpus, or cauda, although silver grains were distributed over the sperm that were located within the lumen of the caput (Figure 2D), corpus (Figure 2E), and cauda epididymidis (Figure 2F). In the ampulla, OPN gene expression was observed in epithelial cells (Figure 2G). OPN transcripts were also observed on the sperm that were located within the lumen of the ampulla (Figure 2H). Transcripts were not detected in tissue sections from the vas deferens, seminal vesicles, prostate, or bulbourethral glands that were probed with the antisense OPN riboprobe (not shown). No specific hybridizations were observed with the sense riboprobes (Figures 2B and I).

Discussion

This is the first study to examine gene expression of OPN in the bull reproductive tract. Based on this study and the study conducted by Cancel et al (1999) we have estab-

Figure 2. Cellular localization of osteopontin (OPN) transcripts in bull reproductive tract tissues by in situ hybridization. Tissues were probed with either a ³⁵S-radiolabeled antisense (A, C, D, E, F, G, H) or negative control sense riboprobe (B, I). Positive signal is represented by the silver grains. In (A), silver grains were distributed over the germ cells within the adluminal compartment of the seminiferous tubules. In (B), no specific hybridization was detected in the seminiferous tubules with the sense riboprobe. In (C), interstitial tissue did not contain OPN transcripts, and silver grains appeared to be restricted to certain stages of the seminiferous epithelium. Silver grains were distributed over the lumen of the caput (D), corpus (E), and cauda (F) epididymidis. In the ampulla (G, H, and I), silver grains were distributed over the epithelium (G) and over luminal sperm when present (H). In (I), no specific hybridization was seen in the ampulla with the sense riboprobe. Magnification: (A) and (B) ×88; (C) ×50; (F) and (G) ×65; (D), (E), (H), and (I) ×130.



lished that the OPN that is present in bull seminal plasma originates from the ampulla and seminal vesicles, with the majority of the protein being synthesized by the epithelial cells of the ampulla. An extensive study conducted in human tissues localized OPN at the luminal surfaces of epithelial cells of the gastrointestinal tract, gall bladder, pancreas, distal tubules of the kidney, the bladder, endocervix, lung, breast, salivary glands, sweat glands, reproductive tracts, and other tissues (Brown et al, 1992). Those researchers also noted bacterial strains that were capable of binding to epithelial cells via integrins and proposed that OPN may prevent attachment of bacteria to the epithelium by competing for the integrin-binding domains that are present on the epithelial cells. In bulls, OPN may protect the epithelial cells from bacterial infections in the accessory sex glands. The most prevalent pathogenic condition of the accessory sex glands is seminal vesiculitis, which can negatively affect semen quality (Cavaliere and Van Camp, 1997). The most common organism isolated in cases of vesiculitis is *Actinomyces pyogenes* (Morris, 1994; Gilbert, 1996), although *Hemophilus somnus* and *Escherichia coli* may also be involved (Morris, 1994). Inflammation of the ampullae can also occur in bulls; however, this condition is considered to be rare (Van Camp, 1997). Although the presence of integrin receptors in the ampulla has not been established, the lower incidence of inflammation in the ampulla may be the result of high levels of OPN expression by the epithelial cells of the ampulla.

OPN transcripts were not observed in the testis by Northern blot analysis, but transcripts were detected in the developing germ cells within the adluminal compartment of the seminiferous tubules by in situ hybridization. One explanation for this discrepancy is that the levels of OPN mRNA were adequate to detect transcripts by in situ hybridization, but the dilution effect of nonexpressing cells following RNA isolation hindered detection by Northern blot analysis (McFadden, 1995). In contrast, Northern blot analyses demonstrated the presence of transcripts in the seminal vesicle, but transcripts were not detected in this tissue by in situ hybridization. In this case, low levels of OPN mRNA may be present throughout the different cell types in the seminal vesicle. Although they were not readily detectable following the 2- to 5-day exposure to emulsion, the concentration of OPN transcripts following RNA isolation may have enabled the detection of such transcripts by Northern blot analysis (McFadden, 1995). An alternative explanation for this discrepancy may be related to the high concentrations of RNase ($1.0\text{--}1.5\ \mu\text{g}/\text{mL}^{-1}$) reported for bull seminal vesicles and the differences in the way the tissues were processed for Northern blot analyses versus in situ hybridization (D'Alessio et al, 1991). For Northern blot analysis, tissues are maintained at -80°C until RNA extraction;

however, cryostat sectioning is performed at -20°C and the tissue warms even more when it is thawed and placed on the slide. Given the high RNase concentrations, the brief warming of tissue during sectioning for in situ hybridization may have resulted in RNase activity that was adequate to eliminate the OPN signal from seminal vesicle tissue.

Although the lack of detail in cryosections prevented definitive identification of cell types within stages expressing OPN transcripts, our observations indicate that OPN expression in the bull testis was present in tubules containing elongated spermatids lining the lumen of the seminiferous epithelium. In contrast, Northern blot analysis and RT-PCR studies in the mouse testis detected mRNA for OPN in separated fractions that were enriched in Sertoli cells, pachytene spermatocytes, or round spermatids (Siiteri et al, 1995).

Studies have suggested the existence of cell adhesion molecules on the surfaces of Sertoli cells as well as on the membranes of germ cells (Newton and Millette, 1992). Adhesion molecules not only anchor cells to their proper locations, they actively mediate the passage of information into the cell. OPN contains a functional Gly-Arg-Gly-Asp-Ser (GRGDS) cell-binding domain (Oldberg et al, 1986). This domain is present in cell adhesion molecules and is involved in cell attachment and spreading reactions in vitro via integrins (Hynes, 1987). Integrins are a large family of cell adhesion receptors that mediate a number of diverse functions, including cell-matrix and cell-cell interactions (Hynes, 1987; Vinatier, 1995). Because cell-cell interactions are necessary to maintain normal tissue function, intricate systems have been developed to ensure cell-cell communications (Jégou and Pineau, 1995). Within the seminiferous tubules, Sertoli cell-germ cell as well as germ cell-germ cell interactions are necessary for the proper development of the germ cells during spermatogenesis. Thus, several structural devices exist that aid in the communication of Sertoli cells with germ cells and vice versa. These forms of intercellular adhesion include the desmosome-like junctions, gap junctions, ectoplasmic specializations, and tubulobulbar complexes (Jégou and Pineau, 1995). In addition, the developing germ cells are also interconnected by intercellular bridges. These bridges are believed to provide a means of communication between the germ cells allowing them to synchronize their development. Considering the role of OPN as a cell adhesion molecule and the importance of cell-cell communication within the testis, it is possible that OPN may play a role in the adhesion of the developing germ cells to the Sertoli cells, the adhesion to other germ cells, or both. OPN may also be involved in the transfer of information between cells in the seminiferous tubules.

These hypotheses make several assumptions. The first

is that the transcripts observed within the germ cells of the adluminal compartment in the bull testis are being translated into protein. This protein could then play a role in cell adhesion within the seminiferous tubules. However, unlike studies performed with the mouse (Siiteri et al., 1995), Cancel et al (1999) were unable to detect OPN protein in the bull testis or on ejaculated spermatozoa using an anti-OPN polyclonal antibody. It is possible that amounts of OPN protein in the testis and on sperm may be too low to be detected by immunohistochemistry. Alternatively, variant forms of OPN may be present on spermatozoa that lack the epitope or epitopes for detection with the antibody used by Cancel et al (1999).

Despite the inability to detect OPN protein on bull sperm, it is intriguing that OPN transcripts were detected in the germ cells within the adluminal compartment of the seminiferous tubules as well as in the sperm located in the lumen of the epididymis and ampulla. Newly formed elongated spermatids are believed to lose their protein synthesizing capabilities; therefore, the presence of OPN transcripts in haploid spermatozoa is an enigma.

Several laboratories have demonstrated the presence of specific mRNAs in the nuclei of mature spermatozoa. Messenger RNAs for the protamines PRM-1 and PRM-2, and the transition protein TP-1 (Wykes et al, 1997), as well as beta-actin, cMYC, and HLA1 (Miller, 1997) have been reported. In addition, nested RT-PCR indicated that ejaculated human sperm contained mRNA transcripts for the $\beta 1$, $\alpha 3$, $\alpha 4$, and $\alpha 6$ chains of the $\beta 1$ integrins (Rohwedder et al, 1996).

Although the presence of RNA transcripts in ejaculated spermatozoa has been reported, little information is available regarding the function of these transcripts within the nucleus or during fertilization (Wykes et al, 1997). Miller (1997) suggested that the transcripts present in ejaculated spermatozoa are remnants of an equilibrium process that is necessary to ensure that the meiotic differences that are generated following spermatogenesis are balanced out. Miller (1997) argued that these transcripts remained in the haploid germ cell either because their degradation would be a wasteful process or because a degradative mechanism was not able to endure the chromatin condensation process.

OPN transcripts expressed within the adluminal compartment of the seminiferous tubules may play a role in the development of elongated spermatozoa. An adhesion molecule such as OPN may aid in the aggregation of vesicles during formation of the acrosome. Alternatively, it is possible that the OPN mRNAs may be stored during spermatogenesis to be available for translation during early embryonic development. Given the novelty of these observations and the potential roles of OPN in reproduction, the presence of OPN mRNAs in spermatozoa warrants further study.

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