CHARACTERIZATION AND HORMONAL REGULATION OF THE PEM HOMEOBOX GENE

by

J. Suzanne Lindsey

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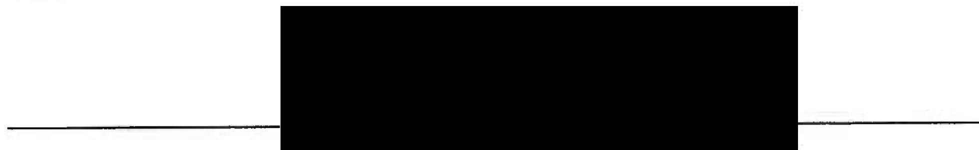

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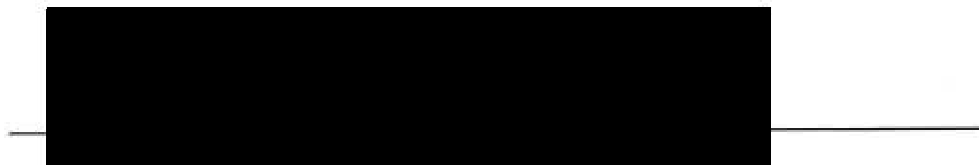

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ABSTRACT

To determine the physiological role for a developmentally regulated gene, the cellular location, temporal expression, and regulation need to be defined. I determined that Pem, a divergent homeobox gene, was expressed in the testis, a site of an adult system of development called spermatogenesis. The epididymis, a site where sperm is matured and stored, also expressed Pem. Spermatogenesis provides a model in which to study Pem expression because of the previous research which has established temporal and hormonal regulation of morphological and molecular events during development, proliferation, and function of this system. I utilized northern blot, RNase protection, and in situ hybridization analyses of mRNA in testes and epididymides of wild type mice and rats as well as mutant mice which were defective in spermatogenesis to show the temporal and specific cellular expression of Pem in the testis and epididymis. Pem was shown to be induced in both testis and epididymis at times in puberty which correlated to increases in androgen for each organ. In the testis, Pem is expressed specifically in androgen-dependent VI, VII, and early VIII stages of the seminiferous tubules in the Sertoli cells. In the epididymis, Pem transcripts were found in the apical principal cells of the corpus and proximal cauda regions which are important for sperm maturation and fertility. In addition, hypophysectomized (HPX) mice and rats and gonadotropin-deficient hypogonadal (hpg) mice were used to determine the hormonal regulation of the Pem gene in this system. HPX and hpg mouse testis and HPX rat epididymides lacked normal levels of Pem transcripts. Luteinizing hormone (LH) or testosterone alone restored Pem expression in HPX testes and epididymides of hypophysectomized animals and induced Pem expression in hpg mouse testes. For the first time, this work provides evidence that Pem is temporally and specifically expressed in androgen-regulated somatic cells of the testes and epididymis that are essential for germ cell development and maturation respectively. In addition, the homeobox gene Pem is the only known putative transcription factor that has been

found to be regulated by testosterone in vivo during spermatogenesis and sperm maturation.

INTRODUCTION

Development.

Several strategies exist to identify murine developmental control genes. A classical genetic route may be taken for determining a gene that contributes to an abnormal phenotype. Another method determines the coding region for a gene expressed during development by some differential screening method. During a search for developmentally regulated genes, subtraction hybridization between two T-cell lymphoma cell lines, SL 12.3 and SL 12.4, was used to isolate the Pem gene (1). Analyses of the protein sequence encoded by Pem cDNA suggest that it encodes a homeodomain (2,3). To determine the function for a gene isolated from a differential screening method a well studied developmental system is required.

One system to examine mammalian gene expression and regulation during development is in the embryo. However, this is a very complex system which involves many genes that are activated and deactivated in many developing organs. In addition, transcription factors that regulate these genes may be found in many different expression patterns and combinations in these embryonic systems. Therefore, for ease of manipulations and isolation of one system an adult developmental system is often preferred. Spermatogenesis provides a well-studied, both morphologically and molecularly, system of adult development in which to study gene expression, regulation, and function.

Spermatogenesis.

The system of spermatogenesis is a complex one. It primarily consists of the hypothalamus-pituitary-gonadal endocrine axis, testis, and epididymis. The hypothalamus secretes gonadotropin releasing hormone (GnRH) which in turn signals the pituitary to secrete the gonadotropins follicular stimulating hormone (FSH) and luteinizing hormone (LH) which exert their effects on the testis (4,5). The testis is made up of two main compartments: the intertubular or interstitial area and the seminiferous tubule. Leydig cells, bone-marrow derived cell types, blood, and lymphatic vessels reside in the interstitial area. Under the influence of LH, Leydig cells secrete and are the major source of androgens (6). In the absence of LH and/or FSH, androgens alone sustain spermatogenesis and maturation of sperm (7-10). More recent evidence shows that testosterone alone is capable of initiating qualitative and quantitative, when normalized to numbers of Sertoli cells, spermatogenesis in the GnRH-mutant mouse, hypogonadal (11). The seminiferous tubules contain myoid cells, basement membrane, Sertoli cells and germ cells. Under androgen stimulation, myoid cells produce P-Mod-S a paracrine factor shown to have effects on Sertoli cell function (12-18). Sertoli cells possess receptors for both FSH (19,20) and androgens (21), hormones which are thought to control germ cell development (22-24). The seminiferous tubule provides a unique microenvironment in which the germ cells are nurtured by Sertoli cells and develop into spermatozoa. This environment is protected by the Sertoli cell barrier, also known as the blood-testis-barrier, which completes formation between Sertoli cells at about day 16 after birth in the rat and day 10 in the mouse (25 -33). During this same time period for the rat the first cell-cell contacts are established between Sertoli cells and the most developed germ cells called spermatids (30) (Fig 1 from ref 31).

Other postnatal developmental events of this system differ slightly chronologically between mice and rats. After birth, secretion of FSH and LH by the pituitary occurs a few days earlier in the mouse when compared to the rat (34). FSH is secreted two

weeks before puberty and precedes LH secretion in the mouse (35). FSH has been shown to increase Sertoli cell (36-38) and spermatogonia (39-43) proliferation and to mature Leydig cells (44). In vivo, Sertoli cell and spermatogonia proliferation is shown to precede Leydig cell proliferation (45). Prenatal intratesticular testosterone levels are thought to drop after birth and start increasing again on day 10 in the mouse (46). However, a drop in total testicular androgen levels including dihydrotestosterone does not occur (35).

This process of developing spermatogonia into spermatozoa may be categorized into three phases: 1) the spermatogonia proliferative phase, 2) the spermatocyte meiotic phase, and 3) the spermatid differentiation (spermiogenic) phase. The proliferative phase provides the vast numbers of spermatogonia that are ready to undergo meiosis and differentiation. The most mature spermatogonia divide to form preleptotene, or resting spermatocytes which proceed through meiosis to form haploid spermatids. Finally, the spermatids undergo tremendous cellular differentiation including specialized packing of the chromatin and flagella formation (Fig. 2 from ref 31). Germ cell development is synchronized within segments of the seminiferous tubule. The organization of these developing germ cells has been categorized (47,48) into stages of the seminiferous tubules of the testes in mice and rats (Fig. 3 and 4 from ref. 31). These staging maps are important to provide investigators with reference points in which to compare their data.

The Sertoli cell is the most important somatic cell for germ cell development. In the testis, only the Sertoli cell forms a barrier which provides a special immunologically privileged site and microenvironment necessary for germ cell maturation. Intimate cell-cell contacts exist in stage-specific patterns to allow the spermatogonia entrance into the adluminal compartment of the tubule for further differentiation. Sertoli cells are columnar epithelial cells with 90% of their lateral and apical surface partitioned off toward the lumen of the tubule by tight junctions at the base of the Sertoli cell. It is

thought that the completion of this barrier at about day 10 in the mouse and day 16 in the rat during the prepubertal period develops the tubular lumen (25,30). Sertoli cell-germ cell contacts and secreted proteins provide the means of communication between these two cell types. Since germ cells have not been shown to have androgen receptors (AR), virtually all androgen effects on germ cell development take place either directly or indirectly through the Sertoli cell (49-51). The main secreted proteins of the Sertoli cell in the rat are inhibin, transferrin, and androgen binding protein (ABP). However, in mouse Sertoli cells ABP levels are 50 to 100 times lower than in Sertoli cells of the rat (52).

Sperm maturation.

Spermatozoa need further maturation in the epididymis before they are capable of fertilization. Development and function of the epididymis is dependent on dihydrotestosterone (DHT)(53). Without DHT, the epididymis does not produce mature sperm capable of fertilization. Under stimulation by DHT, principal cells in the epididymis secrete proteins that promote forward motility and egg recognition by the spermatozoa (54,55). After birth of the rat the epididymis does not complete development until Leydig cells in the testis produce increased testosterone and the first spermatozoa enter the epididymis from the testis (56,57). In addition to circulating androgen, the epididymis must receive testosterone from the testis usually bound to ABP or the principal cells of the initial segment of the epididymis dedifferentiate and the other regions fail to secrete proteins that mature spermatozoa (58). Even though the epididymis and testis have been extensively studied, little is known about expression and regulation of transcription factors during development or processes of spermatogenesis and sperm maturation.

Homeodomain proteins.

DNA-binding proteins, especially regulatory proteins or transcription factors, combine with other transcription regulatory proteins to determine differentiation and development of a particular cell type. Evidence for this role in mammalian development comes from the observation that expression of an abnormal DNA-binding AR causes a mouse with a male (XY) genotype to develop phenotypically as a female (59). Another class of DNA-binding proteins is encoded by homeobox genes. Over thirty murine homeobox genes have been identified and partially characterized in adult and embryonic tissues. These genes are expressed during embryogenesis and have been shown to dictate positional information and cellular differentiation processes in the embryo. Considerably less is known concerning the role of homeobox genes in controlling developmental events in tissues that undergo differentiation, such as spermatogenesis, in adult animals.

Hox genes are a subset of homeobox genes that bear homology to the *Drosophila Antennapedia* gene. The classical mammalian Hox genes are situated in clusters on four different chromosomes (Fig 5 from ref 60). The new nomenclature for the murine Hox gene clusters is Hox-a, -b, -c, and -d whereas the previous nomenclature was Hox 1, 2, 3, 4, respectively. At least six homeobox genes are expressed in murine testicular tissue: Hox-a3, -a4, -a5, -b4, -b7, and -d4. The Hox-a4 gene was originally isolated by screening a mouse testis cDNA library with the 180bp homeobox domain from the Antennapedia gene of Drosophila. The Hox-a4 gene is selectively expressed in adult testes; it is not detectably expressed in various other adult tissues (61-63). Several approaches were used to show that Hox-a4 gene expression is restricted to germ-line cells that have entered meiotic prophase (64,65). First, mutant mice homozygous for the atrichosis mutation (at/at), which renders the mice sterile due to the absence of germ cells, lack Hox-a4 expression in the testis. Second, Hox-a4 transcripts are detected by northern blot analysis only in adult testis, not in embryonic or

neonate testis that lack meiotic germ cells. Specifically, Hox-a4 transcripts are not detectable in day 7 neonate testes that contain spermatogonia but lack mature spermatozoa, although these transcripts are present in day 17 old mouse testes which contain germ cells at all stages of meiotic prophase. Third, in situ hybridization directly shows that Hox-a4 transcripts are specifically present in post-meiotic germ cells (64). Fourth, northern blot analysis of enriched populations of spermatogenic cells indicates that Hox-a4 mRNA is present in germ cells from the time they enter meiotic prophase through to the stage when the cytoplasm is shed as residual bodies (63).

The expression pattern of Hox-a4 during spermatogenesis has been compared with other genes, including the c-mos proto-oncogene. Transcripts encoding both of these proteins are undetectable in mouse mutants that lack germ cells in the gonads. This includes mice homozygous for the dominant white spotting (W/W^V) or sex reversal (Sxr) mutations. In addition, Hox-a4 and c-mos transcripts are undetectable in mouse mutants in which spermatogensis is arrested at the pachytene or diplotene stages (66). In contrast, Hox-a4 and c-mos are both expressed by mouse mutants that are capable of completing spermatogenesis, but produce aberrant sperm incapable of fertilizing eggs. Hox-a4 and c-mos differ, however, in their temporal expression with transcripts for the former found in 20-day old and the latter found in 25-day old mouse testes (65).

Homeobox paralogues are defined as Hox genes that occupy the same relative position in their respective cluster of Hox genes [e.g. Hox-a4 and -b4 are paralogues (see fig 4)]. Paralogous Hox genes display a higher degree of sequence conservation between each other than is exhibited between Hox sub-family members present in the same gene cluster (orthologues). Not only is the sequence of paralogous genes highly related, but their expression pattern during embryonic development is often strikingly similar, presumably due to conserved regulatory elements. For example, gene paralogues located at the 3' end of Hox-a, -b, -c, and -d gene clusters show similar

anterior-posterior boundaries of expression in the developing central nervous system (67).

Since Hox gene paralogues exhibit a strikingly similar pattern of expression in embryos, it is of interest to determine if they also display a similar pattern of expression in differentiating neonate or adult tissues. Thus, two of the paralogues of Hox-a4 have been tested for their expression pattern in postnatal testes. The Hox-b4 and Hox-d4 genes are expressed in adult testis (68), thus superficially supporting the notion that paralogues exhibit similar expression patterns. However, there are marked differences in other aspects of the expression pattern of these three paralogues. For example, while Hox-a4 appears to be expressed exclusively in testis and not other adult tissues (61-63), Hox-b4 and -d4 transcripts are abundant in many different adult organs (68,69). The particular cell types in the testis which express these three paralogues also differ. Germ cell and somatic cell populations both express Hox-b4 at very low levels as determined by in situ hybridization and mRNA analysis from testes of germ cell deficient mice (68). In contrast, the germ cell population solely expresses Hox-a4 (64), The other paralogue, Hox-d4, displays yet another expression pattern: it is expressed only in interstitial cells not in germ cells (68).

Hox-a4 appears to be the only orthologue that displays germ cell-specific expression. The two neighboring genes, Hox-a3 and -a5, are not only expressed in germ cells, but also in many non-testicular somatic tissues, such as kidney and ovary (68,70). However, Hox-a3 and -a5 are not expressed in somatic cells of the testis, as determined by northern blot and in situ hybridization analysis of at/at or +/+ mouse strains (68). Thus, the immediately neighboring orthologues of Hox-a4 display an expression pattern in testis that is more similar to the Hox-a4 gene than the paralogues, Hox-b4 and -d4, both of which are expressed in somatic cells of the testis.

Very few homeobox gene expression patterns have been characterized in the epididymis. Hox-a 4 is not expressed in the epididymis (J.S. Lindsey and M.F.

Wilkinson unpublished results). In contrast Hox-c8 appears to be highly expressed in the epididymis but not the testis based on a study in which transgenic mice were generated by homologous recombination that contained a lacZ gene to disrupt the Hox-c8 coding region (71, unpublished results J.S. Lindsey and M.F. Wilkinson).

Homeobox genes have been classified into many subgroups based on the primary amino acid sequence of the 60 residues in the homeodomain that dictate their DNA-binding specificity. The large Hox gene family is grouped together based on their homology with the classical Drosophila Antennapedia homeodomain and the fact they are clustered on four chromosomes. Other homeobox gene families are dispersed throughout the chromosomes. The paired family has an additional DNA-binding conserved motif of 130 amino acids. Mutations in Pax-6 present a phenotype of severe visual defects in Small eye mice (72,73), rats (74,75), humans (76-78) The Drosophila homologue for Pax-6, *eyeless*, directs ectopic eye development when expressed in imaginal disc primordia (79). To date, of the eight known paired homeobox genes, analyses show Pax-2 expression in the epididymis and Pax-5 (80) expression in the testis of normal male mice. Interestingly, in testicular feminized male mice, the gonads do express Pax-2 (81). The specific cell types that express these genes in the male reproductive system are unknown.

Oct homeobox gene family members encode homeodomains that diverge from classical Drosophila sequences. They also encode another DNA-binding region termed the POU-specific domain. Several Oct family members appear to be expressed in testicular tissue. Oct-6 mRNA locates in the testis but is not found in spermatozoa (82). Oct-6 is identical to Tst-1, isolated by PCR from testis cDNA (83), and SCIP, a cAMP-regulated gene (84). Like virtually all known Hox genes, Oct-6 is expressed in the developing central and peripheral nervous systems, as well as embryonal carcinoma stem cells (83-86). RNase protection and northern blot analyses show an accumulation of Oct-3/4 in testis (87). Another POU-domain gene, Sperm-1, which is most related to

the Oct-3/4 gene, is selectively expressed in male germ cells 36-48 hours prior to meiosis I (88).

Although the Pem homeodomain sequence bears some similarity to those encoded by paired family members, it is sufficiently divergent to be placed in a new homeobox gene family. Surprisingly, Pem transcripts are also expressed in the majority of immortalized and malignant cell lines that we have analyzed , regardless of cell lineage (89). During early gestation of the mouse embryo, primitive endoderm- and trophectoderm-derived cells of the placenta and yolk sac express *Pem* whereas, other embryonic or extra-embryonic cell lineages do not express *Pem* (89,90). In addition, embryonal carcinoma stem cells cultured *in vitro* exhibit this *in vivo* expression pattern: F9 stem cells induced to differentiate into visceral or parietal endoderm up-regulate Pem mRNA expression as assessed by northern blot analysis (3) and in situ hybridization (90,91). However, northern analyses did not detect *Pem* transcripts in any of the adult mouse tissues that have been tested including, liver, stomach, heart, pancreas, intestine, brain, lung, kidney, pituitary, thymus, and spleen (1,89). Pem is the only known homeobox gene shown to reside on the X-chromosome (90). Therefore, Pem differs significantly from other known homeobox genes in expression patterns and in chromosomal location.

Hypotheses.

This work was done to identify an adult developmental system which expressed Pem in order to further investigate specific expression patterns, regulation, and function of this homeobox gene. The hypotheses that Pem mRNA expression is located in specific cell types of the male reproductive system, is expressed during a specific developmental period, and is regulated by developmental signals were addressed.

Studies of gene expression during spermatogenesis generally show what type of cell in this system expresses a given gene. The somatic cells (Leydig, peritubular, and

Sertoli) have been well characterized for temporal gene expression as determined by transcript analyses by in situ hybridization, RNase protection of mRNA from isolated cell populations, and testis from mutant animals deficient in germ cells. The various stages of germ cells (spermatogonia, spermatocytes, and spermatids) have also been similarly studied by separation of the various germ cell populations, by using mutant mice which are defective at a certain stage of germ cell development, and by synchronizing germ cell development by a diet deficient in vitamin A. In the epididymis, cellular locations of gene expression have been determined for many genes. In this organ, newly transcribed RNA is not made in the spermatozoa because the chromatin is tightly wrapped around a special histone called protamine. Therefore, to my knowledge, the mRNA that is expressed in the epididymis strictly locates to somatic cells. Even though these cells are very similar throughout the highly convoluted tubule, genes are expressed in sometimes very limited regions of the epididymis. Since many different genes have been studied in these various cell populations of the testis and epididymis, the hypothesis was made that Pem is expressed by a certain cell population. RNA analyses of separated cell populations of the testis and of mutant mouse testes deficient in germ cells along with in situ hybridization analyses provided evidence that Sertoli cells expressed Pem. In situ hybridization of the epididymis showed Pem transcripts in the somatic cells of a specific region.

The second hypothesis that Pem was expressed during a specific developmental period was based on the fact that most significant developmental genes are expressed during a phase of development when particular morphological and molecular events take place. The determination of this temporal expression may give some insight as to what the function(s) is of the gene of interest. In the testis and epididymis there are five basic phases of development, prenatal, infantile, pubertal, spermatogenesis, and sperm maturation. In addition, spermatogenesis is an ongoing cyclical developmental system. The main focus of this hypothesis was postnatal, pubertal, spermatogenesis, and sperm

maturation. It was determined by time course analyses of mRNA from testes and epididymides from as early as five days after birth into adulthood that Pem expression was coincident with developmental periods in both organs which responded to increased androgen levels. During spermatogenesis, a cyclic pattern of expression was detected. Pem transcripts were localized primarily in the androgen-dependent stage VII seminiferous tubules as determined by in situ hybridization of mouse testes.

This correlation with androgen levels and the data that showed Pem expression in the androgen responsive Sertoli cell population led to the hypothesis that Pem was regulated by testosterone. We tested this hypothesis with a gonadotropin-deficient mutant mouse and with a surgically induced gonadotropin-deficient mouse and rat model which lacked Pem transcripts. Testosterone induced Pem transcripts in both animal models but at different levels between mouse and rat testes. The restored Pem levels in rat epididymis were comparable to normal rats.

Therefore, these results show that the homeobox gene, Pem, is a developmentally regulated gene which is induced by testosterone in Sertoli cells of the testis and somatic cells of the epididymis during spermatogenesis and sperm maturation.

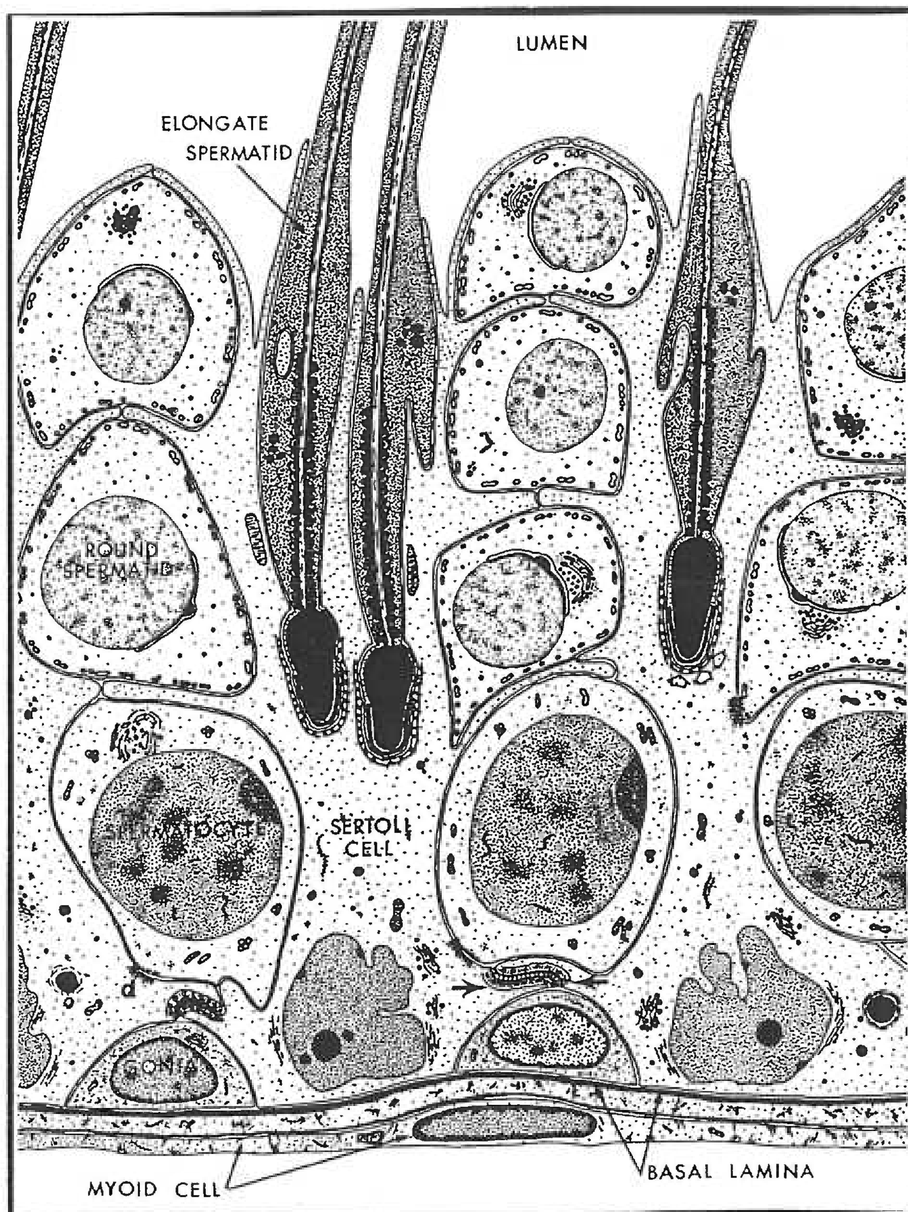


Figure 1. A schematic drawing of a cross-section of the seminiferous tubule. Spermatogonia (gonia) are located close to the basement membrane. Spermatocytes, located between Sertoli cells are more mature toward the lumen. Spermatids begin as round cells then undergo spermiogenesis, dramatic morphological changes of cell structure and shape. Special Sertoli cell-germ cell adhesion molecules (open arrowheads) exist from spermatocyte stage until the elongated spermatid is released to the lumen. Closed arrows beneath spermatocytes point to Sertoli cell-Sertoli cell adhesion structures which form the Sertoli cell barrier. Both of these cell-cell structures are necessary for spermatogenesis. (used with permission from Cache River Press from "The Sertoli Cell" L. Russell and M. Griswold eds.)

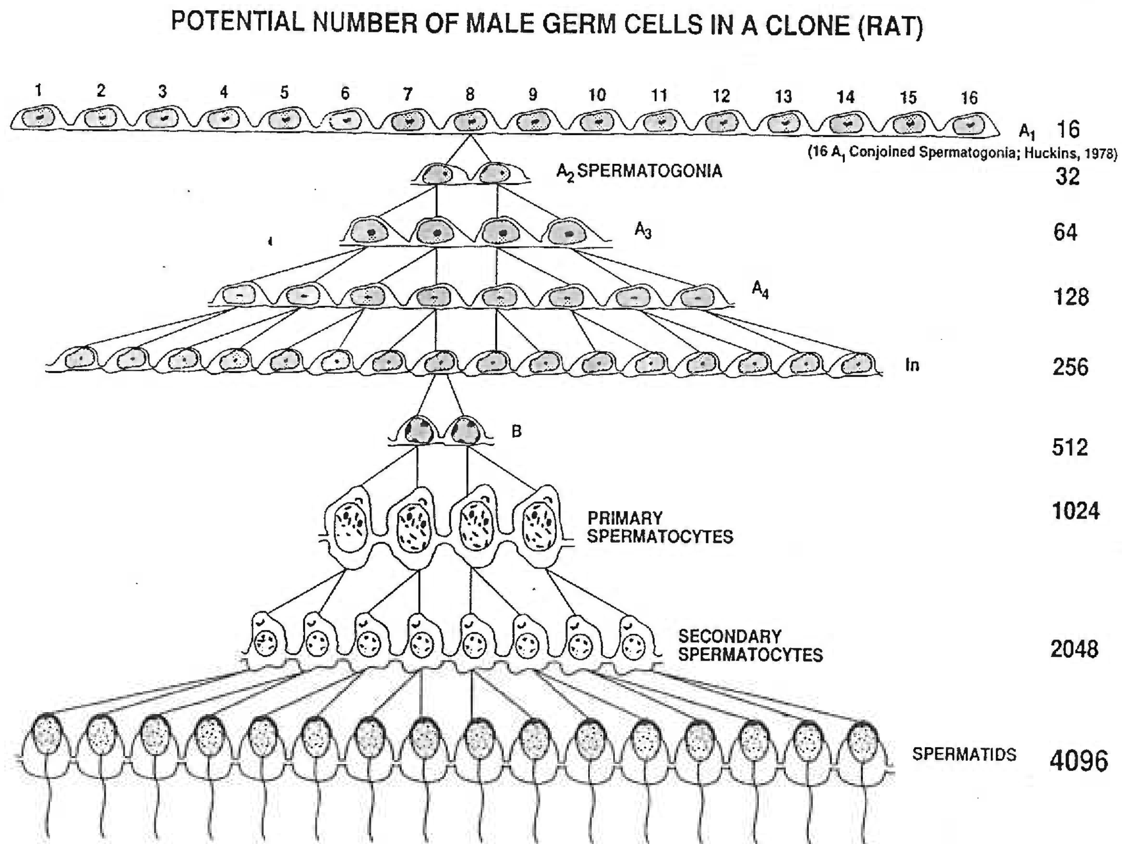


Figure 2. The successive stages of germ cell development. One spermatogonia undergoes four cell divisions to form 16 A_1 spermatogonia which then continue to divide, go through meiosis, differentiate, and potentially form 4,096 spermatids. (used with permission from Cache River Press from "Histological and Histopathological Evualuation of the Testis", L. Russell, R. Ettlin, A. Hikim, E Clegg, eds.)

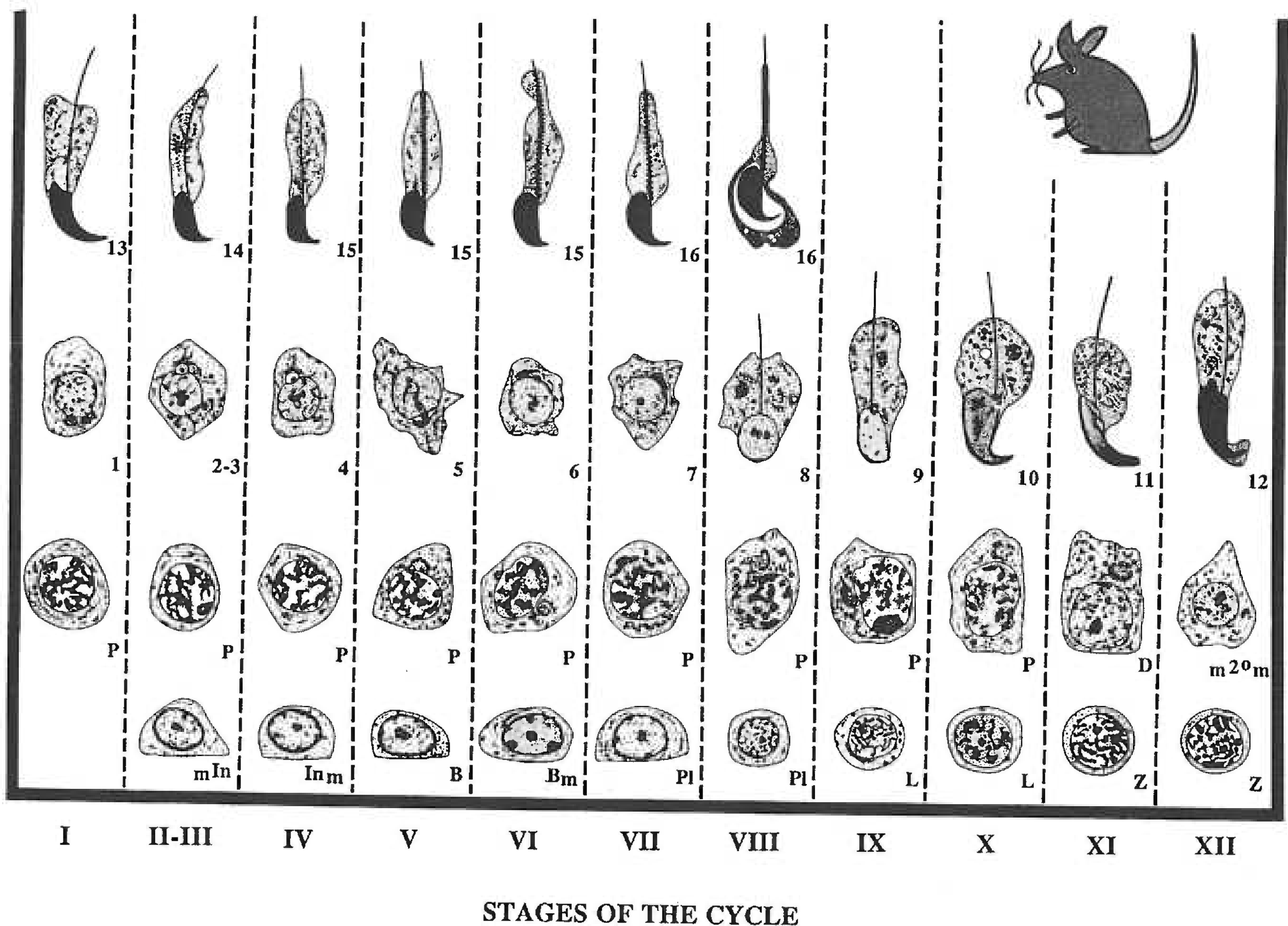
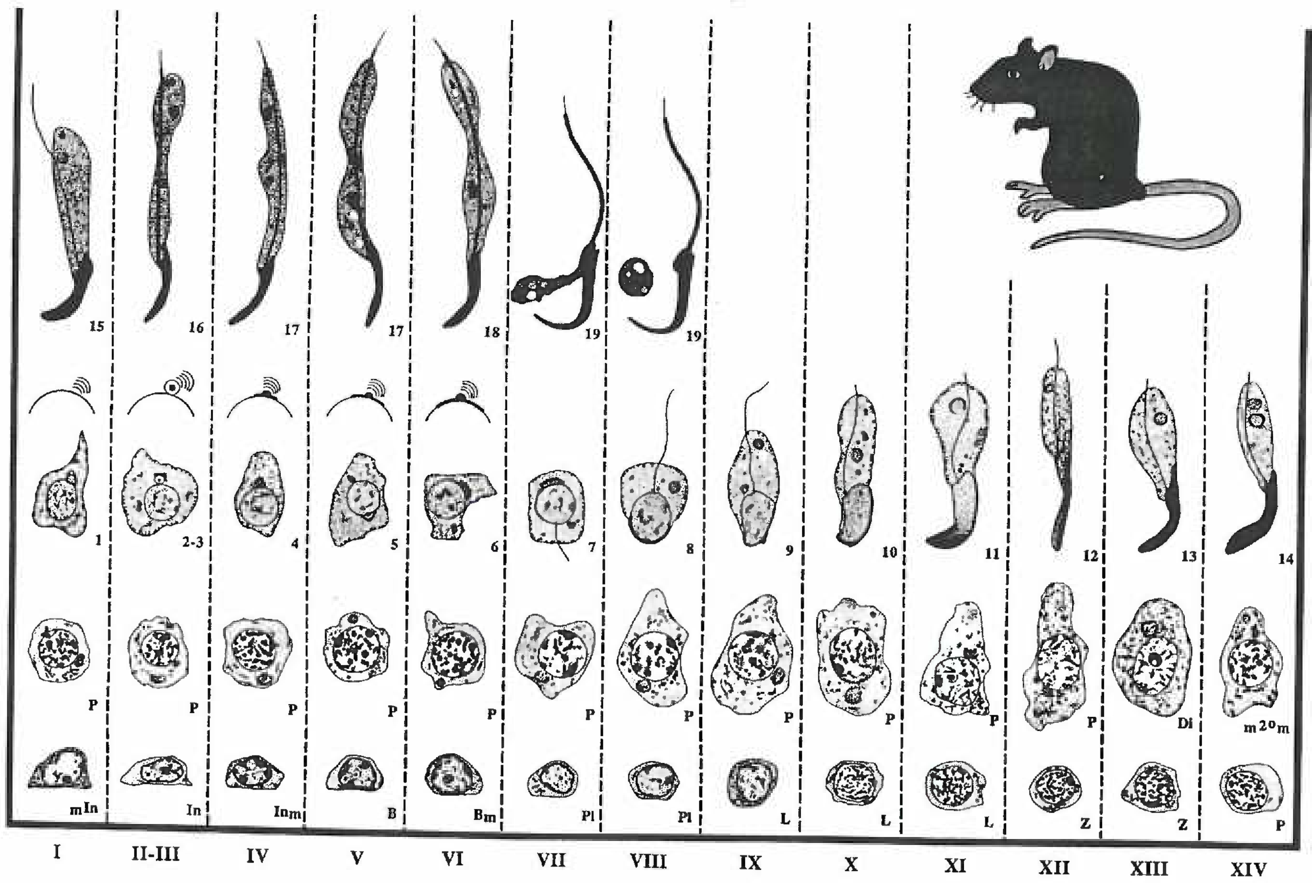


Figure 3. Cycle maps of mouse and rat (page 16) spermatogenesis. The vertical columns show cell associations within a given stage (Roman numerals). The bottom horizontal row gives spermatogonia just from the In stage on to the B stage; divisions of spermatogonia before this have not been shown to be stage related. Second horizontal row depicts: Pl= preleptotene, L=leptotene, Z=zygotene, P=pachytene, Di=diakinesis, m2°m=meiosis II. The third horizontal row shows differentiation of spermatocytes into round spermatids and elongate spermatids which continues into the top horizontal row and ends when the spermatids are released to the lumen. (used with permission from Cache River Press from "Histological and Histopathological Evualuation of the Testis", L. Russell, R. Ettlin, A. Hikim, E Clegg, eds.)



STAGES OF THE CYCLE

MANUSCRIPT #1

Pem: a gonadotropin-regulated homeobox gene expressed in Sertoli cells of the testis before meiosis and in somatic cells of the epididymis

J. Suzanne Lindsey[1] and Miles F. Wilkinson[2*]

1 Department of Molecular Microbiology and Immunology, Oregon Health Sciences University, Portland, OR 97201
2 Department of Immunology, The University of Texas M. D. Anderson Cancer Center, Houston, TX 77030

* Author for correspondence

Running title: *Pem*: a homeobox gene regulated in testis and epididymis

Key words: epididymis, gonadotropins, homeobox genes, luteinizing hormone, spermatogenesis, testis, testosterone

SUMMARY

Pem, an orphan homeobox gene located on the X chromosome, encodes a putative transcription factor that possesses a homeodomain related to those in the *Prd/Pax* gene family. In this communication, we show that the *Pem* gene is expressed in a stage- and cell-type specific manner in the reproductive tissues of adult and postnatal male mice. RNase protection, *in situ* hybridization, and northern blot analyses of wild-type and spermatogonia-deficient mutant mice localized Pem transcripts to Sertoli cells of the testis. Sertoli cells expressed *Pem* primarily in stage VII-VIII seminiferous tubules of adult mouse testis, the stages at which spermatogonia enter meiosis. During prepubertal testicular development, *Pem* expression was dramatically induced on day nine just prior to the first meiotic event in germ cells. *Pem* gene expression in Sertoli cells depended on gonadotropins, as demonstrated by a lack of expression in mutant mice which do not produce these hormones. Injection of luteinizing hormone into homozygous hypogonadal mice induced *Pem* expression in the testes. Germ cells need not be present for expression of *Pem* transcripts in testis as shown in homozygous dominant white spotting (W^V/W^V) mice that lack germ cells. *Pem* transcripts also accumulated in the apical cells of the proximal cauda region of the epididymis, the region where spermatozoa gain forward motility and fertilization competence. *Pem* expression in the epididymis did not depend on spermatozoa in the lumen of the testis, but did depend on the presence of germ cell-induced factors. During prepubertal development, temporal expression of *Pem* in the epididymis differed from that found in the testis and correlated with testosterone levels. Collectively, our results show that discrete cell types in male reproductive tissues transcribe and independently regulate the *Pem* homeobox gene. To our knowledge, *Pem* is the first candidate transcription factor demonstrated to be gonadotropin-regulated in Sertoli cells.

INTRODUCTION

Complex signaling events and cascades of responding messengers dictate mammalian development. Transcription factors, including those encoded by homeobox genes, are part of this cascade. Homeobox transcription factors contain a conserved 60 amino acid motif termed a homeodomain that engenders DNA binding specificity. Many homeobox genes have been classified as members of homeobox gene sub-families on the basis of sequence homology in the homeodomain region (Dubuoule, 1994). Members of the *Hox*, *Prd/Pax*, and *POU* homeobox gene families have been shown to dictate discrete developmental events in *Caenorhabditis elegans, Drosophila melanogaster*, *Xenopus laevis*, and mice (Dubuoule, 1994). In contrast, little is known regarding the function of orphan homeobox genes that display insufficient sequence identity to be classified as members of homeobox gene sub-families. The expression pattern of these orphan homeobox genes has suggested biological systems in which they may dictate developmental events. For example, the *TTF-1* gene encodes a thyroid specific homeodomain protein that binds to promoters expressed exclusively in thyroid follicular cells (Civitareale et al., 1989; Francis-Lang et al., 1992). The *Gtx* orphan homeobox gene is transcribed in glial cells of the brain and germ cells of the testis (Komuro et al., 1993). The *Msx-1* and *Msx-2* genes are expressed in the mesodermal progress zone of the developing limb bud and of the mandibular and maxillary arches (Davidson et al., 1991; Robert et al., 1991). The *Cdx-1* (Duprey et al., 1988) and *HNF-1* genes (Baumhueter et al., 1990; Blumenfeld et al., 1991) are expressed in endoderm-derived tissues.

In a search for developmentally regulated genes, we used the subtraction hybridization approach and isolated the orphan homeobox gene *Pem* (MacLeod et al., 1990; Wilkinson *et al.*, 1990). The mouse Pem gene encodes a 210 amino acid protein that includes a divergent homeodomain in the carboxy terminal portion of the molecule.

The *Pem* homeodomain possesses most of the conserved amino acids known to be important for homeodomain protein folding and DNA-binding, yet it is clearly not a member of any of the known classes of homeodomains since it displays only 30-35% sequence identity with the most closely related *Prd/Pax* homeodomains (Sasaki et al., 1991; Rayle et al., 1991). *Pem* gene transcription is first evident at the morula and blastocyst stages of the pre-implantation embryo (Lin *et al., 1994*). After implantation, trophectoderm and descendent cells in the placenta express *Pem* protein and mRNA (Wilkinson *et al.*, 1990; Lin *et al.,1994*). *Pem* is also expressed by primitive endoderm and its derivatives, including parietal endoderm and the visceral endoderm in the visceral yolk sac (Wilkinson *et al.*, 1990; Lin *et al.,1994*). Embryonal carcinoma stem cells cultured *in vitro* mimic this *in vivo* expression pattern: F9 stem cells induced to differentiate into either visceral or parietal endoderm up-regulate Pem mRNA expression as assessed by northern blot analysis (Sasaki et al., 1991) and *in situ* hybridization (Labosky et al., 1993; Lin et al., 1994). Diploid embryonic stem cells induced to differentiate also display a dramatic up-regulation of *Pem* gene expression (Sasaki et al., 1991). Collectively, these studies show that the *Pem* gene exhibits a unique tissue- and stage-specific pattern of expression during embryogenesis. In contrast, past studies have suggested that the *Pem* gene is not expressed by adult mouse tissues. Northern analyses did not detect *Pem* transcripts in any of the adult mouse tissues that had been tested, including, liver, stomach, heart, pancreas, intestine, brain, lung, kidney, pituitary, thymus, and spleen (MacLeod et al., 1990; Wilkinson et al., 1990).

In this work, we show that the *Pem* gene is expressed in the testis and epididymis during pre- and postpubertal development. The testis and epididymis are derived from the endoderm and make up the primary organs involved in spermatogenesis. This developmental system is well defined morphologically and molecularly and is a logical system within which to explore the expression and

regulation of *Pem*. Other homeobox genes have been shown to be transcribed in the mouse spermatogenic system. The POU-homeobox gene *Sperm-1* is transiently expressed immediately prior to meiosis in germ cells (Andersen et al., 1993). *Hox-a4* is expressed specifically in postmeiotic germ cells of the testes, not in somatic cells of the testis or in other organs, including epididymis (Wolgemuth et al., 1991; Lindsey and Wilkinson unpublished results). In contrast, *Hox-b4* and *-d4* genes are expressed in adult testes as well as many other adult tissues (Featherstone et al., 1988; Graham et al., 1988). The only known homeobox genes to be expressed in the epididymis are *Hox-c8* and *Pax-2* (Mouellic et al., 1992; Fickenscher et al., 1993; Lindsey and Wilkinson unpublished results).

In this study, we demonstrate that the *Pem* homeobox gene is expressed by Sertoli cells in the testis. *Pem* is the first homeobox gene shown to be expressed by Sertoli cells. We also show that *Pem* is expressed by somatic cells in a specific region of the epididymis that studies suggest regulate germ cell maturation (Blaquier *et al.*, 1988). We make use of mutant mice that are defective in the spermatogenic system to determine the specific pathways that regulate *Pem* expression in testis and epididymis. *Pem* expression in these two cell types exhibits unique temporal kinetics in newborn mice and is regulated by distinct factors. The expression of the *Pem* gene in Sertoli cells is dependent on the gonadotropin LH for expression. This property is unique: no other transcription factors are known to depend on this gonadotropin for expression in Sertoli cells. The temporal and spatial pattern of *Pem* expression in testis and epididymis corresponds with several important biological events in spermatogenesis, including meiosis, spermatid release into the lumen of the testis, and spermatozoa maturation in the epididymis.

METHODS AND MATERIALS

Animals

BALB/c and mutant mice were obtained from The Jackson Laboratory (Bar Harbor, Maine). Animals were housed in the Oregon Health Sciences University animal care facility and cared for according to approved protocols. Animals were killed by CO_2 asphyxiation. Testes and epididymides were immediately removed and RNA was extracted. For the gonadotropin experiments, homozygous hypogonadal mice (four in each of the three groups) were injected with 2 µg of purified porcine follicle stimulating hormone (FSH, from Sigma), 40 ng of purified ovine luteinizing hormone (LH, from Sigma), or 0.9% sterile saline twice daily at 12-hour intervals for two or seven days and then killed by CO_2 asphyxiation. We repeated these experiments three times.

We verified the absence of germ cells in the epididymis of homozygous quaking and dominant white spotting mice by mincing the epididymides and viewing them under a microscope.

Riboprobe preparation

For RNase protection and *in situ* hybridization analyses, we prepared ^{32}P-, ^{33}P-UTP- or ^{35}S-ATP-labeled RNA probes with T7 and SP6 RNA polymerase that correspond to nucleotides 6 through 432 of the mouse *Pem* cDNA sequence (Wilkinson et al., 1990). In some experiments, a GAPDH probe was used as a positive control. In others, we utilized a probe corresponding to a portion of the L3 ribosomal protein (cDNA template kindly provided by Dr. Eric Barklis, OHSU). An RNA ladder template (Ambion) was used to generate specific size markers. We used the *in vitro* transcription protocol as described in Current Protocols in Molecular Biology (Ausubel et al., 1987). Probes were purified on a 6% polyacrylamide denaturing gel. After exposure to film, the appropriately sized bands were isolated and placed in individual Eppendorf tubes. The

gel slices were mashed with an RNase-free pestle in 100 μl of diethylpyrocarbonate (DEPC) treated water. To each sample, 600 μl of 1X proteinase K (PK) buffer [0.3 M NaCl, 0.5% SDS, 10 mM Tris (pH 7.5), 200 μg/ml PK, and 20 μg/ml tRNA] was added, vortexed and incubated at 37°C for 15 minutes. After vortexing and a pulse-spin, the suspended probe was filtered through a 0.45 μm filter (Acrodisc). Another 600 μl of the PK buffer was added and incubated for at least 5 more minutes at 37°C and again the suspended probe was filtered through the same 0.45 μm filter. Each sample was extracted with 200 μl of phenol/chloroform. One microliter was taken for determining radioactive counts per minute and the rest was precipitated and stored at -70°C.

RNase protection assays

Total RNA from tissues was prepared as previously described by either guanidinium isothiocyanate lysis and centrifugation over a cesium chloride cushion (Wilkinson, 1991) or by a single-step acid guanidinium thiocyanate-phenol-chloroform extraction (Chomczynski and Sacchi, 1987) and quantified by measuring optical density. RNase protection analyses were performed as described in Current Protocols (Ausubel et al., 1987) with minor modifications. Briefly, sample RNAs or tRNA as a negative control, were centrifuged with the appropriate gel-purified ^{32}P-UTP-labeled probes. The pellet was resuspended in 30 μl of annealing buffer [40 mM PIPES (pH 6.4) 0.4 M NaCl, 1 mM EDTA, 80% formamide] and allowed to hybridize overnight at 42°C. Unhybridized RNA was digested with RNase A (50 μg/ml) and RNase T1 (4 μg/ml) for 30 minutes at 37°C. RNases were then removed by treatment with proteinase K and extraction with phenol/chloroform/isoamyl alcohol. After ethanol precipitation, the RNA pellet was resuspended in 90% formamide loading buffer, denatured at 85°C, electrophoresed on a 6% polyacrylamide denaturing gel, and analyzed by autoradiography.

Northern blot analysis

Ten micrograms per lane of total cellular RNA was electrophoresed on 1% agarose gels. The separated RNA was transferred to Nytran™ paper by capillary blot as described by Maniatis et al. (Maniatis et al., 1982). After transfer, the blot was crosslinked by ultraviolet irradiation (Strata-linker from Stratagene) and stained with methylene blue to evaluate the transfer and loading of RNA in each lane. Blots were then prehybridized in pre-hyb buffer (50% formamide, 5X Denhardt's solution, 5X SSPE, 0.5% SDS, and 100 μg/ml sheared salmon sperm DNA) for 4 hours at room temperature. Blots were then hybridized with random oligomer-primed ^{32}P-labeled cDNA in the presence of hyb buffer (pre-hyb buffer plus 10% dextran sulfate) overnight at 42°C. The cDNAs for mouse *GATA-1* and *CREB* were kindly provided by Drs. Stuart Orkin (Howard Hughes Medical Institute, Boston, MA) and Richard Goodman (Vollum Institute, Portland, OR), respectively.

Cell fractionation

The testes were decapsulated, being careful not to cut the seminiferous tubules. The tubules were separated from the interstitial cells by incubating in a 1 mg/ml collagenase solution [Sigma type 1 collagenase freshly prepared in Dulbecco's Modified Eagle's Medium-Ham's F-12 medium (DMEM-F12)] at 37°C for 25 minutes. Eight decapsulated testes were put in 20 ml of the collagenase solution in a 50 ml screw top conical tube which was placed horizontally on an orbital shaker at 70 rpm (the solution had a 2 cm amplitude). After the incubation, the interstitial cell-tubule mass was gently poured onto a nylon mesh sieve (60 μm mesh size; Nytex, Spectrum, Inc.). The retained tubules were washed three times with 10 ml DMEM-F12 at 37°C and used for RNA preparation. The cell suspension that passed through the mesh was enriched for interstitial cells; the retained tubule mass consisted primarily of seminiferous tubules. The interstitial cell

suspension was pelleted by brief centrifugation (500 g for 5 minutes) for RNA preparation.

In situ hybridization

In situ hybridizations were performed as described (Wilcox, 1993). Briefly, the tissues were removed, rinsed in phosphate buffered saline (PBS) and immersed in 4% paraformaldehyde-0.1 M sodium phosphate buffer, pH 7.4, at 4°C for 3 hours. The tissues were then immersed in a 15% sucrose-PBS solution overnight at 4°C. After embedding the tissue in OCT (Miles), 10 to15 µm cryosections were adhered to Superfrost/Plus microscope slides (Fisher Scientific) and stored at -70°C in a sealed box with desiccant. Slides were thawed at 55°C for 5 minutes and fixed for 10 minutes in 4% paraformaldehyde-0.1 M $NaPO_4$, pH 7.4, at 4°C. Slides were then immersed in 1 µg per ml proteinase K solution (1 µg/ml in 500 mM NaCl,10 mM Tris, pH8.0) for 10 minutes at room temperature. After washing in 0.5X SSC for 10 minutes at room temperature, the slides were prehybridized (hybridization solution: 10 mM 1,4-dithiothreitol (DTT), 0.3 M NaCl, 20 mM Tris, pH 8.0, 5 mM EDTA, 1X Denhardt's solution, 10% dextran sulfate, and 50% formamide) for one hour at 42°C. Hybridization was performed with $6x10^5$ counts per minute ^{35}S-ATP- or ^{33}P-UTP-labeled RNA probe per specimen. After annealing overnight at 55°C, the slides were washed two times for 10 minutes in 2X standard saline citrate (SSC) with 10 mM beta-mercaptoethanol (BME) and 1mM EDTA at room temperature. Unhybridized RNA was digested with an RNase A solution (20 µg/ml RNase A in 500 mM NaCl and 10 mM Tris, pH 8.0) for 30 minutes at room temperature. The slides were then washed as before but for only 2 minutes and then washed for 2 hours in 4 liters of 0.1X SSC with 10 mM BME and 1mM EDTA at 55°C. After this stringent wash, the slides were washed twice for 10 minutes in 0.5X SSC without BME or EDTA at room temperature and then dehydrated for 2 min. each in 50%, 70%, and 90% ethanol containing 0.3 M NH_4Ac. After desiccation, the slides were

dipped in Kodak NTB2 nuclear emulsion diluted 1:1 with water at 42°C. After exposure the slides were developed and counterstained with 0.1% thionin, which stains the nucleus of mammalian cells and weakly stains the cytoplasm of germ cells.

The classification of spermatogenesis used was that of Russell *et al.* (1990). Since not all stages were discernible in frozen sections, the cycle was divided up into three parts to aid in the staging as follows: 1) tubules with condensed elongate spermatids lying at the same level as round spermatids (stages I-VI), 2) tubules with elongating spermatids lining the lumen (stages VII-VIII), and 3) tubules with elongating and condensing spermatids (IX-XIV). Frozen sections were used for the analysis since higher resolution techniques for fixation failed to permit specific hybridization.

RESULTS

Pem expression in mouse testis and epididymis

Northern blot analyses revealed a 1.0-kb *Pem* transcript expressed in mouse testis (Fig. 1A). The size of this transcript in adult testis was identical to the known size of *Pem* transcripts in embryonic tissues (Wilkinson et al., 1990). RNase protection analysis using a riboprobe antisense to the 5' portion of the *Pem* gene (including the amino terminal portion encoding the homeodomain) showed protection of a full-length band (Fig. 1B), implying that this transcript in mouse testis was derived from the *Pem* gene and not from a cross-hybridizing gene. *Pem* transcripts were also present in the epididymis (Figs. 1A & B). Independent analyses of several different testis and epididymides showed that the epididymis consistently expressed *Pem* mRNA at lower levels than did testes.

Pem expression is localized to Sertoli cells

The testis contains three main cells types: germ cells, Leydig cells, and Sertoli cells. Leydig cells are the primary cell type in the interstitial layer of the testis; these cells secrete the testosterone necessary for spermatogenesis. Germ cells are found at different stages of maturation in the seminiferous tubules. Sertoli cells, which also reside in the seminiferous tubule, dictate the development of the adjacent germ cells. To identify the subset of cells that express *Pem*, we took several approaches. We first separated seminiferous tubules from interstitial cells. *Pem* mRNA was expressed in the seminiferous tubule fraction at levels comparable to those of the whole testis but was undetectable in the interstitial fraction (Fig. 2A). This result indicates that *Pem* mRNA is not significantly expressed by Leydig cells and is instead present in the Sertoli cells, the germ cells, or both.

To determine the cellular site of *Pem* expression in the seminiferous tubule, we utilized a homozygous mutant mouse, dominant white spotting (W^V/W^V), that is deficient in germ cells. Although these mutant mice express normal levels of the hormones of the gonadal-pituitary axis, they exhibit impaired proliferation and migration of germ cells during embryonic gestation because of a mutation in the *c-kit* receptor (Coulombre and Russell, 1954). Testis from adult W^V/W^V mice contained *Pem* mRNA (Fig. 2B) which implies that Sertoli cells express the *Pem* gene. RNase protection and Northern blot analyses revealed that the level of expression of *Pem* transcripts was at least twofold higher in homozygotes (without germ cells) than in heterozygotes (with germ cells) (Fig. 2B & 2C). This difference in *Pem* expression levels probably reflects the fact that the ratio of Sertoli cells to spermatogonia changes from 1:1.6 in heterozygotes to 1:0.00005 in homozygotes (Tegelenbosch and Rooij, 1993; Vergouwen et al., 1993). The GATA-1 gene, which has shown to be selectively expressed in Sertoli cells, not germ cells (Yomogida *et al.*, 1994), showed a similar ratio of expression in W^V/W^V and $W^V/+$ testis (Fig. 2C).

To further assess the site of *Pem* expression in the seminiferous tubules, we performed *in situ* hybridization analyses. A *Pem* antisense riboprobe specifically hybridized to the tubular region of the testis (Fig. 3, panels A-C), not the interstitial cells, confirming that the *Pem* gene is not detectably expressed by Leydig cells. Approximately three quarters of the hybridization in the seminiferous tubules was confined to the basal region, a pattern expected of Sertoli cells. A punctate pattern of hybridization was not seen over spermatocytes, spermatids, or spermatogonia, suggesting that these cell types do not express the *Pem* gene. However, since spermatogonia reside near the basal layer where hybridization was most intense, we cannot rule out that the *Pem* gene is expressed by a subset of spermatogonia, in addition to its predominant expression in Sertoli cells.

Spermatogenic stage-specific expression of *Pem* in mouse Sertoli cells

During examination of cross-sections of tubules of the testes, we determined that *Pem* hybridization was preferentially detected in tubules that were beginning to release the oldest generation of differentiating spermatids to the tubule lumen (Fig. 3, panels A-C). These tubules are classified as stages VII and VIII (Russell *et al.*, 1990). Quantitative analysis revealed that of 192 stage VII-VIII tubules analyzed by dark field and light field microscopy, 187 displayed hybridization with the *Pem* probe. *Pem* expression declined precipitously in late stage VIII tubules that contained typical dense cytoplasmic lobes. *Pem* transcripts appeared to be first detectable in a proportion of stage VI tubules that possess elongate spermatids amongst round spermatids, although the precise staging could not be determined under conditions that permitted specific hybridization with the *Pem* probe (see materials and methods). Fig. 4 summarizes the expression pattern of *Pem* during the spermatogenic cycle.

Germ-cell deficient tubules from W^v/W^v mice were examined by *in situ* hybridization since Northern blot and RNase protection analyses showed that *Pem* expression was elevated in these tubules compared to tubules from wild type mice. Seminiferous tubules from W^v/W^v mice have altered morphology compared to +/+ tubules by virtue of the fact that they lack germ cells (Yomogida *et al.*, 1994). *In situ* hybridization analysis revealed *Pem* expression in the majority of the W^v/W^v tubules; *albeit* at different levels in different tubules (Fig. 3, panels D-E). This result suggests the possibility that germ cells normally down-regulate *Pem* gene expression in Sertoli cells of seminiferous tubules other than stages VII-VIII. Similar results and conclusions were drawn from results obtained with the GATA-1 transcription factor (Yomogida *et al.*, 1994).

Temporal *Pem* expression in mouse testis

Developmental events take place in the testis in a defined temporal manner. For example: FSH is first received from the pituitary gland on day-8, the first meiosis occurs on day-10, and LH is first received on about day-11 after birth (Kofman-Alfaro and Chandley, 1970; Selmanoff et al., 1977; Jean-Faucher et al., 1978). To determine if the onset of *Pem* expression in the testis correlated with any of these known events, we extracted total RNA from pooled testes from 5- to17-day-old mice and individual testis from 23- to 60-day-old mice and analyzed them by northern blot and RNase protection analyses. Day nine testes showed a strong induction of *Pem* expression; transcript levels remained elevated throughout adulthood (Fig. 5A). A shorter time course of total RNA from pooled testes from another set of 6- 7- 8- 9- and 10-day-old mice confirmed that *Pem* mRNA levels increased specifically on day 9 (Fig. 5B). Results from RNase protection analyses were corroborated by northern blot analyses which confirmed that *Pem* expression was maintained throughout adulthood. Northern blot analysis also showed that at all time points these transcripts were indistinguishable in size (1.0 kb) from *Pem* transcripts expressed in the placenta (data not shown). Since mouse Sertoli cells are known to cease mitotic proliferation on day-12 post-partum (Kluin *et al.*, 1984), the persistence of *Pem* gene expression after this day demonstrates that the expression of this gene is not necessarily linked to cellular proliferation. Previous studies had shown that the *Pem* gene was expressed preferentially by highly proliferative tissues and cell lines (Wilkinson *et al.*, 1990; Sasaki *et al.*, 1993; Lin *et al.*, 1994).

Gonadotropins induce *Pem* expression in the testis

The spermatogenic system is regulated by hormones in both an endocrine and paracrine manner. To test whether gonadotropins are necessary for *Pem* expression, we utilized the mutant hypogonadal (hpg/hpg) mouse that is deficient in gonadotropins (LH and FSH) because of a recessive genetic defect in the gonadotropin-releasing

hormone gene (Cattanach et al., 1977). We found that testes from hpg/hpg mice expressed almost undetectable levels of *Pem* mRNA, whereas hpg/+ testis accumulated high amounts of *Pem* mRNA (Fig. 6A). This result suggested that gonadotropins are needed for normal Pem expression in the testis.

We injected hpg/hpg mice with purified gonadotropins to determine if LH or FSH deficiency is responsible for the absence of *Pem* expression. Physiological levels of purified FSH (2 μg) or trace amounts of purified LH (40 ng) induced *Pem* expression in testis (Fig. 6B). The purified FSH contained about 20 ng of contaminating LH and induced *Pem* expression at levels twofold lower than those induced with 40 ng of purified LH (in three independent experiments). Collectively, these data indicate that LH induces *Pem* expression in gonadotropin-deficient animals, even at trace doses. Because LH is active at low doses, it is not clear whether FSH (that contains traces of LH) also stimulates *Pem* gene expression.

In parallel experiments, we assessed the expression of the zinc finger GATA-*1* and b-Zip*CREB* transcription factors in hypogonadal mice. In contrast to *Pem* mRNA,*GATA-1* and *CREB* transcripts were abundant in the testis of hpg/hpg mice. Neither LH nor FSH up-regulated the levels of these mRNAs (Fig. 6C). Thus, the gonadotropin-dependence of the Pem gene is a unique feature not displayed by two other transcription factors known to be expressed in the testis.

LH induces testosterone production by Leydig cells (Hall and Eik-Nes, 1962; Hall and Eik-Nes, 1963; Dufau et al., 1983). Therefore, we tested the importance of testosterone for *Pem* expression by employing the testicular feminization (tfm) mutant mouse, an animal that possesses a defective androgen receptor because of a mutation on the X chromosome. This mutant is non-responsive to testosterone but nevertheless produces FSH, LH, and testosterone (Lyon and Hawkes, 1970). We found that tfm testes lacked detectable *Pem* transcripts (Fig. 6D). This result shows that *Pem* expression requires a responsive androgen receptor.

Fetal gonads of normal mice produce testosterone on day 15 of embryonic gestation, probably due to induction by placental hormones (Jean-Faucher et al., 1978). Thus, we considered the possibility that *Pem* gene expression is activated in fetal gonads after the appearance of testosterone. RNase protection analysis of d16-fetal gonads showed trace levels of *Pem* mRNA 85 fold lower than in adult testis (Fig. 6E). This expression in fetal gonads was apparently not restricted to a particular cell type since a specific signal could not be detected by *in situ* hybridization with a *Pem* probe. A control probe corresponding to the L3 ribosomal subunit gene did show specific hybridization with cells in fetal gonads (data not shown). These results suggest either that fetal testosterone levels are insufficient to significantly induce *Pem* gene expression or that fetal gonads have not adequately differentiated to transcribe the *Pem* gene.

Location of *Pem* gene expression in the epididymis

After leaving the testes, spermatozoa enter the epididymis for further maturation. In the epididymis, the spermatozoa acquire the abilities of forward motility and egg recognition. RNase protection and Northern blot analysis showed that *Pem* is expressed in epididymis (Fig. 1). To determine the specific location of *Pem* transcripts in the epididymis, we performed *in situ* hybridization analyses. The hybridization signal was clearly present over the cytoplasm of the somatic apical cells (Fig 7). No detectable signal over background was observed over the spermatozoa in the lumen. The epididymis is typically divided into three regions: caput, corpus, and cauda. The hybridization signal was restricted to the proximal cauda region of the epididymis (data not shown). These results show that *Pem* expression originates exclusively in the somatic cells of a highly specific region of the epididymis, a region where spermatozoa are known to undergo maturation (see discussion).

Temporal pattern of *Pem* expression in mouse epididymis

Time-course analyses of RNA from epididymides revealed that *Pem* gene expression increased between days 12 and 17 after birth (Fig. 8). This increase corresponded with a known increase in testosterone secretion by the Leydig cells and an increase in fluid and protein production by the Sertoli cells (Selmanoff et al., 1977; Jégou et al., 1983). Another increase in epididymal *Pem* expression took place between days 23 and 30, the same temporal phase when testosterone levels further increase and the first wave of spermatozoa and seminiferous fluid enters the epididymis from the testis (Oakberg, 1956; Eleftheriou and Lucas, 1974). These data are consistent with the possibility that testosterone, germ cell factors, or both contribute to *Pem* gene regulation in the epididymis.

Germ cell-dependent regulation of *Pem* in mouse epididymis

In an effort to further understand *Pem* gene regulation in the epididymis, we assessed *Pem* mRNA levels in quaking (qk/qk) and W^v/W^v mutant mice. Qk/qk mice have defective spermatids that are phagocytosed by the Sertoli cells. All preceding stages of germ cells in qk/qk mice are normal (Bennett et al., 1971). Figure 9 shows that the epididymis of qk/qk mice expressed *Pem* transcripts. Since these mice lack spermatozoa in the epididymides, this confirms the *in situ* hybridization results (Fig. 7) that epididymal somatic cells express *Pem* mRNA. *Pem* transcript levels were two-to-three fold lower in qk/qk mice than in qk/+ mice, suggesting that mature germ cells or their products may modestly enhance *Pem* gene expression.

W^v/W^v mice lack germ cells at the spermatogonium stage and, therefore, all succeeding stages. These mice are also deficient in the germ cell-stimulated Sertoli cell products such as androgen-binding protein (ABP), inhibin, and tubule fluid (Jégou et al., 1984). *Pem* mRNA was not detected in epididymides from W^v/W^v mice, implying a requirement for germ cell factors for *Pem* expression (Fig. 9). In contrast, Pem

transcripts were expressed in the epididymis of $W^v/+$ control mice. Collectively, the results from W^v/W^v and qk/qk mutant mice suggest that although *Pem* gene transcription in epididymal somatic cells occurs in the absence of spermatozoa within the lumen of the epididymis, that germ cell factors and/or induced products derived from the testis are an obligate requirement for *Pem* expression in the epididymis.

DISCUSSION

In this report, we determined the cell populations that expressed *Pem* by cellular fractionation, *in situ* hybridization, and analyses of RNA from BALB/c and mutant mouse testes and epididymides. We conclude that *Pem* transcripts are expressed in a stage specific manner in Sertoli cells of the testis. Our studies in mutant mice showed that the expression of *Pem* mRNA in Sertoli cells was dependent on gonadotropins but not germ cells. In the epididymis, *Pem* mRNA was localized to somatic cells of the proximal cauda region. Regulation of *Pem* transcription in the epididymis differed from regulation in the testis as demonstrated by temporal pattern of expression in prepubertal mice and by germ cell dependence. The spatial and temporal expression pattern of *Pem* mRNA in testis and epididymis exhibited a correlation with specific developmental events during spermatogenesis.

Several lines of evidence indicated that testicular Sertoli cells express *Pem* (Figs. 2-3). First, RNase protection analyses showed that the seminiferous tubule fraction of the testis (containing Sertoli and germ cells) expressed high levels of *Pem* mRNA, while few *Pem* transcripts could be detected in the interstitial cell fraction (enriched for Leydig cells). Second, W^v/W^v mice, which lack germ cells, expressed *Pem* mRNA at high levels. *Pem* transcript levels in the W^v/W^v mouse testis were twice as high as the levels detected in heterozygote testis, presumably because of an increased ratio of Sertoli cells to germ cells in the homozygote. Third, *in situ hybridization* analyses showed that *Pem* transcripts displayed a hybridization pattern expected of the distribution of Sertoli cell cytoplasm: a uniform signal strongest around the basal region of the tubule.

Although we cannot rule out the possibility that a small subset of spermatogonia may express *Pem* transcripts, comparison of *Pem* mRNA expression levels in W^v/W^v

and W^v/+ mice is inconsistent with high levels of expression in germ cells. Furthermore, we did not detect *Pem* transcripts in germ cells by *in situ* hybridization. If spermatocytes or spermatids expressed *Pem,* a punctate or adluminal pattern would have been expected over these germ cells. The chromosomal location of the *Pem* gene provides further evidence against the expression of *Pem* in germ cells. The *Pem* gene is located on the X chromosome (Lin et al., 1994). The X-chromosome is transcriptionally inactivated in pre-meiotic germ cells, as assessed by a lack of ^{3}H-UTP incorporation (Henderson, 1964; Monesi, 1965; Kierszenbaum and Tres, 1974). Since *Pem* gene expression is induced concurrently with the onset of meiosis (day 9 after birth; Fig. 5), it is unlikely that *Pem* gene transcription occurs in meiotic or postmeiotic germ cells. Nonetheless, these data do not entirely rule out the possibility that a subset of premeiotic germ cells (i.e., pale-A, dark-A, or B spermatogonia) may express *Pem.*

Injection of purified LH induced *Pem* in the regressed testes of hpg/hpg mice (Fig. 6B). Since Sertoli cells lack LH receptors (Heindel et al., 1975; Dufau et al., 1978) it is clear that the induction of *Pem* gene expression by LH is mediated by an indirect mechanism. LH is known to induce testosterone production by the Leydig cells of the testes and, in turn, testosterone has been shown to bind to androgen receptors on Sertoli cells and regulate their proliferation and differentiation (Lyon et al., 1975; reviewed in Jégou, 1992). Thus, LH is considered to act in a paracrine manner on Sertoli cells. A role of testosterone in *Pem* gene induction is strongly supported by the lack of *Pem* transcripts in testis of mice with a defective androgen receptor (Fig. 6D). Although we hypothesize that LH induces the production of androgens necessary to maintain *Pem* gene expression in adult animals, it is highly unlikely that LH is involved in the initial induction of *Pem* mRNA on day-9 after birth since the pituitary does not start secreting LH until after day-11 (Selmanoff et al., 1977). Prior to the secretion of LH by the pituitary, Leydig cells are known to produce low levels of androgens (Jean-Faucher et al., 1978) which may be sufficient in levels to permit *Pem* gene transcription by Sertoli

cells in newborn animals, provided that other factors necessary for *Pem* gene expression are also present. In contrast, fetal gonads express *Pem* transcripts at nearly undetectable levels (Fig. 6E), presumably because androgen levels in the fetal gonad are insufficient to induce *Pem* expression or because the Sertoli cells in the fetal gonad have not differentiated sufficiently to activate *Pem* gene transcription.

Pem is the first candidate transcription factor shown to be dependent on gonadotropins for expression in Sertoli cells. In contrast, the zinc finger *GATA-1* transcription factor, which is selectively expressed by Sertoli cells of the testis (Yomogida *et al.*, 1994), was transcribed independently of the presence of gonadotropins (Fig. 6C). In addition, we show that the ß-Zip transcription factor CREB is expressed by testis independently of the presence of gonadotropins (Fig. 6C). In contrast, the expression of the CREM-*t.* gene in germ cells depends on gonadotropins. Foulkes et al. show that the expression of this ß-Zip transcription factor is down-regulated in the testis after hypophysectomy. FSH, but not LH, restores *CREM-t* expression in the germ cells of these hypophysectomized mice. Therefore, the LH regulated Pem expression in Sertoli cells is unique among this group of transcription factors.

We found that postpubertal testes preferentially expressed *Pem* in seminiferous tubules at stages VII-VIII (Figs. 3-4). Sertoli cells are smallest during these two stages and possess Golgi that have migrated from the basal region towards the mid and apical cytoplasm (Jegou, 1992). Stages VII and VIII are known as androgen-dependent stages (Sharp *et al.*, 1992; Kerr *et al.*, 1993), in part because androgen binding protein, which acts as an "androgen-sink", is at its lowest level just prior to these stages (Ritz *et al.*, 1982) thus freeing more androgen to bind to the androgen receptor. Therefore, we hypothesize that the cyclical pattern of *Pem* expression in seminiferous tubules is a response of Sertoli cells to androgens. This hypothesis is further supported by our observation that *Pem* mRNA expression in the testis is absolutely dependent on

androgen receptor expression (Fig. 6D). Interestingly, the X-chromosome encoded *GATA-1* transcription factor is expressed at similar spermatogenic stages as *Pem* . Antibodies specific for *GATA-1* detect this protein in stages VII, VIII, and IX (Yomogida et al., 1994). The expression of *Pem* and *GATA-1* in virtually all tubules from W^v/W^v mice (Fig. 3 and Yomogida et al., 1994) suggests that germ cells normally down-regulate the expression of these genes in a stage-specific manner. The similar expression pattern of the *Pem* and *GATA-1* genes suggests that one of these genes may control the expression of the other, or alternatively, a third factor may regulate these genes coordinately. This is unlikely since GATA-1 was not down-regulated in hpg mouse testes suggesting that this transcription factor is not regulated by hormones.

Since the *Pem* gene contains a homeobox and is likely to encode a transcription factor, it is intriguing what spermatogenic events it may regulate. Since the intimate physical contact between Sertoli cells and germ cells is critical for spermatogenesis (O'Brien et al., 1991; Wu et al., 1993), it is reasonable to suppose that the expression of *Pem* by Sertoli cells will influence germ cell maturation events. Events that occur in seminiferous tubules during stages VII-VIII when *Pem* is preferentially expressed include the following: i) initiation of meiosis in spermatocytes; ii) break-down of the Sertoli cell barrier to allow the primary spermatocytes to move from the basal compartment; and iii) release of elongate spermatids from the seminiferous tubular epithelium into the lumen (Russell et al., 1990). *Pem* may regulate the expression of the proteases or adhesion proteins important in these events. For example, one candidate downstream target of *Pem* is the protease plasminogen activator since it is expressed specifically in stage VII-VIII (Bardin, 1988). However, PA has been shown to be induced by FSH or with cyclic AMP derivatives, but not by androgens (Lacroix et al., 1977; Lacroix and Fritz, 1982a; Lacroix et al., 1982b).

Sertoli cell-germ cell adhesion structures called ectoplamic specializations and tubulobulbar complexes are also needed for germ cell maturation. These structures are found during the same stages in which Pem was found to be expressed (Russell, 1977a; Russell, 1977b; Russell et al., 1980; Russell et al., 1986). Ectoplasmic specializations are androgen regulated (Muffly et al., 1993). In addition, one of the few known functions for homeobox genes is their regulation of adhesion molecules. The homeobox proteins *Hox-b8* and *-b9* have been shown to modulate the transcription of the gene encoding the neural cell adhesion molecule N-CAM (Jones et al., 1992). The endoderm specific homeobox gene, *HNF-1*, regulates the expression of the liver cell adhesion molecule, L-CAM, as demonstrated with a reporter construct containing the L-CAM promoter in NIH 3T3 cells (Goomer et al., 1994). Therefore, Pem is a candidate transcription factor that regulates Sertoli cell adhesion molecules.

The pattern of expression and regulation of the *Pem* gene in the epididymis differed from its regulated expression in testis. For example, our work with the qk/qk and W^v/W^v mutant mice showed that a factor induced by germ cells is needed for *Pem* expression in the epididymis (Fig. 9). In contrast, testicular expression of *Pem* did not depend on germ cells. The temporal kinetics of *Pem* expression also differed between the two tissues. In epididymis, *Pem* transcripts increased gradually between days12 and 37 after birth (Fig. 8). In contrast, testicular *Pem* expression was induced dramatically between day-8 and -9 and then remained relatively constant into adulthood (Fig. 5).

Despite the differences between the expression pattern of *Pem* in epididymis and testis, we suggest that androgens regulate the expression of the *Pem* gene in both tissues. In epididymis, the temporal pattern of *Pem* mRNA levels correlates with known changes in testosterone levels in newborn mice (Selmanoff et al., 1977; Jégou et al., 1983). For example, LH is known to cause Leydig cells to produce testosterone between days 11 and 17 after birth of the mouse (Oakberg, 1956; Eleftheriou and

Lucas, 1974) coincident with the first increase in epididymal *Pem* transcript levels. In the rat, we have shown hypophysectomy ablates *Pem* expression in the epididymis and that this expression is restored after addition of exogenous testosterone (Lindsey and Wilkinson, manuscript in preparation). Although several genes encoding structural proteins or enzymes have been shown to be dependent on androgens for expression in the epididymis (Robaire & Hermo, 1988), *Pem* is the first putative transcription factor suggested to be androgen-dependent in the epididymis.

We propose that regulation of *Pem* expression by testosterone in the epididymis occurs through the Sertoli cell-secreted androgen binding protein (ABP). This protein is secreted into the seminiferous tubule lumen after day-8 postnatally and has been shown to provide the necessary testosterone to both germ cells and the epididymal somatic cells through receptor-mediated endocytosis (Byers et al., 1985). Our experiments with mutant mice support the notion that ABP transported in seminiferous fluid provides the testosterone required for *Pem* expression in the epididymis: *Pem* was expressed in the epididymis of qk/qk mice (Fig. 9), in which Sertoli cells produce seminiferous fluid and proteins in response to germ cells up through the spermatid stage. In contrast, W^v/W^v mice, in which seminiferous fluid and proteins are lacking because of a virtual absence of germ cells, did not express epididymal *Pem* transcripts (Fig. 9). Although our results are consistent with ABP as the germ cell-induced factor necessary for *Pem* expression, our results do not rule out regulation by another germ cell-induced factor.

Pem transcripts are specifically localized to principal cells in the proximal cauda region of the epididymis (Fig. 7). This specific localization may be due, in part, to higher dihydrotestosterone (the predominant form of androgen in the epididymis) binding in the cauda region of the epididymis than in the corpus (Purvis & Hansson, 1978). However, the specific localization of *Pem* transcripts in the epididymis cannot be due only to the local concentration of androgens since most androgen-regulated genes are expressed in a broader region of the epididymis than *Pem*. *Pem* may function to regulate genes

that exhibit a similar regional expression pattern in the epididymis; e.g. the proteinase inhibitor-like protein HE4 and superoxide dismutase (Perry *et al.*, 1993; Krull *et al.*, 1993). Since the proximal cauda and the neighboring distal corpus region of the epididymis has been shown to confer fertilization ability to spermatozoa, it is reasonable to suspect that *Pem* may play a role in regulating these processes. Forward motility is gained by spermatozoa in this region of the epididymis, as well as changes in the spermatozoan membrane that permit egg recognition (Blaquier *et al.*, 1988; Moore et al., 1986). In summary, the *Pem* gene exhibits a unique and provocative pattern of expression and regulation in principal cells of the epididymis and Sertoli cells of the testis. The expression pattern of the *Pem* gene suggests that it may act as a transcription factor to regulate important events in the male reproductive system.

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FIGURE LEGENDS

Fig. 1. ***Pem* mRNA expression in mouse testis and epididymis.** (A) Northern blot analysis of 10 μg total cellular RNA isolated from the testis and epididymis of adult BALB/c mice. Methylene blue staining of ribosomal RNA (28S and 18S) on the Nytran blot was used to verify that the amount of RNA loaded was equal (not shown). (B) RNase protection analysis of 40 μg total cellular RNA from adult BALB/c mouse testis and epididymis using a ^{32}P-UTP-labeled antisense *Pem* RNA probe. An L3 ribosomal protein RNA probe was included in each annealing reaction to demonstrate the amount of RNA.

Fig. 2. ***Pem* mRNA expression is localized to Sertoli cells.** RNase protection analyses of 40 μg total cellular RNA isolated from adult BALB/c whole testes, tubule fraction, and interstitial fraction (A) and adult W^V/+ and W^V/W^V whole testes (B). An L3 ribosomal protein RNA probe was included in each annealing reaction to demonstrate the amount of RNA. (C) Northern blot analysis of 10 μg total RNAs isolated from W^V/+ and W^V/W^V testis. Methylene blue staining of ribosomal RNA (28S and 18S) on the blot was used to verify equivalent RNA loading in each lane.

Fig. 3. ***In situ* hybridization localization of *Pem* transcripts in Balb/c and W^v/W^v testis.** (A-C) Balb/c testis sections hybridized with an antisense *Pem* RNA ^{33}P-UTP-labeled probe. Seminiferous tubules that express *Pem* transcripts (as shown by dark grains) are stage VI-VIII (note the characteristic appearance of spermatids aligned at the lumen of these tubules). (D-E) Testis sections from 12-week old W^v/W^v mice. All tubules from these germ cell-deficient mice expressed *Pem* transcripts (as shown by the dark grains); *albeit* at different levels. A *Pem* sense probe did not exhibit hybridization above background levels with either Balb/c and W^v/W^v tubules (data not shown). A 20X

and 10X microscope objective was used for the photography shown in panels A-D and E-F, respectively. Sections were stained with the nuclear stain thionin.

Fig. 4. **Stage-specific expression of *Pem* mRNA during the spermatogenic cycle.** A map of the cycle of the seminiferous epithelium of the mouse is shown overlain by a dense area depicting the stages and the general location in the epithelium of grains corresponding to *Pem* transcripts. A less dense gray zone is indicated extending toward the lumen and represents areas of low level labeling over the region that includes the luminal portion of the Sertoli cytoplasm and adjacent spermatocytes and spermatids (Cycle map from Russell *et al.* (1990) used with permission of Cache River Press).

Fig. 5. **Temporal *Pem* mRNA expression in mouse testis.** (A) RNase protection analysis of 40 µg of total RNA from BALB/c mouse testes on the days indicated after birth. A GAPDH probe was included in each annealing reaction (except for day 60 RNA where the annealing was done in parrallel but in separate tubes) to demonstrate amount of RNA. The histogram shows relative *Pem* mRNA levels normalized against GAPDH mRNA as determined by densitometry. Methylene blue staining of ribosomal RNA on a northern blot verified the amounts and integrity of RNA for each time point (data not shown). (B) RNase protection analysis of BALB/c mouse testes, as in panel A. An L3 ribosomal probe was included in each annealing reaction to demonstrate equivalent amounts of RNA for each time point.

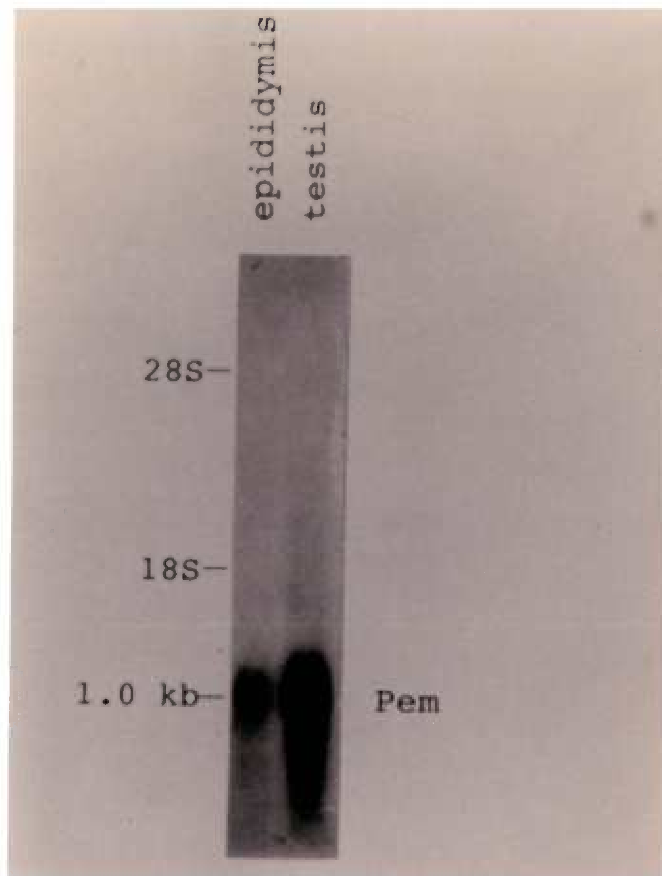
Fig.6. **Dependence of the *Pem* gene on gonadotropins and androgen receptor for expression.** RNase protection analysis of 40 µg total cellular RNA extracted from testes of 5-week-old hpg/+ and hpg/hpg litter mates (A) or of hpg/hpg mice after two

days of injections with the agents shown (B). (C) Northern blot analysis of 10 μg RNA from 5-week-old hpg/hpg mice after two days of injections with the agents shown. (D) RNase protection analysis of 40 μg total cellular RNA from wild-type (Balb/c) and Tfm mouse testes. (E) RNase protection analysis of total cellular RNA from day 16-fetal gonads (40 μg) or adult testis (20 μg). For all RNase protection analyses (A,B,D,E) an L3 probe was included in each annealing reaction to verify the amount of RNA. Equivalent loading for the Northern blot (C) was demonstrated by methylene staining of the 18S and 28S rRNA.

Fig.7. **Location of *Pem* transcripts in mouse epididymis.** *In situ* hybridization of cross-sectioned adult BALB/c mouse epididymis stained with the nuclear stain thionin. Drawing shows the region of the epididymis represented by the micrographs. A ^{33}P-UTP antisense *Pem* RNA probe was used to detect *Pem* transcripts in the cytoplasmic region of apical cells in the proximal cauda. The top and bottom panels are dark-field and bright-field views, respectively, of the same field (20X microscope objective). A sense *Pem* probe did not display specific hybridization (data not shown).

Fig.8. **Temporal pattern of *Pem* mRNA levels in epididymis.** RNase protection analysis of total cellular RNA (40 μg) from mouse epididymides on days indicated after birth. The origin of the smaller *Pem* transcripts detected by the *Pem* probe are not known.

Fig.9. **Germ cell dependent regulation of *Pem* gene expression in epididymis.** RNase protection analysis of 40 μg total cellular RNA from adult heterozygous or homozygous quaking (qk) (A) and dominant white spotting (W^v) (B) mouse epididymides. An L3 ribosomal protein RNA probe was included in each annealing reaction to demonstrate the amount of RNA.



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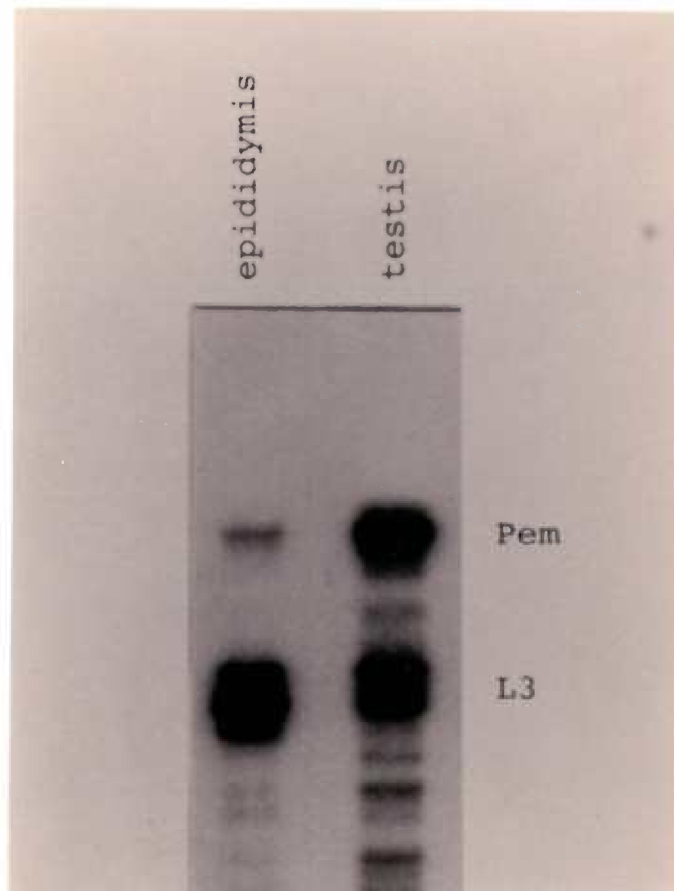

B.

Figure 1

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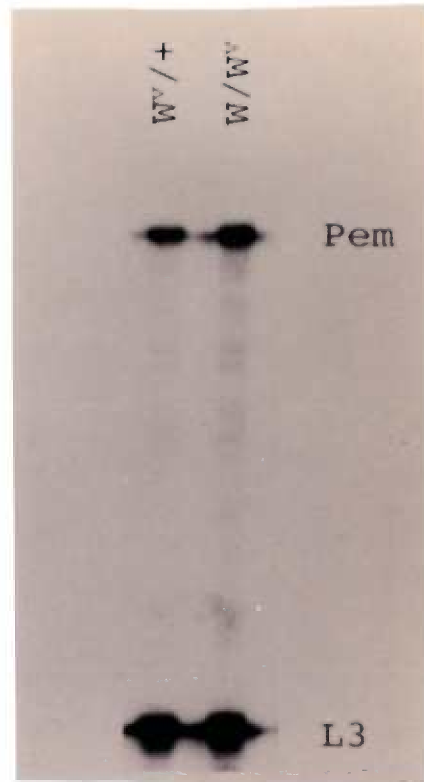

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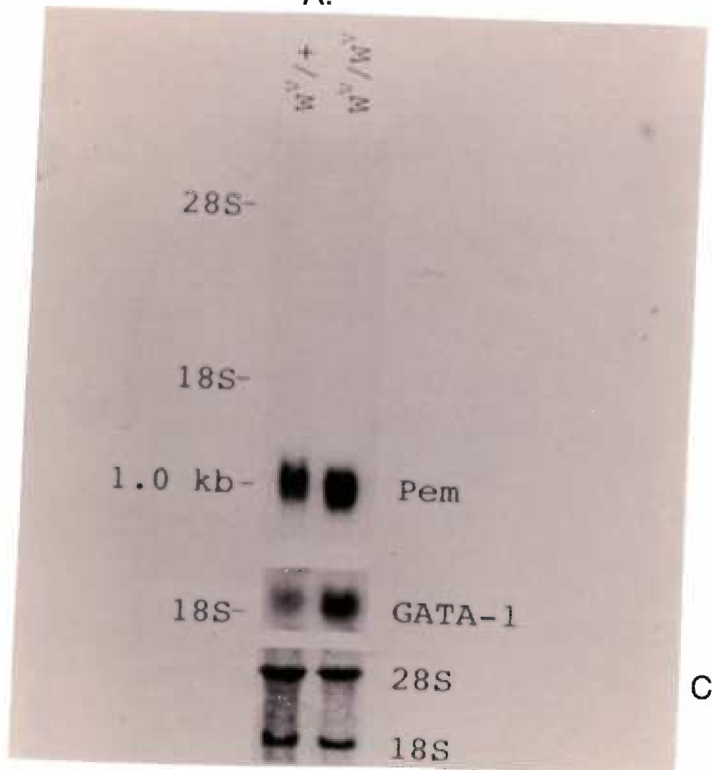
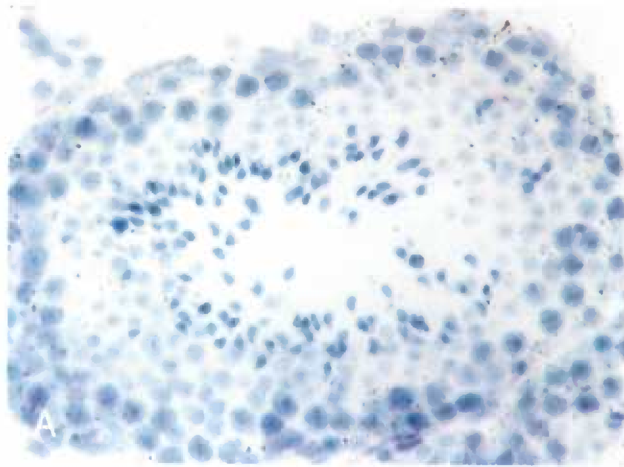
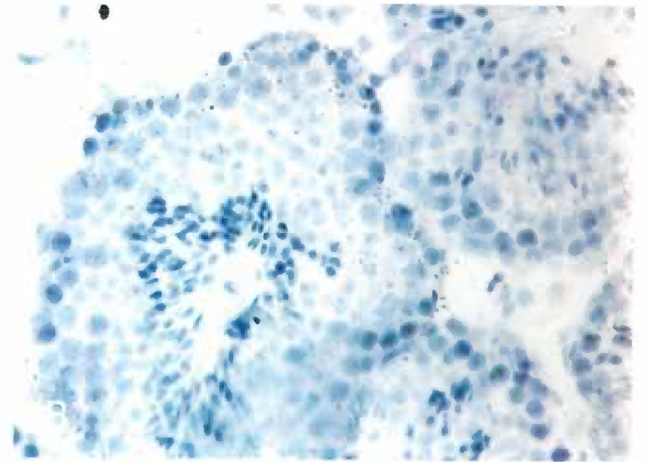
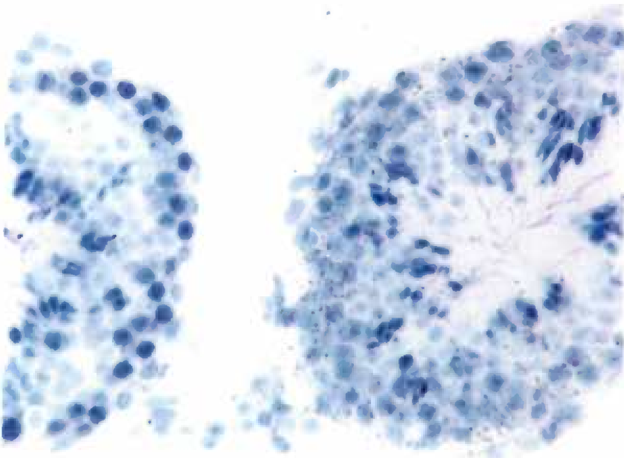
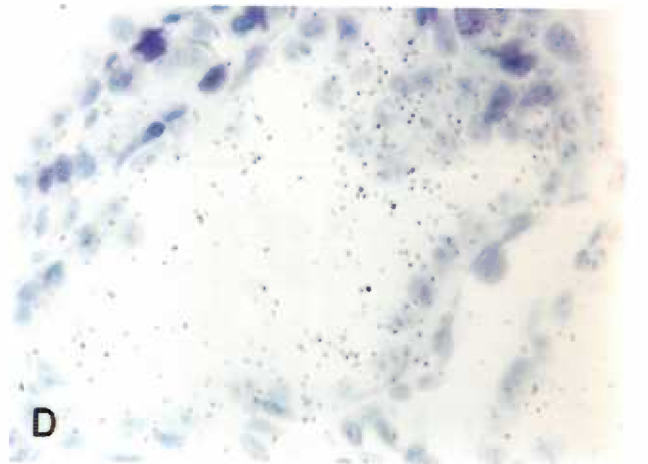


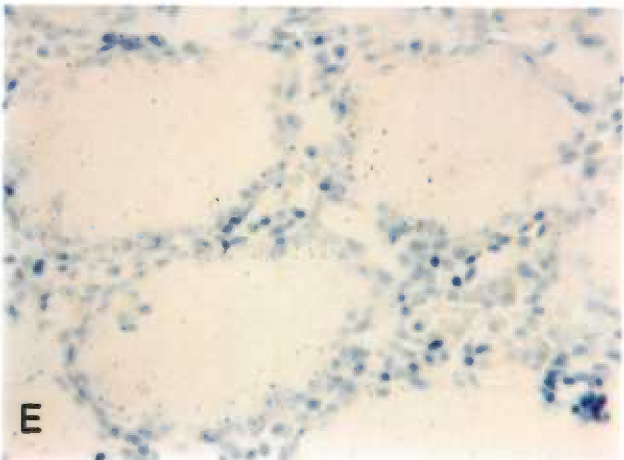
C.

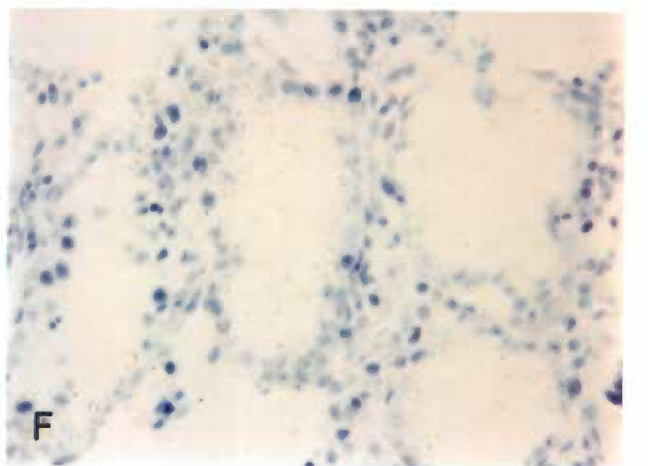
Figure 2









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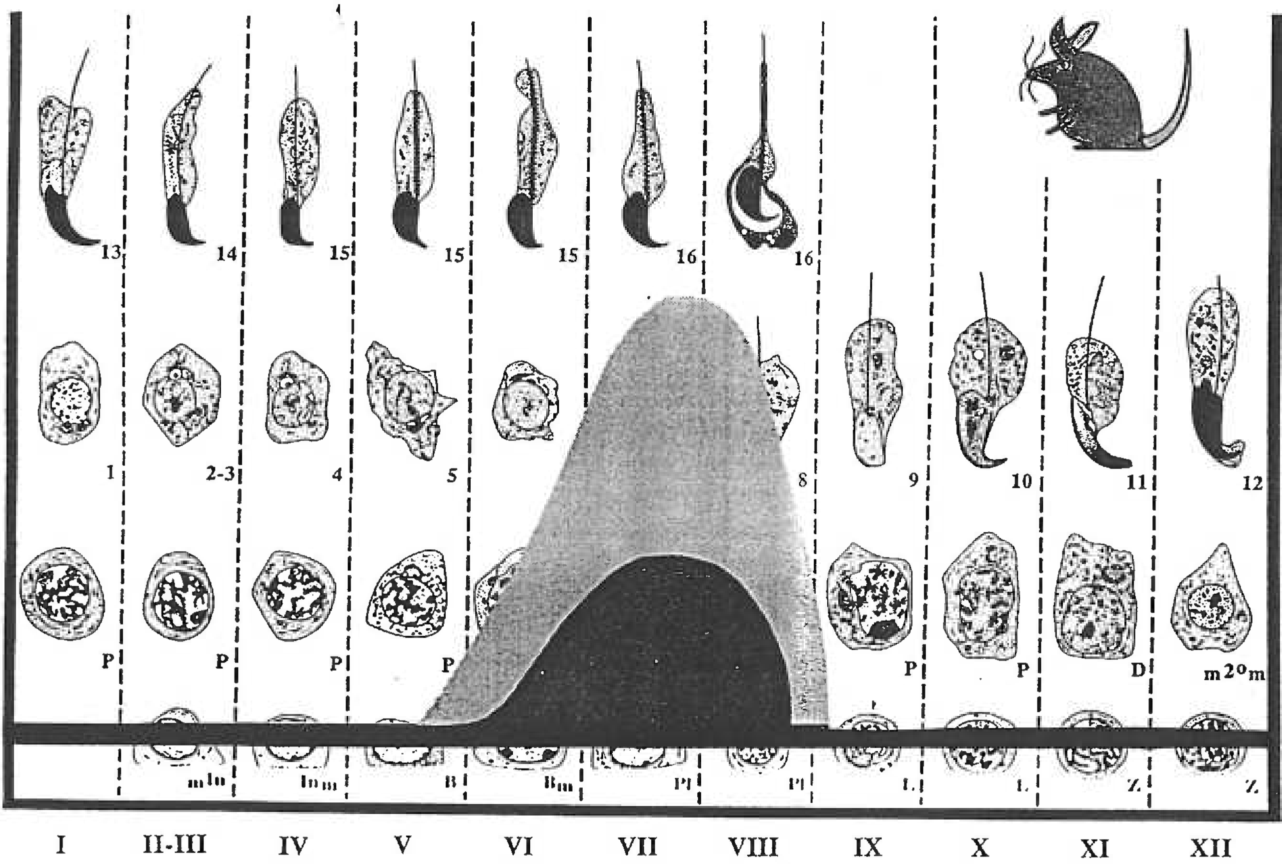

Figure 4

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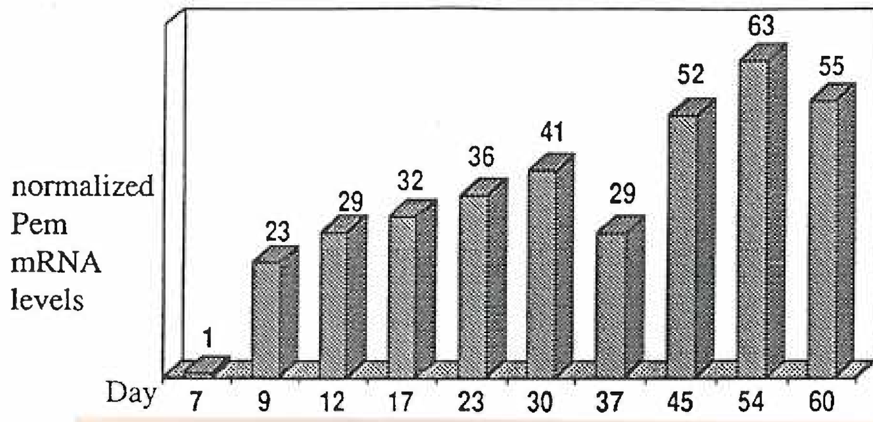

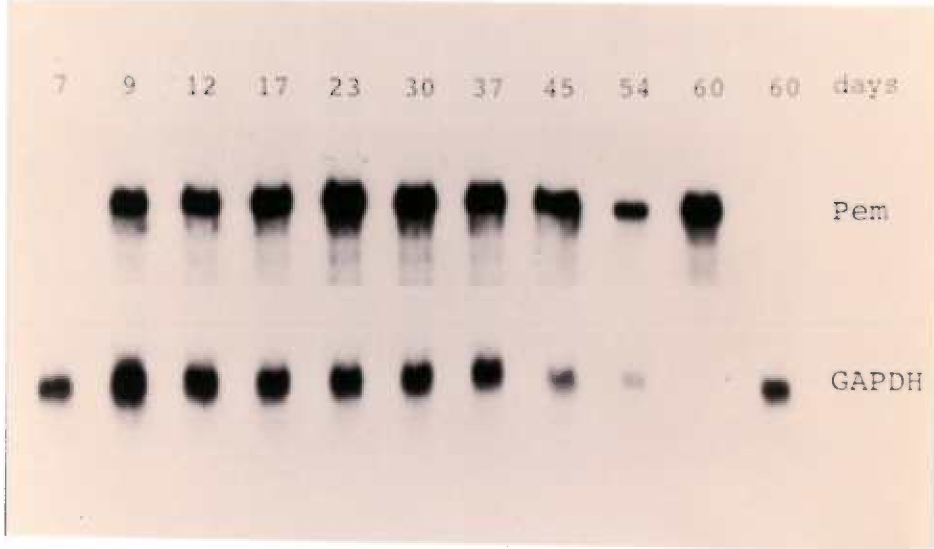

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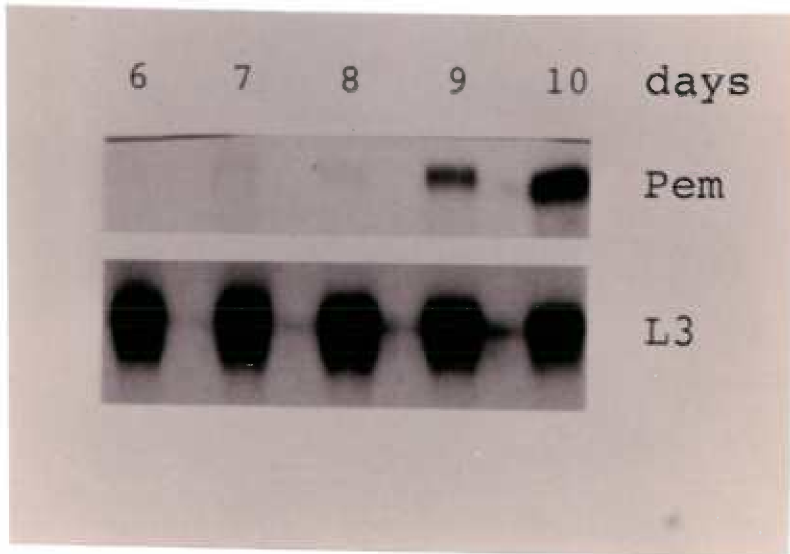

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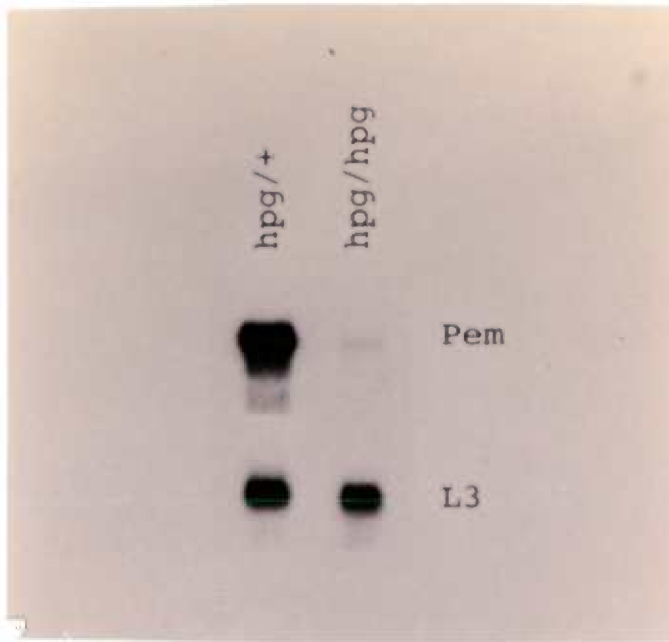

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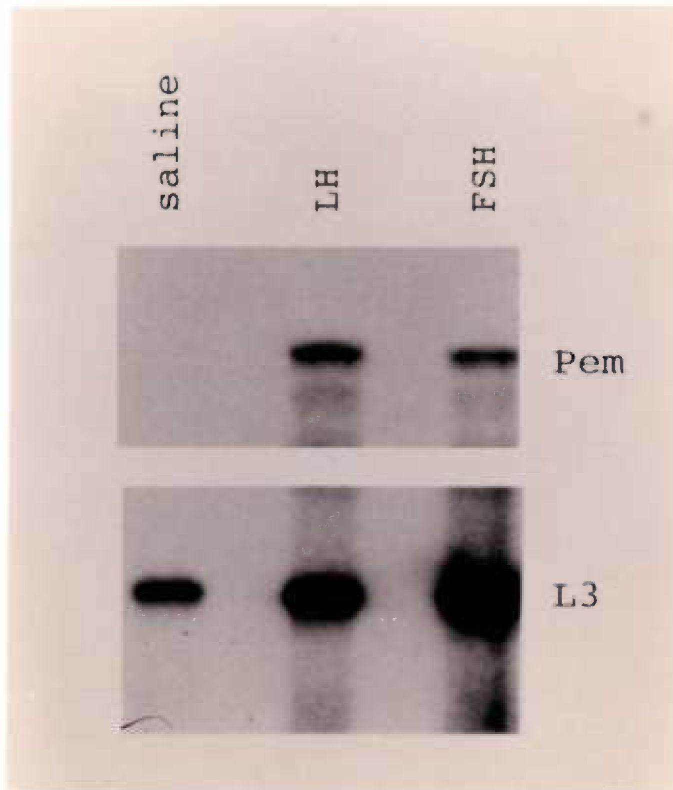

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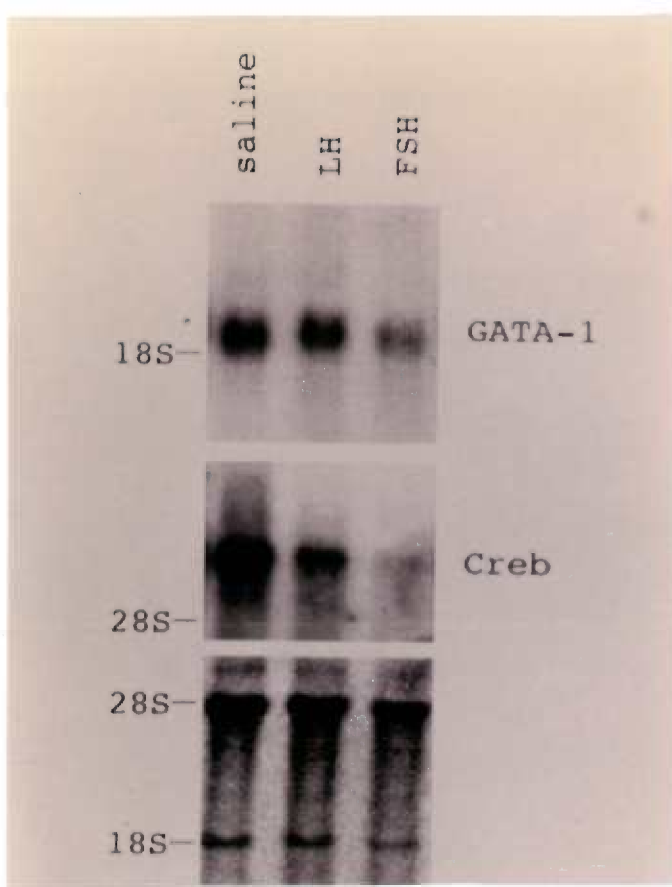
Figure 6



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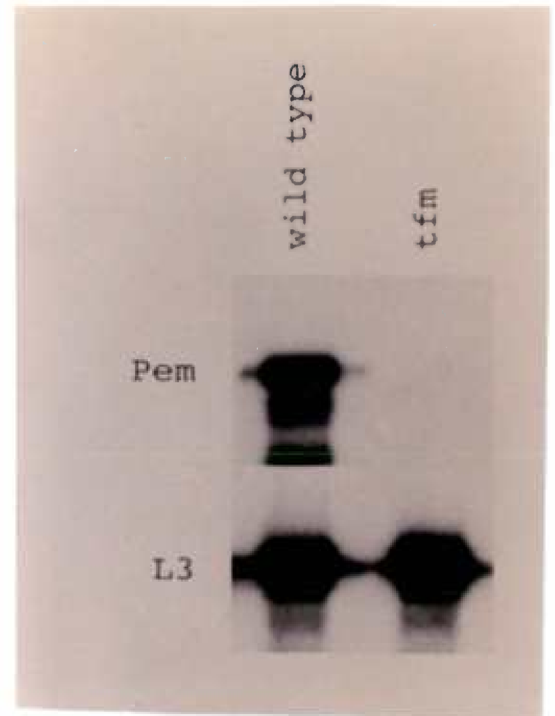

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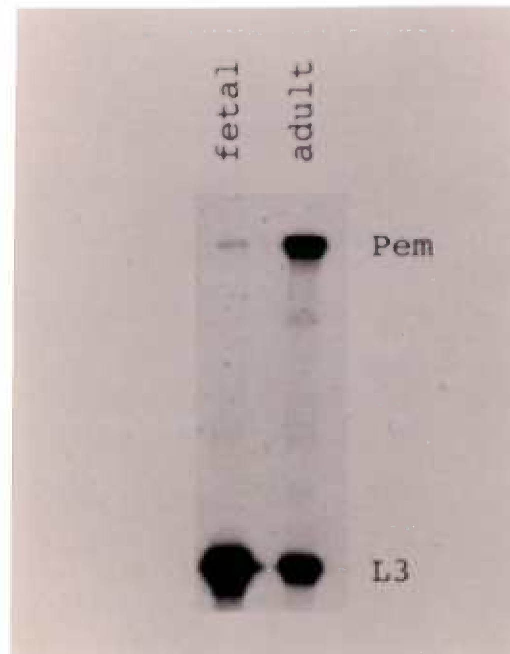
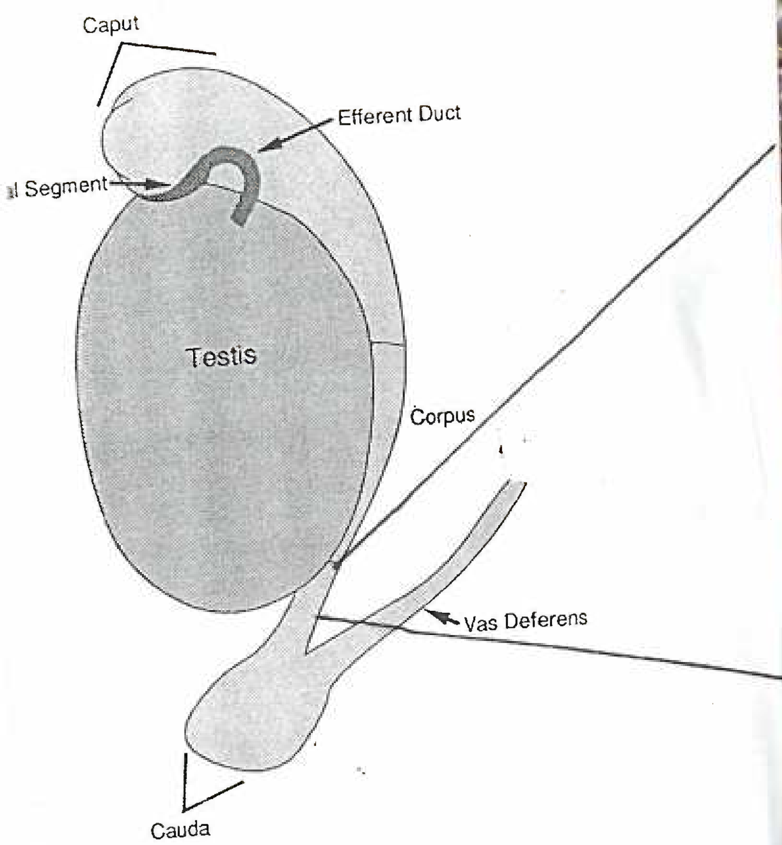
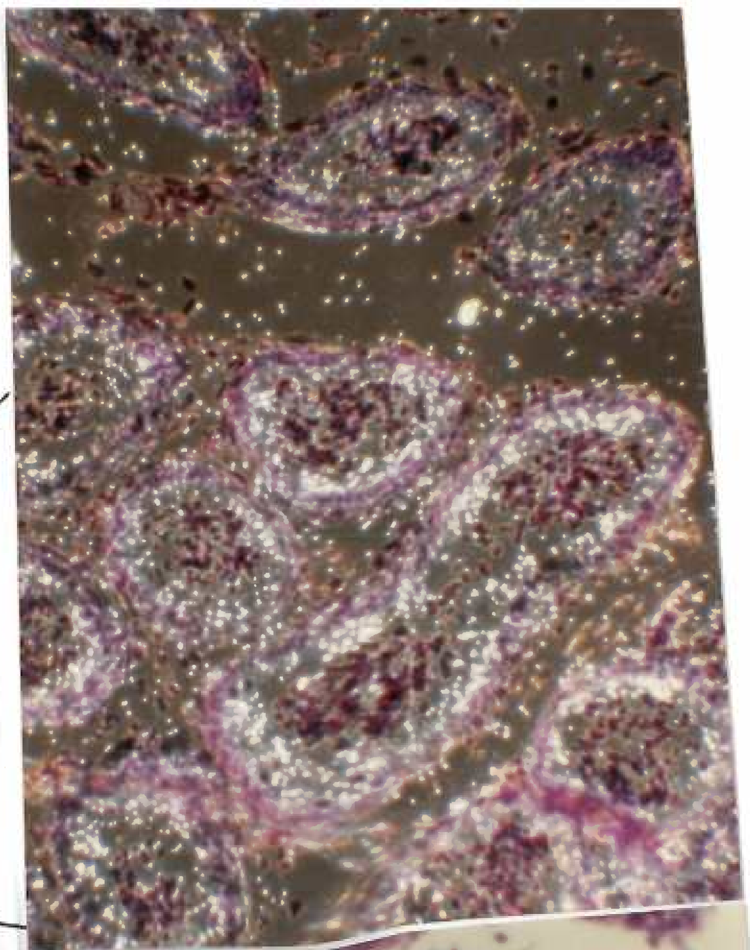


E.

Figure 6





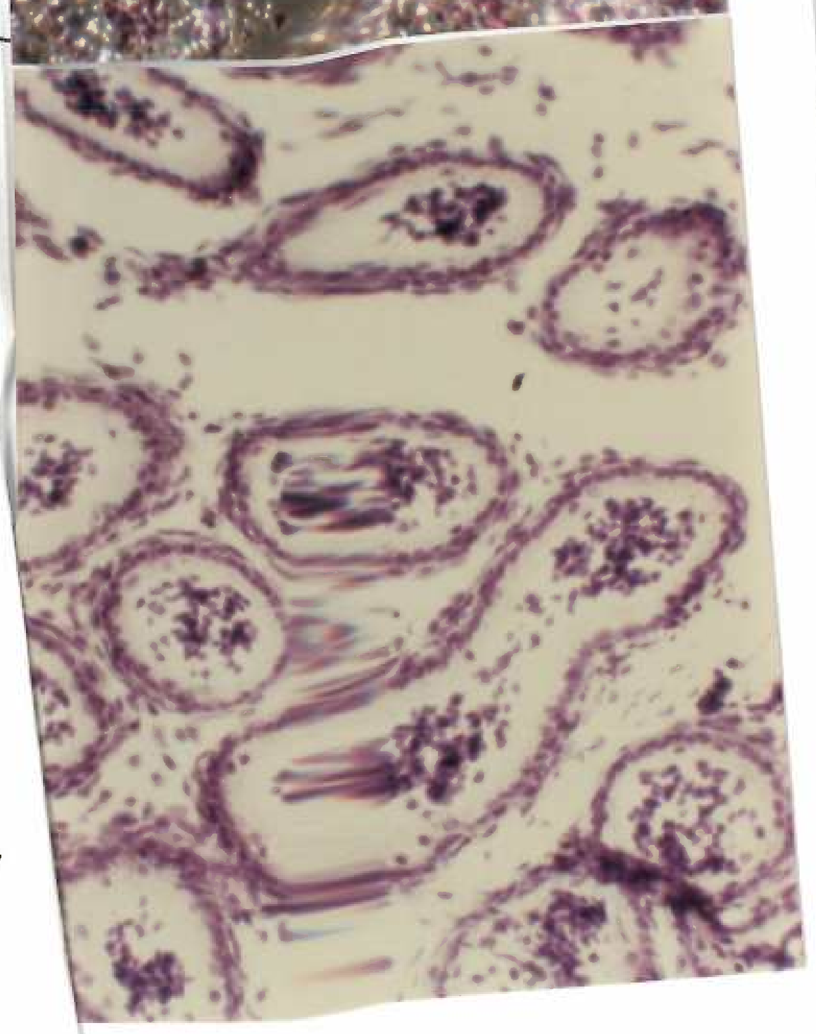


Figure 7

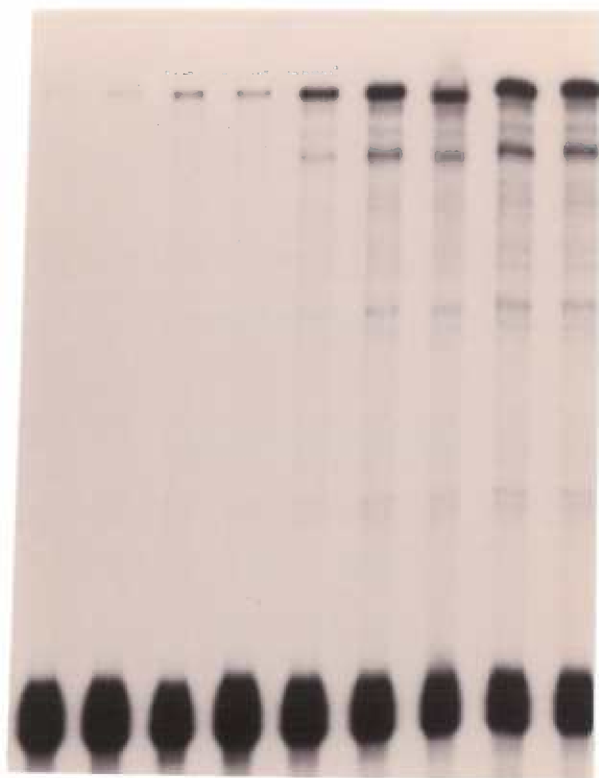
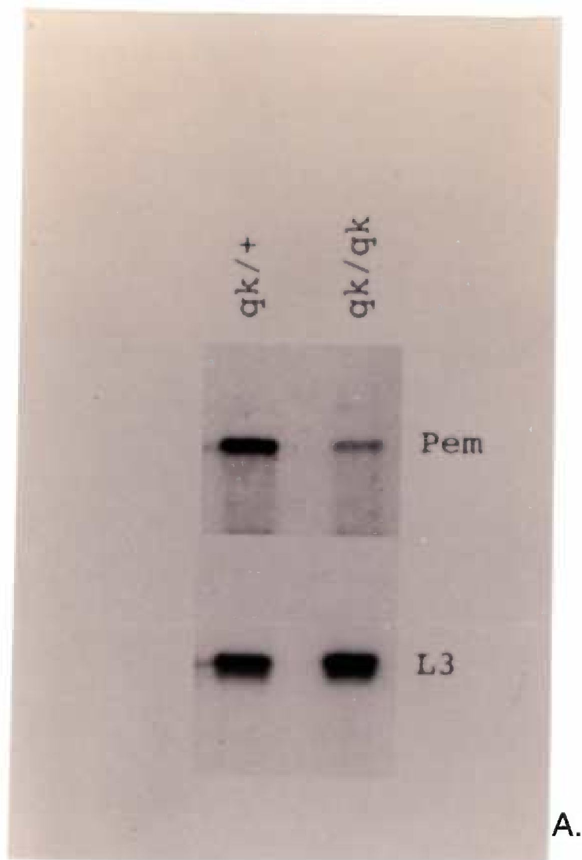


Figure 8



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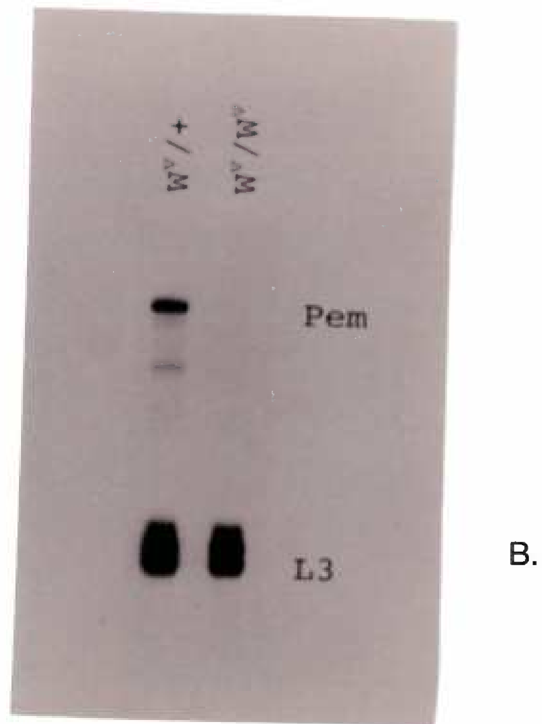

B.

Figure 9

MANUSCRIPT #2

Pem Homeobox Gene regulated by Testosterone in Rat Testis and Epididymis*

J. Suzanne Lindsey and Miles F. Wilkinson

Oregon Health Sciences University, Dept. of Molecular Microbiology and Immunology, Portland, OR 97201 (J.S.L.) and The University of Texas MD Anderson Cancer Center, Houston, TX 77030 (M.F.W.).

Address all correspondence and requests for reprints to : Dr. Miles F. Wilkinson, MD Anderson Cancer Center, Dept. of Immunology, 1515 Holcombe Box 180, Houston TX 77030.

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ABSTRACT

The ovary, testis, and epididymis of the adult mouse are the only tissues that express the divergent homeobox gene, Pem, at detectable levels. We showed that the rat Pem gene (rPem) was expressed in reciprocal amounts in testes and epididymides when compared to the mouse Pem gene (mPem) expression levels in these same organs of the mouse. Therefore, we wanted to further characterize this homeobox gene during testicular and epididymal development in the rat. During a time course of epididymides mRNA after birth, rPem expression occurred at day 23, a period of development when other genes regulated by testosterone are induced. Indeed, Pem expression in the rat epididymis was dependent on testosterone as exhibited by an absence of Pem transcripts in hypophysectomized rats and restoration of Pem expression with testosterone implants. The rat epididymis expressed rPem in apical cells of the distal corpus and proximal cauda regions as determined by analysis of total RNA from sectioned epididymis and by in situ hybridization. In the rat testis, rPem expression increased on day 15 after birth and showed a cyclic pattern of expression after birth into adulthood. This cyclical pattern is consistent with stage VII and VIII seminiferous tubules expression of mPem in mouse testes. Pem represents one of the major differences when comparing mouse to rat gene expression during spermatogenesis.

INTRODUCTION

Spermatogenesis is a well studied adult developmental system. In the testis primordial germ cells proliferate, undergo meiosis, and transform into spermatids. From the testis, spermatozoa enter the epididymis and continue to mature so that they are capable of fertilization. Both paracrine and endocrine hormones as well as a discrete microenvironment are necessary for these events. Gonadotropins, luteinizing hormone (LH) and follicular stimulating hormone (FSH), from the pituitary affect Leydig cells and Sertoli cells respectively. LH has been shown to induce proliferation of Leydig cells and cause their production of testosterone which acts in a paracrine fashion on peritubular cells and Sertoli cells of the seminiferous tubule. FSH causes Sertoli cells to proliferate [1], to become responsive to testosterone [2-4] as well as increase testosterone production by causing release of a factor which acts on Leydig cells [5]. Testosterone causes maturation, directly or through peritubular cells, of Sertoli cells for protein secretion and germ cell interactions [6-13]. Testosterone alone maintains qualitative spermatogenesis in mature testes even in the absence of FSH and LH [14-16].

Many developmental events are regulated by homeobox gene expression such as the Pax-6 gene product during eye development [18] and Hox proteins establishing the rostral-cauda axis [19]. Other homeobox genes have been shown to be transcribed in the spermatogenic system. *Sperm-1* is transiently expressed immediately prior to meiosis in germ cells (20). *Hox-a4* is expressed specifically in postmeiotic germ cells of the testes but not in the epididymides of the adult mouse (21; Lindsey and Wilkinson unpublished results). *Hox-b4* and *-d4* genes are expressed in adult testes as well as other adult organs (22,23). *Hox-c8* and *Pax-2* are transcribed in the epididymis but not in the testes (24,25; Lindsey and Wilkinson unpublished results). The function and regulation of these homeobox genes expressed during spermatogenesis has not been determined.

In a search for developmentally regulated genes, we isolated the divergent homeobox gene (26,27), *Pem,* by subtraction hybridization between two, developmentally different, T-lymphoma cell lines SL 12.3 and SL 12.4 (28). During early gestation of the mouse embryo, primitive endoderm- and trophectoderm-derived cells of the placenta and yolk sac express *Pem* whereas, other embryonic or extra-embryonic cell lineages do not express *Pem* (29,30). In addition, embryonal carcinoma stem cells cultured *in vitro* exhibit this *in vivo* expression pattern: F9 stem cells induced to differentiate into visceral or parietal endoderm up-regulate Pem mRNA expression as assessed by northern blot analysis (26) and in situ hybridization (30,31). However, northern analyses did not detect *Pem* transcripts in any of the adult mouse tissues that have been tested including, liver, stomach, heart, pancreas, intestine, brain, lung, kidney, pituitary, thymus, and spleen (28,29). We have shown that in mouse, gonadotropins induce the expression of Pem in mice lacking gonadotropins. Furthermore, *Pem* is the only homeobox gene known to be expressed in Sertoli cells in a stage-specific manner (32). The rPem homologue varies from mPem in its coding region for the amino terminal end of the homeodomain but chromosomal location, expression location, and gene frequency shows that it is the true rat homologue to mouse Pem (33).

In this work we expanded our search for adult rat expression and regulation of rPem. We isolated mRNA from the uterus, kidney, pancreas, stomach, intestine, spleen, thymus, lung, muscle, testis, heart, liver, brain, epididymis, and ovary and did northern and RNase protection analyses. The temporal expression pattern of Pem was determined for both testicular and epididymal development. We examined the regulation of rPem in the epididymis of hypophysectomized rats with and without exogenous testosterone. Cellular localization of rPem mRNA in the epididymis was determined by in situ hybridization. Rat Pem is the only known homeobox gene to be regulated by testosterone in the rat epididymis.

Methods and Materials

Animals

Normal, sham operated, and hypophysectomized (HPX) Sprague-Dawley rats were obtained from Charles River Laboratories. Oregon Health Sciences University's animal care facility housed these animals according to approved protocols with hypophysectomized animals receiving 5% glucose water ad libitum. Animals were killed by CO_2 asphyxiation. Organs were immediately removed, homogenized, and frozen at -70°C until RNA was extracted.

For the testosterone implant experiments, hypophysectomized rats were anesthetized at one atmosphere isofluorane and the implant was placed subcutaneously along the upper back and neck. Testosterone implants were made with Silastic tubing three centimeters long filled with testosterone proprionate. These implants were made in collaboration with Dr. John Resko, Oregon Health Sciences University, who has shown them to deliver 4ng of testosterone per ml of serum (verbal communication). Implantation was done 12 days post hypophysectomy.

We verified the completeness of the HPX and sham operated animals by determining testosterone levels by chromatography of serum (34). All testosterone assays were done in the laboratory of Dr. David Hess at the Oregon Regional Primate Research Center, Beaverton, OR. Serum was obtained by tail bleeds prior to testosterone implantation. Serum, intratesticular, and intraepididymal testosterone and dihydrotestosterone levels were analyzed by the same method after testosterone treatment and in the untreated animals. Contralateral epididymides from each animal

were immediately collected, weighed, homogenized in phosphate buffered saline, and frozen at -70°C until analyzed.

We repeated the rat epididymis time course and HPX rats with testosterone implants experiments two times with different sets of animals.

Riboprobe preparation

For RNase protection and *in situ* hybridization analyses, we prepared ^{32}P- or ^{33}P-UTP or ^{35}S-ATP labeled RNA probes with T7 DNA-dependent RNA polymerase for anti-sense and SP6 RNA polymerase for sense transcripts corresponding to the middle FokI to FokI region of the rat Pem cDNA (33]) We also utilized GAPDH, a housekeeping gene, template (Ambion) to generate antisense probes as a positive control. An RNA ladder template (Ambion) was used to generate specific size markers. We used the *in vitro* transcription protocol as described in Current Protocols in Molecular Biology (35). Probes were purified on a 6% polyacrylamide denaturing gel. After exposure to film, the appropriate sized bands were cut out and put in individual eppendorf tubes. The gel slices were mashed with an RNase-free pestle in 100 µl of diethylpyrocarbonate (DEPC) treated water. To each mashed sample, 600 µl of 1X proteinase K (PK) buffer (0.3 M NaCl, 0.5% SDS, 10 mM Tris (pH 7.5), 200 µg PK, and 20 µg tRNA) was added, vortexed and incubated at 37°C for 15 minutes. After vortexing and a pulse-spin, the suspended probes were filtered through a 0.45 micron disc (Acrodisc). Another 600 µl of the PK buffer was added and incubated for at least 5 more minutes at 37°C and again the suspended probe was filtered through the 0.45 micron disc. Each sample was extracted with 200 µl of phenol/chloroform. One microliter was taken to determine counts per minute and the rest was precipitated and stored at -70°C.

RNase protection assays

Total RNA from tissues was prepared as previously described by either guanidinium isothiocyanate (36) or by a single-step acid guanidinium thiocyanate-phenol-chloroform extraction (37) and quantified by optical density. RNase protection analyses were performed as described in Current Protocols (35) with some minor modifications. Briefly, sample RNAs, or tRNA as a negative control, were centrifuged with the appropriate gel purified ^{32}P-UTP labeled probes. The pellet was resuspended in 30 μl of annealing buffer (40 mM PIPES, pH 6.4, 0.4 M NaCl, 1 mM EDTA, 80% formamide) and allowed to hybridize overnight at 42° C. Unhybridized RNA was digested with RNase A (50 μg/ml) and T1 (4 μg/ml) for 30 minutes at 37°C. RNases were then removed by treatment with proteinase K, and extraction with Phenol/chloroform/isoamyl alcohol. After ethanol precipitation, the RNA pellet was resuspended in loading buffer, denatured at 85°C, run on a 6% polyacrylamide denaturing gel and analyzed by autoradiography.

Densitometry was done on scanned images with the NIH image 1.54 program.

Northern Blot Analysis

Ten μg per lane of total cellular RNA was electrophoresed on 1% agarose gels at 50 volts in a running buffer . The separated RNA was transferred to Nytran™ paper via overnight capillary blot as described by Maniatis et al. (38). After transfer, the blot was UV cross-linked (Strata-linker) and stained with methylene blue to evaluate the transfer and loading of RNA in each lane. Blots were then prehybridized in pre-hyb buffer (50% formamide, 5X Denhardt's solution, 5X SSPE, 0.5% SDS, and 100 μg/ml sheared salmon sperm DNA) for 4 hours at room temperature. The blot was then hybridized with random oligomer primed ^{32}P-labeled cDNA in the presence of hyb buffer (pre-hyb buffer plus 10% dextran sulfate and 50% formamide) overnight at 42° C. Results were analyzed by autoradiography.

In situ hybridization.

In situ hybridizations were performed as described by Wilcox (39). Briefly, the tissues were removed, rinsed in PBS and immersed in 4% paraformaldehyde-0.1 M sodium phosphate buffer, pH 7.4, at 4°C for 3 hours. Routinely, placenta as the positive control tissue and brain the negative tissue were used as controls. In addition, anti-sense GAPDH transcripts were used as a positive control for the procedure. The tissues were then immersed in 15% sucrose-PBS solution overnight at 4°C. After embedding tissue in OCT (Miles; Elkhart, IN), 10 to15 micron cryosections were adhered to Superfrost/Plus microscope slides (Fisher Scientific; Pittsburgh, PA) and stored at -70°C in a sealed box with desiccant. Slides were thawed at 55°C for 5 min. and fixed for 10 min. in 4% paraformaldehyde-0.1 M $NaPO_4$, pH 7.4, at 4°C. Slides were then immersed in 1 μg per ml proteinase K solution (1 μg/ml in 500 mM NaCl,10 mM Tris, pH8.0) for 10 min. at room temperature. After washing in 0.5X SSC for 10 min. at room temperature, the slides were prehybridized (hybridization solution: 10 mM DTT, 0.3 M NaCl, 20 mM Tris, pH 8.0, 5 mM EDTA, 1X Denhardt's solution, 10% Dextran Sulfate, and 50% Formamide) for one hour at 42°C. Hybridization was performed with 6 x10^5 cpm ^{33}P-UTP labeled RNA probe per specimen. After annealing overnight at 55°C, the slides were washed 2 times for 10 min. in 2X SSC with 10 mM beta-mercaptoethanol (BME) and 1mM EDTA at room temperature. Unhybridized RNA was digested with an RNase A solution (20 ug/ml RNase A in 500 mM NaCl and 10 mM Tris, pH 8.0) for 30 min. at room temperature. The slides were then washed as before but only for 2 min. then a stringent wash for 2 hours in 4 liters of 0.1X SSC with 10 mM BME and 1mM EDTA at 55°C. After the stringent wash, the slides were washed 2 times for 10 min. each in 0.5X SSC without BME or EDTA at room temperature and then dehydrated for 2 min. each in 50%, 70%, and 90% ethanol containing 0.3 M NH_4Ac. After desiccation, the slides were dipped in Kodak NTB2 nuclear emulsion diluted 1:1 with water at 42°C. After exposure the slides were developed and counter stained with 0.1% thionin.

RESULTS

Pem expression in rat adult tissues

We first examined the expression of Pem mRNA in adult tissues of the rat by Northern analyses (data not shown) and by RNase protection analyses. Uterus, kidney, pancreas, stomach, intestine, spleen, thymus, lung, muscle, testis, heart, liver, and brain lacked detectable Pem transcripts. We found that only placenta, ovary, and epididymis expressed Pem transcripts at detectable levels in this assay (Fig. 1A). Since mouse testis express mPem (32), we analyzed rPem expression with a higher specific activity probe and longer exposure during autoradiography. We detected rPem transcripts in rat testis at very low levels. Several independent analyses showed that rPem mRNA levels were consistently higher in epididymides than in testes (Fig 1B).

Localization of Pem expression in rat epididymis

The epididymis is typically divided into several regions; the initial segment, the caput, the corpus, and the cauda. To determine the region that expressed Pem transcripts, we dissected the epididymis into three main regions; initial segment, caput/corpus, and corpus/cauda (see diagram Fig. 2A). Pem mRNA levels in each region were assessed by RNase protection analysis. The initial segment did not express Pem mRNA. Whereas, the caput/corpus and the cauda/corpus segments both expressed Pem transcripts (Fig. 2B).

To further define the exact location of Pem transcripts in the epididymis we did in situ hybridization analyses of cross-sectioned rat epididymis. Specific hybridization of the Pem antisense probe was found in the proximal cauda region. Pem-positive cells were strictly somatic apical cells and no signal was detected above background levels over the germ cells (Fig. 2C).

Temporal expression of Pem in the epididymis

To determine if the regulation of Pem expression correlates with known developmental events in the epididymis we analyzed total mRNA on various days from epididymides of 8- to 44-day old rats. Pem expression increased significantly between days 23 and 26 after birth, a time that is known to correspond to increased testosterone levels in the epididymis of the rat (41,42). Pem transcript levels remained constant through day 44 (Fig. 3A).

To compare this expression pattern with other known transcript patterns which are expressed in the epididymis, a northern blot analysis was done. This time course was of epididymides from day-5 to day-90 rats after birth. We first probed the blot with a Pem cDNA probe and the blot was subsequently stripped and probed for proenkephalin, BC1, and another homeobox transcript, Hoxc-8. Pem expression was as demonstrated in the RNase protection analysis and remained upregulated into adulthood. Hoxc-8 transcripts were high on day-5 after birth and gradually decreased in amount up to adulthood. Proenkephalin mRNA did not increase until day 44 after birth a period in epididymal development when the first wave of spermatozoa enter the epididymis which is the same expression pattern obtained by Garret et al. (40). BC1, a testosterone regulated gene (40), was upregulated at the same time as Pem expression (Fig. 3B).

Testosterone regulates Pem expression in the rat epididymis

Since Pem transcripts first appear at a time known for testosterone responsive gene regulation in the epididymis, we tested the possibility that Pem is regulated by testosterone. We utilized hypophysectomized rats which no longer produce the gonadotropins necessary for testosterone production. An absence of testosterone was tested by serum and intratesticular testosterone assays. In hypophysectomized rats, epididymides no longer expressed Pem transcripts as compared to sham operated rat

epididymides (Fig. 4), suggesting that Pem was regulated by gonadotropins secreted by the pituitary. Pem expression was restored as early as two days after testosterone administration in hypophysectomized rats (Fig. 4). These results strongly suggest that epididymal Pem expression is regulated by a gonadotropin induced hormone, testosterone.

Pem expression in rat testis

Since mouse Pem is expressed at greater levels in the testis than in the mouse epididymis, we used a high specific activity Pem antisense probe to determine if Pem was developmentally regulated in rat testis. A time course of mRNA from day-5 to day-104 after birth rat testes was analyzed by RNase protection and densitometry of the Pem bands normalized to the GAPDH bands. Pem transcripts increased at day-15 to a peak at day 21. Pem expression in the testis then declined to a low level on day 26 but started to increase again as early as day 30 and levels again peaked on day 78 after birth (Fig. 5).

DISCUSSION

In this paper we report that the rat Pem homologue is expressed in the same adult tissues as the mouse homologue. Analyses of mRNA from uterus, kidney, pancreas, stomach, intestine, spleen, thymus, lung, muscle, testis, heart, liver, brain, epididymis, and ovary confirmed detectable rPem expression exclusively in adult reproductive tissues. This expression occurred in developing testis and epididymis at periods of time known for gonadotropin and testosterone action in these tissues (41-43). Indeed, in hypophysectomized rats, rPem expression is ablated in the testis and epididymis. Most importantly, exogenous testosterone restores rPem transcript levels in testes and epididymides of hypophysectomized rats. Pem transcripts were located by in situ hybridization in a very precise region of the distal corpus and proximal cauda in the epididymis and by the more sensitive assay of RNase protection in larger regions of the caput/corpus and corpus/cauda. The highly specific expression pattern and hormonal regulation of the Pem gene suggests that Pem may be a transcription factor which directs gene expression important in spermatogenesis and sperm maturation.

Rat epididymis expressed Pem mRNA developmentally. Pem transcripts were not detected prior to day 23 in the epididymis. Pem mRNA levels increased on days 23 and 30; a period of time that has been shown by others when increases in testosterone and dihydrotestosterone levels in the epididymis occur (41, 42) and testicular fluid first enters the epididymis (43). When rPem expression was compared to other known epididymal transcripts the pattern was consistent with testosterone induction of gene transcription. We detected a lack of Pem transcripts in epididymides of HPX rats. HPX

rat epididymides do not mature spermatozoa. In HPX rats with testosterone implants, epididymal rPem expression was restored. Androgens are essential for epididymal development as well as for the microenvironment needed for sperm maturation and protection (44). Therefore, we speculate that Pem is a transcription factor that may regulate some of the testosterone regulated genes expressed in the epididymis.

The precise cellular location of rPem mRNA in the epididymis is consistent with the location of epididymal mouse Pem mRNA (32). The more sensitive RNase protection analysis showed Pem transcripts over a broader area, the caput/corpus and the corpus caput. Whereas, the less sensitive RNA analysis of in situ hybridization located Pem transcripts to the proximal cauda region. Therefore, it is likely that Pem levels were greater in this area. The proximal cauda region has been shown to be important for maturation of the spermatozoa, specifically in forward motility and egg recognition (45,46). Most importantly, two proteins, "forward motility" and carboxypeptidase y-like (D/E) proteins, shown to be important for spermatozoa to gain these abilities, are specifically found in this region and are regulated by testosterone (47,48). Other genes which encode superoxide dismutase (49) and proteinase inhibitor-like protein (50) are highly expressed in the cauda and are thought to play a protective role for spermatozoa during their storage in the cauda. Therefore, rPem may be a transcription factor that regulates these genes.

There are very few known genes expressed by both the testis and the epididymis. Rat Pem may be regulating different genes in the two organs. One protein in common between testis and epididymis is dimeric acidic glycoprotein (DAG). Interestingly, DAG is secreted by Sertoli cells of the testis where Pem is expressed, however, it is also secreted from the caput region of the epididymis (51) where Pem transcripts are not detected at high levels . Therefore, it is unlikely that Pem regulates the DAG gene in the epididymis. Alternatively, since epididymal gene regulation and expression have not been researched as extensively as that of the testis, it remains

possible that Pem may regulate the same gene(s) in each organ. For example, ABP is secreted by the Sertoli cells of the testis primarily stimulated by testosterone (52). ABP is then transported to the epididymis where it has been localized by immunohistochemistry to endocytic vesicles of the initial segment and caput principal cells (53,54). However, ABP is immunolocalized to endocytic vesicles of epithelial cells in the proximal prostate (55) which is downstream of the cauda epididymis, so perhaps ABP is secreted upstream of the prostate. Pem could be regulating the production of ABP in both the testis and the epididymis but mRNA for ABP has not been localized in the epididymis.

In rat testis, Pem mRNA increased by day 15 after birth. This testicular developmental time period has been shown to be influenced by increased binding of follicular stimulating hormone (FSH) to receptors on Sertoli cells and increased binding of luteinizing hormone (LH) to receptors on Leydig cells with a resultant increase in testosterone production (56). In addition, FSH binding to Sertoli cells has been shown to increase gonadal sensitivity to LH (57). Mitoses of the Sertoli cells, the cell type identified to express Pem in the mouse, decrease at about day 14 after birth in the rat (58-60). So, it is most likely that Pem expression does not regulate Sertoli cell proliferation.

The cyclical pattern of Pem expression in the rat testis is reminiscent of the spermatogenic cycle which is every 12.9 days in the rat [61]. The increased Pem transcripts began between days 12 and 15 and the second increase started around day 26; the two increases are about 13 days apart. The first spermatozoa do not reach the lumen of the seminiferous tubule until day 45 after birth (58). Since Pem transcripts in the rat testis peaked at day 21 and decreased by day 26 this suggests that Pem transcription is down-regulated well before the first spermatozoa are released. However, Pem protein levels during these cycles still need to be determined. The timing of Pem expression during the seminiferous cycle suggests that Pem may be

needed for gene regulation of early- and mid-spermatogenic events. Indeed, we have shown that mPem is specifically expressed late in stage VI, most highly in Stage VII, and decreases in early stage VIII seminiferous tubules of the mouse (32); stages that have been shown to be regulated by androgens (62) and correlate to the same stages in the rat (63).

We conclude that Pem is the first testosterone regulated homeobox gene to be localized primarily in the proximal cauda region in the epididymis and expressed in a stage-specific manner in the testis. Therefore, Pem may be a key transcription factor which directs testosterone regulated gene expression in the epididymis and is necessary for spermatogenesis.

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FIGURE LEGENDS

Fig. 1. Pem mRNA Expression in Adult Rat Tissues.

(A) RNase protection analysis of 20 µg total cellular RNA from the tissues shown. *Pem* probe A was used to detect *Pem* transcripts and an 18S rRNA probe was included in each annealing reaction to demonstrate the amount of RNA. Note that in the case of epididymis RNA, the two probes were hybridized in separate annealing reactions. The protected RNA fragment size for all tissues is 210 nt. as judged by comparison with the migration of the RNA Century Ladder. (B) RNase protection analysis of total cellular RNA (amounts are indicated) hybridized with *Pem* probe B. The protected fragment size is approximately 220 nt. (the slight difference in migration of some of the protected RNA fragments seen in this gel was not observed in other experiments).

Fig. 2. Localization of *Pem* Transcripts in the Epididymis.

(A) Diagram of the testis and epididymis showing the regions that were dissected from the epididymis [see ref. 66 for more detailed description of sections]. (B) RNase protection analysis of total cellular RNA (20 µg) from the initial segment, caput/corpus, and corpus/cauda A GAPDH probe was included in each annealing reaction to demonstrate the amount of RNA. (C) *In situ* hybridization analysis of a 10 µm cross-section of the rat epididymis hybridized with a ^{33}P-ATP-labeled *Pem* (antisense) probe A. Dark-field and bright-field views are shown of a portion of the distal corpus. Note the specific hybridization over the principal cells and a lack of hybridization above background levels over the spermatozoa. A *Pem* (sense) probe did not exhibit hybridization above background levels in the same region of the distal corpus (data not shown).

Fig. 3. Temporal Expression of Pem mRNA in the Rat Epididymis.

(A) RNase protection analysis of 40 μg of total cellular RNA from rat epididymides on the indicated day after birth. *Pem* probe A was used for hybridization. A GAPDH probe was included in each annealing reaction to demonstrate the amount of RNA. The data represents one of three time courses performed - each of which demonstrated the same temporal pattern of *Pem* mRNA expression. (B) Northern blot analysis of other known genes expressed in the epididymis during its development. Total cellular RNA (10 μg) from epididymides of 5- to 90-day old Sprague Dawley rats was separated in a formaldehyde gel and transferred to a membrane for hybridization. The same blot was sequentially hybridized, stripped, and re-hybridized with *Hoxc-8*, BC1, proenkephalin, and *rPem* cDNA probes (the data with the *Pem* probe is not shown). Equivalent loading of RNA in each lane was demonstrated by methylene blue staining of the 18S and 28S rRNA (data not shown).

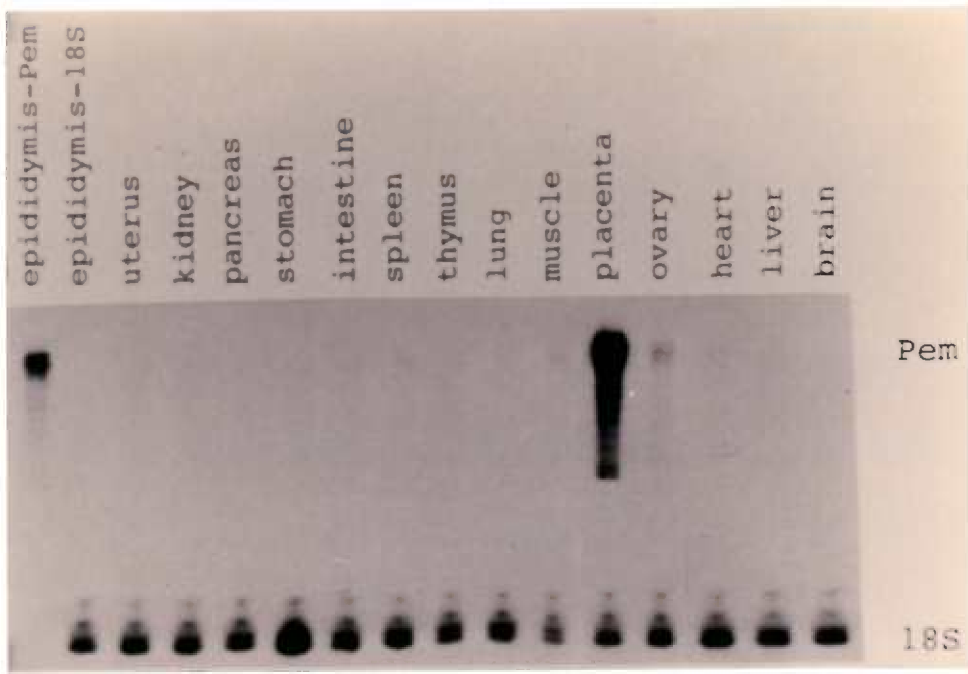
Fig. 4. Testosterone-dependent *Pem* mRNA Expression in Rat Epididymis.

RNase protection analysis of total cellular RNA (40 μg) from the epididymides of rats that had been sham operated, hypophysectomized, or hypophysectomized with testosterone implanted for two days. *Pem* probe A was used for hybridization. A GAPDH probe was included in each annealing reaction to demonstrate the amount of RNA. The same results were obtained with multiple animals in experiments performed on two separate occasions.

Fig 5. Developmental Regulation of Pem mRNA Levels in Rat Testis.

(A) RNase protection analysis of total cellular RNA (40 μg) from rat testes at the indicated days of age. *Pem* probe A was used for hybridization. A GAPDH probe was included in each annealing reaction to demonstrate the amount of RNA. (B) Relative

Pem mRNA levels determined by densitometry and normalization against GAPDH mRNA levels (the data from panel A was used for this analysis).



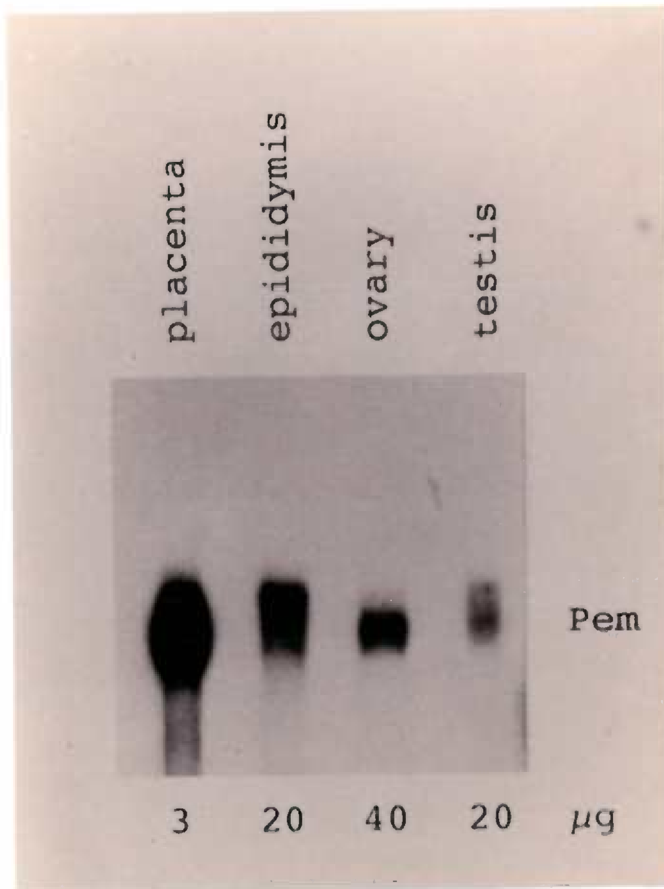
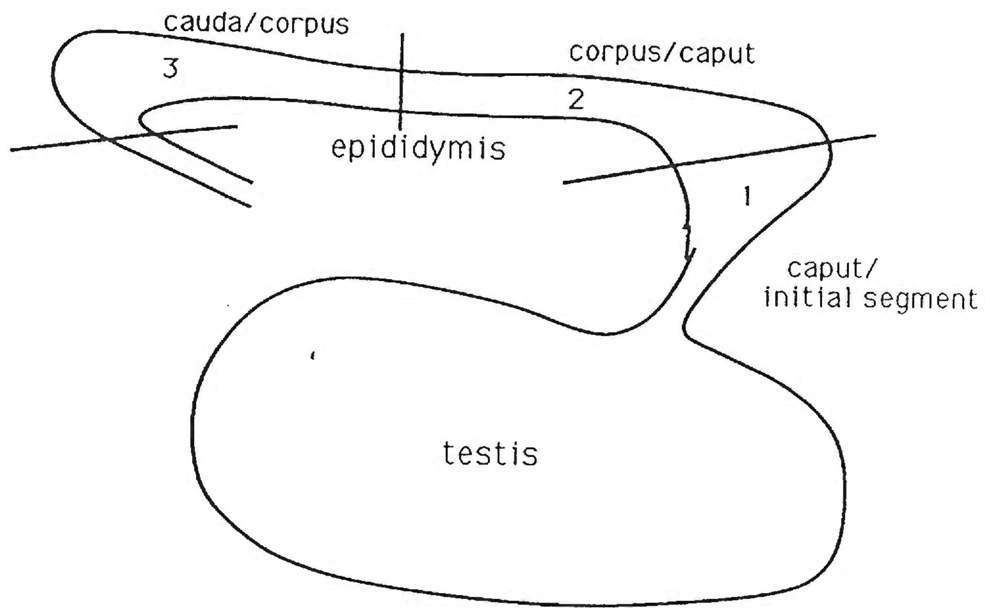


Figure 1



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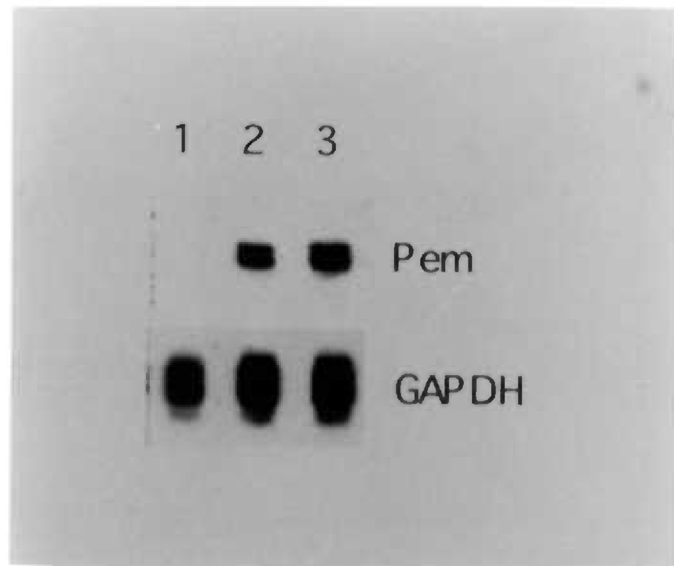
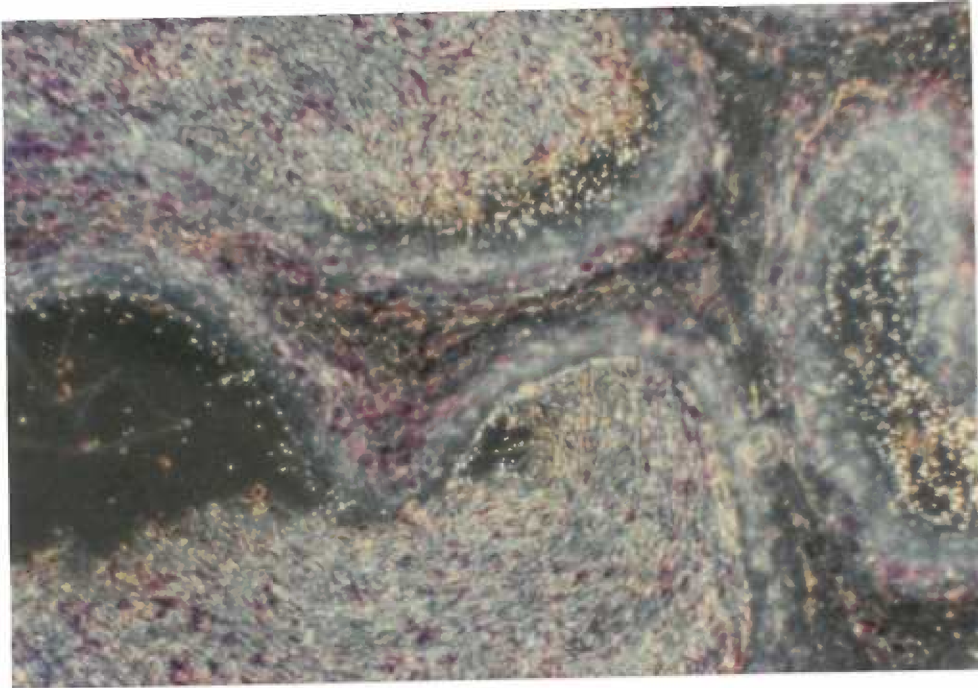


B.

Figure 2



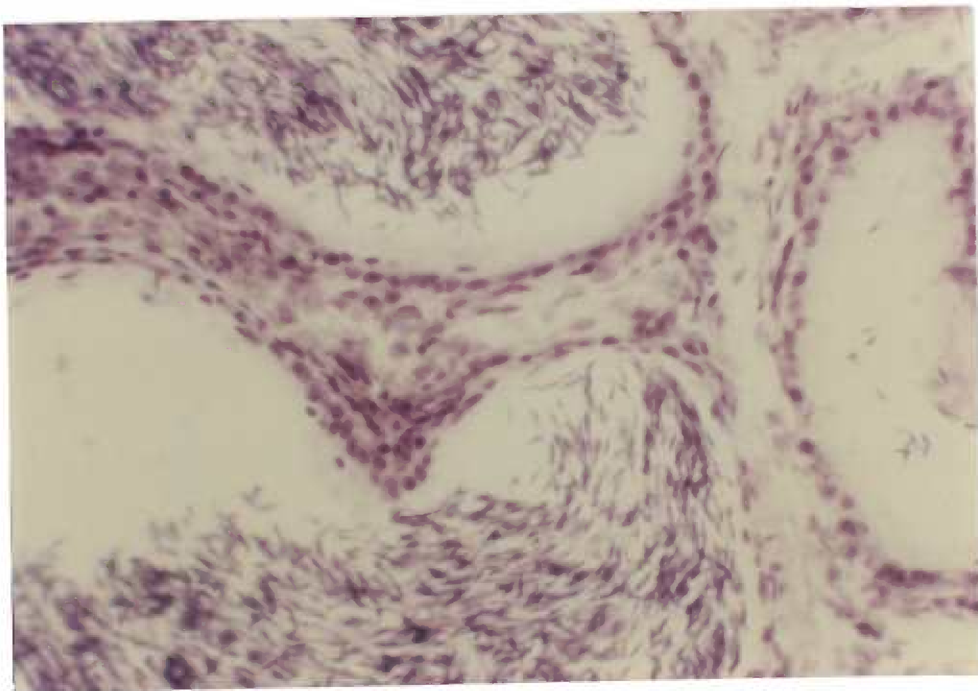
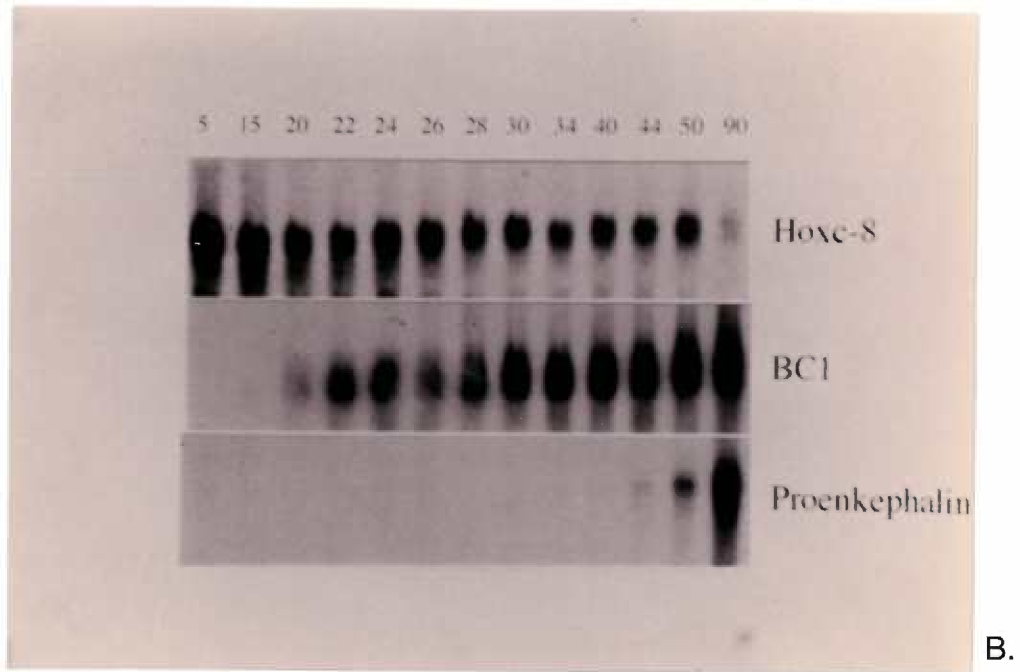


Figure 2 C

A.



B.

Figure 3

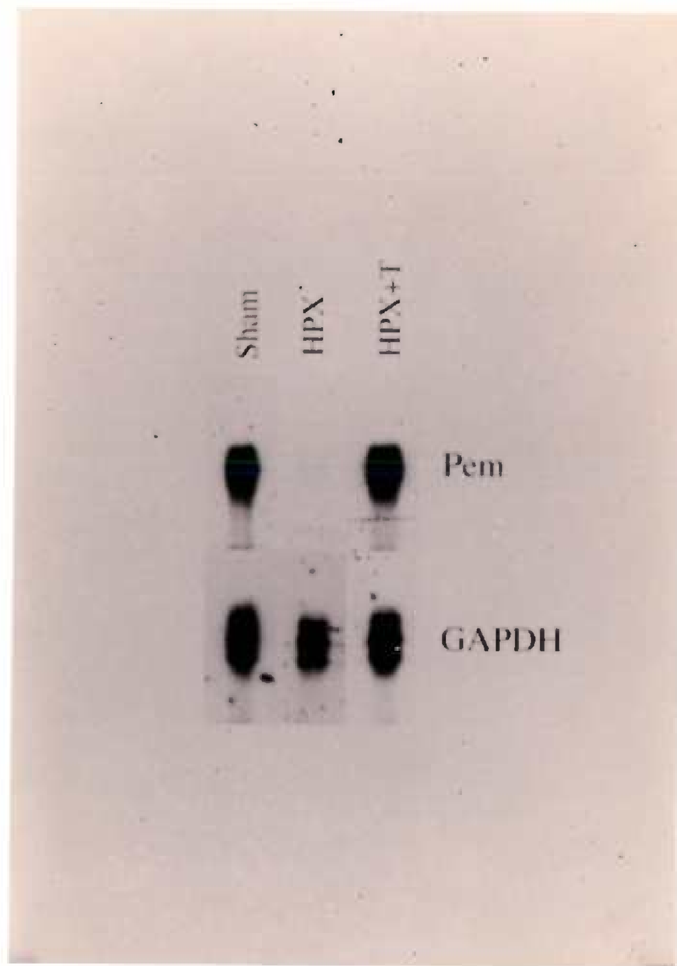
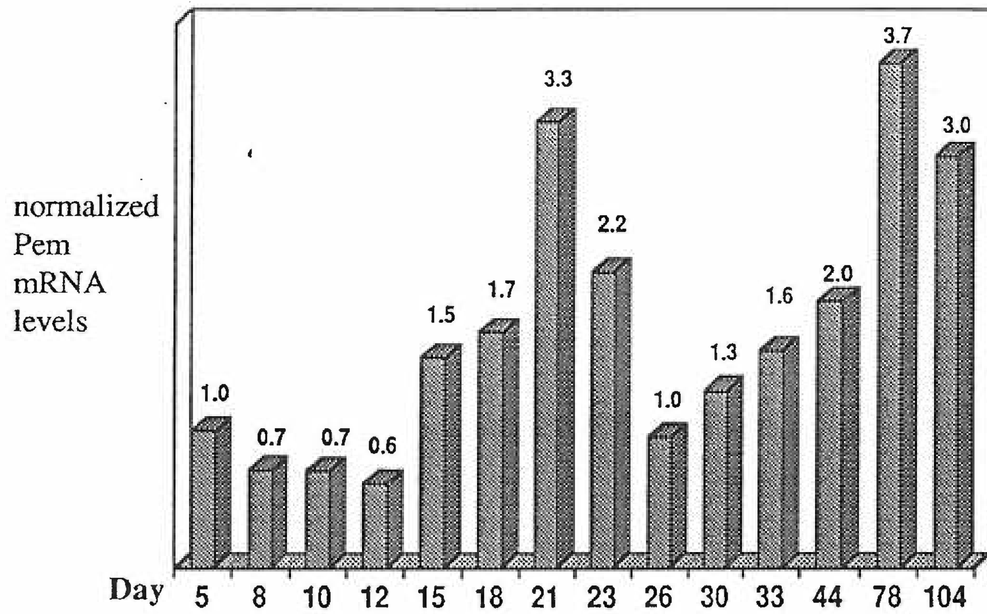


Figure 4



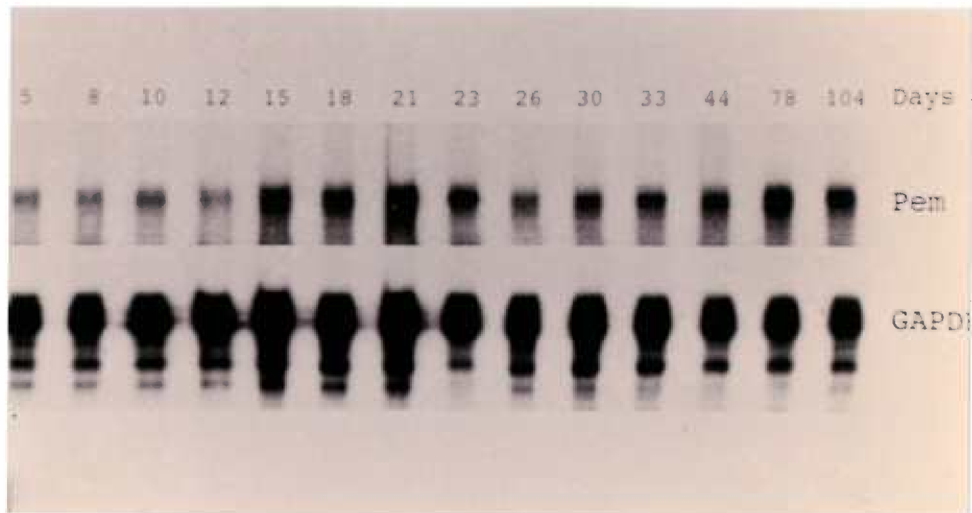


Figure 5

MANUSCRIPT #3

Testosterone induced Pem expression in Testes of Hypophysectomized and Hypogonadal mice and in Epididymides of Hypophysectomized Rats*

J. Suzanne Lindsey and Miles F. Wilkinson

Oregon Health Sciences University, Dept. of Molecular Microbiology and Immunology, Portland, OR 97201 (J.S.L.) and The University of Texas MD Anderson Cancer Center, Houston, TX 77030 (M.F.W.).

Address all correspondence and requests for reprints to : Dr. Miles F. Wilkinson, MD Anderson Cancer Center, Dept. of Immunology, 1515 Holcombe Box 180, Houston TX 77030.

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Abstract

Androgens are the main hormones needed for maintaining spermatogenesis. Even though widely studied, virtually no transcription factors have been shown to be regulated during spermatogenesis by androgens. This study was designed to determine the effects of FSH, LH, and testosterone proprionate on the regulation of a Sertoli cell-expressed homeobox gene, Pem. We studied this expression in testes and epididymides of hypophysectomized (HPX) mice and rats as well as the gonadotropin-deficient mouse, hypogonadal (hpg). Androgen levels of the serum, testes, and epididymides were determined by chromatography. Testosterone proprionate restored Pem expression in mouse testis and rat epididymides. Testosterone converted to dihydrotestosterone was the main regulator of Pem expression in the rat epididymis. In HPX rat testis, the regulation of this homeobox gene by testosterone was less distinct. However, in HPX mouse testis Pem levels induced by LH or testosterone alone increased over 20 fold and over five to ten fold respectively compared with controls. In hpg testes, LH or testosterone alone induced Pem expression over 20 fold compared with controls. In both HPX and hpg mouse testes, FSH suppressed Pem induction by testosterone. Therefore, Pem is the first known homeobox gene that may act as a transcription factor during spermatogenesis that is up-regulated by testosterone and suppressed by FSH . Differences between the mouse and rat Pem expression are discussed.

Introduction

Spermatogenesis is the process of developing spermatogonia into spermatozoa and may be categorized into three phases: 1) the spermatogonia proliferative phase, 2) the spermatocyte meiotic phase, and 3) the spermatid differentiation (spermiogenic) phase. This system consists of the hypothalamus-pituitary-gonadal endocrine axis, the testis, and the epididymis. The hypothalamus secretes gonadotropin releasing hormone (GnRH) which in turn signals the pituitary to secrete the gonadotropins follicular stimulating hormone (FSH) and luteinizing hormone (LH) which exert their effects on the testis (1,2). The testis is made up of two main compartments: the intertubular or interstitial area and the seminiferous tubule. Leydig cells, bone marrow-derived cell types, blood, and lymphatic vessels reside in the interstitial area. Under the influence of LH, Leydig cells secrete and are the major source of androgens (3). The seminiferous tubules contain primarily myoid cells, basement membrane, Sertoli cells and germ cells.

FSH initiates germ and Sertoli cell divisions in the immature rat testis. Experiments with hypophysectomized or estrogen-treated animals treated with FSH or by selective immunization against FSH provide clear evidence for the proliferative effect of FSH on germ cells (1-6). Cultured primary Sertoli cells from immature rats treated with FSH increase in DNA synthesis and in mitoses (7). However, the formation of fully developed spermatozoa in the testis could not be obtained with FSH alone (8).

Testosterone alone maintains qualitative spermatogenesis (i.e.; produces fully developed spermatozoa) in testes of hypophysectomized adult rats (9,10). In addition,

when spermatogenesis is interrupted by estrogen or antibodies to gonadotropin releasing hormone, treatments which leave an intact pituitary gland, testosterone alone will maintain quantitative daily sperm production even though serum FSH is not detected by radioimmunoassay (11,12). More recent evidence shows that testosterone alone is capable of initiating qualitative and quantitative, when normalized to numbers of Sertoli cells, spermatogenesis in the GnRH-deficient mouse, hypogonadal (13).

Androgens bind to the AR which is an intracellular receptor. The androgen-receptor complex then binds with high affinity to an androgen response element and acts as a transcription enhancer on target genes (14). Since antibodies to androgen receptors (AR) do not detect their presence in germ cells (15), it is thought that testosterone exerts its effects on spermatogenesis through peritubular and Sertoli cells which do posses AR (16). Testosterone effects on cultured immature Sertoli cells are mediated by peritubular cells (17, 18). Upon testosterone stimulation (19), the peritubular cells secrete P-Mod-S protein which, in turn, has been shown to increase Sertoli cell secretion of transferrin and ABP; two proteins used as indicators of Sertoli cell function (18,20-22). In addition, androgen has direct effects on Sertoli cells. Testosterone induces two abundant proteins in cultured Sertoli cells from 40 day old rats as shown by two-dimensional gel analysis (23).

Spermatozoa need further maturation in the epididymis before they are capable of fertilization. Development and function of the epididymis is dependent on dihydrotestosterone. Without DHT, the epididymis does not produce mature sperm capable of fertilization. Under stimulation by DHT, principal cells in the epididymis secrete proteins that promote forward motility and egg recognition by the spermatozoa (24). After birth of the rat the epididymis does not develop until the Leydig cell in the testis produces increased testosterone and the first spermatozoa enter the epididymis from the testis (25, 26). In addition to circulating androgen, the epididymis must receive testosterone from the testis usually bound to ABP or the principle cells of the initial

segment of the epididymis dedifferentiate and the other regions fail to secrete proteins that mature spermatozoa (27).

No transcription factors have been shown to be regulated by testosterone in Sertoli cells of the testis during spermatogenesis or in principle cells of the epididymis during sperm maturation. Homeodomain-encoding genes are a group of transcription factors. In a search for developmentally regulated genes, we isolated the divergent homeobox gene (28,29), *Pem,* by subtraction hybridization between two T-lymphoma cell lines SL 12.3 and SL 12.4 (30). During early gestation of the mouse embryo, primitive endoderm- and trophectoderm-derived cells of the placenta and yolk sac express *Pem* whereas, other embryonic or extra-embryonic cell lineages do not express *Pem* (31,32). In addition, embryonal carcinoma stem cells cultured *in vitro* also exhibit this *in vivo* expression pattern: F9 stem cells induced to differentiate into visceral or parietal endoderm, up-regulate Pem mRNA expression as assessed by northern blot analysis (28) and in situ hybridization (28,29). Only ovary, testis, and epididymis of the adult mouse and rat express Pem at detectable levels (30,31,34). In rat, testosterone restores rPem levels in epididymides of hypophysectomized rats (34). In mouse, we have shown that gonadotropins induce Pem expression in mice lacking gonadotropins (35). Furthermore, *Pem* is the only homeobox gene known to be expressed in Sertoli cells in specifically stages VII and VIII seminiferous tubules (35), stages shown to be androgen-dependent (36).

The work presented here investigates further the testosterone regulation of Pem in mouse and rat male reproductive tissues. We used gonadotropin-deficient mice, hypogonadal (hpg), in addition to hypophysectomized (HPX) mice and rats which were depleted of pituitary hormones to determine the effects of FSH, LH, and testosterone on Pem expression. Since hpg mice lack FSH and LH due to a mutation in the gonadotropin releasing hormone gene (37), the testes are not exposed to FSH which is thought to be required for Sertoli cell proliferation and maturation. Whereas, the

hypophysectomized animal testes are exposed to FSH prior to surgery so the Sertoli cells should be more mature. Therefore, these two animal models enabled us to determine if FSH must be present before Pem is induced. In addition, using both mice and rats provided a comparison of known differences in Sertoli cell gene regulation between the two species. Testosterone proprionate restored Pem expression in mouse testis and rat epididymides. In HPX rat testis, the regulation of this homeobox gene by testosterone was less distinct. However, in HPX mouse testis Pem levels induced by LH or testosterone alone increased over 20 fold and over five to ten fold respectively compared with controls. In hpg testes, LH or testosterone alone induced Pem expression over 20 fold compared with controls. In both HPX and hpg mouse testes, FSH suppressed Pem induction by testosterone. Therefore, the homeobox gene Pem was regulated by the hormones that regulate spermatogenesis.

Methods and Materials

Animals

Hypogonadal mice were obtained from Jackson Laboratories when they were six weeks or older. Normal, sham operated, and hypophysectomized (HPX) adult Sprague-Dawley rats and adult mice were obtained from Charles River Laboratories. Oregon Health Sciences University's animal care facility housed these animals according to approved protocols with hypophysectomized animals receiving 5% glucose water ad libitum and housed at 28-30°C. Animals were killed by CO_2 asphyxiation. Organs were immediately removed, homogenized, and frozen at -70°C until RNA was extracted.

For the testosterone implant experiments, rats 12 days after hypophysectomy were anesthetized at one atmosphere isofluorane, a blood sample drawn, and the implant was placed subcutaneously along the upper back and neck. Testosterone implants were made with Silastic tubing three centimeters long filled with testosterone proprionate. These implants were made in collaboration with Dr. John Resko, Oregon Health Sciences University, who has shown them to deliver 4ng of testosterone per ml of serum (verbal communication). The implants remained for two, four, or eight days after which three animals of each group were killed and organs removed for RNA extraction and androgen assays. In addition, three sham and HPX animals were killed on corresponding days.

Hormone treatment of the HPX and hypogonadal mice was done by subcutaneous injection into the scruff of the neck. The mice had been hypophysectomized for 36 days. The LH (rat LH-I-9 lot number AFP-10250C) and FSH

(ovine FSH-19-SIAFP lot number AFP-4117A) were obtained from the National Hormone and Pituitary Program, National Institute of Diabetes and Digestive and Kidney Diseases. Quantities of each injection were as follows: in controls: 50 μl sesame oil (Sigma) alone, and 0.6 mg flutamide, an antiandrogen, in sesame oil with 2.0 μg FSH in saline; non-controls: 5.3 μg LH in saline. These injections were made twice daily at 12 hour intervals for two days. The testosterone (kindly provided by Dr. John Resko, Oregon Health Sciences University) suspended in sesame oil was injected once at 2.5 mg per animal and testes were harvested two days later. All HPX mice were tested for completeness of hypophysectomy by radioimmunoassay of serum corticosterone levels done by the laboratory of Dr. David Hess at the Oregon Regional Primate Research Center, Beaverton, OR. Only HPX mice with less than 20 ng corticosterone per ml serum were used. Normal corticosterone levels for sham operated mice were in the 300 ng/ml range. Mice were hypophysectomized for five weeks before hormone treatment. We verified the completeness of the HPX and sham operated rats by determining testosterone levels by chromatography of serum (38). All testosterone assays were done in the laboratory of Dr. David Hess at the Oregon Regional Primate Research Center, Beaverton, OR. Serum was obtained by tail bleeds prior to testosterone implantation or injection. Serum, intratesticular, and intraepididymal testosterone and dihydrotestosterone levels were analyzed by the same method after testosterone treatment and in the untreated animals. Contralateral testes and epididymides from each animal were immediately collected, weighed, homogenized in phosphate buffered saline, and frozen at -70°C until analyzed.

We repeated the HPX rats with testosterone implants experiments two times with different sets of animals.

Riboprobe preparation

For RNase protection and *in situ* hybridization analyses, we prepared ^{32}P- or ^{33}P-UTP or ^{35}S-ATP labeled RNA probes with T7 DNA-dependent RNA polymerase for anti-sense and SP6 RNA polymerase for sense transcripts corresponding to the middle FokI to FokI region of the rat Pem cDNA (39) or corresponding to nucleotides 6 through 432 of the mouse *Pem* cDNA sequence (31). We also utilized glyceraldehyde phosphate dehydrogenase (GAPDH), a housekeeping gene, template (Ambion) to generate antisense probes as a positive control. An RNA ladder template (Ambion) was used to generate specific size markers. We used the *in vitro* transcription protocol as described in Current Protocols in Molecular Biology (40). Probes were purified on a 6% polyacrylamide denaturing gel. After exposure to film, the appropriate sized bands were cut out and put in individual eppendorf tubes. The gel slices were mashed with an RNase-free pestle in 100 µl of diethylpyrocarbonate (DEPC) treated water. To each mashed sample, 600 µl of 1X proteinase K (PK) buffer (0.3 M NaCl, 0.5% SDS, 10 mM Tris (pH 7.5), 200 µg PK, and 20 µg tRNA) was added, vortexed and incubated at 37°C for 15 minutes. After vortexing and a pulse-spin, the suspended probes were filtered through a 0.45 micron disc (Acrodisc). Another 600 µl of the PK buffer was added and incubated for at least 5 more minutes at 37°C and again the suspended probe was filtered through the 0.45 disc. Each sample was extracted with 200 µl of phenol/chloroform. One microliter was taken to determine counts per minute and the rest was precipitated and stored at -70°C.

RNAse protection assays

Total RNA from tissues was prepared as previously described by either guanidinium isothiocyanate (41) or by a single-step acid guandinium thiocyanate-phenol-chloroform extraction (42) and quantified by optical density. RNase protection analyses were performed as described in Current Protocols (40) with some minor modifications. Briefly,

sample RNAs, or tRNA as a negative control, were centrifuged with the appropriate gel purified ^{32}P-UTP labeled probes. The pellet was resuspended in 30 μl of annealing buffer (40 mM PIPES, pH 6.4, 0.4 M NaCl, 1 mM EDTA, 80% formamide) and allowed to hybridize overnight at 42° C. Unhybridized RNA was digested with RNase A (50 μg/ml) and T1 (4 μg/ml) for 30 minutes at 37°C. RNases were then removed by treatment with proteinase K, and extraction with Phenol/chloroform/isoamyl alcohol. After ethanol precipitation, the RNA pellet was resuspended in loading buffer, denatured at 85°C, run on a 6% polyacrylamide denaturing gel and analyzed by autoradiography.

Densitometry was done on scanned images with the NIH Image 1.54 program.

Northern Blot Analysis

Ten μg per lane of total cellular RNA was electrophoresed on 1% agarose gels at 50 volts in a running buffer . The separated RNA was transferred to Nytran™ paper via overnight capillary blot as described by Maniatis et al. (43). After transfer, the blot was UV cross-linked (Strata-linker) and stained with methylene blue to evaluate the transfer and loading of RNA in each lane. Blots were then prehybridized in pre-hyb buffer (50% formamide, 5X Denhardt's solution, 5X SSPE, 0.5% SDS, and 100 μg/ml sheared salmon sperm DNA) for 4 hours at room temperature. The blot was then hybridized with random oligomer primed ^{32}P-labeled cDNA for GATA-1 (generously provided by Dr. Stuart Orkin, Howard Hughes Medical Institute, Boston, Massachusetts) or for CREB (kindly provided by Dr. Richard Goodman, Vollum Institute, Portland, OR) in the presence of hyb buffer (pre-hyb buffer plus 10% dextran sulfate and 50% formamide) overnight at 42° C. Results were analyzed by autoradiography.

Results

Testosterone restores Pem expression in rat epididymides.

Previously, we showed that Pem was regulated by exogenous testosterone in HPX rat epididymis (34). To determine if the restoration of Pem transcripts in HPX rat epididymides correlated to testosterone levels we tested epididymal levels of testosterone and dihydrotestosterone (DHT). HPX rat epididymides lacked normal levels of Pem transcripts and DHT when compared to sham operated rats. Animals 7 and 12 (HPX 7 and HPX 12) were not truly HPX since the epididymal DHT level was almost the same as the sham operated animals and the HPX labeled animals were not treated with testosterone (Fig. 1A, 1B, and data not shown from a total of 6 sham operated animals). In addition, when exogenous testosterone was administered for two, four, or eight days to HPX rats, Pem expression in the epididymis was restored when compared to control HPX epididymides removed on corresponding days. The levels of Pem expression were as great or greater than levels found in epididymides of sham operated rats for any level of DHT over 2.5 pg per mg of epididymal tissue (Fig 1B). Animals T3+2 and T5+4 were not truly hypophysectomized as determined by pre-treatment serum testosterone levels (data not shown).

Down-regulation of Pem expression in HPX rat testes.

To determine if restoration of Pem expression in testes of HPX rats correlated with testosterone we used testes from HPX rats with or without treatment for two, four, or eight days. In this RNAse protection analysis we used total RNA extracted from completely HPX rat testes as determined by testicular testosterone levels. The animals that were treated with testosterone were completely hypophysectomized prior to treatment as determine by serum testosterone levels except for animals T2+3 and T4+5. Only one HPX rat testis showed Pem induction by testosterone, T4+4.

Completely hypophysectomized animals treated for two days, T2+1 and T2+2, did not show an increase in Pem expression. In addition, animals treated for longer (T8) did not show an increase in Pem expression (Fig. 2B). In this experiment, elimination of testosterone and gonadotropins in general suppressed Pem expression in HPX rat testes. However, in completely hypophysectomized rats treated with testosterone, only one, T4+4, out of seven testes contained normal levels of Pem transcripts (Fig. 2). Another sample, T8+7 showed a low level of Pem transcripts even though its intratesticular testosterone levels were higher than those found in the testis from T4+4 (Fig. 2 A & B)

Testosterone restores high levels of Pem expression in HPX mouse testes. Since mouse testes express much higher levels of Pem than do rat testes (34), we decided to look at Pem regulation by testosterone in mouse testes. We used completely hypophysectomized mice as determined by serum corticosterone levels (see methods and materials). After waiting 36 days post surgery, we injected the animals for two days with testosterone alone, LH alone, FSH with testosterone, and FSH with the antiandrogen flutamide. In control animals that were either not injected or injected with vehicle, Pem expression was absent. Testosterone alone induced Pem expression (Fig 3A). Pem transcript levels in testes from animals treated with LH were most like those seen in testes from sham operated animals (Fig 3A) probably due to the higher intratesticular testosterone levels (Fig 3B). In FSH administered in conjunction with flutamide Pem transcripts were barely detectable. Flutamide blocked testosterone, that was probably produced by contaminating levels of LH in the FSH, since the same amount of intratesticular testosterone (Fig. 3B) gave a clear induction of Pem expression (Fig 3A). Testosterone with FSH seemed to suppress Pem expression even though the intratesticular testosterone levels were five times higher than testosterone alone (Fig. 3A and 3B).

Expression profile of Sertoli cell transcription factors in hormone treated HPX mouse testes.

To analyze other Sertoli cell-expressed transcription factors, we did a northern blot of the same RNA from the HPX and hormone treated HPX mice and probed it sequentially with probes for GATA-1 and CREB. Yomogida et al. showed that GATA-1 is developmentally and specifically expressed in only Sertoli cells of the testis (44). CREB is expressed in most somatic and germ cells of the testis and has been shown in vitro to be transcriptionally regulated by FSH in Sertoli cells (45). GATA-1 transcript levels in testes from HPX mice were not down-regulated. Furthermore, no effect on GATA-1 expression was detected with LH, testosterone alone, testosterone with FSH, or FSH with flutamide treatment when compared with controls (Fig. 4). In addition, CREB expression in vivo was not detectably regulated in testes by a lack of pituitary hormones nor by administration of these same hormones in testes of HPX mice (Fig 4).

Pem expression does not require maturation by gonadotropins.

Hypogonadal mice have very small immature testes due to a lack of GnRH and subsequently lack endogenous LH and FSH (37,46). Therefore, the testes of these animals unlike those of the HPX animals have not been exposed to developmentally significant levels of gonadotropins. In addition, the levels of androgens are very low in hpg mice (47,48). We injected hormones as before with the HPX mice. The testes of uninjected hpg mice showed a slightly higher level of Pem expression than the control testes of vehicle injected hpg mice which may have been due to procedure-induced stress. Still, testosterone alone induced Pem transcripts in testes 90 fold over vehicle (oil) injected animals (Fig. 5A). Surprisingly, the regulation of Pem transcription in hpg mice was very similar to that shown for HPX mice. However, induction by testosterone was four times greater and induction by testosterone with FSH was two times greater in

hpg mice than in HPX mice for similar amounts of intratesticular testosterone (Fig. 3A, 3B, 5A, & 5B). The same down-regulation of Pem transcripts was detected in testes of hpg mice injected with FSH and testosterone (Fig. 5A and 5B).

DISCUSSION

These studies show that testosterone regulated Pem expression in epididymides and testes of HPX rats as well as in testes of two mouse models, HPX and hpg. Analyses of total RNA from epididymides of HPX rats and testosterone treated HPX rats revealed a clear absence of Pem transcripts in HPX rats with full restoration in HPX rats treated with testosterone after two days of treatment. The induction of Pem transcription in HPX rat testes is far less dramatic than the induction in either HPX or hpg mouse testis. Hypogonadal mouse testes do not mature due to a lack of exposure to gonadotropins; yet, testosterone alone induced Pem expression to levels seen in normal mouse testes. These findings support the proposition that the homeobox gene Pem is regulated by androgens; it is the first homeobox gene for which such regulation has been demonstrated.

Androgens are essential for the function and development of the epididymis. The epididymis rapidly converts testosterone into dihydrotestosterone (49,50). Exogenous radioactive testosterone is found in the epididymis as dihydrotestosterone (51-53). This conversion is thought to occur when Sertoli cell-secreted androgen binding protein (ABP) brings bound testosterone to the initial segment of the epididymis where it is taken up by endocytosis. It is in the initial segment cells of the rat epididymis where 5-alpha-reductase activity primarily resides (54). The initial segment epithelial cells convert testosterone to dihydrotestosterone by 5-alpha-reductase thereby providing DHT to the rest of the epididymal caput, corpus, and cauda regions. Pem mRNA was restored in HPX rat epididymides by exogenous testosterone. Pem transcripts localize to the corpus and cauda regions (34); regions important to sperm motility and fertility (55,56). We obtained results consistent with previous results showing that exogenous testosterone restores epididymal Pem expression (34). The present study showed that Pem expression correlated with DHT levels in HPX rat epididymides that had been

treated with testosterone. Pem expression starts increasing dramatically between day 20 and 23 after birth (34), a period of epididymal development that shows an increased entry of testicular fluid that contains ABP bound to testosterone (57) and conversion of testosterone to DHT primarily in the initial segment of the epididymis (58). Therefore, Pem is the only known homeobox gene that may act as a transcription factor under androgen regulation in the corpus to cauda region of the epididymis.

In the testis, the expression of the androgen receptor (AR) in Sertoli cells is regulated in a stage-specific manner. Nuclear AR levels reach a peak in stage VII and decline rapidly in stage VIII Sertoli cells as determined by immunohistochemistry (59). In the mouse, Pem mRNA levels increase during stage VI and peak during stage VII (35) just before the new wave of spermatogenesis in stages VIII-XIV. In addition, ABP, which is secreted from Sertoli cells and acts as an androgen sink, is at its lowest level of secretion in stage VI (60) making androgen more available to bind AR. Indeed, stages VII and VIII are known as the androgen-dependent stages (61,62). Therefore, this period of testosterone stimulation of Sertoli cell function is consistent with androgen restoration of Pem expression in HPX mouse and rat testes. It is intriguing that rat testis showed less Pem induction than mouse testis. However, since the rat is known to have 50 to 100 times more ABP than mouse (63), this result is consistent with this protein acting as an androgen sink which may alter Pem expression in rat testes.

In HPX rat testis, a defect in binding of step 8 spermatids to Sertoli cells and a cessation of further maturation of spermatids takes place. This can be prevented by administration of testosterone soon after hypophysectomy (64). This restoration by testosterone of advanced spermatids has also been shown in the azoospermic rat model by immunizing against LH or GnRH (12). This binding to spermatids is through ectoplasmic specializations which form 16 days after birth of the rat (65) during the same time frame as rPem expression (15).

In hpg mice, that posses a defective GnRH gene (46) and therefore, are not exposed to LH or FSH, we detected a dramatic increase in Pem expression with injected LH or testosterone alone. Indeed, within one hour of treatment, LH alone induces detectable levels of testicular androgens in hpg mice (49). In addition, AR is found in peritubular cells and not in Sertoli cells of 10 day old rat testes (19) suggesting this cell population is responsive to androgens before Sertoli cells of the immature testis. This result suggests that prior maturation of the Sertoli cells by gonadotropins is not necessary for Pem expression. Alternatively, if maturation is needed, it may take place through the peritubular cells which have been shown to have a differentiating effect on Sertoli cells through a testosterone-induced secreted protein, P-Mod-S (16,17,66). Although testosterone levels in hpg mice are very low (48,49) we did detect a very low level of Pem expression in saline or uninjected hpg mouse testes. Obviously, further studies are required to determine if testosterone induction of Pem expression is through P-Mod-S from the peritubular cells or through a direct effect of androgens on Sertoli cells.

Hypogonadal mouse testes show a four fold greater induction of Pem transcripts than in HPX mouse testes with testosterone alone. This result was probably due to a lack of the Sertoli cell barrier in the FSH-deficient hpg mouse testes because the formation of this barrier requires FSH (67,68). Without a barrier more of the testosterone or testosterone induced P-Mod-S from peritubular cells would be able to reach the Sertoli cells of hpg mouse testes to induce Pem expression. In addition, both HPX and hpg mouse testes showed a suppression of Pem expression by FSH when administered with testosterone. These results are interesting since the lowest levels of FSH binding to its receptor is found in stages VI to VIII (69,70); the stages found to be the highest for Pem expression in the mouse. We have also detected a decrease in Pem expression in testes from inhibin knock-out mice that overexpress FSH (unpublished results). These results are consistent with the cyclic expression pattern of

Pem seen during an RNase protection analysis of total RNA taken from a time course of 5- through 104-day old rat testes (34) and with the in situ hybridization analysis of mouse testes (35).

Two other known transcription factors, GATA-1 and CREB, are also expressed in Sertoli cells during spermatogenesis (44,45). Neither GATA-1, which is thought to be a negative regulator of alpha inhibin (44), or CREB increased or decreased in expression levels in testes of HPX and hpg mice that were treated with testosterone alone or testosterone with FSH. Although, FSH induces CREB expression in vitro in cultured Sertoli cells (45), this induction in our experiments was probably masked by other cells that express CREB in the testis and the fact that Sertoli cells only make up 5-10% of the cell population in this organ. It has been shown in vitro that testosterone regulated P-Mod-S transiently upregulates the transcription factor c-fos which in turn regulates transferrin production in Sertoli cells (71,72). In vivo, c-fos protein is also found in other cells of the testis such as stage I and VIII Sertoli cells, primary pachytene spermatocytes, round spermatids, and stage II-V peritubular cells (73). Pem expression was controlled in vivo by testosterone. Indeed, testosterone alone produced spermatozoa capable of egg fertilization in hpg mice (13). Pem mRNA is expressed specifically in late VI, VII, and early VIII androgen-dependent stages of the seminiferous tubule (35). Therefore, Pem expression is unique since expression is tightly controlled during the cycles of the seminiferous tubules in Sertoli cells and clearly regulated by testosterone in vivo. We postulate that the homeobox gene, Pem, may be an important regulator of Sertoli cell function and spermatogenesis.

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FIGURE LEGENDS

Figure 1. Testosterone regulated Pem expression in hypophysectomized rat epididymides. (A) RNase protection analysis of 40 μg of total RNA isolated from epididymides of hypophysectomized rats for 12 days and another group of hypophysectomized rats for 12 days then treated with testosterone propionate. HPX 1, HPX 2, HPX 7, HPX 9, HPX 11, and HPX 12 are all individual animals killed at 14 days post HPX (HPX 1 & 2), 16 days post HPX (HPX 7 & 9) and 20 days post HPX (HPX 11 & 12); 7 and 12 were not truly HPX since their serum, testicular, and epididymal androgen levels were similar to sham operated animals. T2/1, T2/2, T2/3, T4/4, T4/5, T4/6, T8/7, T8/8, T8/9 all represent individual animals treated with testosterone for 2 (T2), 4 (T4), or 8 (T8) days beginning at day-12 post HPX. Sham operated animals were used for controls. Antisense RNA probes to GAPDH and to rat Pem were made as described in Methods and Materials. Pem expression levels were normalized to GAPDH and densitometry. (B) Graph of pg DHT per mg epididymal tissue for each individual animal in (A). DHT was measured as described in Methods and Materials and performed after testosterone was administered for the T2-T8 animals.

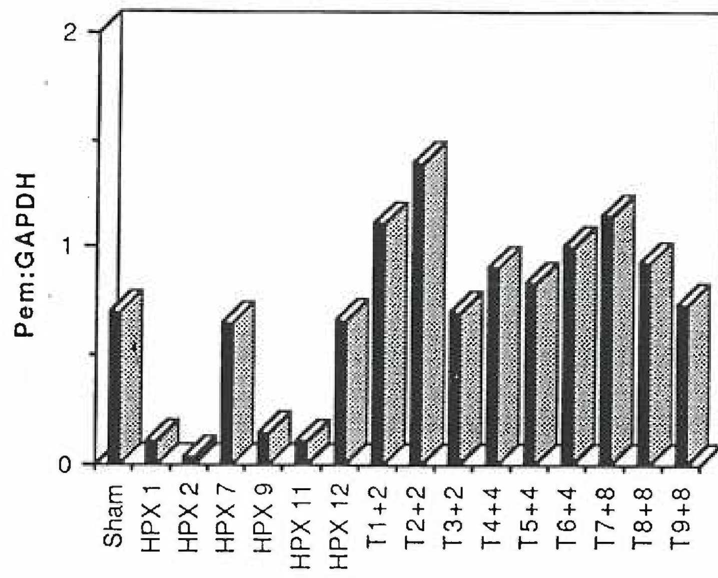
Figure 2. Testosterone regulates Pem expression in rat testis. RNase protection analysis of Pem transcripts normalized to GAPDH in total RNA from testes of HPX and testosterone treated HPX rats. HPX 1, HPX 2, HPX 4, and HPX 11 are all individual animals. T2/1, T2/2, T2/3, T4/4, T4/5, T4/6, T8/7, T8/8, T8/9 all represent individual animals treated with testosterone for 2 (T2), 4 (T4), or 8 (T8) days. Sham operated

animals were used for controls. Note the bottom band marked Pem is the testosterone induced Pem transcript. Upper bands were specific to the Pem probe, not undigested probe which may depict a basal level that is not regulated. Very basa levels are seen for Pem expression after very long autoradiography which in this experiment was 14 days. (B) Graph of pg testosterone per mg epididymal tissue for each individual animal in (A). Testosterone was measured as described in Methods and Materials and performed after testosterone was administered for the T2-T8 animals. DHT levels were barely deteced (data not shown).

Figure 3. Testosterone restores high levels of Pem expression in HPX mouse testes. (A) RNase protection analysis of Pem normalized to L3 transcripts in 10 μg total RNA from pooled testes of HPX mice. (B) Nanograms of testosterone per mg of testicular tissue from each animal which was pooled for each group of hormone, vehicle, or no injection in (A).

Figure 4. Expression profile of Sertoli cell transcription factors in hormone treated HPX mouse testes. Northern blot analysis of GATA-1 and CREB normalized to methylene blue staining of ribosomal RNA (28S and 18S) on the Nytran blot in 20 μg total RNA from pooled testes of HPX mice. The Blot was stripped and re-probed with each ^{32}P-ATP-labeled cDNA probe for GATA-1 and CREB.

Figure 5. Pem expression does not require maturation by gonadotropins. (A) RNase protection analysis of Pem normalized to L3 transcripts in 10 μg total RNA from pooled testes of hpg mice. (B) Nanograms of testosterone per mg of testicular tissue from each animal which was pooled for each group of hormone, vehicle, or no injection in (A).



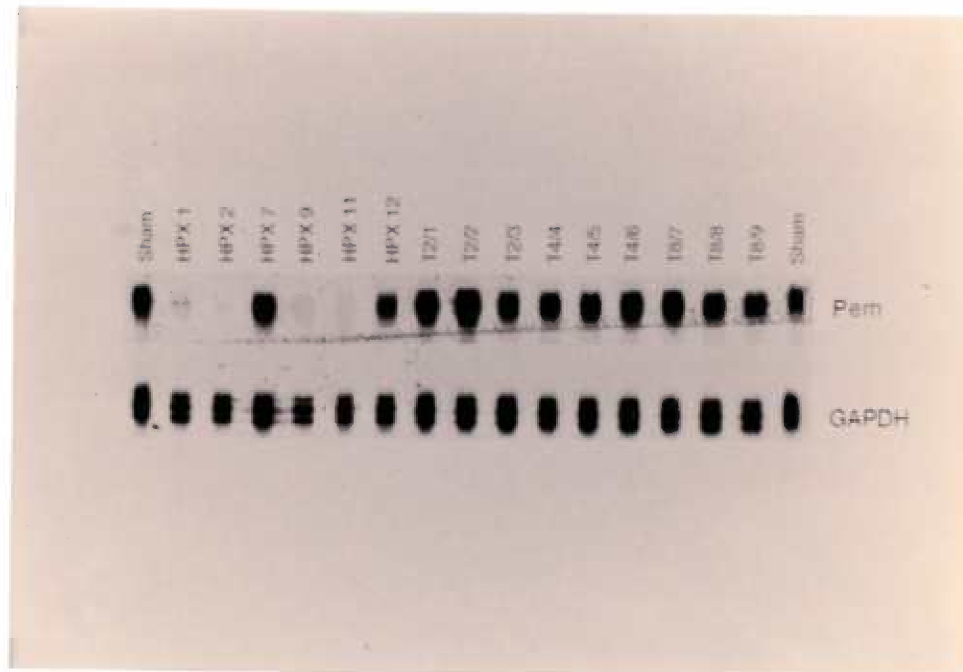


Figure 1 A

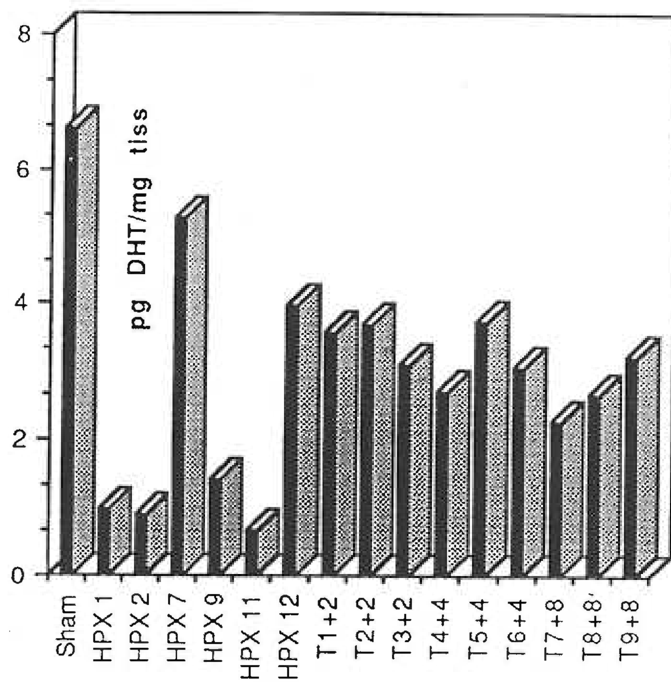
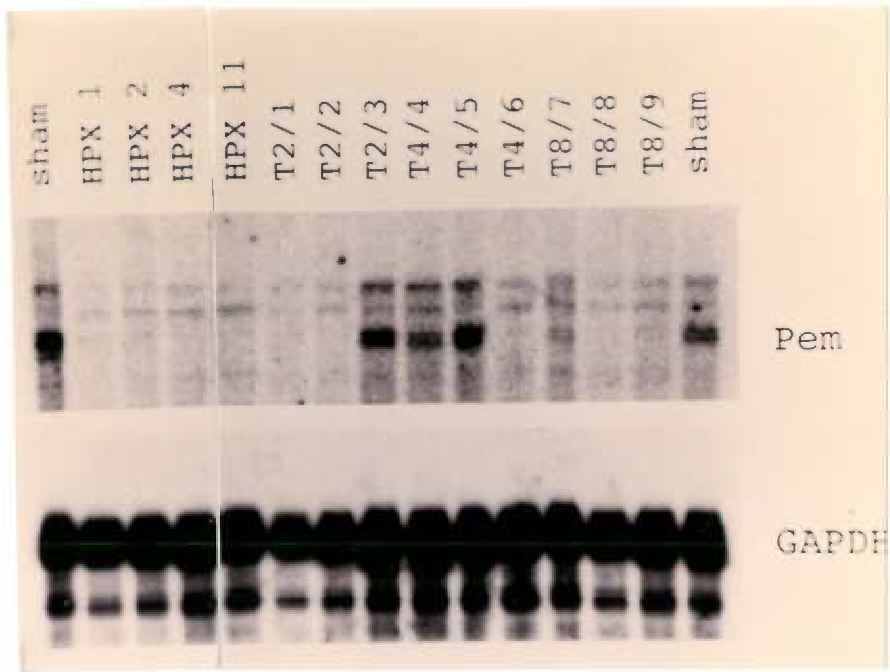


Figure 1B



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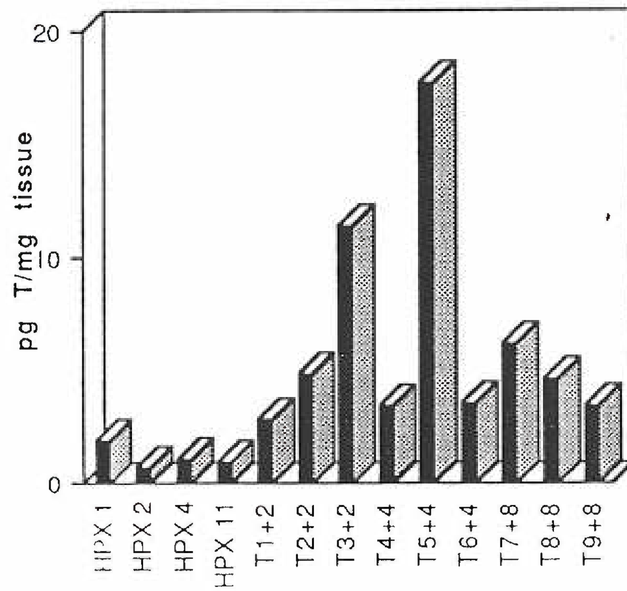
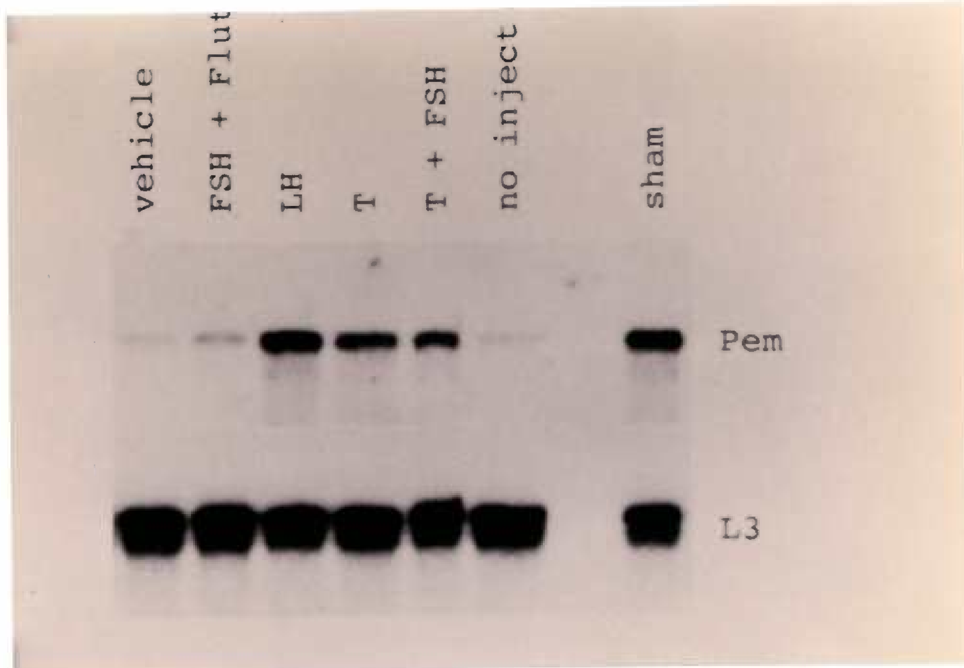
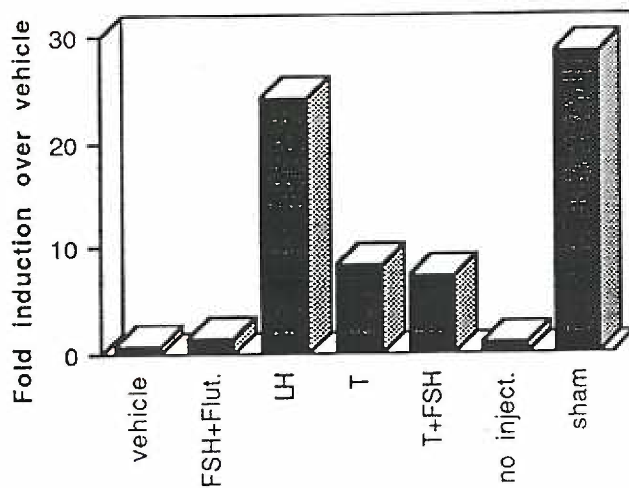


B.

Figure 2





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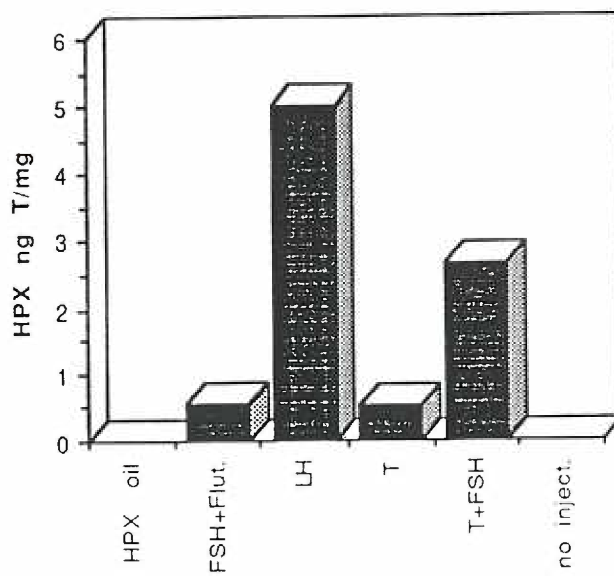

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Figure 3

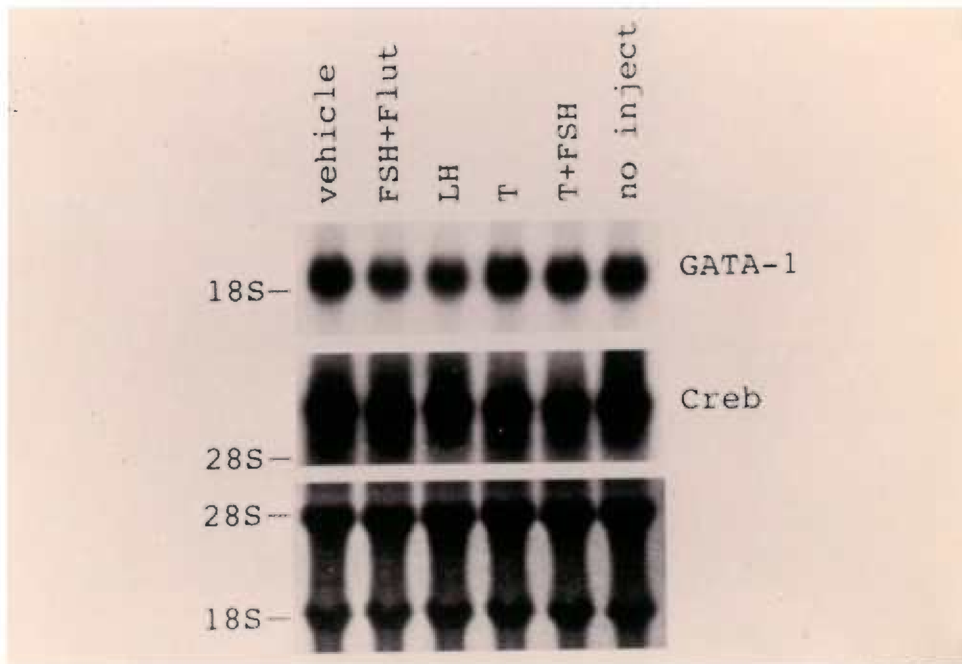
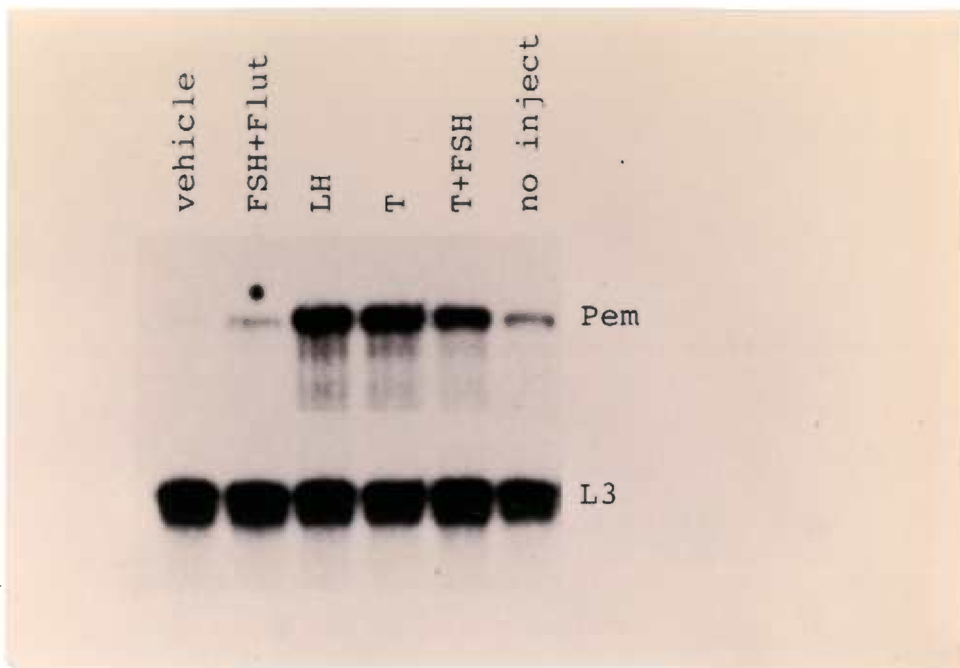
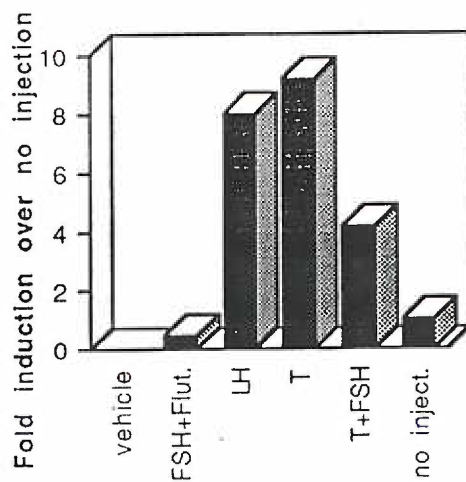
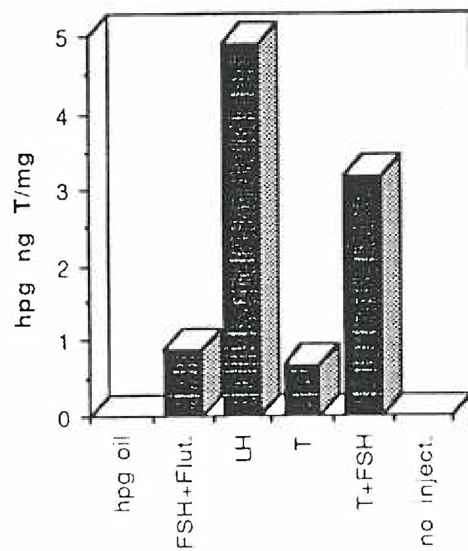


Figure 4





A.



B.

Figure 5

DISCUSSION AND CONCLUSIONS

In this report, the cell populations in testis and epididymis that expressed *Pem* were determined by cellular fractionation, *in situ* hybridization, and analyses of RNA from BALB/c and mutant mice testes and epididymides. *Pem* transcripts localized to Sertoli cells of mouse testes. My studies of RNA from testes of mutant mice showed that the induction of *Pem* mRNA in Sertoli cells was dependent on gonadotropins but not germ cells. In the epididymis, somatic cells of the proximal cauda expressed *Pem* mRNA in both mice and rats. Temporal regulation of *Pem* transcription in the epididymis differed from temporal regulation in the testis as demonstrated by a different developmental period of expression in prepubertal mice and by a difference in germ cell dependence. However, the temporal expression pattern of *Pem* mRNA in testes and epididymides of both species exhibited an excellent correlation with increasing levels of androgen in these tissues and with developmental events during spermatogenesis. Additionally, in hypophysectomized mice and rats, Pem expression was ablated in the testis and epididymis. In HPX animals, LH or testosterone alone restored normal Pem transcript levels in mouse testes and rat epididymides. Whereas, in HPX rat testes upregulation was not as distinct. Hypogonadal mouse testes have not been exposed to prior maturation by gonadotropins yet testosterone alone induced Pem expression to levels seen in normal mouse testes. Pem is the first homeobox gene found to be expressed in both the testis and the epididymis. Also, Pem is the first potential transcription factor expressed during spermatogenesis shown to be regulated by testosterone. The highly specific expression pattern and hormonal regulation of the Pem gene suggests that Pem may be a transcription factor which directs gene expression important in spermatogenesis and sperm maturation.

Expression patterns.

Other homeobox genes are transcribed in the mouse spermatogenic system. *Sperm-1* is transiently expressed immediately prior to meiosis in germ cells (88). *Hox-a4* is expressed specifically in postmeiotic germ cells of the testes but not in the epididymides of the adult mouse (65; Lindsey and Wilkinson unpublished results). *Hox-b4* and *-d4* genes are expressed in adult testes as well as other adult organs (69, 91,92). *Hox-c8* and *Pax-2* are transcribed in the epididymis but not in the testes (71,81, Lindsey and Wilkinson unpublished results).

Pem transcripts were found at higher levels in 9-day old and 15-day old mouse and rat testes respectively. Because of the longer experimental time course of rat testes, a cyclical pattern of Pem expression was evident and reminiscent of the spermatogenic cycle which begins every 12.9 days in the rat (93). The increased *Pem* transcripts began between days 12 and 15 and the second increase started around day 26; the two increases are about 13 days apart. The first spermatozoa do not reach the lumen of the seminiferous tubule until day 44 after birth (94). Since Pem transcripts in the rat testis peaked at day 21 and decreased by day 26 this suggests that Pem transcription is down-regulated well before the first spermatozoa are released. *Pem* expression increased during the first pachytene differentiation and decreased during the first round of spermatid formation (95). Even though *Pem* protein levels during these cycles still need to be determined, it has been shown that most mRNA in the Sertoli cell is translated within the same or very next stage of the spermatogenic cycle (96). The timing of *Pem* expression during the seminiferous cycle implies that *Pem* may be needed for gene regulation of early- and mid-spermatogenic events. Indeed, *mPem* was specifically expressed late in stage VI, most highly in stage VII, and decreased in early stage VIII seminiferous tubules of the mouse (97); stages that are regulated by androgens (96) and correlate to the same stages as those found in the rat (31).

Pem expression was determined by RNase protection analyses of RNA from fractionated Balb/c mouse testes to reside in the tubule fraction. By in situ hybridization, Pem transcripts were confirmed to be in the seminiferous tubules primarily over Sertoli cells. In addition, RNase protection analyses and northern analyses of RNA from dominant white spotting mice which lack germ cells shows an increased Pem expression when compared to wild type levels. This increase is due to the increase ratio of somatic cells to germ cells which in normal mice make up the majority of the cell population. This is the same two-fold increase that was seen for another Sertoli cell-specific transcription factor, GATA-1, which has been shown by in situ hybridization and immunohistochemistry to be expressed only in Sertoli cells of the testes (98). These results strongly suggest that the presence of germ cells is not necessary for Pem expression in the testes.

Pem transcripts were found to increase in the epididymides of 17-day and 23-day old mice and rats respectively. In the well studied rat epdididymis, this is a developmental period of time when increases in testosterone and dihydrotestosterone levels occur in the epididymis (99,100) and testicular fluid first enters the epididymis (26). The first spermatozoa enter the epididymis at day-33 and -44 after birth in the mouse and rat respectively (101,102). Therefore, Pem is upregulated well before spermatozoa enter the epididymis. However, Pem transcripts were diminished in homozygous quaking mouse testes which have a defect in spematogenesis in which only greatly reduced numbers of defective spermatozoa reach the epididymis (103). Additionally, Pem transcripts were absent in epididymides of dominant white spotting mice which have virtually no germ cells in the testes but normal hormone levels (104). These results suggested that at least germ cells *in the testis* were necessary for Pem expression in the epididymis which is consistent with previous studies which show that germ cells at certain stages affect Sertoli cell function.

When rPem expression was compared to other known epididymal transcripts the pattern was consistent with testosterone induction of BC1 gene transcription (99). In addition, another homeobox gene shown to be expressed in epididymides, Hoxc-8 (71), was expressed at very high levels in immature rat epididymides then decreased in expression into adulthood. Virtually no Pem expression was detected in the initial segment where the proenkephalin gene is expressed and is upregulated around day 44 after birth (105). By RNA analyses of sectioned epididymides from rat and in situ hybridization, it was determined that Pem transcripts located to the caput/corpus and corpus/cauda regions with higher expression in the proximal cauda. This region has been associated with maturing spermatozoa (106-107).

In both the testes and the epididymides, Pem expression resides in similar cell types. Sertoli cell and principal cells are both epithelial and columnar in shape. Like most epithelial cells they are polarized, have cell-cell junctions, and secrete, primarily from their apical surface, proteins into the lumen of the tubules in which they reside. One of the primary differences between these two epithelial population is the cyclic morphology and function of the Sertoli cell. The cyclic expression of Pem in Sertoli cells is consistent with this distinguishing characteristic.

Regulation of transcription factors

Few studies of the regulation of transcription factors in testes have been completed. Very little information exists on regulation of transcription factors in the epididymis. The PEA3 transcription factor is detectably expressed in brain and epididymis. In retinoic acid differentiated embryonic carcinoma cells, PEA3 is downregulated. Regulation of PEA3 in epididymis has not been investigated (108). Peritubular cell secreted protein, P-Mod-S, has been shown to transiently upregulate c-fos in cultured Sertoli cells (109). However, c-fos is expressed by other testicular cells (110). FSH also induces c-fos in cultured Sertoli cells (111). GATA-1, a trancription factor originally identified in the

erythropoietic developmental system, was not regulated by gonadotropins in HPX or hpg mice but has been shown to be stage specific in stage VII, VIII, and IX seminiferous tubules (98). Cyclic adenosine 3',5' -monophosphate (cAMP) response element-binding protein (CREB) is expressed in many somatic and germ cells of the testis but is induced by FSH only in cultured Sertoli cells (112). Also, in vitro, the proto-oncogene, c-myc, has been shown to be induced by testosterone in immature but not mature Sertoli cells (113).

Data on regulation of homobox genes during spermatogenesis does not exist. Some studies on regulation of homeobox genes in other systems are done. For example, retinoic acid specificallly activates the expression of all four hoemobox gene (Hox) clusters in the human embryonal carcinoma cell line NTERA-2 clone D1 (114). It is surprising that no information exists regarding retinoic acid regulation of Hox genes expressed in the testis since vitamin A (retinol) and its metabolites are essential for spermatogenesis (115-117). Retinoic acid has also been shown to induce LFB1, also known as HNF1, in F9 embryonic carcinoma cells (118) Specialized epithelia cells of liver, intestine, and kidney express LFB/HNF mRNA and protein (119) The two eye development homeobox genes, Pax-2 and -6, are regulated by Shh, a protein secreted from axial midline mesoderm and a *Drosophila* hedgehog homologue (120).

Two other molecules that act as both receptors and transcription factors in Sertoli cells are the AR and retinoic acid receptors. The AR is thought to be regulated by FSH (121-123). Immunohistochemical localization of the AR shows increasing nuclear AR in Sertoli cells from stage II, peaking in stage VII seminiferous tubules and declining rapidly in stage VIII. High levels of AR immunoreactivity are detected in peritubular cells throughout all of the stages (124). The retinoic acid receptor -alpha$_1$ (RAR-∂_1) is expressed in both Sertoli cells and germ cells whereas RAR-∂_2 is strictly expressed in Sertoli cells. Both are found in stage VIII and IX seminiferous tubules. Both retinoic

acid and retinol are found to increase the transcription of RAR-∂_1 and -∂_2 genes 2-3 fold in vitamin A depleted rats (125,126).

Regulation of Pem expression.

The Pem homeobox gene was expressed in a developmental pattern which implied hormonal regulation of this gene. The mutant mouse, hypogonal (hpg), which possesses a mutated GnRH gene causing an absence of gonadotropins (127), was used to determine if hormones might play a role in Pem expression. These animals lacked Pem expression in the testes. In addition, testicular feminized mice, which have a defective AR (128) lacked Pem expression.

Pem expression was ablated in testes of HPX rats and mice. These animals have had the pituitary surgically ablated and no longer secrete pituitary hormones as detected by assays of corticotropin or testosterone serum levels. Most importantly, treatment for two days with exogenous LH or testosterone alone restored Pem expression in HPX mouse testes to levels detected in normal mice. This result is consistent with Pem expression primarily in stage VII seminiferous tubules which are androgen-dependent (129,130). These results strongly suggest that testosterone is either directly or indirectly regulating Pem expression in mouse testes.

In contrast, exogenous testosterone did not give a distinct induction of Pem transcript in the testes of HPX rats. The difference in Pem expression in normal rat (low expression) and normal mouse (high expression) testes was inversely related to ABP levels found in the testes of each animal (52). These data imply that Pem may be downregulated by ABP. This is consistent with ABP acting as an androgen sink (131), and therefore, less androgen would be available to upregulate Pem.

In hpg mice, LH or testosterone induced Pem transcript levels to those detected in wild-type mice. This strongly suggests that prior maturation of the Sertoli cells by FSH was not needed. In addition, both HPX and hpg mouse testes showed a

suppression of Pem expression by FSH when administered with testosterone. These results are interesting since the lowest levels of FSH binding to its receptor is found in stages VI to VIII (132,133); the stages found to be the highest for Pem expression in the mouse. A decrease in Pem expression was also detected in testes from inhibin knock-out mice that overexpress FSH (Maiti & Wilkinson, unpublished results). These results are consistent with the cyclic expression pattern of Pem detected with an RNase protection analyses of total RNA taken from a time course of 5- through 104-day old rat testes and with in situ hybridization analyses of mouse testes.

Since hpg and chronically HPX mice do not have a detectable epididymis, hormonal regulation of Pem in this organ was investigated by using HPX rats. HPX rat epididymides lacked detectable Pem expression. Exogenous testosterone restored Pem transcript levels to those found in sham operated rat epididymides. In addition, the levels of Pem transcripts that were restored correlated to dihydrotestosterone (DHT) levels, a 5-∂ reduced form of testosterone that is found in the epididymides after exogenous treatment and in normal rat epididymides (131,134-136). During development there is a five fold increase in principal cell nuclear DHT in the caput/cauda during the same time period when Pem is upregulated (134). Since the first spermatozoa enter the epididymis at day 33 in the mouse and day 44 in the rat after birth (94) and Pem expression started at day 17 and day 23 in the mouse and rat respectively; Pem expression is not regulated by a spermatozoa associated molecule or signal. However, since Pem transcripts are absent in epididymides from mutant mice lacking germ cells, these results suggest that germ cells are required *in the testis* and may induce a Sertoli cell protein which then regulates Pem in the epididymis. ABP is such a candidate protein. The Sertoli cell-secreted protein, ABP, also increases in the epididymis around day 20 (26) when Pem transcripts increased and is thought to be necessary to provide the testosterone which is then converted to DHT (137-139).

The role for DHT in facilitating prostate cancer has been well documented (for review see ref. 140). However, the molecular mechanism of action of this androgen during cancer is not well understood. Since Pem is expressed in about 80% of immortalized cell lines tested, regardless of lineage, in sites of high cell proliferation (placenta and testis), and is induced by testosterone and DHT, it is tempting to speculate that Pem may be involved in the processes of immortalization and/or proliferation of cells. The immortalized Sertoli cell lines, 15P-1 and TM4, did not express Pem transcripts (data not shown). The 15P-1 cell line, when treated with testosterone, did not express Pem (data not shown). However, it is not known if these cells possess AR. Yet when stable transfections were made with the 15P-1 cell line using two different Pem expression constructs, three out of four clones that expressed Pem had four fold higher numbers of viable cells when compared with Pem antisense transfected clones (data not shown). In addition, germ cells increase in numbers in testes of hpg mice treated with testosterone in the absence of detectable FSH (11). Therefore, Pem expression may regulate genes necessary to specific cell immortalization and/or proliferation.

Pem as a candidate regulator of genes.

The difference in Pem expression in rat and mouse testis was inversely related to ABP levels in each animal (52). Additionally, Tfm mice did not express Pem and they also express high levels of ABP (141) Therefore, it is unlikely that Pem regulates ABP which is secreted by Sertoli cells. Mitoses of the Sertoli cells, the cell type identified to express Pem in the mouse, decrease at about day 14 after birth in the rat (94,142,143) and before Pem expression. Additionally, in hypogonadal mice, exogenous LH does not induce Sertoli cells to proliferate (144) but did induce Pem transcription. So, it is most likely that Pem expression does not regulate Sertoli cell proliferation.

The retinoic acid nuclear receptor alpha-1 (RAR-∂_1) mRNA levels increase seven fold in stage VIII and IX seminiferous tubules (125) stages right after Pem transcripts were detected. Pem may be a transcription factor that regulates the essential RAR-∂_1 although evidence for testosterone regulation of RAR-∂_1 is lacking. Another nuclear receptor, the AR is also regulated during spermatogenesis. However AR mRNA starts to increase in stage II seminiferous tubules suggesting regulation by FSH. Indeed, AR mRNA is up-regulated by FSH (121-123). Another candidate downstream target of *Pem* is the protease plasminogen activator (PA) since it is expressed specifically in stage VII-VIII (24). However, our results with HPX animals in combination with the results obtained by Muffly et al (145) and Cameron et al. (146) suggest that cell-cell contacts do break down upon withdrawal of testosterone without PA although the experimental breakdown may not be exclusive of a protease breakdown physiologically. In addition, PA has been shown to be induced by FSH or with cyclic AMP derivatives, but not by androgens (146-148).

In HPX rat testis, a defect in binding of step 8 spermatids to Sertoli cells and a cessation of further maturation of spermatids takes place. This can be prevented by administration of testosterone soon after hypophysectomy (144). This restoration by testosterone of advanced spermatids has also been shown in the azoospermic rat model by immunizing against LH or GnRH (10). This binding to spermatids is through ectoplasmic specializations (149-151) which form 16 days after birth of the rat (30) during the same time frame as rPem expression during development. Ectoplasmic specializations are found during the mid-cycle first associated with pachytene spermatocytes (152-155) and also with the Sertoli cell barrier junction (156-159). It has been shown that testosterone alone, if administered soon after hypophysectomy in mature rats, is sufficient to sustain daily sperm production (110). Experiments done by Cameron et al. also show that the ectoplasmic associations, needed for cell-cell interactions between the Sertoli cell and the step eight spermatids, are disrupted in HPX

rat testes. Even though the f-actin and vinculin proteins involved in this association were still made they were highly disorganized (145). Pem may be a transcription factor that regulates the gene(s) needed to be expressed for the organization of this association which is essential for spermatozoan development.

In addition to ectoplasmic specializations, another structure found at the Sertoli cell barrier junctions and Sertoli cell-germ cell adhesion points is called tubulobulbar complex (157-158) (Fig. 5). These structures are also associated with actin and do not form in cytochalasin-D injected testes (160). These complexes may function to internalize junctions prior to two major events; release of the most mature spermatids and the advancement of spermatocytes from the basal to the adluminal compartments (160-162). In addition, tubulobulbar complexes are formed in stage VII and degraded in late stage VIII seminiferous tubules (163,164); stage VII Sertoli cells expressed the highest levels of Pem mRNA. The actin expression also correlates with these stages. Specifically, actin muscle isoforms are found during stages VII and VIII of the cycle. It is hypothesized that late spermatids are actively forced from the crypts of the Sertoli cell by contractile actins found at the ectoplasmic specializations (165). Since Pem is found in very low levels in skeletal muscle, it is possible that expression of this homeobox gene regulates muscle-type actin.

The most well defined molecular function of homeobox gene expression is that they have been implicated in the regulation of cell adhesion molecules. Mutations in the Pax-6 gene result in impaired migration of neual crest cells from the anterior midbrain (75); migration has been shown to be mitigated by cell-cell contacts. In vitro analyses show that Hox-d9 and HNF-1 regulate the liver cell adhesion molecule, L-CAM, as demonstrated by transient co-transfections of choramphenicol acetyltransferase gene reporter constructs containing the L-CAM promoter and enhancer regions along with expression contructs for these two homeobox genes (166). Similar co-transfection experiments provide evidence for Hox-b8, -b9, Cux and Phox2 homeodomain proteins

in regulation of the neural cell adhesion molecule, N-CAM (167). Therefore, the correlations of the temporal and spatial Pem expression regulated by testosterone suggests that Pem may be a candidate regulator of adhesion molecules that are required for spermatogenesis (168,169). Of course, Pem, in concert with other transcription factors, may regulate more than one testosterone directed process.

It might be postulated that the cycles of spermatogenesis along the seminiferous tubule are necessary for long-term spermatogenesis. Perhaps the cycles prevent too many spermatids being released at the same time in the same area. Induction of Pem expression by testosterone and suppression of Pem expression by FSH would fit nicely with the speculation that Pem expression may regulate the cycles of spermatogenesis. Since testosterone alone has been shown to be sufficient for initiation of qualitative and quantitative spermatogenesis in hpg mice (11), why is FSH necessary? One concept is that FSH increases Sertoli cell proliferation perinatally and this increased number can support more germ cell development (170). But why would adult expression be needed for spermatogenesis? It might be speculated that FSH is needed to down-regulate spermatogenesis so that too many developing germ cells do not accumulate in the same area of the semiferous tubule. Since Pem is regulated by testosterone and testosterone has been shown to up-regulate FSH mRNA in Sertoli cells of stage VII seminiferous tubules (171), Pem is a candidate transcription factor that regulates FSH receptor gene expression. In addition, FSH suppressed Pem expression in testes of HPX and hpg mice, therefore, if Pem regulates spermatogenesis, FSH would down-regulate spermatogenesis. Obviously, more research needs to be done to determine the adult function for FSH and for the necessity of cycles during spermatogenesis.

In HPX rat epididymides testosterone restores Pem expression. Pem may be a transcription factor that regulates testosterone induced gene transcription. The proximal cauda region, the region that had the highest levels of Pem expression, has been shown to be important for maturation of the spermatozoa specifically in forward motility

and egg recognition (106,107). Most importantly, two proteins, "forward motility" and carboxypeptidase y-like (D/E) proteins, shown to be important for spermatozoa to gain these abilities, are specifically found in this region and are regulated by testosterone administration (54,55). In addition, a blood-epididymal barrier exists at the apical region of the principal epithelial cells similar to those found at the base of the Sertoli cell in the testes (172,173). Whether or not Pem regulates secretion of any these proteins or formation of the barrier will be the subject of future investigations.

Differences between mouse and rat Pem expression.

Pem expression levels were inversely related in mouse compared with rat testis and epididymis. Mouse Pem expression was higher in the testis than in the epididymis and rat Pem expression was higher in the epididymis than in the testis. In HPX rat testes, Pem induction was far less distinct than in HPX mouse testes. One main cause might be the levels of ABP which acts as an androgen sink in the testes and carries androgen to the epididymis where the complex is primarily internalized and converted into DHT. ABP levels are 50 to 100 fold higher in mouse testes compared to rat testes (52). Testosterone and DHT regulate Pem in the testes and epididymis respectively. Therefore, with less ABP to bind to testosterone in mouse testis more testosterone would be available to upregulate Pem. Also, with less ABP to transfer testosterone to the epididymis, less DHT would be made and available for Pem regulation in mouse epididymis although we could not show this experimentally since the epididymides of hpg and chonically HPX mice are nonexistent. In rat, the opposite would be hypothesized, higher testicular ABP would make less testosterone available in the rat testis and therefore lower Pem expression levels. In addition, ABP is at its lowest levels in early stage VI right before Pem is expressed in rat testis. In the rat epididymis, a higher production of ABP would carry more testosterone to the epididymis and convert into DHT therefore inducing higher levels of Pem expression in rat epdidymides. In

transgenic mice that specifically overexpress the rat ABP in the testes degenerating Sertoli cells and interrupted spermatogenesis are found (174). This suggests that mouse testis Sertoli cells need more androgen and perhaps more Pem expression in order to be functional. This significant expression difference in a protein known to be important in rat spermatogenesis, ABP, and the difference in expression and regulation of a homeobox gene thought to be important in spermatogenesis is intriguing.

In addition, helices I and II of the homeodomain encoded by the rat Pem gene when compared to the mouse Pem gene are only 44% identical even though the rest of the gene is 87% to 96% homologous (175). The amino terminal of the homeodomain interacts with other proteins (176,177). This difference may direct some physiological difference that exists between these two species. Alternatively, this difference may act to maintain a species barrier. The latter hypothesis is especially appealing since Pem is expressed at very high levels in the placenta. Hypothetically, expression of mouse Pem in the embryonic portion of the placenta may prevent implantation and placentation in a rat. As a putative transcription factor this highly divergent region may not bind with the necessary proteins to transactivate genes when made from the other rodent genome.

Summary

Since it is generally accepted that FSH initiates spermatogenesis and testosterone maintains spermatogenesis in mature animals, the results presented here suggest that the Sertoli cell-expressed Pem gene may act as a transcription factor induced by testosterone and downregulated by FSH in a cyclical manner for maintenance of spermatogenesis. Additionally, the DHT-regulated expression of Pem in a region of the epididymis known to mature sperm suggests that Pem is a candidate transcription factor that regulates sperm maturation proteins. Very little is known about the molecular mechanisms of androgen action in the seminiferous tubules of the testis and principal cells of the epididymis compared with other androgen-dependent tissues (178).

Therefore, for the first time, a candidate transcription factor encoded by the Pem homeobox gene that is regulated by androgens in a temporal and spatial manner during spermatogenesis and sperm maturation has been determined.

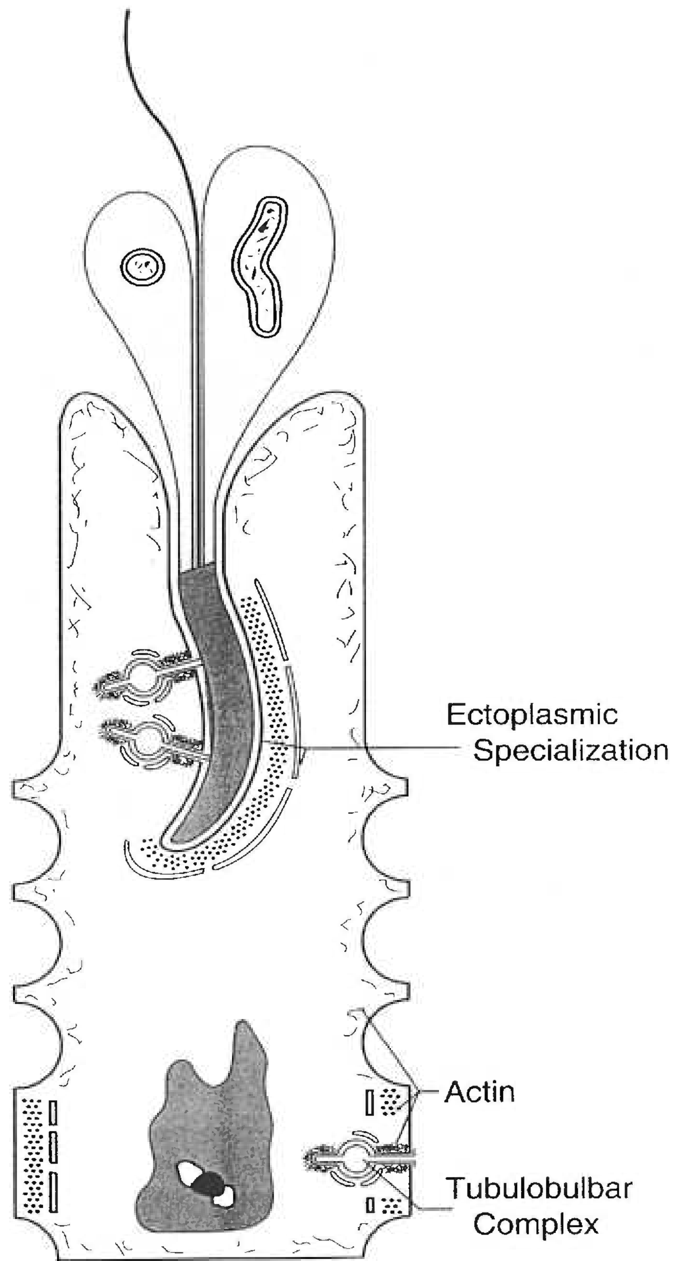


Figure 5. Schematic drawing of the location of ectoplasmic specializations, tubulobulbar complexes, and actin in the Sertoli cell. (used with permission from Cache River Press from "The Sertoli Cell" L. Russell and M. Griswold eds.)

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