CHARACTERIZATION AND REGULATION OF EPIDIDYMAL 4-ENE STEROID 5α-REDUCTASE MESSENGER RIBONUCLEIC ACIDS AND PROTEIN

by

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Abstract

Tissue distribution, endocrine, developmental, and aging studies were used to characterize the regulation of the 5α-reductase mRNAs, types 1 and 2, in the rat epididymis. 5α-Reductase type 1 mRNA was predominantly localized to the initial segment of the epididymis. Bilateral orchidectomy markedly reduced type 1 mRNA levels; testosterone replacement could maintain these levels in all epididymal regions with the exception of the initial segment. Unilateral orchidectomy or efferent duct ligation caused a sharp decrease in type 1 mRNA concentrations but only in the initial segment of the epididymis. 5α-Reductase type 1 mRNA expression was developmentally regulated; age-dependent changes in type 1 mRNA expression were specific to the initial segment of the epididymis. Thus, the regulation of epididymal 5α-reductase type 1 mRNA was multifactorial and segment-specific. In contrast to the type 1 mRNA, the 5α-reductase type 2 transcript was primarily found in the caput epididymidis. Moreover, its expression did not change as a function of increasing postnatal age and was increased after efferent duct ligation in the initial segment of the epididymis. Hence, the 5α-reductase mRNAs, types 1 and 2, were differentially regulated in the rat epididymis. Aging (between 6 and 12 months) altered gene expression in the Brown Norway rat epididymis. Changes in the expression of the mRNAs for the 5α-reductase isozymes, types 1 and 2 and proenkephalin were identified as early markers for aging in the epididymis. A specific anti-5α-reductase type 1 serum was developed to study the localization and regulation of the type 1 protein in the rat epididymis. This protein was intensely localized to a discrete lobule of the initial segment of the epididymis (the proximal initial segment). In all regions of the tissue, the type 1 protein was specific to epithelial principal cells and had distinct intracellular localizations. The intracellular localization of the type 1 protein was regulated in an age-dependent and region-specific manner. Using these novel observations, a scheme for the regulation of 5α-reductase and androgen action in the rat epididymis was proposed.

Résumé

Ce travail avait pour objectif de caractériser la distribution et la régulation des ARNms codant pour les isotypes 1 et 2 de la 5α-réductase dans l'épididyme du rat. Chez le rat adulte, l'ARNm de la 5α-réductase de type 1 a été principalement localisé dans le segment initial de l'épididyme. L'orchiectomie bilatérale a considérablement réduit les quantités d'ARNm de type 1; à l'exception du segment initial de l'épididyme, le remplacement par la testostérone a pu maintenir l'expression de l'ARNm de la 5α-réductase de type 1. L'orchiectomie unilatérale ou la ligation des canaux efferents a entrainé une diminution très nette des quantités d'ARNm de la 5α-réductase de type 1 uniquement dans le segment initial de l'épididyme. Au cours du développement postnatal, une variation de l'expression de l'ARNm de type 1 a été observée au niveau du segment initial de l'épididyme. Ces résultats ont indiqué que la régulation épididymaire de l'ARNm de la 5α-réductase de type 1 était liée à plusieurs facteurs et dépendait de la région du tissu étudié. Contrairement à l'ARNm de type 1, l'expression de l'ARNm de la 5α-réductase de type 2 se trouvait essentiellement dans la tête de l'épididyme (le caput). De plus, cette expression n'a pas varié au cours du développement postnatal et a augmenté dans le segment initial de l'épididyme après ligation des canaux éfferents. Les isotypes 1 et 2 de la 5α-réductase étaient donc régulés différemment dans l'épididyme du rat. Le vieillissement (entre 6 et 12 mois) a modifié l'expression de certains gènes dans l'épididyme. Au cours du vieillissement, l'altération de l'expression des ARNm codant pour les isotypes 1 et 2 de la 5α-réductase, et la proenkephaline ont permis d'identifier ceux-ci comme marqueurs précoces du vieillissement épididymaire. Un sérum polyclonal dirigé contre la 5α-réductase de type 1 a été développé pour permettre de localiser et d'étudier la régulation de cette protéine dans l'épididyme. Chez le rat, la protéine 5α-réductase de type 1 était fortement exprimée dans la région proximale du

segment initial de l'épididyme. Dans toutes les régions du tissu, cette protéine se trouvait uniquement dans les cellules principales et présentait des localisations intracellulaires distinctes. La localisation intracellulaire de la protéine 5α-réductase de type 1 était régulée de façon différente le long de l'épididyme du rat. Les résultats de ce travail ont permis de proposer un schéma pour la régulation de la 5α-réductase et le mode d'action des androgènes dans l'épididyme du rat.

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Preface

Format of the Thesis

This thesis comprises five papers which are included almost entirely in the form in which they were submitted for publication. Connecting texts are provided in compliance with section B.2/ of the "Guidelines Concerning Thesis Preparation", Faculty of Graduate Studies and Research, McGill University. These guidelines state:

"Manuscripts and Authorship:

> Candidates have the option, subject to the approval of their Department, of including, as part of their thesis, copies of the text of a paper(s) submitted for publication, or the clearly-duplicated text of a published paper(s), provided that these copies are bound as an integral part of the thesis. If this option is chosen, connecting texts, providing logical bridges between the different papers, are mandatory. The thesis must still conform to all other requirements of the "Guidelines Concerning Thesis Preparation" and should be in a literary form that is more than a mere collection of manuscripts published or to be published. The thesis must include, as separate chapters or sections: (1) a Table of Contents, (2) a general abstract in English and French, (3) an introduction which clearly states the rationale and objectives of the study, (4) a comprehensive general review of the background literature to the subject of the thesis, when this review is appropriate, and (5) a final overall conclusion and/or summary. Additional material (procedural and design data, as well as descriptions of equipment used) must be provided where appropriate and in sufficient detail (eg. in appendices) to allow a clear and precise judgement to be made of the importance and originality of the research reported in the thesis. In the case of manuscripts co-authored by the candidate and others, the candidate is required to make an explicit statement in the thesis of who contributed to such work and to what extent; supervisors must attest to the accuracy of such claims at the Ph.D. Oral Defense. Since the task of examiners is made more difficult in these cases, it is in the candidates's interest to make perfectly clear the responsibilities of the different authors of the co-authored papers."

The introduction, Chapter I, includes a general introduction to the male reproductive system, a comprehensive review of the background literature on the

epididymis and 5α-reductase, and provides the rationale for the studies presented in this thesis. Chapters II (128:2407-2414, 1991), III (131:1534-1540, 1992), and VI (134:2298-2306, 1994) are published in ENDOCRINOLOGY; appendices are attached to chapters II and VI. The appendix to chapter II establishes the linearity of mRNA quantitation via laser densitometric scanning. The data given in the appendix to chapter VI are an extension to the findings presented in chapter VI. Chapters IV and V have been submitted for publication.

All experiments presented in the studies of this thesis were done by the candidate with the exception of the perfusion, embedding, and cutting of some the tissue sections used in chapter VI and its appendix. These tissue sections were kindly provided by Dr. L. Hermo of the Department of Anatomy and Cell Biology at McGill University. The aging samples used in chapter V were kindly provided by Drs. B. Zirkin and W. Wright of the Department of Population Dynamics, School of Hygiene and Public Health, at Johns Hopkins University.

Chapter VII contains a general discussion of the results; these data are integrated into a proposed scheme for the regulation of epididymal 5α-reductase. The last section, List of Original Contributions, summarizes the major findings of Chapters II-VI.

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Chapter I

Introduction

A. General Introduction: The Male Reproductive System

Over the past four decades, major advances in the field of male reproduction have been made possible by the intense research efforts of many laboratories worldwide. In recent years, this knowledge base has expanded rapidly with the development and availability of new methodologies in the areas of cellular and molecular biology. Male reproduction has been the subject of numerous review articles. A compilation of these articles have appeared in many excellent books such as in *Section 7 of the Handbook of Physiology in 1975* and *Volume 1 of the Physiology of Reproduction* in 1988 (1,2). Thus, the objective of the following section is not to present a comprehensive review of all the information pertaining to male reproduction. Rather, the intent is to provide a brief overview of the major components of the male reproductive system as a basis for the subsequent in-depth presentation of epididymal physiology, structure, and function.

Anatomically, the male reproductive tract is comprised of two testes, the excurrent ducts, and the sex accessory tissues:

1. The Testis

The mammalian testis is an encapsulated organ whose size in relation to total body mass is species-dependent (3,4). The testicular capsule, also known as the tunica albuginea, is a tough outer covering composed of thick connective tissue and variable numbers of smooth muscle fibres which are capable of spontaneous contractile activity (5-8). In some species such as the rabbit, the rhythmic contractions of the smooth muscle cells have been suggested to be important for the transport of spermatozoa out of the testis and into the epididymis (6,9). The testicular capsule displays considerable innervation (10-13).

Consequently, the contractility of the capsule can be modulated by sympathetic nerve stimulation and by different pharmacological stimuli (14-21).

The testis is composed of two main compartments: the seminiferous tubules and the interstitial tissue; the major functions of these two compartments are the production of male germ cells, i.e., spermatozoa, and steroid biosynthesis and secretion, respectively. The seminiferous epithelium is the site where spermatozoa are formed through the process of spermatogenesis. Spermatogenesis involves a complex series of different cellular events, i.e., proliferation and mitosis, meiosis, and differentiation, whereby diploid precursor cells (spermatogonia) are progressively transformed into highly specialized haploid germ cells (spermatozoa). Pioneering studies by Leblond and Clermont done in the early 1950s have established much of our current understanding of the process of spermatogenesis (22,23) and its timing (24,25) in the mammalian testis.

Throughout their development and differentiation within the seminiferous epithelium, germ cells are in intimate contact with a population of non-dividing, supporting cells, the Sertoli cells. Several functions have been ascribed to the Sertoli cell, many of which are related to the developing germ cell. These functions include a supportive or "sustentacular" role which was described by Sertoli himself more than one hundred years ago, endocytosis, the formation of the blood-testis barrier through intercellular tight junctions between adjacent Sertoli cells, the delivery of nutrients to the germinal cells, spermiation (release of spermatozoa from the seminiferous epithelium), and secretion. A number of reviews concerning the different functions of the Sertoli cell and their regulation have appeared in a recent publication (26). It is important to point out that apart from these functions being important in mediating the development and

differentiation of germ cells within the seminiferous epithelium of the testis, many Sertoli cell products have also been postulated to have equally relevant extratesticular roles. Androgen binding protein (ABP), Müllerian inhibiting substance (MIS), inhibin, and sulfated glycoprotein-2 (clusterin) are good examples of Sertoli cell secretory products that have been hypothesized to have significant functions outside the testis (27-34).

The space between the seminiferous tubules which contains blood vessels, lymphatics, and numerous cells is known as the interstitial tissue of the testis. The most abundant cell type found in the testicular interstitium is the steroid-producing Leydig cell (35,36). In many species, Leydig cells are often found clustered around blood vessels or in small groups near the seminiferous epithelium (36). In the testis, testosterone, which is the main androgen synthesized and secreted by the mature Leydig cell, is produced in locally high concentrations. These elevated testosterone concentrations are essential for spermatogenesis. Other cell types found in the testicular interstitium, but which are present in smaller numbers than Leydig cells, are macrophages, monocytes, mast cells, and fibroblast-like cells. Peritubular myoid cells are identified by their close circular arrangement around the walls of the seminiferous tubules; they are separated from Sertoli cells by extracellular matrix. The close proximity of myoid cells to the seminiferous epithelium is consistent with their proposed role in the paracrine regulation of Sertoli cell function and differentiation (37-40).

The hormonal regulation of testicular function has been the subject of extensive study by many investigators. The fact that testicular function is under hormonal control has been known for more than sixty years. In a report published in 1930, Smith demonstrated that the removal of the pituitary gland in the rat results in a complete suppression of the formation of spermatozoa in the testis

(41). Presently, a large body of data would suggest that the key regulators of testicular function are the circulating gonadotropins, luteinizing hormone (LH), and follicle stimulating hormone (FSH), which are synthesized and secreted by the anterior pituitary. The release of gonadotropins from the anterior pituitary is controlled by the pulsatile secretion from the hypothalamus of the hormone, luteinizing hormone releasing hormone (LHRH). Since LHRH can stimulate the release of both LH and FSH from the anterior pituitary, LHRH has also been referred to as gonadotropin-releasing hormone (GnRH).

In the testis, the direct action of LH is the stimulation of testosterone synthesis by Leydig cells whereas FSH is thought to act directly on the germinal epithelium (42-44). In turn, the testosterone that is produced by the testicular Leydig cell under LH stimulation, negatively controls LH and FSH secretion by the pituitary. The simplified scheme described above illustrates the fundamental concept of *negative feedback* in the regulation of testicular function. In reality, the regulation of the hypothalamic-pituitary-gonadal axis in the male is complex, involving numerous factors acting at different levels of the axis; a number of excellent reviews, devoted to different facets of this topic, have been published (45-50).

It is well-established that spermatogenesis is a testosterone-dependent process (51-53). Since LH stimulates testosterone synthesis, then the importance of LH in the regulation of spermatogenesis is clear. In contrast to LH, however, the relative contribution of FSH in the control of spermatogenesis has been highly controversial. In humans, both hormones have been suggested to be required for quantitatively normal spermatogenesis (54). However, in the GnRH-immunized rat model, i.e., under conditions where serum LH and FSH levels are undectable, testosterone alone has been reported to be able to quantitatively restore and

maintain spermatogenesis in these animals (55-57). This result was substantiated by the finding that passive immunization against FSH has no substantial effect on spermatogenesis (58). Using a similar animal model, the recent study of McLachlan et al., however, is in disagreement with the reports cited above (59). In their study, testosterone was only capable of qualitative restoration of spermatogenesis in GnRH-immunized rats (59). Thus, it appears that the question of the exact role of FSH in the control of spermatogenesis will continue to be an issue of heated debate.

In mammals, the seminiferous tubules are arranged as coiled loops that terminate at both ends in the rete testis. The rete testis, which is represented by a complicated series of anastomotic ducts, lies in a region of the testis termed the mediastinum. The lumen of the rete testis is lined with squamous and low cuboidal epithelial cells (60) which are highly active in the processes of absorption and fluid phase endocytosis (61-64). Spermatozoa and testicular fluid that are formed in the seminiferous tubules are transported through the rete testis and into the testicular excurrent duct system. Many cholinergic nerve fibres penetrate into the testicular capsule forming a broad network in the mediastinum that ends beneath the rete testis epithelium where actin-containing myofibroblasts reside (13). The colocalization of cholinergic fibres and contractile cells among the rete testis ducts have raised the possibility that the contractile activity of these cells may be important in pumping the luminal contents of the rete testis into the efferent ducts (13).

2. The Excurrent Duct System

The testicular excurrent duct system is represented by the efferent ducts (ductuli efferentes), epididymis, and vas deferens (ductus deferens). In the

following section, a brief description of the structure and function of the efferent ducts and vas deferens is presented. The epididymis is temporarily omitted at this time since it will be the subject of considerable review later in this introductory chapter.

Anatomically, the rete testis is connected to the epididymis via the **efferent ducts**. The branching pattern of the efferents ducts, i.e., the number of ducts connected to the rete testis, is highly variable within and between different species; the range observed is between 4 and 20 tubules (67). In the rat, the presence of blind-ending tubules (ductuli abberantes) are common. In contrast to the normally open-ended duct, the lumina of the blind-ended tubules are constricted and devoid of sperm (67). The epithelium of the efferent ducts is primarily composed of ciliated and non-ciliated columnar cells, although halo cells can also be occasionally found (68). Like their cuboidal counterparts in the rete testis, the tall columnar epithelial cells of the efferent ducts are actively involved in both adsorptive and fluid phase endocytosis (69,70).

In contrast to the highly convoluted tubule of the epididymis, the **vas deferens** is a relatively straight tube that is surrounded by a thick layer of smooth muscle. The functionally mature spermatozoa that enter the vas deferens from the epididymis are transported to the outside of the body via a connection with the urethra. The vas deferens can be divided into proximal, distal, and terminal segments based on the ultrastructural differences of the epithelial principal cells that line its lumen (71); histologically, the rat has been the most studied species (71-74). The principal cell is the most abundant epithelial cell type found in the vas deferens; clear, basal, and halo cells, and unusually shaped pencil cells can also be found (68,71). Functionally, the epithelium that lines the vas deferens has both absorptive and secretory properties (68,75). Additionally, the capacity of the

vas deferens to phagocytose spermatozoa (spermiophagy) by specialized groups of epithelial cells in the terminal segment of its tissue in the rat and other species has been demonstrated (76-79). However, whether this process occurs normally in the vas deferens or only under situations that disrupt the tissue (abnormal conditions such as vasectomy) has been the subject of controversy.

3. The Sex Accessory Tissues

The male sex accessory tissues are represented by the prostate, seminal vesicles, bulbourethral (Cowper's) glands, and ampulla. The well-established function of these glands in male reproduction is that their secretions form the bulk of the volume of the seminal plasma. In the ejaculatory ducts, the secretions of the sex accessory tissues combine with spermatozoa from the vas deferens to form semen. A characteristic feature of these glands is that their development, growth, function, and the maintenance of their size is dependent on the presence of androgens (80).

Of the different male sex accessory tissues, the one that has been most intensely studied is the prostate. The great interest in understanding prostate physiology has been due to the high incidence of two different pathologies of the tissue in men: benign prostatic hyperplasia (enlargement of the prostate) and prostatic cancer. Embryologically, the prostate develops from the cranial portion of the urogenital sinus; this differentiation is under hormonal control and is thought to be mediated by the potent androgen, dihydrotestosterone (81). The prostate is composed of stroma and epithelium. The stroma contains smooth muscle cells, fibrocytes, and macrophages whereas the glandular epithelium is lined with luminal and basal cells (82-87). The primary secretory cell of the prostate is the luminal epithelial cell; the basal epithelial cells have been suggested to be

precursors of these cells (88). The production of paracrine factors by the stromal cells of the prostate has been proposed to be important in both the morphogenesis of the prostatic epithelial cells and the control of their secretory processes (89-92).

Unlike the prostate, the seminal vesicle, bulbourethral gland, and ampulla are not found in all mammals; when present they occur as paired organs. The ampulla, which is located at the terminal portion of the vas deferens, joins with the seminal vesicles to form the initial part of the ejaculatory ducts. During ejaculation, the secretory products of the ampulla and seminal vesicles are combined with those of the prostate as the ejaculatory ducts pass through this tissue; together, the sum of these secretions empty into the urethra. The contents of the bulbourethral glands are added to the ejaculate through a direct ductal connection with the urethra. The histology of the seminal vesicle, bulbourethral gland, and ampulla has been described for a number of different species (93-99).

B. The Epididymis

The mammalian epididymis is the major component of the testicular excurrent duct system. One of the major functions of this tissue is the functional maturation of spermatozoa. The fact that spermatozoa do mature in the epididymis, i.e., acquire the ability to swim and fertilize eggs, was demonstrated by Bedford (100) and Orgebin-Crist (101,102) more than 25 years ago. Since then, it has been clearly established that the maturation and storage of spermatozoa in the epididymis are androgen-dependent processes (103). Further, it has become apparent that the main androgen(s) responsible for maintaining epididymal structure and functions, is(are) the 5α-reduced metabolite(s) of the testosterone, 5α-dihydrotestosterone (DHT) and/or 5α-androstan-3α,17β-diol (3α-diol). In the epididymis, as well as in many other androgen-sensitive target tissues, the

rate limiting step in the pathway leading from testosterone to its 5α-reduced metabolites is the reaction catalyzed by the enzyme 4-ene steroid 5α-reductase (EC 1.3.1.22, 5α-reductase). Since both spermatozoal maturation and storage are important functions of the tissue, then the 5α-reductase enzyme has to be considered as a potentially important target for regulating fertility in the male.

The first step in attaining this long term goal, however, is to thoroughly understand the regulation and properties of epididymal 5α-reductase. Since this objective is the focus of my thesis, the rest of this introduction will be devoted to the epididymis and the 5α-reductase enzyme. First, the structure and functions of the epididymis, and their regulation, will be described. Second, the regulation of 5α-reductase enzyme activity in several rat tissues will be presented; these data will then be compared to those for the epididymis. Finally, the recent molecular aspects pertaining to the 5α-reductase enzyme will be introduced. These recent findings form the basis of the design and rationale of the experiments that are presented in this thesis.

1. Embryonic Sexual Differentiation and Development of the Epididymis

In mammals, gonadal sex is normally determined by the genetic sex; that is, the development of a testis is normally associated with the presence of a Y chromosome and conversely, an ovary develops in its absence (104). More specifically, the regulation of human sex differentiation is dependent on a specific gene, encoding a testis-determining factor (TDF), which is found on Yp, the short arm of the Y chromosome (105,106). This gene has recently been identified and termed SRY (for sex-determining region Y; 107,108). The SRY gene is deleted and/or mutated in many sex-reversed XY females (thus accounting for their female phenotype) and translocated to the X chromosome in some XX males (thus

accounting for the male phenotype in some XX males) (105,109-112). The SRY gene product is a sequence-specific DNA-binding protein (113,114).

Prior to gonadal sex differentiation, the genital ducts of the developing embryo are undifferentiated; both the female (Müllerian duct) and male (Wolffian duct) structures are present in the urogenital ridge (115). In mammals, masculinization of the undifferentiated reproductive tract is directly dependent on the presence of the testes. In the male, the fetal testis synthesizes androgens and SRY. Androgens act to prevent the programmed cell death-mediated degeneration of the Wolffian ducts (115), whereas the DNA-binding region of SRY specifically recognizes and activates the proximal upstream element in the promoter of the gene encoding Müllerian inhibiting substance (MIS) (114). In turn, MIS is secreted by the fetal testis and causes regression of the Müllerian ducts (115). In the female, the lack of SRY prevents the testes from being formed and thus, there is no secretion of androgens or MIS (115). As a result, the Wolffian ducts regress and the Müllerian ducts are allowed to develop.

In the male, the Wolffian (mesonephric) ducts give rise to the efferent ducts, epididymides, vasa deferentia, and seminal vesicles; the common mesonephric origin of these structures is also shared by the kidney (115). The first two tissues arise from the cranial portion of the mesonephric duct, the vasa deferentia from the middle portion, and the seminal vesicles from the caudal portion (116). Although the efferent ducts and the entire epididymis have been generally considered to develop from the mesonephric duct, there is evidence to suggest that the efferent ducts and the proximal portion of epididymis develop from a different embryological origin. The data supporting this argument have been provided by the ACI rat which is an inbred strain that manifests a high incidence of ipsilateral agenesis of the mesonephric duct (117-119). Thus, as would be

predicted, ACI rats have absent kidneys, seminal vesicles, and vasa deferentia (117-119). Unexpectedly, however, the efferent ducts and the proximal portion of the epididymis are still apparent in these animals (119). Thus, this finding led to the proposal that the efferent ducts and the proximal portion of the epididymis develop from the mesonephric tubules and not the mesonephric duct (119).

Over the years, it has been become generally accepted that the differentiation of the Wolffian ducts is dependent on the presence of testosterone that is secreted by the fetal testis (120,121). In contrast, the differentiation of the urogenital sinus into the prostate and bulbourethral glands is induced by DHT due to the presence of high levels of 5α-reductase in these embryonic tissues (116, 120,121). Recently, however, the concept that testosterone is the major androgen responsible for the differentiation of all the Wolffian duct derived tissues (epididymis, vas deferens, seminal vesicles) has been put into question by the findings of Tsuji et al., which have clearly indicated a more prominent role of DHT in the development of the seminal vesicles (116).

2. Structure of the Epididymis

Anatomically, epididymides occur as paired organs; one epididymis is located adjacent to each testis (hence its name from the Greek: "epi", near; "didymos", testis). The epididymis is held to the testicular capsule, the tunica albuginea, by connective tissue and is covered, in some species, by a considerable amount of adipose tissue, the epididymal fat pad. Testicular input to the tissue is conveyed via the efferent ducts which anastomose to form the single, convoluted epididymal duct. The highly convoluted nature of the epididymal duct is clearly reflected by its length when completely stretched out; in mammals, the elongated tubule can measure from 3 to 80 meters (122,123).

Morphologically, the epididymis can be divided into three gross anatomical regions: the head (caput), body (corpus), and tail (cauda). In 1926, Benoit introduced the concept of an initial segment, located between the efferent ducts and the caput epididymidis, that has a characteristic visual and histological appearance (124). The different segments of the epididymis have also been defined based on functional and histological differences, and more recently, on the immunocytochemical localization of many regionally expressed epididymal proteins. The histological characterization and functional properties of the epididymis are discussed in the next and subsequent sections of this introduction.

3. Histology of the Epididymis

The mammalian epididymis can be divided into two main compartments: the epithelium and the lumen. In normal adult animals, the epididymal lumen contains spermatozoa which are bathed in luminal fluid that changes markedly in composition as one moves down the tissue (125).

The size of the epididymal lumen is region-specific. In the initial segment, the lumen is small, circular and is sparsely populated with spermatozoa. In the rat, the very proximal region of the initial segment, i.e., the region that connects with the efferent ducts, is frequently found devoid of spermatozoa. The reason for this observation is unknown, but the absence of spermatozoa is often a good means of identifying the proximal initial segment region of the epididymis in adult animals. The width of the epididymal lumen increases dramatically and is characterized by a more irregular shape in the distal regions of the tissue. Many of the features described above are apparent in the immunocytochemical studies presented in chapter VI and its appendix.

The intertubular space of the epididymis is composed of connective tissue

and is occupied by blood and lymphatic vessels, nerve and smooth muscle fibres, and variable numbers of fibroblasts and macrophages. The epididymal tubule is lined with a pseudostratified epithelium that contains five major cell types: the principal, basal, clear, halo, and narrow cells. The regional changes in the histology of the epididymis at the level of the light microscope have been described for a number of mammalian species (68,124,126-134). The histological characterization of the most often studied species, the rat, has been reported by Reid and Cleland in 1957 (126), and reviewed by Robaire and Hermo in 1988 (134). In the next section, a brief description of the light and electron microscopic appearance of the epithelium of the adult and developing rat epididymis will be presented. The histological data for the adult rat were primarily drawn from the review of Robaire and Hermo (134).

In contrast to the efferent ducts, ciliated cells are not present in the epididymal epithelium (126,134). The most abundant cell type found in the epididymal epithelium is the principal cell which bears prominent stereocilia (microvilli) that extend into the lumen. In rats, principal cells constitute a minimum of 65% of the total epithelial cell population in the cauda epididymidis and as much as 80% in the initial segment (134). The light and electron microscopic appearance of the epithelial principal cells manifest important differences along the epididymal duct (126,134).

In the initial segment, principal cells are tall, columnar in shape, and have a basally located, round nucleus; the infranuclear region of the cell is densely packed with rough endoplasmic reticulum (126,134). The most prominent ultrastructural feature of the apical region of principal cells in the initial segment is the abundant presence of large, dilated membranous elements whose surfaces show the occasional ribosome (135). These structures have been termed the

sparsely granulated endoplasmic reticulum (SER) and are unique to principal cells of the initial segment of the epididymis (134,135). The SER is often found in close proximity to the apical plasma membrane (134,135). Although the functional significance of the SER is presently unknown, it has been proposed to be a potential site for the metabolism of androgens (28). The supranuclear cytoplasm of principal cells is also characterized by the presence of large stacks of Golgi saccules, mitochondria, multivesicular bodies, and smooth surfaced vesicles (134). Studies in which tracers were injected into the epididymal lumen have demonstrated that principal cells of the initial segment manifest considerable endocytic activity (135). Moreover, the presence of numerous coated pits and vesicles, multivesicular bodies, and endosomes in the apical region of these cells is consistent with the fact that these cells are actively involved in endocytosis (134,135).

In contrast to the initial segment, principal cells of the caput, corpus, and cauda epididymidis are shorter in height and have irregularly shaped nuclei that are often lobulated (126,134). The infranuclear cytoplasm of principal cells in these regions of the epididymis contains abundant cisternae of rough endoplasmic reticulum, numerous mitochondria, and lipid droplets (134). In the apical and supranuclear regions of these cells, a well-developed Golgi apparatus, lysosomes, multivesicular bodies, mitochondria, endosomes, and polysomes are apparent (134). A prominent feature of the supranuclear cytoplasm of principal cells in the corpus epididymidis that is not found in the other epididymal regions is the abundant presence of lipid deposits (134). As in the initial segment, principal cells of the caput and corpus epididymidis have endocytic properties (134,136,137).

An important ultrastructural finding demonstrated by Friend and Gilula was the existence of adluminal tight junctions between adjacent principal cells (138).

Such junctional complexes at the luminal surface of principal cells lining the epididymal duct act as a barrier preventing the transfer of substances from the general circulation into the epididymal lumen (139,140). The importance of this barrier, termed the blood-epididymis barrier, will be described later under the section "functions of the epididymis".

The second most abundant cell type found in the epididymal epithelium is the basal cell (134). Basal cells are flat elongated cells that reside near the base of the epithelium where they make contact with the basement membrane (126,134). Like principal cells, basal cells are found throughout the epididymis. Basal cells usually manifest a large elongated or round shaped nucleus with a proportionally small amount of cytoplasm (126,134). Since basal cells are located between principal cells at the base of the epithelium, they were originally thought not to extend to the luminal surface. However, a recent report by Veri et al. has suggested otherwise (141). In their study, Y_f, a subunit of glutathione-S-transferase P, was intensely immunolocalized throughout the cytoplasm of epididymal basal cells (141). Interestingly, some immunoreactive basal cells had long processes extending between principal cells, occasionally reaching the luminal surface (141). At present, the exact functional role of these cells in the epididymis is unknown. Recent findings, however, would suggest that epithelial basal cells may play an important protective role in the epididymis (28,141).

Clear cells of the epididymis are so named because of their characteristic pale-staining cytoplasm at the level of the light microscope (68,134); they have been identified in only a limited number of species (68, 132,142-145). In the rat, clear cells are found in moderate numbers in the caput and corpus regions and are most abundant in the cauda epididymidis; they are not present in the initial segment (134). Clear cells can be identified by the presence of numerous

apically-localized pale-staining vesicles and dense granules which are located above or below the nucleus (134). The basal region of clear cells also contains variable amounts of lipid (68,134,142).

Tracer studies have been used to demonstrate that clear cells are active in endocytosis, particularly in the cauda epididymidis (137,146). Functionally, clear cells have been proposed to play a major role in the uptake of luminal components (134). For example, clear cells of the corpus epididymidis have been suggested to recognize and internalize the cytoplasmic droplet of spermatozoa since their contents can been observed in the endocytic organelles of these cells (147).

Halo and narrow cells are the least common cells found in the epididymal epithelium (134). Halo cells, which are found at all levels of the epididymal epithelium, are identified by their characteristic dark-staining nucleus surrounded by a pale-staining cytoplasm (68,134). These cells are frequently referred to as intraepithelial lymphocytes due to their similarity to the circulating monocytes of the human (148). Finally, narrow cells are exclusively found in the initial segment of the epididymis (134). Their name stems from the fact that their intracellular width is narrower than the neighbouring principal cells. Narrow cells are also identified by their deep-staining cytoplasm and elongated, apically localized nuclei (134). These cells have been reported in only a limited number of species (133,143,145,148,149); at present, their function remains undefined.

The postnatal development and structural differentiation of the epithelial cell types lining the epididymal duct of the rat have been studied by a number of investigators (150-153). In the immature rat, between birth and postnatal day 21, the epithelial cells of the epididymis are generally uniform in appearance and are said to be in an undifferentiated state (150). On postnatal day 14, halo cells are evident in the epididymal epithelium (150). Their differentiation period occurs

between the ages of 16 and 44 days postpartum (150).

Barin et al. reported that principal cells of all regions of the rat epididymis become structurally differentiated (adult in appearance) by postnatal day 39 (152); this age is consistent with the time-point that has been given by others (150,151). Since spermatozoa were not present in the epididymal lumen at this time, it was concluded that the structural differentiation of principal cells in the rat epididymis was independent of the presence spermatozoa or a factor carried by them (152). Further, since androgens are elevated at this age, they were proposed to play a significant role in the structural differentiation of epididymal principal cells (152). A similar role of androgens has also been proposed for the clear cells of the cauda epididymidis since these cells also become structurally mature on postnatal day 39 (152). In contrast to the cauda epididymidis, clear cells of the caput and corpus epididymidis only become structurally differentiated on postnatal day 49 (152). Thus, luminal factors and/or spermatozoa, but not androgens, have been implicated in the structural differentiation of clear cells in these two regions of the epididymis (152).

A study done by Sun and Flickinger revealed that narrow cells first become apparent in all regions of the epididymis on postnatal day 16 (150). They also reported that narrow cells in the corpus and cauda epididymidis disappeared on postnatal day 35 (150). Interestingly, this disappearance coincided with the appearance of clear cells (150). Thus, based on these observations, they concluded that narrow cells may be the precursors of clear cells in the rat epididymis (150).

4. Vascularization and Innervation of the Epididymis

The epididymis receives its vascular supply from two sources. The vascular

supply to the caput, corpus, and proximal cauda segment of the epididymis is provided by the spermatic artery which branches into the superior epididymal artery (serving the initial segment and caput), the inferior epididymal artery (serving the corpus and proximal cauda) and the testicular artery which provides the main blood supply to the testis (154). Most of the cauda epididymidis, however, receives its blood supply from the vas deferential artery which is a branch of the internal iliac artery (154). The venous drainage of the different parts of the epididymis occurs in a similar pattern to that described for the arterial supply but is mediated by the superior and inferior epididymal veins and the vas deferential vein (154). The vascularization of the testis-epididymis as presented above is found in several mammalian species including the rat, rabbit, mouse, and man (155-158).

The epididymis receives innervation from branches of the autonomic nervous system and contains both adrenergic and cholinergic nerve fibres (159-164). The distribution of these nerve fibres in the epididymis are either peritubular, perivascular, or intramuscular; no intraepithelial localization of neurons has ever been reported (159,165). Innervation of the epididymis is provided by two main nerves: the middle and inferior spermatic nerves (159).

The degree to which the different regions of the epididymis are innervated is apparently related to the amount of smooth muscle present. Thus, in many mammalian species, the initial segment, caput, corpus, and proximal cauda epididymidis are sparsely innervated, whereas the innervation of most of the cauda epididymidis and vas deferens is more prominent due to the increasing prevalence of smooth muscle fibres in these two tissues (160-166). The inferior spermatic artery contains both sympathetic and parasympathetic nerve fibres (159). These nerves form a considerable plexus around the vas deferens. Consequently, the

vas deferens has been a very frequently used tool of neuropharmacologists.

Functionally, innervation of the epididymis is important for both the movement of spermatozoa through the epididymal lumen via spontaneous tubular contractions and the expulsion of spermatozoa from the cauda epididymidis into the vas deferens and subsequently outside the body during ejaculation. The notion that innervation of the epididymis is important for its contractility is supported by the findings that the tissue can respond to adrenergic drugs and other pharmacological stimuli (167-171).

5. Functions of the Epididymis

It has been recognized for more than sixty years, that spermatozoa leaving the testis do not have the capacity to fertilize eggs. In studies done between 1929 and 1931, Young had proposed that the site for the attainment of full spermatozoal maturity was in fact the epididymis (172-174). However, at that time, the epididymis was regarded as a simple conduit for the passive transport of spermatozoa; the key event in the maturation of spermatozoa in the epididymis was perceived to be the passage of time and not specific functions of the tissue. In the late 1960s, the important studies of Orgebin-Crist (101,102) and Bedford (100) revealed that spermatozoa were not simply retained in the epididymis to allow for the "aging" of spermatozoa but rather that the functional maturation of spermatozoa in the epididymis was the result of their exposure to the luminal microenvironment. Thus, the ability of the epididymis to provide the appropriate milieu for the maturation of spermatozoa is considered one of its most important functions. This milieu is created by several processes, most notably the highly active absorptive and secretory activities of the epithelial cells that line the duct. These processes and others are described in the next sections.

a. Absorption

The epithelium of the epididymis is responsible for the absorption of fluid, ions, and numerous micro- and macromolecules that are produced and secreted by the seminiferous tubules. The capacity of the epididymis to absorb material from the lumen was alluded to earlier when endocytic ultrastructural features were described for principal and clear cells of the epididymal epithelium. The most evident feature for the existence of absorption in the mammalian excurrent duct system is the large volume of fluid that is taken up by the efferent ducts and proximal portion of the epididymis. The extent to which fluid is absorbed is remarkable; greater than 90% of the fluid secreted by the rete testis is absorbed by the efferent ducts/proximal epididymis in species such as the bull (175). In the rat, fluid absorption in this region was initially reported to be somewhat lower at around the 50% level (176). However, a more recent study done by Yamamoto et al. (177), would suggest a significantly higher number in the rat, comparable to that seen in other species. Using micropuncture techniques, the amount of rete testis fluid resorbed in the rat was estimated to be 89% and 96% between the efferent ducts and the caput and cauda epididymidis, respectively (177).

The most noticeable effect of the absorption of luminal fluid in the efferent ducts/proximal epididymis is the multifold increase in the concentration of spermatozoa that occurs by the time they reach the cauda epididymidis (125,178,179). Wong et al. have suggested that the mechanism of water transport in the epididymis is one of passive diffusion (180). Further, the proposed ions mediating the absorption of water in the caput and cauda epididymidis are chloride and sodium, respectively (180-182). The fact that the absorption of fluid in the epididymis is an ion-dependent process is consistent with the observation that individuals afflicted with cystic fibrosis (CF) have marked abnormalities at the level

of the excurrent ducts which are characterized by their excessively viscous contents in the epididymis and the absence of the vasa deferentia (183). Individuals with CF present a defect in the gene for the cystic fibrosis transmembrane conductance regulator (CFTR), a type of regulated chloride channel (184). Recent data by Tizzano et al. have indicated that CFTR is expressed in the epididymal epithelium of normal humans (185).

Micropuncture studies have revealed that the ionic composition of epididymal luminal fluid is markedly different in the different regions of the tissue (154,178,186). Thus, these data would dictate that ions are absorbed and/or secreted by the epididymis. The decreases in the intraluminal concentrations of sodium, chloride, and bicarbonate ions that occur from the proximal to the distal segments of the epididymis (154,178) are consistent with the notion that these ions are absorbed. The removal of bicarbonate anion from the luminal fluid of the caput may account for the marked decrease in pH that also occurs in this epididymal segment (154,178). In the rat and boar, the acidification of the epididymal fluid and decrease in bicarbonate anion concentration, that is first observed in the caput epididymidis, is maintained through to the cauda region of the tissue (154,178,187). Wong et al. have proposed that the uptake of sodium and chloride ions in epididymis is hormonally regulated (188,189). Many recent studies, however, have postulated several other factors which can modulate electrolyte transport in the epididymis (183,190-193).

Hinton and Hernandez reported that certain organic molecules, namely L-carnitine and alpha-aminoisobutyric acid (AIB), can be absorbed from the lumen of the epididymis (194,195). First, L-carnitine is selectively taken up from the lumen of the proximal portion of the rat epididymis (194). In the proximal caput epididymidis, its uptake was time-dependent, saturable and predominantly sodium-

independent (194). However, a facilitated transport system for this molecule was not observed in the distal caput epididymidis; in this epididymal region, the absorption of L-carnitine was attributed to passive diffusion (194). The absorption of AIB from the lumen of the caput, corpus, and cauda epididymidis was also demonstrated to be saturable and time-dependent (195). In contrast to L-carnitine, however, the transport mechanism for this neutral amino acid was different depending on the epididymal region studied (195). Thus, these results indicate that in addition to electrolyte transport, the epididymis possesses specific transport mechanisms for the absorption of several organic solutes.

As previously described, the epididymis can take up certain small molecules that are injected into its lumen. In addition to its ability to absorb non-specific markers, the in vivo uptake of specific luminal proteins has also been demonstrated. Data to support this statement come from reports by Turner (123) and Dacheux and Volgamyr (196), showing a general disappearance of luminal proteins between the proximal and distal segments of the epididymis, and others (197-203), demonstrating the endocytosis of specific proteins by epithelial cells in different locations along the duct. Good examples of the latter are found in studies which have characterized the endocytosis of androgen binding protein (ABP), clusterin, oxytocin, and transferrin in the ram (200-202), and alpha-2 macroglobulin (197), ABP (199), clusterin (64), and transferrin in the rat (198). Interestingly, the presence of ABP in the epididymal lumen, has also been demonstrated to be an important determinant in the net uptake of androgens by epididymal tubules in vitro and proposed to be a critical regulator of epididymal nuclear 5α-reductase activity (27,28).

b. Secretion

In addition to absorption, an important contributor to the establishment and maintenance of the epididymal microenvironment is the process of secretion. Similar to its absorptive capacity, the mammalian epididymis secretes various ions, small organic molecules, and a variety of proteins and/or glycoproteins. The most notable ions secreted are potassium and phosphate since their luminal concentrations increase markedly between the rete testis and the cauda epididymidis (178,186). The underlying mechanisms for the secretion of these two ions, however, are poorly understood.

Three low molecular weight organic compounds are secreted by the epididymis: carnitine, glycerylphosphocholine and inositol. Carnitine is found at very high levels in the epididymal lumen (203-205). In the epididymis, this molecule is neither synthesized nor provided by direct testicular secretions via the efferent ducts (206,207). Rather, carnitine is actively taken up from the peripheral circulation and is concentrated in the epididymal luminal fluid (206,208). Carnitine has the potential to be acetylated to acetylcarnitine by both the epididymal epithelium and spermatozoa (205,209). Although the exact reason for the concentration of carnitine in luminal fluid remains unclear, a potential role in the maturation of spermatozoa has been proposed (194,208,209). Further, the total carnitine content of spermatozoa (carnitine and acetylcarnitine) as measured in boars, has been proposed as a useful marker to assess the maturation of spermatozoa (210).

Unlike carnitine, inositol and glycerylphosphocholine can be synthesized by the epididymal epithelium (211,212). In addition to being synthesized by this tissue, inositol can also be transported across the epididymal epithelium and secreted into the lumen of the epididymis thus resulting in very high concentrations

of this sugar in luminal fluid (213-215). The transport of inositol against a concentration gradient has been proposed to be mediated by a specific carrier located at the basolateral membrane of the epididymal epithelium (214). Like many other processes that occur in the epididymis, the secretion of inositol is under the control of androgens (215).

One of the most intensely studied properties of the epididymis is its ability to secrete a variety of proteins and/or glycoproteins. In 1966, Neutra and Leblond were the first to present autoradiographic data that labeled sugars could be synthesized and/or incorporated into glycoproteins by the epididymis (216); subsequent studies by others (217-219) provided similar evidence. Suggestive data that the epididymis was indeed capable of secreting proteins into its lumen also came from several studies which showed that the electrophorectic patterns of proteins found in the epididymis differ markedly from those found in plasma (196,220-222). Brooks (223-225) and others (226-237) have demonstrated that the secretion of many of these proteins is in fact androgen-dependent in many mammalian species. Some of these proteins are able to bind or interact with spermatozoa as they traverse the epididymis and thus, have been proposed to play a role in the maturation of spermatozoa (238). It is important to emphasize, however, that the identity and function of the majority of the proteins that are secreted by the epididymis remain undefined. These proteins are the subject of intensive study at the present time.

c. The Blood-Epididymis Barrier

For many mammalian species, the existence of a morphological and functional blood-testis barrier has been well-established (239,240). As previously described, the tightness of the blood-testis barrier is conveyed via the presence of tight

junctions between adjacent Sertoli cells (239,240). In 1972, Friend and Gilula gave the first ultrastructural evidence for a similar type of barrier in the rat epididymis (138). In the epididymis, tight junctions are present in the adluminal area between adjacent principal cells (138,241-243); their number decreases progressively from the proximal to distal regions of the tissue (241). The development of tight junctional complexes in the epididymis has been described for certain mammalian species such as the mouse and rat (244,245). Hoffer and Hinton reported that the blood-epididymis barrier was effective in blocking the entry of the electron-opaque marker, lanthanum, into the lumen of the epididymal duct (243). Other studies have revealed that in addition to lanthanum, the integrity of the barrier is also resistant to a number of other markers and/or compounds (140,246-248). Importantly, the selective permeability characteristics of the epididymal epithelium have been proposed to play a significant role in establishing its specialized luminal microenvironment (247). However, other important physiological considerations such as the role played by the barrier in protecting epididymal spermatozoa from cytotoxic substances and immunoglobulins remain poorly understood. Although the regulatory mechanisms involved in the formation of cell-cell contacts in the epididymis are poorly defined, recent data by Cyr et al. would suggest that certain cadherins (E-cadherin and P-cadherin) may play important roles in the formation and maintenance of the blood-epididymis barrier (249-251).

d. Transport, Maturation, and Storage of Spermatozoa

Based on its localization within the testicular excurrent duct system, it is not surprising that the most easily recognized function of the epididymis is to provide a physical link for the transport of spermatozoa from the testis to the vas deferens.

As described earlier, the epididymis was originally believed to act only as a passive conduit for the transport of spermatozoa, and that the ability of spermatozoa to mature in the epididymis was a function inherent to spermatozoa themselves and not to some specific function of the tissue (172-174).

The transit time for the passage of spermatozoa through the epididymis has been determined for a number of mammalian species (134); the average epididymal transit time for spermatozoa is approximately 10 days. Many of the factors that contribute to the movement of spermatozoa along the epididymal duct have already been alluded to in this introduction. They include spontaneous contractions of the epididymal tubule in the proximal segments of the epididymis (252,253), muscular contractions occurring during ejaculation in the cauda epididymidis (254), hydrostatic pressure (255), and the beating action of cilia. As previously mentioned, neuronal input to the epididymis plays a significant role in regulating tubular contractility. It is important to note that the relative time that spermatozoa spend in the cauda epididymidis is longer than the other segments of the tissue (134). The reason for this observation is presumably due to the fact that transit time through the cauda epididymidis is directly dependent on ejaculatory frequency whereas in the proximal portions of the tissue, transit time is dependent on spontaneous contractions (256).

Prior to their entry into the epididymis, spermatozoa that have been released by the testis are immotile and are characterized by their inability to fertilize eggs. In contrast, spermatozoa obtained from the cauda epididymis have acquired these functions. Thus, spermatozoa mature (acquire the ability to swim and fertilize eggs) during their transit through the epididymis. Data compiled by Orgebin-Crist have revealed that the epididymal location where spermatozoa first acquire their fertilizing ability is different for different mammalian species (257).

An important feature of these data, however, was that the passage of spermatozoa through the caput epididymidis was critical for the development of fertilizing potential (257). During epididymal transit, a number of structural and biochemical changes in spermatozoa occur in tandem with their acquisition of progressive motility and fertilizing ability. In the rat, the most apparent morphological change is the shedding of the cytoplasmic droplet (258). When spermatozoa are released from the seminiferous epithelium (spermiation), spermatozoa retain a small amount of cytoplasm, termed the cytoplasmic droplet, that surrounds their neck region. As spermatozoa traverse the epididymis, the cytoplasmic droplet migrates down the tail and eventually disappears. The absence of cytoplasmic droplets observed in most spermatozoa found in the cauda epididymidis are an attestation to this fact. After being shed by spermatozoa, the cytoplasmic droplet has been proposed to be endocytosed and subsequently degraded by the clear cells of the corpus epididymidis (134,147).

Biochemically, spermatozoa manifest a marked increase in the number of disulfide bonds during epididymal transit (258-261). Intra- and inter protein disulfide bonds cross-links aid in maintaining the integrity and overall stability of the nuclear structure of spermatozoon. The highly compact nature of the nuclear structure of the male germ cell after epididymal transit must invariably be an important factor in protecting it from untoward damage by certain cytotoxic agents. In addition to changes in disulfide content, alterations in cell surface components of spermatozoa also occur (238,262). Many of these changes are hypothesized to be important in post-epididymal events such as sperm-egg recognition, binding, and gamete fusion. A number of techniques such as the radiolabelling of sperm surface antigens (263-265), lectin binding (264,266), direct biochemical analyses of sperm membranes (265,267,268), and the use of monoclonal and/or polyclonal

antibodies (269-275) have been useful in studying the changes in sperm surface composition that occurs during maturation in the epididymis. The types of changes reported include the addition of epididymal secretory proteins to the sperm surface, the addition, loss or alteration of preexisting sperm surface moieties (e.g., glycosylation), and the masking or unmasking of preexisting sperm surface epitopes.

Complementary to the development of sperm fertilizing ability during passage through the epididymis is the acquisition of progressive motility. Spermatozoa isolated from the caput epididymidis demonstrate circular motion in the absence of forward movement. On the other hand, spermatozoa obtained from the cauda epididymidis manifest considerable forward motility in combination with significant rotational motion around their longitudinal axis. Although the mechanisms underlying the acquisition of motility are unknown, a number of factors, such as cyclic AMP, forward motility protein, carnitine, acidic epididymal glycoprotein and substance P, have been proposed (276-281).

The major site for the storage of spermatozoa in the testicular excurrent duct system of mammals is the cauda epididymidis. Amann reported that in several mammalian species, between 50 to 80% of spermatozoa present in the excurrent ducts are found in this region of the tissue (282). Thus, the cauda epididymidis acts as a reservoir for sperm which is available during ejaculation. Though spermatozoa have the potential to be motile in the cauda epididymidis, they are, in fact, held in an immotile state until ejaculation. In the rat, the factor responsible for the immotility of spermatozoa in cauda epididymal fluid is a high molecular weight protein that has been termed immobilin (283). Immobilin is thought to protect sperm from the mechanical shearing forces that are experienced at the time of ejaculation (284).

C. Regulation of Epididymal Functions

1. The Importance of Androgens

It is well-established that the epididymis is dependent on the presence of the testis for the maintenance of its structure and functions. The dependence of the epididymis on testicular secretions is clearly exemplified by the observation that the tissue shrinks dramatically after castration (removal of the testes). Apart from spermatozoa, an important constituent of testicular secretions are the high levels of androgens, most notably testosterone. Testosterone concentrations in rete testis fluid, which the epididymis directly receives via the efferent ducts, are approximately ten fold higher than those found in the peripheral circulation (125,179,285). In a castrate animal, the ability of the epididymis to respond to exogenously administered testosterone is clearly reflected by the significant increase in tissue weight that is observed. The androgen dependence of epididymal structure and function has been the subject of intensive study. Epididymal processes that have been demonstrated to be under androgen control include the transport of ions (188,189), carnitine (209,286), and inositol (215) across the epididymal epithelium, the synthesis and secretion of many epididymal proteins (223-236), the activity of several enzymes (287-293), the expression of numerous genes (250,294-309), and importantly, the maturation and storage of spermatozoa (103). The fact that the epididymis can mediate an androgenic response is because the tissue possesses androgen receptors. The presence of a specific receptor for androgens in the rat epididymis was first demonstrated by Blaquier in 1971 (310) and confirmed by Ritzen et al. (311) shortly thereafter.

2. Androgen Action in the Epididymis

More than twenty-five years ago, Gorski et al. (312) and Jensen et al. (313)

independently proposed a model describing the basic mechanism by which steroid hormones exert their biological effects on target tissues. Although many revisions of this model have been made since their initial proposal, it is important to point out that many of the elements of their original model are still valid today. Updated models for the mechanism of steroid hormone action have been recently provided by Landers and Spelsberg (314), Carson-Jurica et al. (315), and Rories and Spelsberg (316). A simplified summary of the model presented by Landers and Spelsberg is given below.

In brief, steroids are believed to enter cells by passive diffusion. Intracellular retention of steroids occurs through the formation of stable complexes with specific intracellular receptor proteins. Some of these receptors can be found in the cytoplasm (e.g., the glucocorticoid receptor), however most are intranuclear. The binding of the steroid to its receptor occurs at high affinity, is reversible, and induces a conformational/oligomeric activation of the receptor protein. The "activated" receptor has DNA-binding properties that allows it to recognize and bind with high affinity to specific locations on the chromatin (the nuclear acceptor site). The activated steroid-receptor complex can then act as a transcriptional regulator, initiating a cascade of events that ultimately leads to the synthesis or modification of proteins. This model, although generalized for all steroid hormones, can be applied to androgen action in the epididymis and many other androgen-sensitive target tissues. It must be emphasized, however, that the scheme presented above is simplified in that many other important aspects of the mechanism of steroid hormone action, such as the complex structure of the inactivated hormone receptor which involves heat shock proteins, are omitted. These aspects and others are available in the reviews cited above.

In addition to the androgen receptor acting as a high affinity receptor for

androgens in the epididymis (317), in 1973, Ritzen et al. reported that rat epididymal cytosol contained a second high affinity binding protein, namely androgen-binding protein (ABP) (318). ABP is synthesized and secreted by Sertoli cells (319). This molecule, whose properties are quite different from those of the androgen receptor (311,317), enters the epididymis via the efferent ducts (320). In the ram, ABP is endocytosed by the efferent ducts and proximal segments of the epididymis (201,202). In the rat, ABP has been reported to be present in the proximal region of the epididymis where it is specifically taken up by the principal cells (199,321). Recent in vivo and in vitro data by Felden et al., have suggested that the uptake of ABP from the epididymal lumen occurs via a receptor-mediated mechanism (322,323). In the testis, ABP has been proposed to act as a sink for androgens and then as their carrier to the epididymis (324). In the epididymis, ABP has been shown to be required for the proluminal movement of tritiated testosterone in the caput region of the tissue (27) and has been proposed to be a regulator of nuclear 5α-reductase enzyme activity (28). Further, it has been hypothesized that in the epididymis, ABP acts to deliver androgens directly to the androgen receptor (325).

3. The Role of 5α-Reduced Androgens

Since the initial observations of Benoit, more than six decades ago, which had established that the epididymis is dependent on the presence of testicular androgens (124), several complementary lines of evidence have come together to indicate that the main androgen(s) responsible for maintaining epididymal structure and functions, is(are) the 5α-reduced metabolite(s) of testosterone, DHT and/or 3α-diol. This evidence has arisen from studies which have identified DHT as the active androgen present in epididymal cell nuclei after injection of radiolabelled

testosterone (326), the capacity of the tissue to synthesize 5α-reduced metabolites from testosterone in vitro (327-329), micropuncture studies showing the concentrations of different steroids in epididymal luminal fluid (179), in vivo experiments on the effects of 5α-reductase inhibitors on epididymal function (330), and in vitro experiments indicating that the 5α-reduced metabolites of testosterone are more potent than testosterone itself in maintaining epididymal functions (103). In terms of the maturation and storage of spermatozoa in the epididymis, studies done by Lubicz-Nawrocki (331) and Orgebin-Crist (332) revealed that the 5α-reduced metabolites of testosterone, DHT and 3α-diol, were more potent than testosterone itself in regulating the maturation and storage of spermatozoa in the epididymis.

The rate limiting step in the pathway leading from testosterone to its 5α-reduced metabolites is the reaction catalyzed by the enzyme 4-ene steroid 5α-reductase (EC 1.3.1.22). The enzymatic conversion of testosterone to dihydrotestosterone is manifested over a broad range of rates in several tissues of the male rat (327,333-336). To date, many studies, using enzyme activity as a marker for the protein, have provided a detailed analysis of the regulation of 5α-reductase in different androgen-sensitive male rat tissues (28,337-340). Besides differing rates of conversion, inspection of these studies reveals the *tissue-specific nature* of the regulation of the 5α-reduction of testosterone in the male rat. The following section will provide a tissue-based comparison of the major factor(s) which have been implicated in the regulation of rat 5α-reductase. Due to the prevalence of the enzyme in male rat tissues and the scope of the studies describing its regulation therein, the emphasis will be placed on the testis, pituitary, prostate, and finally the epididymis.

D. Regulation of 5α-Reductase in Male Rat Tissues

1. Cellular Localization of 5α-Reductase

The major approach taken to date to identify the cellular localization of 5α-reductase has been to physically separate the cellular components of a given tissue by enzymatic digestion and then differential centrifugation and to use enzymatic activity as a marker for the protein. This approach has provided fairly clear answers for the pituitary where a detailed cellular fractionation has been done (341,342). 5α-Reductase activity is preferentially localized in rat pituitary fractions enriched in gonadotrophs (341,342). In contrast to the pituitary, there has been considerable controversy as to the cellular localization of 5α-reductase activity in the rat testis and prostate (343-350).

In the testis, several studies have identified 5α-reductase activity in two testicular compartments: the seminiferous tubules and the interstitium (343-348). Most of these studies have indicated that 5α-reductase activity is predominantly localized to the interstitial cell compartment of the testis, particularly in immature rats (344,347,348,351-353). The strongest lines of evidence supporting the premise that 5α-reductase is localized to the interstitial cells are the demonstrations that purified immature Leydig cells contain considerable 5α-reductase activity (354), and that 90% of testicular 5α-reductase activity in the mature rat is lost following Leydig cell destruction by treatment with ethylene-1,2-dimethane sulphonate (EDS) - an effect that was reversed during Leydig cell repopulation (355). It remains unclear, however, as to which other testicular component(s) are responsible for the small percentage of activity that cannot be attributed to Leydig cells. It has been proposed that there is 5α-reductase activity in the seminiferous epithelium (343,346), and specifically in the Sertoli cells and spermatocytes (346).

With respect to the prostate, the controversy has centered on the epithelial versus stromal localization of 5α-reductase. Some studies have indicated that 5α-reductase activity resides in the prostatic epithelium (350,356,357), while others have presented convincing evidence that the stroma contains most of the activity in this tissue (349). Using the technique of isopycnic centrifugation and selective attachment of fibroblast-type cells to plastic dishes, Orlowski and Clark showed an overwhelming predominance of 5α-reductase activity in the epithelium of immature rat prostate (357). In contrast, Djøseland et al. (349) reported higher activity in the stroma when using homogenates of physically separated epithelial and stromal fractions. The discrepancies between these studies may be a result of the method employed in the physical separation of epithelial and stromal tissue fractions (357).

2. Subcellular Distribution of 5α-Reductase

The issue of the subcellular distribution of 5α-reductase has also been controversial. The enzyme is clearly membrane-bound and can be identified primarily in two subcellular compartments: the microsomal and nuclear fractions. Furthermore, the presence of this activity in one or both subcellular fractions is tissue specific. In the prostate, 5α-reductase activity can be found in both of these subcellular fractions (333,358-360). In comparison, most of the studies of the pituitary enzyme report that the activity is selectively localized to the microsomal fraction (361-364); activity in the nuclear fraction, however, has also been reported (365). Similarly, testicular 5α-reductase activity has been identified primarily in the microsomal fraction (366-368); most studies, however, which have characterized testicular 5α-reductase have been limited to using homogenates of whole testis, seminiferous tubules, interstitial tissue, or isolated microsomes. No detailed subcellular fractionation of this enzyme for the testis has yet been

reported.

3. The Role of Androgens in the Regulation of 5α-Reductase

With the notable exception of the testis, 5α-reductase activity in the pituitary and prostate has been shown to be regulated by androgens (338,340). This androgenic regulation is direct in the prostate (338) but only partly direct in the pituitary (369). In the prostate, it has been well-established that testosterone is the prime regulator in the recovery of 5α-reductase enzyme activity following bilateral orchidectomy (338). In contrast to the prostate, orchidectomy results in an increase in 5α-reductase activity in the pituitary, especially in the immature rat; this effect can be reversed by testosterone administration (362-364,370-372). The 5α-reduced metabolites of testosterone, DHT and 3α-diol, are also effective in offsetting the orchidectomy-induced increase in pituitary 5α-reductase activity (362).

Hypophysectomy dramatically decreases testicular 5α-reductase activity in immature and adult rats (366,373). Many studies have indicated that LH treatment can increase testicular 5α-reductase activity in hypophysectomized rats (366,373-375). It remained possible, however, that the stimulatory effect of LH could be mediated by an increase in testosterone production by Leydig cells. This possibility was eliminated by studies by Nayfey et al. (376) and Chase and Payne (375) which demonstrated that the increase in testicular 5α-reductase activity generated by LH is independent of testosterone production. Therefore, in the testis it is clear that androgens do not have a predominant role in the regulation of 5α-reductase activity.

4. The Influence of Pituitary Hormones on the Regulation of 5α-Reductase

In the testis, it is well-established that LH has a strong influence on the regulation of 5α-reductase activity (366,374-376). The stimulatory effect of LH on testicular 5α-reductase activity occurs in both immature and mature hypophysectomized rats (366,374), and has been reported to be potentiated by prolactin (374,375). Furthermore, the LH-induced increase in prepubertal testicular 5α-reductase activity is a result of direct stimulation of Leydig cells by LH - an effect that can be mimicked by 8-bromo-cAMP (354). In contrast to LH, the role of FSH has been controversial (354,376). It is clear, however, that FSH does not directly stimulate 5α-reductase activity in Leydig cells, at least in the immature rat (354). It has been suggested that the reported stimulation of testicular 5α-reductase by FSH in vivo (376) may be the result of an indirect mechanism occurring through Sertoli cells (354).

Similarly, pituitary 5α-reductase activity has been reported to be correlated with FSH levels in vivo (370,377) and stimulated by exposure to this gonadotropin in vitro (340). Neither LH nor prolactin, however, have effects on pituitary 5α-reductase activity when given in vitro or in vivo (340). In marked contrast to the testis and pituitary, little role, if any, has been attributed to pituitary hormones in the direct regulation of 5α-reductase activity in the prostate (338).

5. Regulation of 5α-Reductase During Sexual Maturation

It has been well-established that the ability of rat tissues to convert testosterone to DHT does not remain constant during sexual maturation. Characteristically, age-dependent fluctuations in 5α-reductase activity in the rat are tissue-specific (327,340). In the prostate, 5α-reductase activity increases post-pubertally and reaches a stable plateau in adulthood (327). In sharp contrast

to this pattern, 5α-reductase activity in the pituitary decreases dramatically from birth onwards (372). In the testis, 5α-reductase activity is undetectable shortly after birth, reaches a sharp peak between days 25 and 35, and then declines to almost undetectable levels by adulthood (345,352). As previously described, LH has been suggested to be the major factor directly regulating the early maturational rise in this activity (354). To date, however, the factor(s) regulating the post-pubertal decline in 5α-reductase activity remain elusive.

6. The Physiological Relevance of Tissue-Specific Regulation

It is evident that the regulation of the conversion of testosterone to DHT in the male rat is a tissue-specific phenomenon. This revelation raises an important physiological question: Why is there a need for such disparate regulatory mechanisms in different rat tissues? The answer to this question can be partly answered by the hypothesis that the 5α-reduction of testosterone serves different functions in different tissues.

In the testis, it has been suggested that the conversion of testosterone to DHT may play an important role in the initiation of spermatogenesis and the onset of puberty in the rat (376). Consistent with this hypothesis is the rise of 5α-reductase activity and its potential to be stimulated by LH just prior to the onset of puberty. In the mature rat, testosterone is the main testicular androgen secreted into the circulation. Testosterone is subsequently 5α-reduced to the active androgen, DHT, at its site of action. Therefore, the regulation of testicular 5α-reductase is also compatible with the ability of testosterone to act as a prehormone, since at the time when Leydig cells begin actively secreting testosterone, 5α-reductase activity is already rapidly decreasing. In androgen dependent sex accessory tissues, regulation of 5α-reductase by androgens in a

positive, feed-forward mechanism, such as that which occurs in the prostate, would be entirely consistent in a tissue whose size and functions are so tightly linked to the presence of androgenic hormones.

E. Regulation of Epididymal 5α-Reductase Enzyme Activity

The ability of the rat epididymis to synthesize the biologically active androgen, DHT, from testosterone has been well-documented (327-329,378-380). At the subcellular level, it has been established that the activity of this enzyme is present in both the nuclear and microsomal fractions of epididymal homogenates (381). 5α-Reductase enzyme activity is expressed in a striking positional gradient in the adult rat epididymis (337). The activity associated with the nuclear fraction is highest in the proximal part of the epididymis (initial segment of the caput epididymidis) and declines dramatically as one moves distally along the tissue.

The regulation of epididymal 5α-reductase activity is complex. The activity of the enzyme found in the nuclear fraction is markedly decreased after bilateral orchidectomy, and simultaneous, exogenous testosterone administration can only partially offset this observed decrease, even when given at very high doses (328). Efferent duct ligation and unilateral orchidectomy both result in a dramatic decrease in epididymal 5α-reductase activity, especially in the proximal portion of the tissue (328,382). Thus, it has been proposed that nuclear epididymal 5α-reductase activity is regulated in a paracrine manner by a substance directly entering the epididymis via the efferent ducts and not the general circulation (28,337).

Several studies by Robaire and his collaborators were undertaken to identify the origin of the paracrine substance regulating 5α-reductase enzyme activity (28,383,384). In the first study, administration of testosterone to intact

adult rats at a dose which suppressed epididymal sperm reserves to near zero in the caput-corpus epididymidis had no effect on nuclear 5α-reductase activity when compared to control (28). This indicated that the paracrine factor regulating epididymal nuclear 5α-reductase activity was not spermatozoa or a factor associated with spermatozoa. This was further confirmed through developmental studies which showed that the profile of nuclear 5α-reductase enzyme activity was not associated with the appearance of spermatozoa as they entered the tissue (383). In a final study, adult rats were hypophysectomized and then simultaneously treated with different doses of exogenously administered testosterone (384). After hypophysectomy, nuclear 5α-reductase activity was markedly decreased; this decrease, however, was preventable by the administration of testosterone (384). Importantly, the dose which maintained nuclear epididymal 5α-reductase activity also maintained spermatogenesis and consequently, the secretions from Sertoli cells (384). Taken together, these data allowed them to propose that the paracrine factor regulating nuclear epididymal 5α-reductase activity is most likely of Sertoli cell origin and that the synthesis of this factor is testosterone-dependent (28,337,384). Furthermore, three complementary lines of evidence had led them to speculate that the paracrine factor in question was in fact androgen binding protein (ABP) (28). First, this evidence stems from the fact that the expression of epididymal nuclear 5α-reductase enzyme activity and ABP show similar developmental profiles (383,385). Second, ABP is a Sertoli cell product whose synthesis is under the control of androgens (386). Third, ABP is actively endocytosed in the proximal segment of the epididymis through a potential receptor mediated mechanism (199,322,323), and hence, the epididymal region where nuclear 5α-reductase activity is highest and most sensitive to the paracrine regulation.

In marked contrast to the nuclear activity, microsomal 5α-reductase activity is found throughout the epididymis and is expressed at a lower level (337). The results of a developmental study which followed the profile of epididymal microsomal 5α-reductase activity revealed that the developmental pattern of expression was coincidental with the pattern for serum androgens in the male rat (381). As a result, epididymal microsomal 5α-reductase enzyme activity was proposed to be regulated by the level of circulating androgens (381).

F. Molecular Characterization 5α-Reductase

For many years, the progress towards understanding the molecular properties and regulation of 5α-reductase has been slow due to the difficulty in purifying the 5α-reductase enzyme. Though numerous attempts have been made at purifying this enzyme, this has not been achieved successfully. 5α-Reductase is a membrane-bound enzyme. The inherent problem in purifying 5α-reductase has been the observation that its activity is rapidly lost under conditions which remove the enzyme from its native membrane-bound environment. Unfortunately, the solubilization of a membrane-associated protein is an absolute prerequisite in the classical approach of protein purification. Thus, the development of the necessary probes (gene, cDNA, and specific antibodies) to study the molecular properties and regulation of 5α-reductase have presented a considerable challenge for researchers specifically interested in 5α-reductase and those interested in the field of androgen action in general.

However, in the late 1980s, the road to obtaining genetic and immunocytochemical probes for 5α-reductase was paved by the results of a novel study by Farkash et al. (387) which clearly demonstrated that 5α-reductase activity could be detected in a stable form in Xenopus oocytes that were injected with

poly(A) mRNA obtained from rat liver. Thus, using the technique of expression cloning in Xenopus oocytes, the cloning of a cDNA for rat and human 5α-reductases were reported by Andersson and Russell shortly thereafter (388,389). Sequence analysis of the rat cDNA revealed that the 5α-reductase encoded was a highly hydrophobic protein with a predicted molecular weight of 29 kilodaltons (388). Later, this figure was revised to 26 kilodaltons when the actual size of the expressed protein in mammalian cells was determined (390). Further, the activity of the expressed enzyme was found to be inhibited by a number of known 5α-reductase inhibitors, thus confirming that the isolated cDNA did, in fact, encode a 5α-reductase enzyme (389,391). Northern blotting experiments revealed that this activity was encoded by an mRNA of approximately 2.5 kb in rat liver and prostate (388). The regulation of this mRNA in the prostate was subsequently shown to be regulated by testosterone in a type of feed-forward mechanism (392).

In the human, interest in understanding the genetic regulation of 5α-reductase stems from its proposed role in several endocrine abnormalities such as benign prostatic hyperplasia, male pattern baldness, acne, and hirsutism. In addition to these disorders, deficiency of the 5α-reductase enzyme leads to a rare from of male pseudohermaphroditism in which affected males develop normal internal urogenital tracts but fail to develop normal male external genitalia (392). Thus based on the latter observation, it was surmised that Southern blot analyisis of genetic material from 5α-reductase deficient individuals using the 5α-reductase cDNA probe would show a marked defect (deletion) in the 5α-reductase gene. To their astonishment, however, the 5α-reductase gene was apparently unaffected (393). This observation led these investigators to the conclusion that perhaps more than one 5α-reductase gene was expressed in the human. Other evidence to suggest that there was, in fact, more than one 5α-reductase in the human was

the observation that 5α-reductase blockers (e.g., finasteride), which are apparently effective at decreasing intraprostatic and serum DHT levels in humans (394,395), poorly inhibit the expressed human 5α-reductase (389).

In 1991, evidence for a second 5α-reductase gene was confirmed by the cloning of another human cDNA that encoded 5α-reductase enzyme activity (396). Unlike the first 5α-reductase gene, this second gene was deleted and/or mutated in 5α-reductase deficient individuals (396-399). When expressed in mammalian cells, this isozyme was strongly inhibited by finasteride (396). One year later, a second 5α-reductase gene was also identified in the rat (400). Thus at present, at least two cDNAs encoding for different 5α-reductase isozymes exist in the human and the rat. Based on their chronological order of identification, these isozymes have been termed 5α-reductase types 1 and 2. Since their discovery, the basic gene structure and chromosomal locations of the human 5α-reductase isozymes have been reported (397,401,402). Although the isolation of the rat genes have been mentioned in the literature, their sequences have not yet been provided. It is important to emphasize, however, that the mechanisms that control 5α-reductase gene expression in different mammalian tissues has only begun to be examined. Moreover, the physiological roles played by each of the two 5α-reductase isozymes in the human and rat also remain largely unknown.

G. Formulation of the Project

Several complementary lines of evidence in the literature have come together to indicate that the primary androgen(s) responsible for maintaining epididymal structure and function is(are) the 5α-reduced metabolites of testosterone DHT and/or 3α-diol. In the epididymis and many other androgen-sensitive target tissues, the rate limiting step in the formation of DHT and 3α-diol

is the enzymatic conversion catalyzed by 5α-reductase. Since the early to mid 1970s, a large body of data has accumulated on the properties of this enzyme and the way its activity is regulated in the epididymis and other tissues. Despite the numerous studies done to understand 5α-reductase enzyme activity, the underlying molecular mechanisms that control the expression of this enzyme have remained elusive. It is important to emphasize that the elucidation of these mechanisms is essential for a complete understanding of the regulation of 5α-reductase, whether this be in the epididymis or in other tissues. The recent cloning of the cDNAs and genes for 5α-reductase has provided some of the tools which can be used to gain insight into certain molecular aspects of the enzyme.

The goal of the present thesis is to use the techniques of molecular biology to extend our knowledge of epididymal 5α-reductase through studies which will characterize the regulation of its messenger ribonucleic acids and protein. Such studies have been done only in a very limited number of androgen-dependent tissues but not in the epididymis. The first two data chapters of this thesis (chapters II and III) will characterize the expression, endocrine dependence, and developmental regulation of the 5α-reductase type 1 mRNA in the rat epididymis. In chapter IV, a similar strategy will be used to characterize the regulation of the type 2 transcript. Chapter V will examine the effect of aging on the gene expression of several markers of epididymal function. In chapter VI and its subsequent appendix, the characterization and regulation of epididymal type 1 protein expression will be presented. Thus, these studies should provide some insight into the molecular mechanisms that control the expression of epididymal 5α-reductase.

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Chapter II

Differential Regulation of Steady State 4-Ene Steroid 5α-Reductase Messenger Ribonucleic Acid Levels along the Rat Epididymis

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Abstract

Epididymal nuclear 5α-reductase enzyme activity is regulated by a testosterone-dependent factor from the testis. Regulation at the mRNA level, however, has not been investigated. Endocrine manipulation experiments were designed to determine whether 5α-reductase is regulated at the steady state mRNA level. Steady state mRNA concentrations were assessed using the full-length cDNA for 5α-reductase type 1. Longitudinal distribution showed that the highest mRNA concentrations were present in the initial segment of the caput epididymidis and were 3-7 fold higher than in the other tissue segments. The androgen dependence of the mRNA levels for 5α-reductase type 1 was assessed by bilateral orchidectomy and simultaneous testosterone replacement therapy. One week after surgery, mRNA concentrations in orchidectomized rats were decreased to 15% of control levels in the initial segment of the caput epididymidis and 40-50% of control levels in the remaining epididymal segments. Administration of testosterone at a dose which mimics normal serum concentrations (2.5 cm Silastic implant) restored 5α-reductase type 1 mRNA concentrations to control levels in the corpus and cauda epididymidis but were not significantly different from orchidectomized levels ($P \geq 0.05$) in the initial segment and caput epididymidis. Administration of testosterone at a dose designed to approximate 5-8 fold normal serum concentrations (18.6 cm implant) maintained 5α-reductase type 1 mRNA concentrations at only 50% of control levels in the initial segment while complete maintenance was observed in the rest of the tissue. The effects of unilateral orchidectomy revealed that 5α-reductase type 1 mRNA concentrations decrease selectively in the initial segment of the orchidectomized side. This is the first report that epididymal 5α-reductase is regulated at the mRNA level and that the regulation is different with respect to the

segment being studied.

Introduction

The mammalian epididymis is the site where spermatozoa mature and are then stored (1,2). The maintenance of epididymal functions is known to be directly dependent on the presence of androgens (3-5). This androgenic control is mediated by the 5α-reduced metabolites of testosterone, 5α-dihydrotestosterone (DHT) and 5α-androstan-3α,17ß-diol (3α-diol), and probably not testosterone itself (6,7). The conversion of testosterone to DHT in the rat epididymis is catalyzed by the enzyme 4-ene steroid 5α-reductase (EC 1.3.1.22) (5α-reductase) (8,9); this activity is present in both the nuclear and microsomal fractions of epididymal homogenates (10,11).

The endocrine regulation of epididymal 5α-reductase activity has been well described (12-18). The activity of epididymal nuclear 5α-reductase decreases markedly after bilateral orchidectomy and simultaneous testosterone replacement therapy can only partially offset the observed decrease (12). It was thus proposed, that some factor other than testosterone regulated epididymal nuclear 5α-reductase (14). The results of experiments on the effect of unilateral orchidectomy on epididymal 5α-reductase indicated that an intact connection between the testis and epididymis was necessary to maintain its activity, and that the regulatory factor was of testicular origin (14). The profile of enzymatic activity with respect to development indicated that the factor was not carried by spermatozoa as they enter the epididymis (15). After hypophysectomy with testosterone replacement therapy, it was found that epididymal 5α-reductase was not directly controlled by gonadotropin-mediated Leydig cell secretions, but rather by a factor from the testis which was itself under androgen control and was probably of Sertoli cell origin (16).

To date, our understanding of the regulation of epididymal 5α-reductase

has not gone beyond the level of enzyme activity. Knowledge of any regulation beyond this level has been limited due to the lack of specific probes for the enzyme (cDNA or antibody). The recent cloning of the rat 5α-reductase type 1 cDNA from female rat liver (19) has made it possible now to understand regulation of the enzyme at the mRNA level. Therefore, the goal of the present study was to determine whether epididymal 5α-reductase is regulated at the mRNA level.

To answer this question, we examined the endocrine dependence of the steady state mRNA concentrations for the type 1 enzyme. Our results indicate that the epididymal enzyme is regulated at the mRNA level; further, the nature of this regulation is dependent on the segment of the epididymis being studied.

Materials and Methods

Animals

Adult male Sprague-Dawley rats weighing 250-300g (Charles River (Canada), St. Constant, QC) were used for all experiments. They were maintained on a 14-h light, 10-h dark schedule and were given food and water ad libitum.

Experiment 1 - Tissue Distribution

Animals were killed by decapitation and the epididymides, vasa deferentia, ventral prostate, pituitary, liver, kidneys, and seminal vesicles were removed and immediately frozen in liquid nitrogen. Epididymides were dissected free of fat and sectioned into different segments prior to freezing as previously described (20). The initial segment of the caput epididymidis is represented by section 1, the caput by 2 and 3, the corpus by 4, and the cauda epididymidis by sections 5 and 6. Five groups, each consisting of tissues pooled from three rats, were used. Tissues were stored at -70°C prior to RNA isolation.

RNA Isolation

Total cellular RNA was isolated according to the method described by Sambrook with slight modifications (22). Frozen tissues were homogenized in at least five volumes of guanidinium thiocyanate solution (4.0 M guanidine thiocyanate, 0.1 M Tris (pH 7.5), 1% ß-mercaptoethanol) using a Brinkman Polytron (Rexdale, ON). The homogenate was then layered onto a cushion of cesium chloride (CsCl), (5.7 M CsCl, 0.01 M EDTA, pH 7.5) and centrifuged at 25°C in a Beckman SW 60 rotor for at least 17 hours at 40,000 rpm. Following the centrifugation, the RNA pellet was resuspended in 60 µl of 0.01 M Tris, 0.001 M EDTA, and 0.1% sodium dodecyl sulfate (SDS), (TE/SDS, pH 7.5). The total cellular RNA was reprecipitated by adding 3 M sodium acetate, pH 5.2, to a final concentration of 0.3 M, and 3 volumes of ethanol and was stored for at least 2 hours at -20 C before pelleting. The RNA sample was washed with 70% ethanol, resuspended in diethyl pyrocarbonate-treated water (ICN Biomedicals Canada, Mississauga, ON) and stored at -80 C until further use. The total cellular RNA concentration was measured by the absorbance at 260 nm (Beckman DU7 Spectrophotometer).

Northern Blot Analysis

Twenty-five micrograms of total cellular RNA were denatured prior to electrophoresis by adding formaldehyde, formamide, and borate buffer (0.1 M sodium borate, 0.1 M boric acid, 0.001 M EDTA, pH 8.0) to final concentrations of 6%, 40%, and 0.02 M respectively, and heating the mixture for 5 minutes at 65°C. The denatured RNA samples were separated in a 1% agarose gel containing 0.02 M borate buffer and 16% formaldehyde. The gel was electrophoresed in recirculated 0.02 M borate buffer at 20 V for 17 hours at 25°C. The separated

RNA was subsequently transferred by vacuum blotting for 2 hours using the Vacugene blotting system (Pharmacia-LKB Biotechnology, Uppsala, Sweden) to Genescreen Plus nylon hybridization membranes (DuPont Canada, Mississauga, ON). To facilitate the vacuum transfer, the agarose gel was pretreated with 0.05 M NaOH, 0.01 M NaCl for 20 minutes, and was then neutralized with 0.1 M Tris, pH 7.5 for 10 minutes. The final transfer buffer was 1.5 M NaCl, 0.15 M sodium citrate (10xSSC, pH 7.0). The membrane was rinsed in transfer buffer, allowed to air-dry and was baked at 80 C for 2 hours in a vacuum oven before hybridization.

Membrane hybridizations were done according to the manufacturer's recommendations with slight modifications. The membranes were prehybridized for at least 1 hour at 42°C in a solution consisting of 50% formamide, 10% dextran sulfate (Pharmacia Canada, Baie D'Urfe, QC), 1% SDS, and 1 M NaCl. Denatured salmon sperm (200µg/ml) and radiolabelled probe were then added to the hybridization solution.

The probe used was a complementary DNA (cDNA) corresponding to the full length mRNA transcript for female rat liver 5α-reductase type 1 (19). The cDNA probe was radiolabelled by random priming using an oligolabelling kit (Pharmacia Canada) and 3000 Ci/mmol dCTP (Amersham Canada) to a specific activity of (1-5)x10^8 dpm/µg DNA. Labelled probe was added to the hybridization solution at 10^6 cpm/ml and was allowed to incubate for 16 hours at 42°C. The membranes were washed twice at 25°C with 2xSSC, twice at 65°C with 2xSSC, 1% SDS, once with 0.1xSSC at 25°C, and once with the same buffer at 65°C.

To monitor the amount of total RNA loaded in each lane, the filters were hybridized with a synthetic ^{32}P-labelled oligonucleotide which is specific for 18S ribosomal RNA (23). The oligonucleotide was end-labelled by standard procedures (22). Hybridization conditions were 6xSSC, 5x Denhardt's solution,

0.5% sodium pyrophosphate, 1% SDS, 100 µg/ml denatured salmon sperm, and 18S probe (10^6 cpm/ml) at 42°C for 17 hours. The filters were washed twice with 6xSSC, 0.5% pyrophosphate at 25°C followed by two washes with 6xSSC, 0.5% pyrophosphate, and 1% SDS at 55°C.

Between hybridizations, the probes were removed by boiling the filters in 0.01 M Tris (pH 7.5), 0.001 M EDTA, and 1% SDS for at least 30 minutes. The membranes were exposed to Kodak X-Omat AR film (Eastman Kodak, Rochester, NY) with intensifying screens (DuPont Canada) at -70°C.

RNA Quantitation

The autoradiograms were scanned using an LKB Ultrascan XL Laser Densitometer (Pharmacia-LKB Biotechnology, Uppsala, Sweden). To confirm the total amount of RNA present in each lane, the integrated areas obtained for the 5α-reductase type 1 probe were normalized to the areas obtained for the 18S ribosomal RNA oligonucleotide probe. The scanning values obtained were within the range of values shown to be linear with a slope of 1 when using defined amounts of total liver RNA hybridized to the 5α-reductase type 1 probe. The mRNA concentrations presented herein are representative of steady state mRNA values; these values are a result of the production (transcription) and degradation (stability) of the mRNA.

Statistical Analysis

Statistical analysis was done by one-way analysis of variance followed by a Newman-Keuls test to identify significant differences (24).

Results

Distribution of 5α-Reductase Type 1 mRNA in Adult Rat Tissues.

The presence of the mRNA for 5α-reductase type 1 was assessed by Northern blot analysis as seen in Fig. 1. The Northern blot showed the mRNA transcript in several tissues which are also known to possess 5α-reductase enzyme activity (8). The size of the hybridizing bands were all identical and at approximately 2.5 kb; this is the same as that which has been reported for female rat liver (19).

The most intense hybridizing signal was observed in the liver, followed by the initial segment of the caput epididymidis. Quantitation of the Northern blots by laser densitometry (Fig. 2) showed that the highest steady state 5α-reductase type 1 mRNA concentration, observed in the liver, was just under 3 fold higher than the epididymal initial segment concentration.

In the epididymis, the steady state 5α-reductase type 1 mRNA concentrations were spatially distributed. The initial segment concentration was 3-7 times higher than that of the other epididymal segments. The steady state concentrations of mRNA in the caput, corpus, and cauda epididymidis, however, were not significantly different from each other ($P \geq 0.05$). The ventral prostate and pituitary showed similar steady state mRNA concentrations; these were comparable to that of the caput epididymidis. The vas deferens and kidney had low amounts of the mRNA for the enzyme while in the seminal vesicle, the level was barely detectable.

Effect of Orchidectomy and Simultaneous Testosterone Replacement Therapy on Steady State 5α-Reductase Type 1 mRNA Concentrations

Accessory sex tissue weights were measured as an index of biologically

active circulating androgens; the weights of these tissues and of the epididymis are shown in Table 1. One week after bilateral orchidectomy, a significant decrease in tissue weight ($P \leq 0.05$) was observed in the ventral prostate, seminal vesicles and epididymides of the Orc-0 group of animals when compared to the controls. In the Orc-1 group, ventral prostate and seminal vesicle weights were not significantly different from control ($P \geq 0.05$). Epididymal weight, however, was significantly lower than control ($P \leq 0.05$); an effect that is consistent with a previous report (12). In the Orc-3 group, ventral prostate and seminal vesicle weights were significantly elevated ($P \leq 0.05$) when compared to either the control or Orc-1 groups.

The effect of bilateral orchidectomy and simultaneous testosterone replacement therapy on 5α-reductase type 1 mRNA was determined by Northern blot analysis (Fig. 3); the steady state mRNA concentrations obtained by laser densitometric scanning of these Northern blots are shown in Fig. 4. In the initial segment of the caput epididymidis, steady state 5α-reductase type 1 mRNA concentrations were dramatically decreased to 15% of control values in the Orc-0 group. The small and large testosterone implants, Orc-1 and Orc-3, did not maintain steady state mRNA concentrations at control levels. Concentrations were less than 20% of control values in the Orc-1 group (not significantly different from the Orc-0 group ($P \geq 0.05$)). In the Orc-3 group, however, steady state mRNA concentrations were only 50% of control levels, but were significantly higher than the Orc-1 group ($P \leq 0.05$).

In the caput epididymidis, steady state mRNA concentrations were one-half of control levels in the Orc-0 group, and only 62% of control in the Orc-1 group. Steady state mRNA concentrations were 87% of control levels in the Orc-3 group (not significantly different from control ($P \geq 0.05$)).

In the corpus and cauda epididymidis, steady state mRNA concentrations were half of control levels in the Orc-0 group. They were maintained in the corpus at 80% and 90% of control levels in the Orc-1 and Orc-3 groups, respectively and in the cauda at 92% and 115% of control levels in the Orc-1 and Orc-3 groups respectively; none of these values significantly differed from control or each other ($P \geq 0.05$).

Effect of Unilateral Orchidectomy on Steady State 5α-Reductase Type 1 mRNA Concentrations

The effect of unilateral orchidectomy, three days post-surgery, on 5α-reductase type 1 mRNA in the initial segment and caput epididymidis is shown in Fig. 5. The Northern blot clearly demonstrates that a dramatic decrease in 5α-reductase type 1 mRNA occurred in the initial segment of the caput epididymidis on the side ipsilateral to the unilateral orchidectomy when compared to the contralateral side or the control animals. There was also no evidence of right-left differences in the control animals.

The decrease in 5α-reductase type 1 mRNA in the initial segment was in marked contrast to the effect in the caput epididymidis, where after three days post-unilateral orchidectomy, there was no change in steady state 5α-reductase type 1 mRNA (Fig. 5). The absence of an effect was also observed for the corpus and cauda epididymidis (data not shown).

To confirm these observations at three days post-surgery, the effect of unilateral orchidectomy was also investigated after seven and fourteen days. The results, summarized in Fig. 6, demonstrate that the results obtained three days after surgery are maintained for at least a two-week period. Steady state 5α-reductase type 1 mRNA concentrations in the ipsilateral initial segment were

10-18% of the average mRNA concentrations in the control initial segments. In the ipsilateral caput epididymidis, no change occurred at three days, but at seven and fourteen days, 75% of control levels was observed. In the ipsilateral corpus and cauda epididymidis, no changes in steady state 5α-reductase type 1 mRNA concentrations occurred at any time point.

Discussion

The present study was designed to determine whether epididymal 5α-reductase is regulated at the mRNA level. This is the first report that the steady state mRNA concentrations for the type 1 enzyme are differentially regulated along the rat epididymis.

It has been well established that 5α-reductase enzyme activity is not uniformly distributed along the epididymis in most species examined thus far (25-28). In the rat epididymis, nuclear 5α-reductase enzyme activity is found primarily in the initial segment of the caput epididymidis, whereas the microsomal form of the enzyme is more evenly distributed (26). In addition, the specific and total nuclear 5α-reductase enzyme activity is higher than the microsomal activity (11). We observed that the distribution of steady state 5α-reductase type 1 mRNA concentrations was qualitatively similar to what has been reported for the activity of the nuclear enzyme. This close association suggests that the longitudinal distribution of 5α-reductase enzyme activity in the rat epididymis is determined to a large extent by the steady state type 1 mRNA concentrations.

Longitudinal distributions of steady state mRNA concentrations in the rat epididymis have been reported for other genes (29,30). The necessity for epididymal 5α-reductase to produce 5α-dihydrotestosterone for the maturation of spermatozoa is clear (3). Since maturation is essentially complete by the time

spermatozoa reach the cauda epididymidis (31), it is not surprising that the mRNA for 5α-reductase is preferentially concentrated in the initial segment of the caput epididymidis.

It is of interest to note that the initial segment of the caput epididymidis had the second highest steady state 5α-reductase type 1 mRNA concentration of the tissues examined. This is surprising since Gloyna and Wilson (8) reported that 5α-reductase enzyme activity in the prostate is greater than in the epididymis. The exact reason for this observation is unknown but it clearly underscores the differential regulation of 5α-reductase which exists in these two tissues.

Bilateral orchidectomy and testosterone replacement therapy were used to determine whether the steady state mRNA for epididymal 5α-reductase type 1 is under androgen control. Since the epididymis receives rete testis fluid which has 10-12 fold higher androgen concentrations than normal serum (32), bilaterally orchidectomized rats were not only given implants which mimicked normal serum testosterone concentrations (Orc-1) but were also given implants that result in highly elevated serum testosterone concentrations (Orc-3) (12).

The observation that bilateral orchidectomy was more effective in decreasing 5α-reductase type 1 mRNA concentrations in the initial segment of the caput epididymidis when compared to the rest of the tissue is consistent with what has been reported for enzyme activity (12). Interestingly, low dose testosterone replacement therapy could maintain control steady state 5α-reductase type 1 mRNA concentrations in the corpus and cauda epididymidis. For the caput epididymidis, maintenance occurred only with the high testosterone dose. However, it was not possible to maintain the steady state 5α-reductase type 1 mRNA concentrations in the initial segment of the caput epididymidis. Therefore, this is the first report to indicate steady state 5α-reductase type 1 mRNA

concentrations are differentially regulated along the rat epididymis.

It has been previously shown that testosterone replacement therapy in the orchidectomized rat could not maintain 5α-reductase enzyme activity at control levels in either the caput-corpus or cauda epididymidis, four weeks after surgery (12). The apparent difference between the steady state mRNA and enzyme activity levels suggests that translational and/or post-translational regulation may also be occurring. It cannot however yet be ruled out that this difference is due to the fact that the effects on steady state mRNA were measured after one week, while those on enzyme activity were determined after four weeks of treatment.

The inability of testosterone replacement therapy to completely maintain steady state 5α-reductase type 1 mRNA at control levels in the initial segment of the caput epididymidis is also consistent with the hypothesis that a factor other than testosterone can regulate epididymal 5α-reductase (26,27). Therefore, testosterone alone can regulate the steady state 5α-reductase type 1 mRNA concentrations in the corpus and cauda of the rat epididymis, but in the initial segment of the caput epididymidis another factor is also responsible for the regulation.

It has been suggested by Fawcett et al. (33), that an intact connection between the testis and epididymis is required to maintain the morphology of principal cells, more so in the initial segment of the caput epididymidis than in the rest of the tissue. Therefore, the necessity for a direct conduit between the testis and epididymis to maintain steady state 5α-reductase type 1 mRNA was assessed through unilateral orchidectomy. We demonstrate that only the steady state 5α-reductase type 1 mRNA concentrations in the initial segment of the caput epididymidis were dramatically affected by unilateral orchidectomy. The consistent decrease to 10-18% of control values at all three time points was similar to the

decrease that was observed in the bilaterally orchidectomized rat and reinforces the argument that a factor, other than testosterone but which is of testicular origin, is the prime regulator of the steady state mRNA levels for 5α-reductase type 1 in the initial segment of the caput epididymidis.

The 25% decrease in steady state 5α-reductase type 1 mRNA concentrations in the caput epididymidis at seven and fourteen days post-unilateral orchidectomy is consistent with the observation that normal serum testosterone in the low dose testosterone replacement did not maintain steady state 5α-reductase type 1 mRNA concentrations at control levels. The lack of a decrease in steady state 5α-reductase type 1 mRNA concentrations in the corpus and cauda epididymidis was also consistent with the testosterone replacement experiment. Therefore, the results of the unilateral orchidectomy experiment support the differential and multifactorial regulation of 5α-reductase that is present along the rat epididymis.

Unilateral orchidectomy decreases 5α-reductase enzyme activity in the ipsilateral caput-corpus and cauda epididymidis five weeks after surgery (14). Again the difference between enzyme activity and steady state mRNA concentrations suggests that regulation at other levels may also be occurring. It is of interest to note that a lack of correlation between the steady state mRNA level and the protein level has been reported for other types of proteins in male reproductive tissues (34).

We can speculate that the differential regulation is caused by selectivity of the testicular factor for the initial segment of the caput epididymidis. The mechanism of this selectivity is unknown but may reside in the stability of the factor along the length of the epididymis. In this case, movement of the factor beyond the initial segment of the caput epididymidis would be associated with increased

instability. The factor would then only impart its regulatory effects on 5α-reductase in the initial segment of the caput epididymidis. Since most of the epididymal 5α-reductase type 1 message and enzyme is located in this segment, the 5α-dihydrotestosterone produced and released in the lumen in this particular segment could regulate the mRNA for the enzyme in the other segments.

The present data on the regulation of steady state 5α-reductase type 1 mRNA concentrations in the rat epididymis is markedly different from what has been reported for the liver and ventral prostate (19). In the liver, bilateral orchidectomy increased mRNA concentrations, an effect which was not decreased by testosterone replacement. In the ventral prostate, bilateral orchidectomy was shown to have little or no effect but testosterone replacement resulted in a dramatic increase in mRNA concentrations for type 1 5α-reductase. This gives strong evidence that the regulatory differences in 5α-reductase enzyme activity in the liver, ventral prostate, and epididymis are due, in part, to differences in regulation at the steady state mRNA level.

Together, these experiments give conclusive evidence that epididymal 5α-reductase is regulated at the mRNA level and that the nature of this regulation is dependent on the epididymal segment being studied. The fact that a testicular factor and testosterone can regulate steady state type 1 mRNA concentrations for epididymal 5α-reductase suggests that the regulation is multifactorial; presumably acting at the levels of mRNA transcription and/or stability. Furthermore, the differences between steady state mRNA concentrations and enzyme activity after bilateral orchidectomy and testosterone replacement or unilateral orchidectomy strongly suggest that regulation at other levels may also be occurring.

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Table 1. Accessory sex tissue weights 7 days after bilateral orchidectomy and testosterone replacement therapy.

	Treatment groups			
	Co	Orc-0	Orc-1	Orc-3
Ventral prostate	363 ± 63	74 ± 12	383 ± 90	628 ± 21
Paired seminal vesicles	322 ± 39	142 ± 17	426 ± 32	651 ± 30
Paired epididymides	882 ± 29	332 ± 37	724 ± 41	820 ± 51

Rats were either left intact to serve as controls (Co), or were bilaterally orchidectomized and simultaneously implanted with blank PDS capsules (Orc-0) or testosterone-filled capsules measuring 2.5 cm (Orc-1) and 18.6 cm (Orc-3), respectively. Values are expressed in milligrams as the mean ± SEM (n=4).

Figure 1. A, Northern blot of adult rat tissues. Twenty-five micrograms of total cellular RNA were electrophoresed in a 1% agarose, formaldehyde-containing gel and transferred to nylon membrane, as described in *Materials and Methods*. A cDNA probe corresponding to the full-length mRNA transcript for 4-ene steroid 5α-reductase type 1 was used in the hybridizations. Lanes 1-4 correspond to the epididymis. Lane 1, initial segment; 2, caput; 3, corpus; 4, cauda; 5, vas deferens; 6, ventral prostate; 7, pituitary; 8, liver; 9, kidney; 10, seminal vesicle. The 28S and 18S ribosomal RNA markers are indicated on the *left*. The blots were autoradiographed for 3 days at -70 C. B, Reprobe of the Northern blot with a synthetic oligonucleotide complementary to the 18S ribosomal RNA.

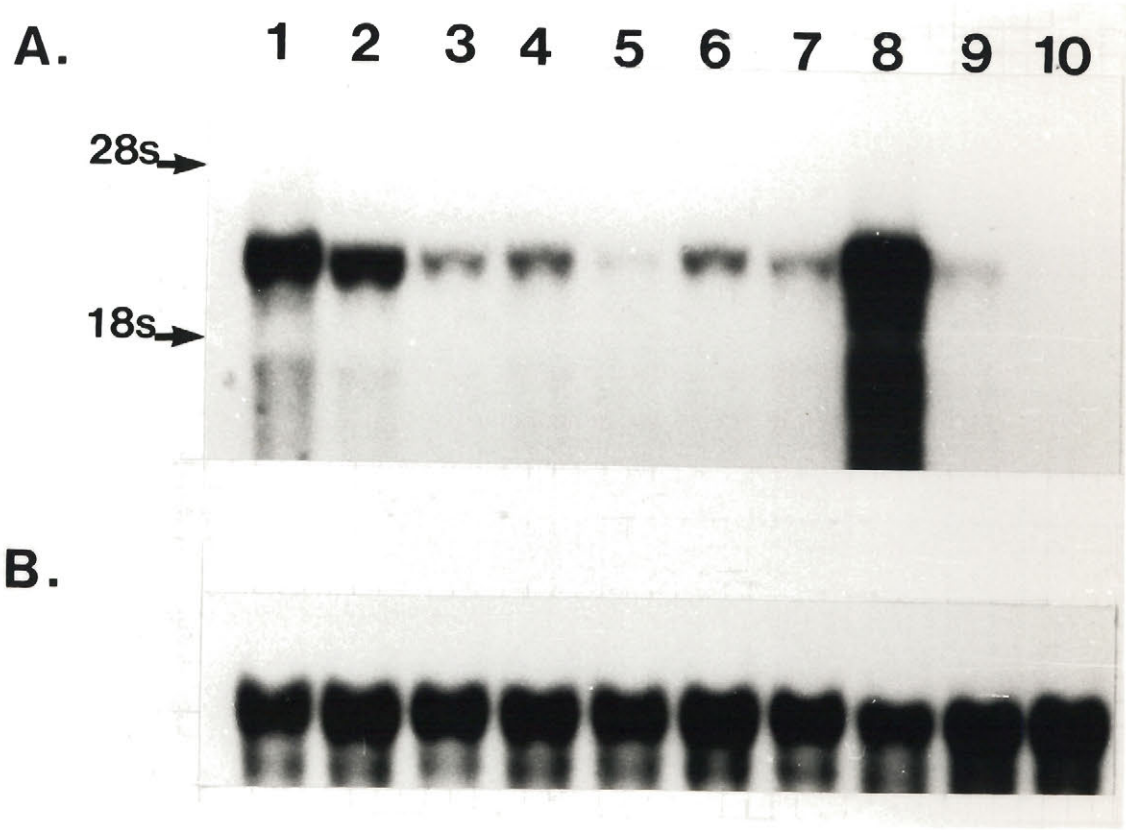

A.
1 2 3 4 5 6 7 8 9 10
28s
18s
B.

Figure 2. Steady state 4-ene steroid 5α-reductase type 1 mRNA concentrations in adult rat tissues. The hybridizing bands obtained in the Northern blots were scanned by laser densitometry and normalized to the corresponding 18S ribosomal RNA. Values are expressed as the mean percentage of the initial segment concentration ± SEM (n=5).

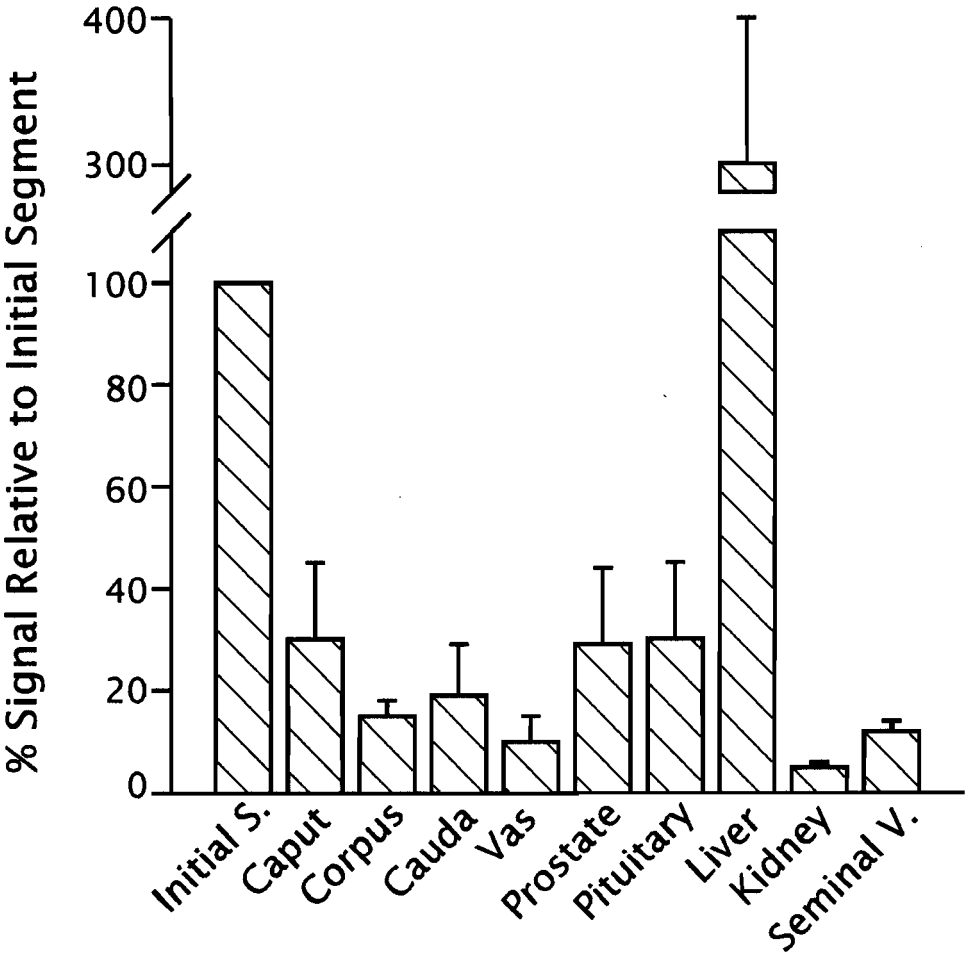

% Signal Relative to Initial Segment
400
300
100
80
60
40
20
0
Initial S.
Caput
Corpus
Cauda
Vas
Prostate
Pituitary
Liver
Kidney
Seminal V.

Figure 3. A, Northern blot analysis of total cellular RNA obtained from epididymal tissues 7 days after bilateral orchidectomy and testosterone replacement. Twenty-five micrograms of total cellular RNA were present per lane and hybridized with the 4-ene steroid 5α-reductase type 1 cDNA probe. The 28S and 18S ribosomal RNA markers are indicated on the *left*. The blots were autoradiographed for 3 days at -70 C. B, Reprobe of the Northern blots with the 18S ribosomal oligonucleotide probe.

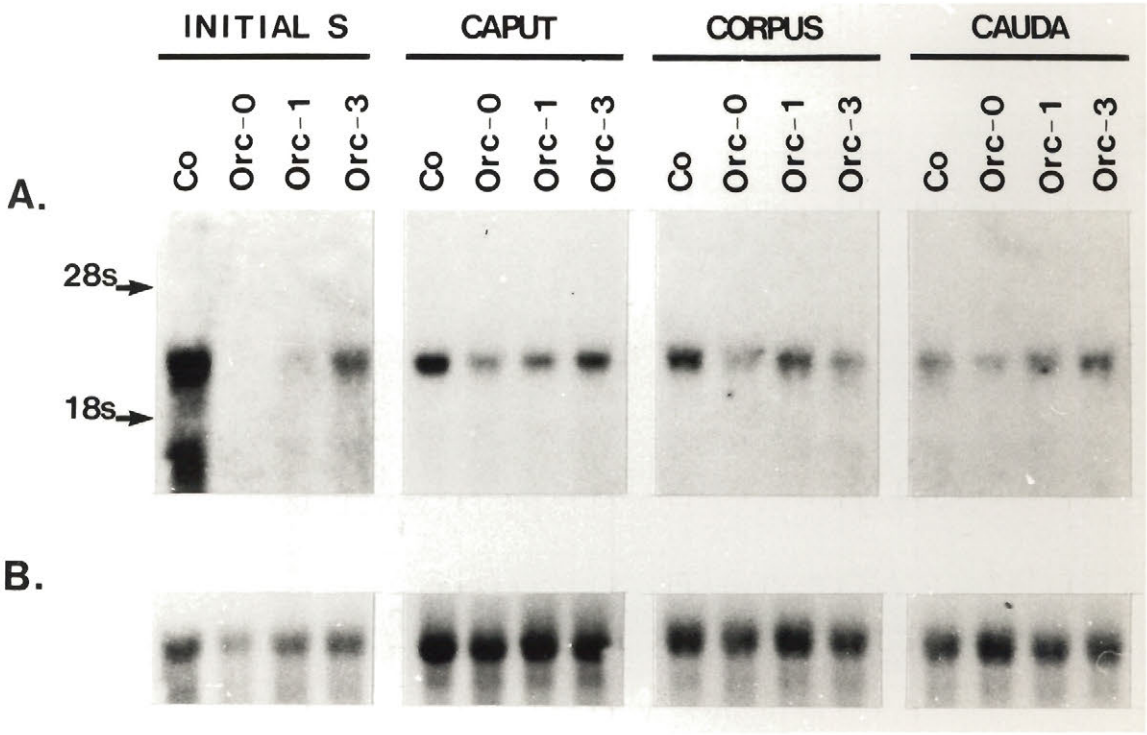
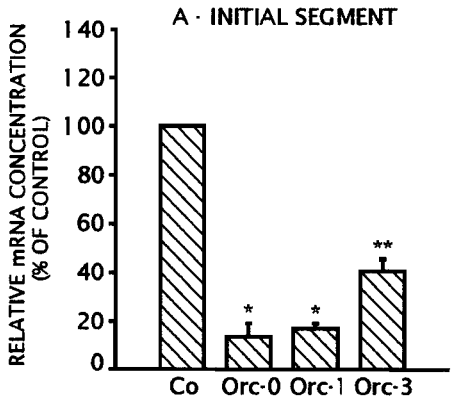
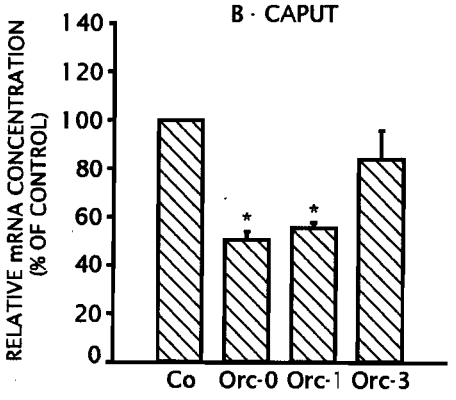
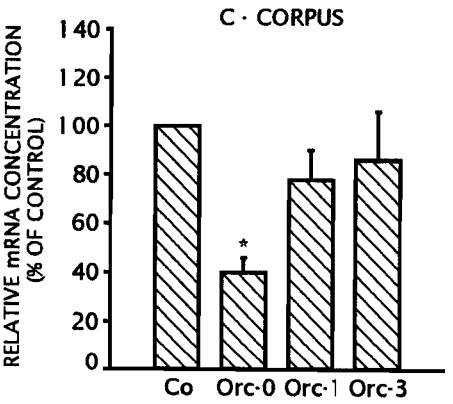

INITIAL S
CAPUT
CORPUS
CAUDA
Co
Orc-0
Orc-1
Orc-3
A.
28s
18s
B.

Figure 4. Steady state 4-ene steroid 5α-reductase type 1 mRNA concentrations in the sectioned rat epididymis 7 days after bilateral orchidectomy and testosterone replacement. The Northern blots were scanned by laser densitometry and normalized to the corresponding 18S ribosomal RNA values. Results are expressed as the mean percentage of the control value ± SEM (n=3). (*) significantly different from control ($P \leq 0.05$), (**) significantly different from control and Orc-1 ($P \leq 0.05$).


A · INITIAL SEGMENT
RELATIVE mRNA CONCENTRATION (% OF CONTROL)
140
120
100
80
60
40
20
0
*
*
**
Co
Orc-0
Orc-1
Orc-3


B · CAPUT
RELATIVE mRNA CONCENTRATION (% OF CONTROL)
140
120
100
80
60
40
20
0
*
*
Co
Orc-0
Orc-1
Orc-3


C · CORPUS
RELATIVE mRNA CONCENTRATION (% OF CONTROL)
140
120
100
80
60
40
20
0
*
Co
Orc-0
Orc-1
Orc-3

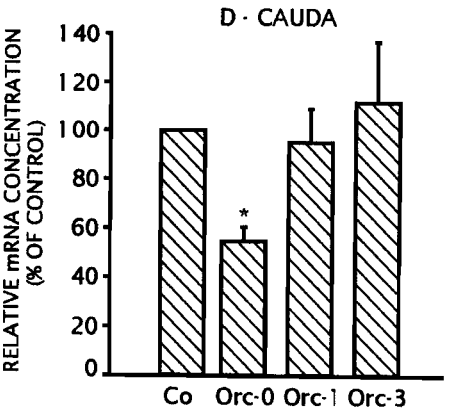

D · CAUDA
RELATIVE mRNA CONCENTRATION (% OF CONTROL)
140
120
100
80
60
40
20
0
*
Co
Orc-0
Orc-1
Orc-3

Figure 5. A, Northern blot analysis of total cellular RNA from the initial segment and caput epididymidis three days following unilateral orchidectomy. Twenty-five micrograms of total cellular RNA were present per lane and hybridized with the 4-ene steroid 5α-reductase type 1 cDNA probe. The 28S and 18S ribosomal size markers are indicated on the *left*. The blot was autoradiographed for 3 days at -70 C. B, Reprobe of the Northern blots with the 18S ribosomal oligonucleotide probe.

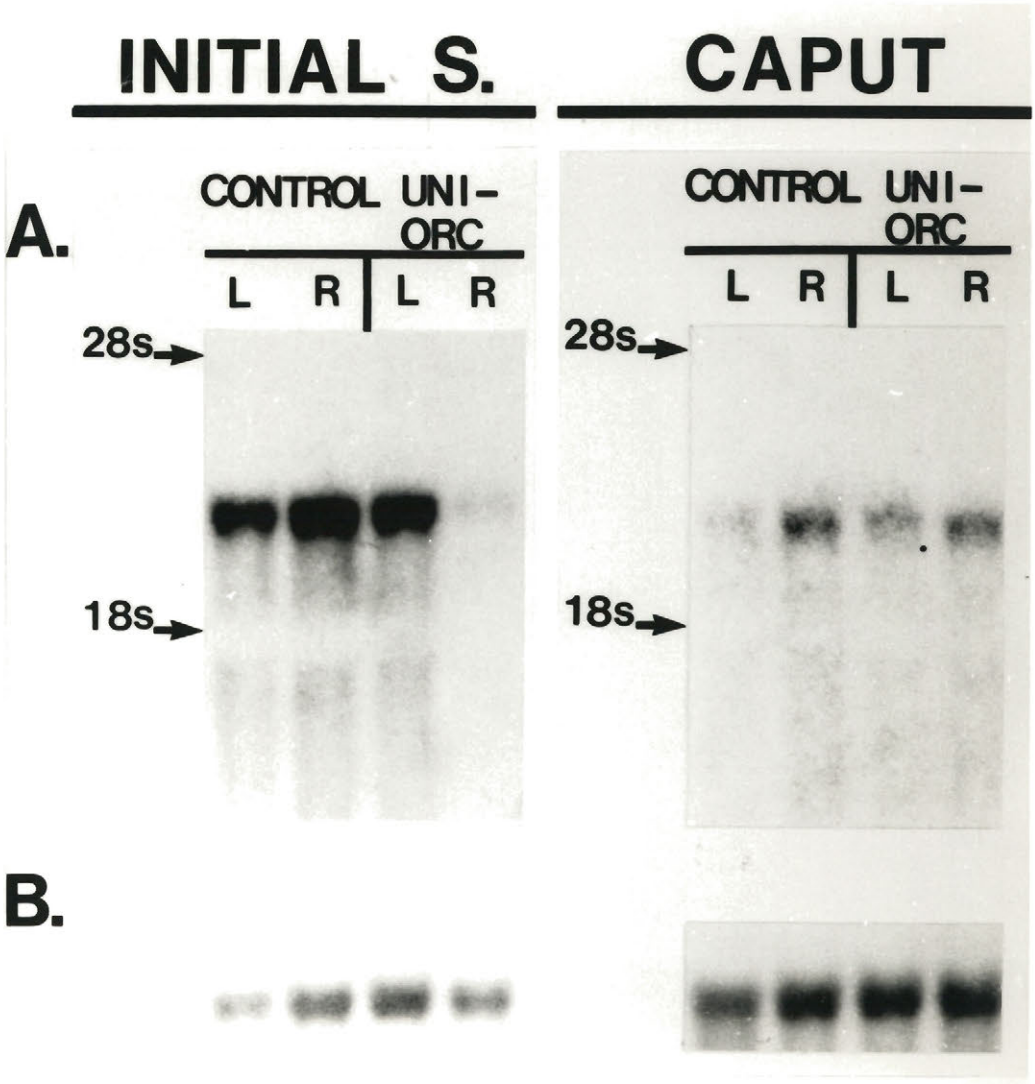
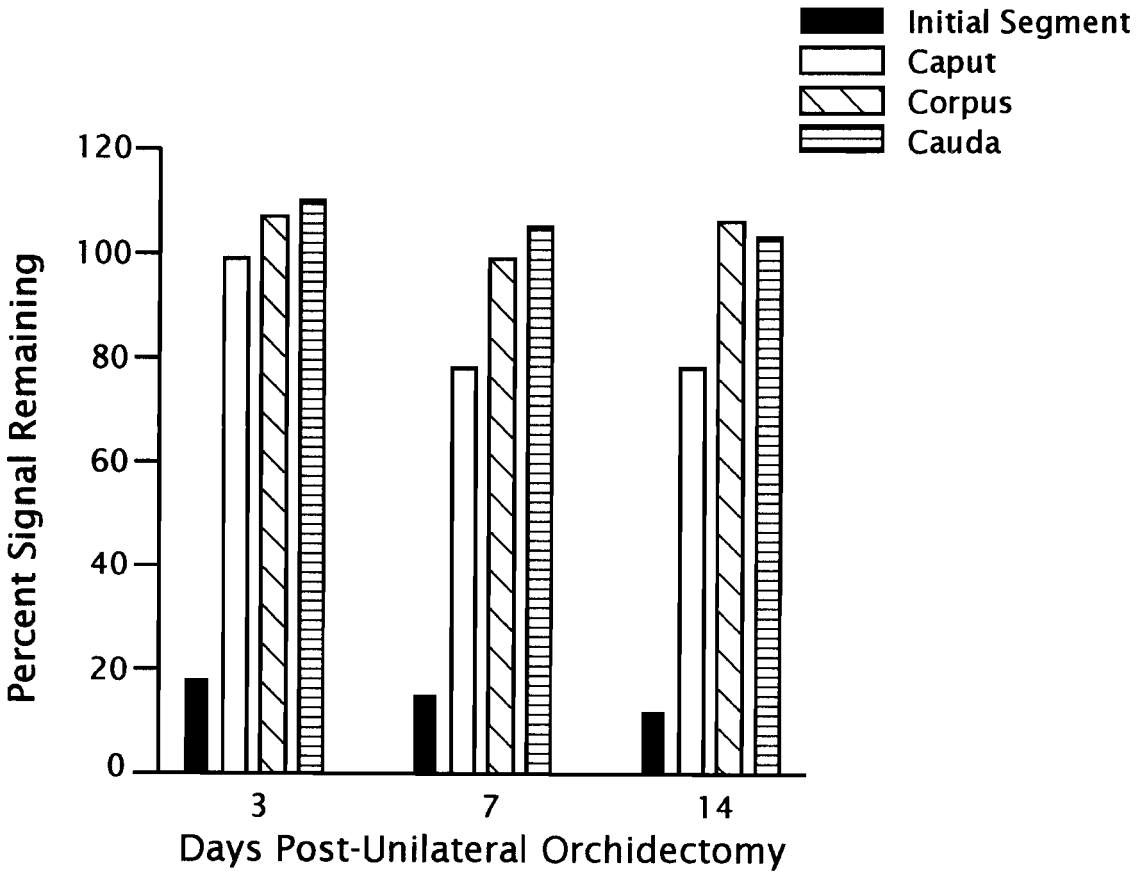

INITIAL S.
CAPUT
A.
CONTROL
UNI-ORC
L R L R
28s
18s
CONTROL
UNI-ORC
L R L R
28s
18s
B.

Figure 6. Time-course of the effect of unilateral orchidectomy on steady state 4-ene steroid 5α-reductase type 1 mRNA concentrations in the sectioned rat epididymis. Northern blots were scanned by laser densitometry and normalized to the corresponding 18S ribosomal RNA values. The results are expressed as the percent signal remaining in the ipsilateral side of the experimental animal (UNI-ORC, right side) when compared to the average of the right and left sides of the control animal.


Initial Segment
Caput
Corpus
Cauda
Percent Signal Remaining
120
100
80
60
40
20
0
3
7
14
Days Post-Unilateral Orchidectomy

Appendix

Northern Blot Quantitation: Linearity of Laser Densitometer

Several methodologies exist which can be used to measure the relative concentration of a particular mRNA species in a sample of total cellular RNA. The different mRNA detection methods that have been described include the techniques of Northern, slot-blot, and solution hybridization (1-4). Solution hybridization is a sensitive detection method designed primarily for the rapid quantitation of specific mRNAs in multiple samples (3,4). In contrast, the more time-consuming slot-blot and Northern hybridization methods permit the screening of several mRNAs from a single RNA sample through multiple reprobings of the same membrane (1,2). It is important to note, however, that of the different mRNA detection methods available, only Northern blot analysis allows for the simultaneous determination of molecular size and assessment of RNA integrity. Thus, in the studies presented in this thesis, Northern blot analysis was the method chosen to characterize and quantitate relative mRNA expression in the rat epididymis.

Northern blots were quantitated with the aid of an LKB Ultrascan laser densitometer. To accurately compare relative mRNA levels between different experimental groups, the linearity of the densitometer with respect to increasing RNA concentrations had to be first established. For this purpose, a series of Northern blots containing defined amounts of total cellular RNA obtained from adult male rat liver was constructed. The 5α-reductase type 1 cDNA was used as hybridization probe. Different absorbance ranges were obtained by varying the exposure time of the autoradiograms; representative exposures are shown in Fig. 1. It is clear from Fig. 1 that both signal intensity (absorbance) and signal size increase as a function of increasing RNA amount. Therefore, relative mRNA concentrations were expressed in arbitrary units as the integrated areas under the curve (absorbance x band width). The results for the low and high area ranges are presented in Tables 1 and 2, respectively. To validate the quantitated data, two

criteria had to be fulfilled: (1) the measured mRNA concentration must increase linearly with increasing RNA amount and (2), the slope of this linear increase must approach the value of 1, that is, a doubling of RNA amount should result in a doubling of the measured mRNA concentration.

To test these criteria, the data were analyzed by simple linear regression. Before the regression analysis, however, the data had to be adjusted by a common factor in order to define 1 µg RNA equal to 1 unit of concentration; this adjustment was done separately for each area range. The adjusted concentration values were plotted as a function of increasing RNA amount; three representative regression curves are shown in Fig. 1. Thus, under these conditions absolute linearity is reflected by how close the slope of the regression curve with a Y intercept of 0 approaches the value of 1. The slopes obtained from the regression curves for the low and high area ranges are given in Tables 1 and 2, respectively.

The results obtained from the regression analysis conclusively demonstrate that the quantitation of Northern blots by laser densitometry conforms to the stipulated criteria for linearity. First, it is apparent from the regression curves shown in Fig. 1 that the measured mRNA concentrations increase linearly with increasing RNA amount. Second, the slopes of the regression curves from both the low and high area ranges were close to the ideal value of 1 (Tables 1 and 2). This range was found be: $0.04 \leq$ absolute integrated area ≤ 15.31. Therefore, the scanning values obtained in the mRNA expression studies presented in chapter I and the subsequent chapters of this thesis were consistently restricted to this linear range.

References

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3. **Durnam DM, Palmiter RD** 1983 A practical approach for quantitating specific mRNAs by solution hybridization. Anal Biochem 131:385-393
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Table 1. Linearity of laser densitometer with respect to mRNA concentrations (low range).

Amount of RNA (μg)	mRNA Concentration (arbitrary units)	Adjusted value (arbitrary units)	Regression Analysis Slope	R Squared
Range 1				
1	0.040	1.00		
3	0.128	3.20		
5	0.276	6.90	1.16 ± 0.04	0.97
7.5	0.362	9.05		
10	0.443	11.07		
Range 2				
1	0.073	1.00		
3	0.129	1.87		
5	0.315	4.24	0.84 ± 0.03	0.98
7.5	0.531	6.85		
10	0.576	8.15		
Range 3				
3	0.290	3.00		
5	0.544	5.63	1.11 ± 0.05	0.99
7.5	0.846	8.75		
10	1.048	10.84		

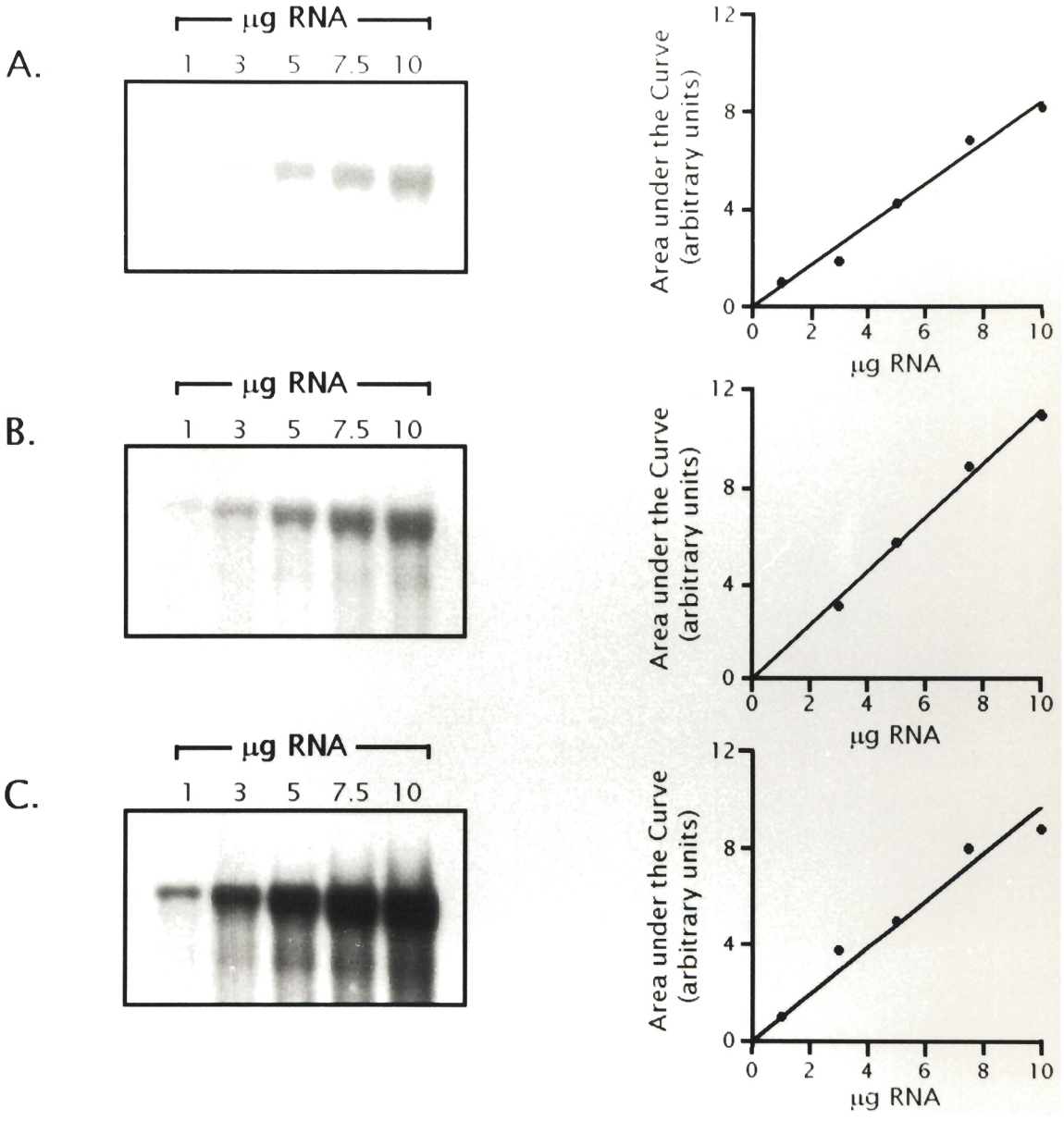
Adult male rat liver RNA samples (1-10 μg) were Northern blotted using the 5α-reductase type 1 cDNA probe. A series of different absorbance ranges were obtained by varying exposure time. For each range, absolute mRNA concentrations (the integrated peak areas in arbitrary units) were adjusted by a common factor in order to define 1 μg RNA equal to 1 concentration unit. Using the adjusted values, the linearity of the densitometric readings (proximity to slope =1) was calculated by simple linear regression.

Table 2. Linearity of laser densitometer with respect to mRNA concentrations (high range).

Amount of RNA (µg)	mRNA Concentration (arbitrary units)	Adjusted value (arbitrary units)	Regression Analysis Slope	Regression Analysis R Squared
Range 1				
1	0.452	1.00		
3	1.697	3.75		
5	2.270	5.02	0.97 ± 0.05	0.95
7.5	3.615	7.99		
10	3.978	8.80		
Range 2				
3	1.335	3.00		
7.5	3.702	8.31		
10	4.331	9.73	0.83 ± 0.05	0.89
15	5.357	12.03		
20	6.744	15.15		
Range 3				
3	2.540	3.00		
7.5	7.400	8.74		
10	10.390	12.27	1.00 ± 0.09	0.79
15	15.310	18.08		
20	13.680	16.16		

Adult male rat liver RNA samples (1-10 and 3-20 µg) were Northern blotted using the 5α-reductase type 1 cDNA probe. A series of different absorbance ranges were obtained by varying exposure time. For each range, absolute mRNA concentrations (the integrated peak areas in arbitrary units) were adjusted by a common factor in order to define 1 µg RNA equal to 1 concentration unit. Using the adjusted values, the linearity of the densitometric readings (proximity to slope =1) was calculated by simple linear regression.

Figure 1. Expression of 5α-reductase type 1 mRNA as a function of increasing total cellular RNA obtained from adult male rat liver. *Left panel*, Northern blots indicating the hybridizing 5α-reductase type 1 message. *Right panel*, quantitated data, as determined by laser densitometric scanning of the corresponding Northern blots. The curves were obtained by simple linear regression. A and B represent data taken from low ranges 1 and 3, respectively, of Table 1; C represents data taken from high range 1 of Table 2.


A.
µg RNA
1 3 5 7.5 10
B.
µg RNA
1 3 5 7.5 10
C.
µg RNA
1 3 5 7.5 10
Area under the Curve (arbitrary units)
µg RNA
0 2 4 6 8 10
0 4 8 12
Area under the Curve (arbitrary units)
µg RNA
0 2 4 6 8 10
0 4 8 12
Area under the Curve (arbitrary units)
µg RNA
0 2 4 6 8 10
0 4 8 12

Connecting Text - Chapter II to Chapter III

In chapter II, three complementary studies were used to characterize the endocrine regulation of 5α-reductase type 1 mRNA in the rat epididymis. This regulation was clearly demonstrated to be multifactorial and epididymal segment-specific. In the next chapter, postnatal development will be used to further characterize the regulation of 5α-reductase type 1 mRNA expression in the rat epididymis where many key developmental events are well-defined. These events include the formation of the blood-epididymal barrier, the rise in serum androgens, and the first appearance of spermatozoa. Therefore, postnatal development has proven to be an invaluable tool for elucidating potentially important regulatory factors in the expression of many epididymal proteins. In chapter III, the age dependence of 5α-reductase type 1 mRNA expression will be described using two separate developmental studies. In the first, the epididymis will be grossly subdivided into only two regions and many different postnatal ages will be examined in order to identify a developmental effect. In the second study, fewer ages will be selected but the epididymis will be sectioned into many regions in order to characterize both inter- and intra-segment specific changes in 5α-reductase type 1 mRNA expression.

Chapter III

Expression of 4-Ene Steroid 5α-Reductase Messenger Ribonucleic Acid in the Rat Epididymis during Postnatal Development

Robert S. Viger and Bernard Robaire

Abstract

The regulation of epididymal 5α-reductase type 1 mRNA is multifactorial and segment-specific. In order to further investigate the regulation of the message for the enzyme, the expression of 5α-reductase type 1 mRNA in the rat epididymis was studied as a function of postnatal development. Developmental changes in 5α-reductase type 1 mRNA concentrations were assessed by probing Northern blots with the full-length cDNA for rat steroid 5α-reductase type 1. In the first experiment, the effect of postnatal age on 5α-reductase type 1 mRNA concentrations in the caput-corpus and cauda epididymidis was studied. Male rats, taken at one week intervals between the ages of 7 and 91 days, were used. In both epididymal regions, the mRNA for 5α-reductase type 1 was present at all ages examined; it appeared in the immature animal at least two weeks before detectable 5α-reductase enzyme activity. In the caput-corpus epididymidis, mRNA levels for 5α-reductase type 1 decreased by one-half between postnatal days 7 and 21, rose 5 fold by day 56 and then remained constant through to day 91. No change with postnatal age, however, was observed in the cauda epididymidis. In the second experiment, the longitudinal distribution of 5α-reductase type 1 mRNA at postnatal days 21, 42, 49, 56, 77, and 91 was studied. The mRNA levels for 5α-reductase type 1 increased remarkably, by 6-7 fold, in the initial segment of the caput epididymidis between postnatal days 21 and 42 and stayed constant thereafter. However, no significant change comparable to that found for the initial segment was observed in the adjacent proximal caput region or in any of the other epididymal segments. Thus, the 5 fold rise in 5α-reductase type 1 mRNA concentrations that occurred in the caput-corpus epididymidis in the first experiment can be attributed solely to changes in the initial segment. We conclude that steady state concentrations of epididymal 5α-reductase type 1

mRNA vary dramatically at different postnatal ages and are highly specific with respect to epididymal segment.

Introduction

Spermatozoa are transported from the testes to the vas deferens via the epididymis in mammals. During transit, spermatozoa are rendered mature in the caput-corpus epididymidis and are then stored in the cauda epididymidis (1,2). Although the mechanisms underlying spermatozoal maturation and storage in the epididymis have yet to be fully resolved, these processes have been shown to be dependent on androgens (1,3). Androgenic control of these epididymal functions is mediated by the 5α-reduced metabolites of testosterone, dihydrotestosterone (DHT) and androstan-3α,17ß-diol (3α-diol), and apparently not by testosterone itself (4,5).

Testosterone is converted to the more potent androgen, DHT, by the enzyme 4-ene steroid 5α-reductase (5α-reductase; EC 1.3.1.22). The ability of the epididymis to metabolize testosterone to DHT has been well-documented (6-11); this activity is present in both the nuclear and microsomal fractions of epididymal homogenates (9,12). The endocrine regulation of epididymal 5α-reductase enzyme activity has been investigated by a number of authors (10,13-16). Regulation of the enzyme found in the microsomal fraction has been suggested to be androgen-dependent (12). Regulation of the enzyme found in the nuclear fraction, however, has been shown to be critically dependent upon a testicular factor secreted directly into the epididymis via the efferent ducts (17,18).

The cloning of the cDNA for rat 5α-reductase type 1 has allowed for the extension of our understanding of the regulation of 5α-reductase to the mRNA level (19). A previous study from our laboratory demonstrated the presence and endocrine regulation of the mRNA for 5α-reductase type 1 in the rat epididymis (20). A single hybridizing species of 2.5 kilobases was observed; this is identical to the 5α-reductase type 1 message that has been reported in other rat tissues

(19-22). The endocrine regulation of epididymal 5α-reductase type 1 mRNA was shown to be both multifactorial and epididymal segment-specific (20); the multifactorial nature of this regulation is consistent with what has been described at the enzyme activity level (23).

In the present study, postnatal development is used as a means to delineate additional factors in the regulation of epididymal 5α-reductase type 1 at the mRNA level. Our results indicate that the expression of 5α-reductase type 1 mRNA in the rat epididymis does change significantly during postnatal development. A segment by segment analysis revealed that this change was most striking in the initial segment of the epididymis.

Materials and Methods

Animals

Timed-gestation pregnant Sprague-Dawley rats, purchased from Charles River Canada (St. Constant, Québec, Canada) were maintained on a 14-h light, 10-h dark lighting schedule and given food and water *ad libitum*. One day after birth, female offspring were removed and the remaining males were randomized throughout the litters. Animals were weaned on postnatal day 21. Normal development of the animals was monitored by assessing gains in body weight, paired testicular, paired epididymal, and accessory sex tissue weights. These growth curves were comparable to those previously observed (24).

Experimental Design

Experiment 1. Effect of Postnatal Age on 5α-Reductase Type 1 mRNA Concentrations in the Caput-Corpus and Cauda epididymides

Animals were ether anaesthetized and then killed by cervical dislocation.

Epididymides were dissected free of fat and sectioned into caput-corpus and cauda regions as shown in Fig. 1A. The total number of animals used at postnatal days 7, 14, 21, 28, 35, 42, 49, 56, 63, 77 and 91 were the following: 30, 30, 21, 15, 12, 9, 9, 6, 6, 6, and 6, respectively. The number of animals used at each postnatal age was optimized to obtain sufficient tissue for RNA isolation. Epididymal tissues obtained from each postnatal age were pooled into three distinct groups. Therefore, each age was examined in triplicate. Epididymal tissues were frozen in liquid nitrogen and stored at -80 C prior to RNA isolation.

Experiment 2. Effect of Postnatal age on the Longitudinal Distribution of 5α-Reductase Type 1 mRNA in the Epididymis

Animals were ether anaesthetized and killed by cervical dislocation. Epididymides were obtained and sectioned into 6 regions as depicted in Fig. 1B. The vas deferens was also used in this study. The total number of animals used at postnatal days 21, 42, 49, 56, 77, and 91 were the following: 21, 12, 9, 9, 6, and 6, respectively. The time-points were chosen to coincide with known developmental events: Postnatal days 21 and 42 reflect periods before and after the rise in serum androgens (24-26). At day 42, specific 5α-reductase enzyme activity is at its peak (24). Postnatal days 49 and 56 are marked by the first appearance of spermatozoa in the caput and cauda epididymides, respectively (24). By day 77, specific 5α-reductase enzyme activity is rapidly declining (24). Finally, postnatal day 91 was used to represent the adult animal. The number of animals used at each postnatal age was optimized to obtain sufficient tissue for RNA isolation. Epididymal and vas deferens tissues obtained from each postnatal age were pooled into three separate groups. Therefore, each age was studied in triplicate. Tissues were stored at -80 C prior to RNA isolation.

RNA Isolation

Total cellular RNA was isolated by the guanidinium thiocyanate/CsCl method as previously described with slight modifications (20). In brief, frozen epididymal tissues were normally homogenized in 5 volumes of guanidinium thiocyanate solution. There were two exceptions to this rule. Firstly, for the cauda epididymidis, 10 volumes of homogenization solution were used. Secondly, a minimum of 1 ml of homogenization solution was used for the initial segments and particularly for epididymal tissues from the immature animals. Total cellular RNA was then obtained by centrifugation through a CsCl gradient. Under these conditions, RNA yields were consistently ≥ 50 µg RNA/ml homogenate.

Northern Blot Analysis

Fifteen micrograms of total cellular RNA were denatured prior to electrophoresis by adding formaldehyde, formamide, and borate buffer (100 mM sodium borate, 100 mM boric acid, and 1 mM EDTA, pH 7.6) to final concentrations of 6%, 40%, and 20 mM, respectively, and heating the mixture for 5 minutes at 65 C. The denatured RNA samples were separated in 1% agarose-formaldehyde containing gels. Electrophoresis was done in recirculated 20 mM borate buffer at 20 V for 17 h at 25 C. Subsequent transfer to Genescreen Plus nylon membranes (DuPont Canada, Mississauga, Ontario, Canada) was done as previously described (20).

Membrane hybridizations with the 5α-reductase type 1 cDNA were performed as previously described (20). The probe used was a cDNA corresponding to the full-length mRNA transcript for rat steroid 5α-reductase type 1 (kindly provided by Dr. D. Russell, University of Texas, Southwestern Medical Center) (19).

To monitor the amount of total RNA loaded in each lane of the Northern blots, all hybridization membranes were reprobed with a synthetic ^{32}P-labelled oligonucleotide which is specific for 18S ribosomal RNA (27). The oligonucleotide was end-labelled by standard procedures (28). Hybridization conditions were as previously described (20). Membranes were autoradiographed by exposure to Kodak X-Omat AR film (Eastman Kodak, Rochester, NY) with intensifying screens (Fisher Biotech, Montréal, Québec, Canada) at -80 C.

RNA Quantitation

Autoradiograms were scanned using an LKB Ultrascan XL Laser Densitometer (Pharmacia-LKB Biotechnology, Uppsala, Sweden). To confirm the total amount of RNA present in each lane of the Northern blots, the integrated areas obtained for the 5α-reductase type 1 probe were normalized to the areas obtained for the 18S ribosomal RNA oligonucleotide probe. To determine the linear range (slope = 1) of scanning values, a standard curve consisting of defined amounts of liver RNA hybridized to the 5α-reductase type 1 probe was constructed. All the scanning values reported are within the linear range. The mRNA concentrations presented herein are representative of steady state mRNA values; this is understood as being a result of the production (transcription) and degradation (stability) of the mRNA.

Statistical Analysis

Statistical analysis was done by one-way analysis of variance followed by a Newman-Keuls test to detect significant differences (29). The analyses were done with the aid of a CSS (Complete Statistics System) computer program (Statsoft Inc., Tulsa, Oklahoma). A value of $P < 0.05$ was considered significant.

Results

Expression of 5α-Reductase type 1 mRNA in the Caput-Corpus and Cauda Epididymides during Postnatal Development

Developmental changes in 5α-reductase type 1 mRNA concentrations were assessed by Northern blot analysis. The results obtained for the caput-corpus epididymidis are shown in Fig. 2A. A single hybridizing mRNA species of 2.5 kilobases was apparent at all postnatal ages examined. This band was identical to the 5α-reductase type 1 mRNA species which was previously shown to exist in the rat epididymis (20). Interestingly, 5α-reductase type 1 mRNA was clearly present in the caput-corpus epididymidis at all ages, from the immature rat at postnatal day 7 to the adult rat (day 91).

The developmental pattern for 5α-reductase type 1 mRNA concentrations in the caput-corpus epididymidis following quantitation by laser densitometric scanning is shown in Fig. 2C. In this region of the epididymis, the developmental pattern was multiphasic. In the first phase, 5α-reductase type 1 mRNA concentrations decreased by one-half between postnatal days 7 and 21. This was followed by a striking 5 fold increase in mRNA concentration between postnatal days 21 and 56. This increase was interrupted, however, at day 49. At this age, 5α-reductase type 1 mRNA concentrations had declined to levels comparable to those observed at day 35. The last phase of the developmental pattern was a plateau between postnatal days 56 and 91.

The developmental changes in 5α-reductase type 1 mRNA that occur in the cauda epididymidis are shown in Fig. 3A. The Northern blot in Fig. 3A was similar to that obtained for the caput-corpus epididymidis with respect to the presence of a single hybridizing mRNA species at all postnatal ages. However, quantitation revealed that the developmental pattern of 5α-reductase type 1 mRNA

concentrations in this region was markedly different from that in the caput-corpus epididymidis (Fig. 3C). Here, 5α-reductase type 1 mRNA concentrations did not change significantly with age ($P > 0.05$).

Longitudinal Distribution of 5α-Reductase Type 1 mRNA in the Epididymis at Different Postnatal Ages

The longitudinal distribution of 5α-reductase type 1 mRNA in the epididymis was determined in order to study inter-segment specific alterations in mRNA expression during postnatal epididymal development. The mRNA distributions for epididymal 5α-reductase type 1 at four discrete ages during postnatal development are shown in Fig. 4.

At day 21, the highest concentrations of 5α-reductase type 1 mRNA were found in the initial segment and proximal caput epididymidis (Fig. 4A). The mRNA levels found within these two epididymal segments were not significantly different from each other ($P > 0.05$). Within the caput epididymidis, 5α-reductase type 1 mRNA concentrations were not homogeneous; they decreased by one-half between the proximal and distal regions of this segment. A trough in 5α-reductase type 1 mRNA concentrations was observed in the corpus epididymidis. In this epididymal segment, 5α-reductase type 1 mRNA concentrations were one-fourth of the initial segment level. Finally, in the distal cauda epididymidis and vas deferens, 5α-reductase type 1 mRNA concentrations rose once again to reach levels that were three-fourths of those found in the initial segment and proximal caput epididymidis.

A dramatic shift in longitudinal distribution occurred at postnatal day 42 (Fig. 4B). At this age, 5α-reductase type 1 mRNA became predominantly localized to the initial segment; here, 5α-reductase type 1 mRNA concentrations were 3-7 fold

higher than those in the rest of the epididymis and the vas deferens. The 5α-reductase type 1 mRNA concentrations in the distal cauda epididymidis were the second highest; mRNA concentrations in this segment were 2 fold higher than the distal caput, corpus, and proximal cauda epididymidis. The longitudinal distribution for 5α-reductase type 1 mRNA was also investigated at postnatal day 49. Results at this time-point were indistinguishable from those given at day 42 (data not shown).

The predominance of 5α-reductase type 1 mRNA in the initial segment was maintained at day 56 (Fig. 4C). A small peak in mRNA concentration found in the proximal cauda epididymidis, similar to the one observed for the distal cauda epididymidis at day 42, was apparent at this age. Apart from the initial segment and proximal cauda epididymidis, mRNA concentrations in the other segments of the epididymis did not differ significantly from one another ($P > 0.05$).

The longitudinal distribution for epididymal 5α-reductase type 1 mRNA changed slightly at day 91 (Fig. 4D). Although the preponderance of 5α-reductase type 1 mRNA in the initial segment remained, the small peaks observed in the proximal and distal caudae epididymidis at days 56 and 42 respectively, had disappeared. Furthermore, 5α-reductase type 1 mRNA concentrations in the initial segment were now 8-10 fold higher than the rest of the tissue. This increase was also evident two weeks earlier, at postnatal day 77 (data not shown). Therefore, the stark difference in 5α-reductase type 1 mRNA concentrations between the initial segment and the rest of the epididymis, already apparent at postnatal day 42, was potentiated by day 77.

Effect of Postnatal Age on 5α-Reductase Type 1 mRNA Expression within each Epididymal Segment

An independent set of Northern blots was generated to assess intra-segment specific fluctuations in 5α-reductase type 1 mRNA expression. In this case, the developmental patterns for 5α-reductase type 1 mRNA within each epididymal segment were studied over the six postnatal ages chosen. The results for two of these segments are shown in Fig. 5. The initial segment displayed an overwhelming change in 5α-reductase type 1 mRNA expression with development; mRNA concentrations within this segment increased 6-7 fold between postnatal days 21 and 42. Thereafter, 5α-reductase mRNA concentrations remained constant with age through to the adult animal at postnatal day 91.

The developmental pattern for 5α-reductase type 1 mRNA in the initial segment was in marked contrast to its adjacent section, the proximal caput epididymidis. In this segment, 5α-reductase type 1 mRNA concentrations did not change significantly with age ($P > 0.05$). Similarly, a lack of significant change, comparable to that of the initial segment, was also observed throughout the rest of the epididymis (data not shown). These data are consistent with the previous results given in this report for the entire cauda epididymidis.

Discussion

Previous studies from this laboratory (12,24), and others (30,31), have established that the ability of the rat epididymis to produce 5α-reduced steroids changes during sexual maturation. These studies have involved direct measurements of 5α-reductase enzyme activity and analyses of 5α-reduced steroid metabolites (12,24,30,31). In the present report, we conclusively demonstrate that the mRNA for epididymal 5α-reductase type 1 is also regulated

during postnatal development.

In the caput-corpus epididymidis, the developmental pattern for 5α-reductase type 1 mRNA was multiphasic. The rise and plateau phases of this pattern are coincidental with the developmental pattern for androgens in the male rat (24-26). The coincidence of these two developmental patterns suggests a role for either androgens or an androgen-dependent factor in the regulation of the developmental expression of 5α-reductase type 1 mRNA in the caput-corpus epididymidis. An analogous role for androgens in the expression of other mRNAs during postnatal development in the rat epididymis has also been suggested (32).

The absence of any significant changes in 5α-reductase type 1 mRNA concentrations during postnatal development in the cauda epididymidis was in marked contrast to the mRNA pattern in the caput-corpus epididymidis. This was surprising based on our previous interpretation of the role of androgens in this epididymal region (20). In that study, exogenous testosterone administration maintained 5α-reductase type 1 mRNA concentrations in the orchidectomized animal (20). At present, the reason for this discrepancy is not known. However, this finding does suggest that the role of androgens during development in the cauda epididymidis is different from their role in the same segment in an orchidectomized animal model. It is possible that androgens could increase the stability of the message in the cauda epididymidis of orchidectomized animals; clearly this situation is not paralleled at any time during postnatal development. Message stability experiments are required to resolve this possibility.

The fact that epididymal 5α-reductase mRNA concentrations increase during postnatal development is consistent with previous enzyme activity data from our laboratory (12,24). In spite of this similarity, comparison of the present mRNA data with that observed for the enzyme activity does indicate some conspicuous

differences. First, the message for the enzyme is present in the epididymis at least two weeks before its activity is detected (12). Second, the mRNA for 5α-reductase type 1 does not decline with age as the animals approach adulthood, whereas the enzyme activity does, after an initial rise to peak (12,24). The exact reason for these apparent discrepancies is not known, but they provide further evidence for the regulation of epididymal 5α-reductase at levels other than at the message, as suggested in a previous report (20). Brooks et al. (33) have suggested the possible existence of factors which prevent the translation of the mRNA for epididymal protein B/C. Post-transcriptional regulation has also been hypothesized for other epididymal proteins (34-36), and other proteins of the male reproductive tract (37,38). Therefore, a similar occurrence for epididymal 5α-reductase would not be unlikely.

The developmental alterations that we have observed in the longitudinal distribution of epididymal 5α-reductase type 1 mRNA indicate that the changes that occurred in the caput-corpus epididymidis are not characteristic of this entire region. The overwhelming shift in 5α-reductase type 1 mRNA concentrations between postnatal days 21 and 42 in the initial segment suggest that this segment is responsible for the changes observed in the caput-corpus epididymidis. Alterations in expression in the remainder of the tissue appear to be minor in comparison to those in the initial segment.

The striking increase in 5α-reductase type 1 mRNA concentrations in the initial segment between postnatal ages 21 and 42 may be a reflection of the state of differentiation of the epithelial cells. Epididymal principal cells, which harbour 5α-reductase enzyme activity (39), pass from an undifferentiated state to one manifesting their mature appearance between these two developmental time-points (40-42). Poorly differentiated principal cells may simply be unable to

express 5α-reductase type 1 mRNA at their full potential. Alternatively, the dramatic increase in 5α-reductase type 1 mRNA concentration may be due to the appearance, during postnatal development, of some critical factor regulating expression of the message for the enzyme in the initial segment of the epididymis. Garrett et al. (32) have suggested a role for a spermatozoa-associated factor in the expression of proenkephalin mRNA in the initial segment. Clearly, this cannot be the case for 5α-reductase type 1 since entry of spermatozoa into the epididymis does not occur until day 49 (24), one full week after the surge in 5α-reductase type 1 mRNA concentrations in the initial segment.

Although androgens have been shown to have a role in the regulation of the mRNA for 5α-reductase type 1 in the initial segment of the epididymis, the prime regulator in this epididymal region is a factor of testicular origin which is dependent on testosterone but not testosterone itself (20,43). The present association of 5α-reductase type 1 mRNA in the initial segment with androgens during postnatal development is consistent with the testosterone dependency of the testicular factor. At the mRNA level, this factor has been shown to regulate the type 1 message only in the initial segment (20). Therefore, the lack of correlation with androgens during development observed in the other epididymal segments is consistent with this hypothesis.

In conclusion, we have demonstrated that the expression of the mRNA for 5α-reductase type 1 is regulated during postnatal development in the rat epididymis. The fact that these developmental changes were limited to one region of the epididymis unequivocally illustrates the segment-specific nature of this regulation. Furthermore, differences between the present mRNA data and previous findings at the enzyme activity level suggest that post-transcriptional regulation may also have an inherent role in the regulation of epididymal 5α-

reductase activity.

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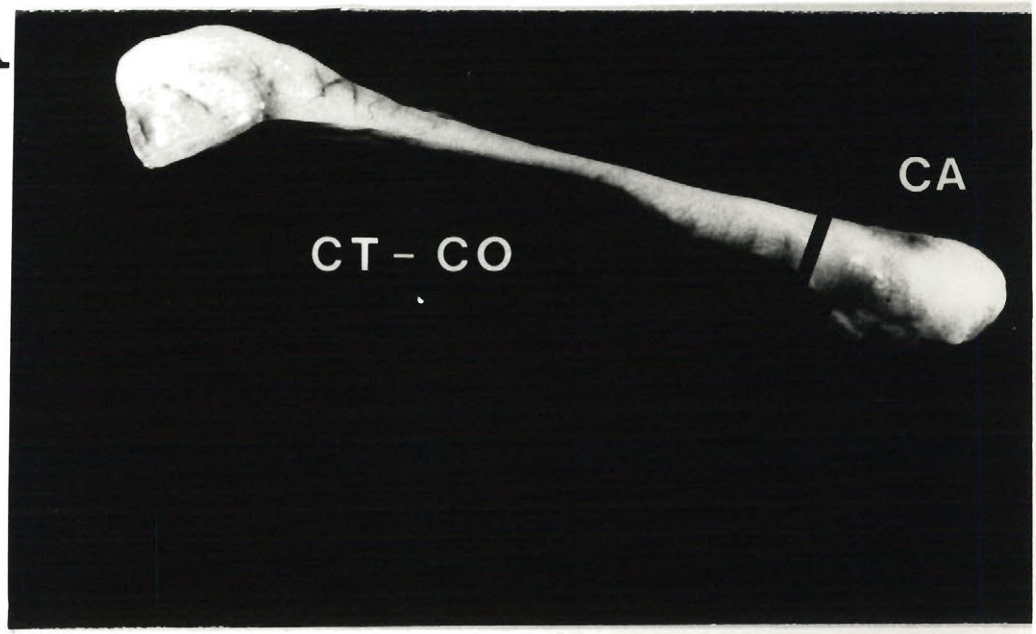
Figure 1. Photographic representation of the rat epididymis depicting its different regions. A: CT-CO, caput-corpus epididymidis; CA, cauda epididymidis. B: IS, initial segment; PCT, proximal caput; DCT, distal caput; CO, corpus; PCA, proximal cauda; DCA, distal cauda; VAS, vas deferens.

A



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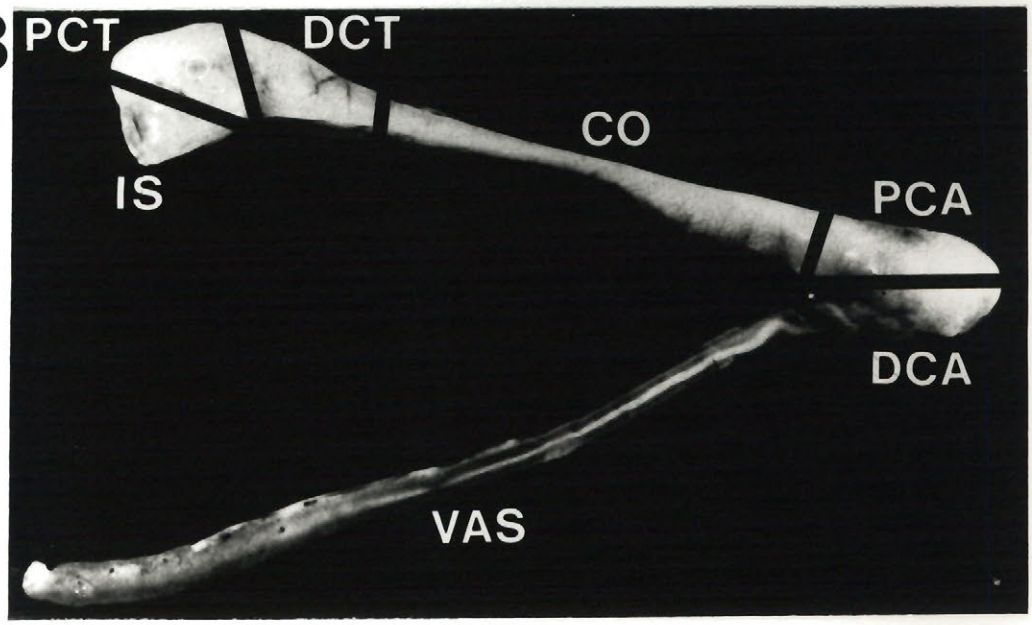
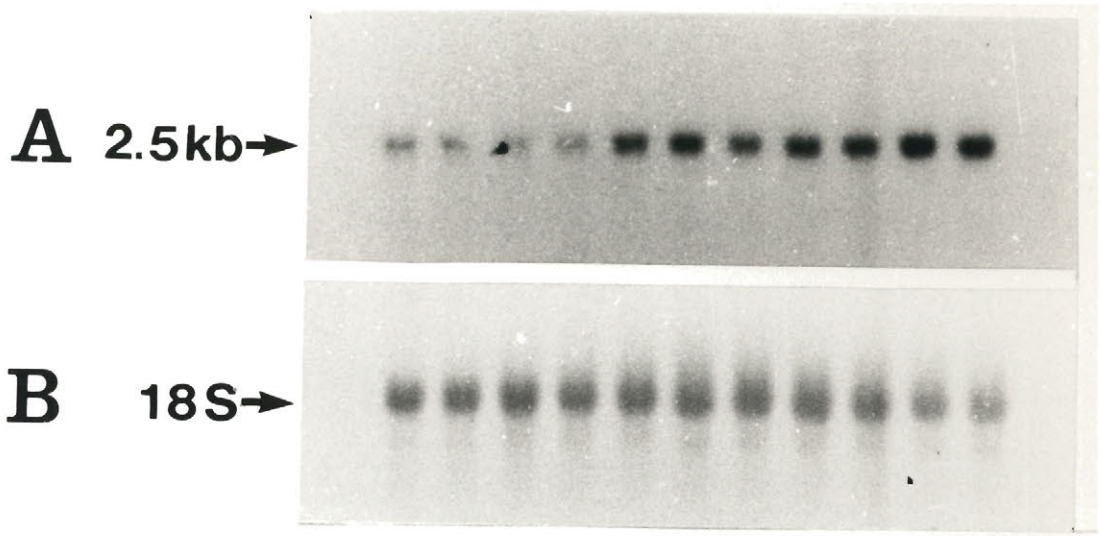


Figure 2. A, Northern blot analysis of total cellular RNA obtained from the caput-corpus epididymidis during postnatal development. Fifteen micrograms of total cellular RNA are present per lane. The size of the 5α-reductase type 1 message is indicated on the *left*. B, Reprobe of the Northern blot with an oligonucleotide hybridizing to the 18S ribosomal RNA. C, Steady state 5α-reductase type 1 mRNA concentrations in the caput-corpus epididymidis during postnatal development. Values are expressed as a percentage of the value in the adult animal (day 91) ± SE (n=3). *, Significantly different from days 56-91 ($P < 0.05$), **, significantly different from days 56-91 and days 7 and 42 ($P < 0.05$).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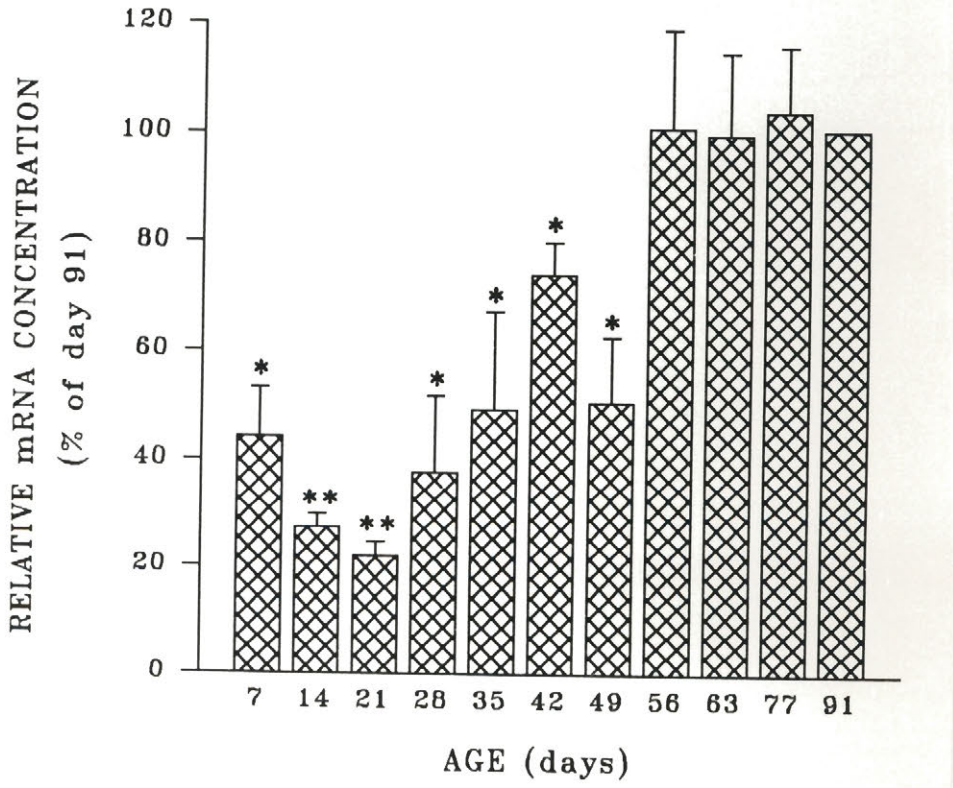
C


RELATIVE mRNA CONCENTRATION
(% of day 91)
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40
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21
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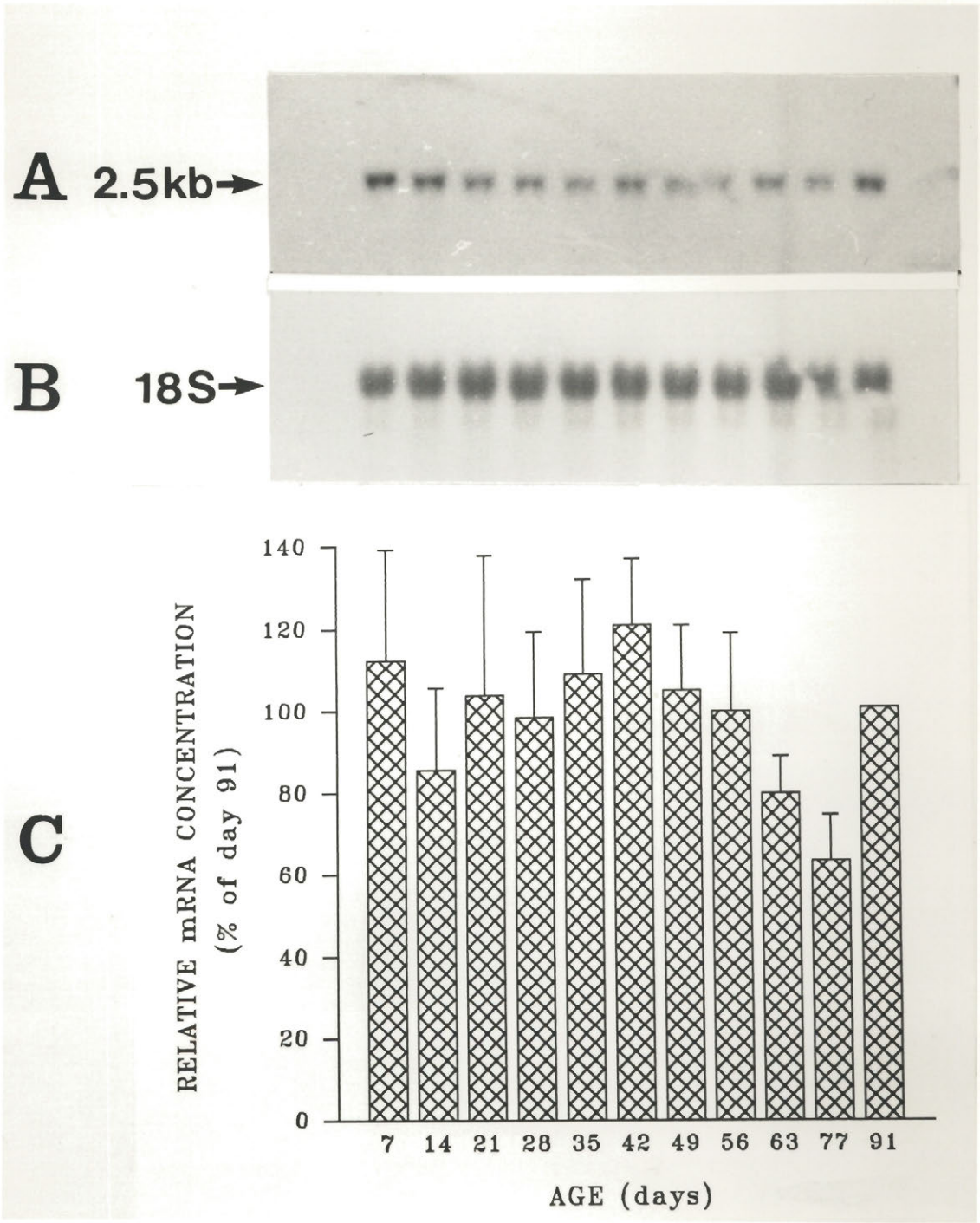
42
49
56
63
77
91
AGE (days)

Figure 3. A, Northern blot analysis of total cellular RNA obtained from the cauda epididymidis during postnatal development. Fifteen micrograms of total cellular RNA are present per lane. The size of the 5α-reductase type 1 message is indicated on the *left*. B, Reprobe of the Northern blot with an oligonucleotide hybridizing to the 18S ribosomal RNA. C, Steady state 5α-reductase type 1 mRNA concentrations in the cauda epididymidis during postnatal development. Values are expressed as a percentage of the value in the adult animal (day 91) ± SE (n=3). All values did not differ significantly from each other ($P > 0.05$).


A 2.5kb
B 18S
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RELATIVE mRNA CONCENTRATION
(% of day 91)
140
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100
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60
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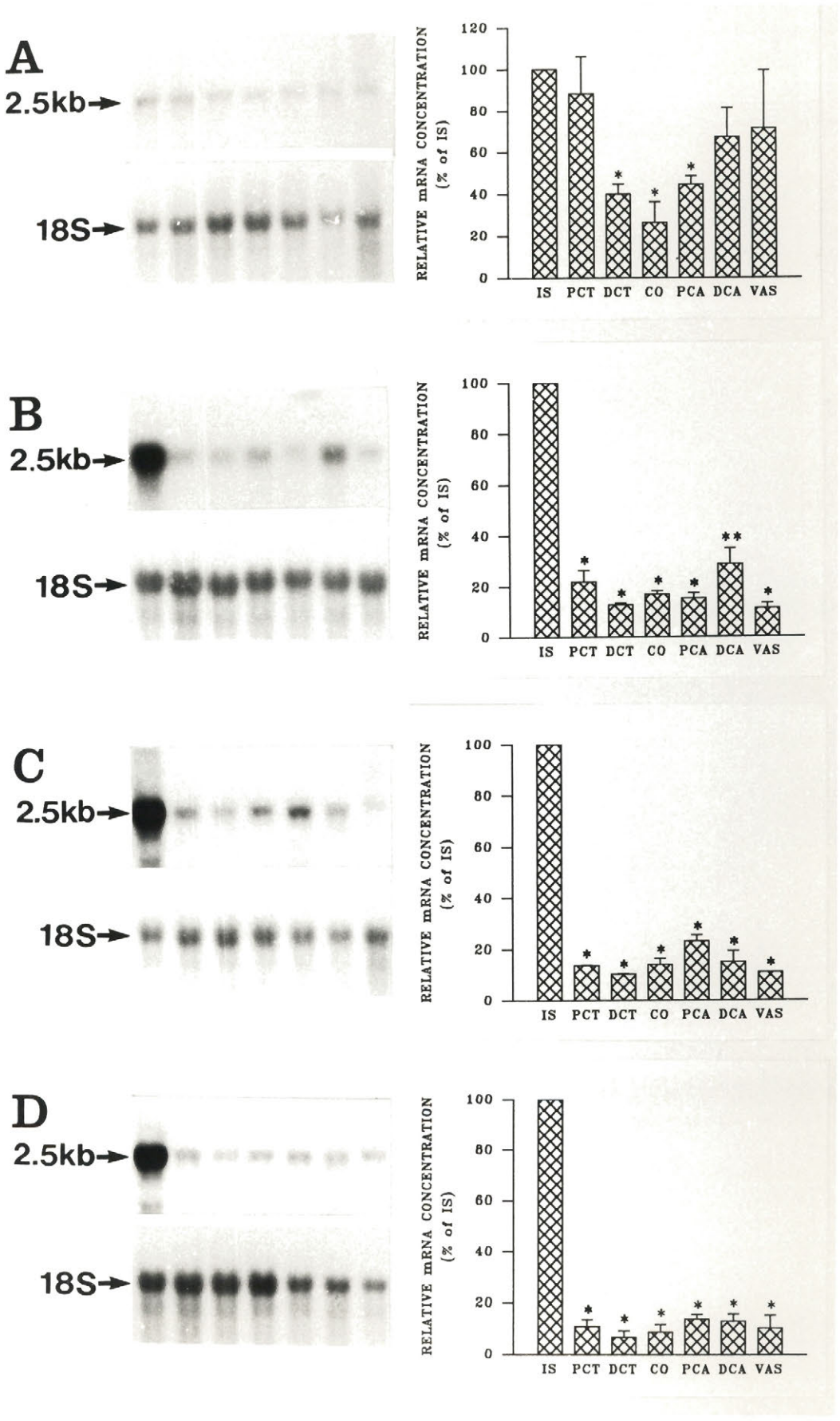
7 14 21 28 35 42 49 56 63 77 91
AGE (days)

Figure 4. 5α-Reductase type 1 mRNA expression during postnatal development in the rat epididymis and vas deferens as a function of longitudinal distribution. A, Day 21; B, day 42; C, day 56; D, day 91. Fifteen micrograms of total cellular RNA are present in each lane. The corresponding 2.5 kilobase hybridizing 5α-reductase type 1 message, 18S ribosomal RNA, and quantitated graph are shown for each age. The values for 5α-reductase type 1 mRNA concentrations are given as a percentage of the value in the initial segment (IS) ± SE (n=3). *, significantly different from IS ($P < 0.05$); **, significantly different from IS, DCT, and VAS ($P < 0.05$).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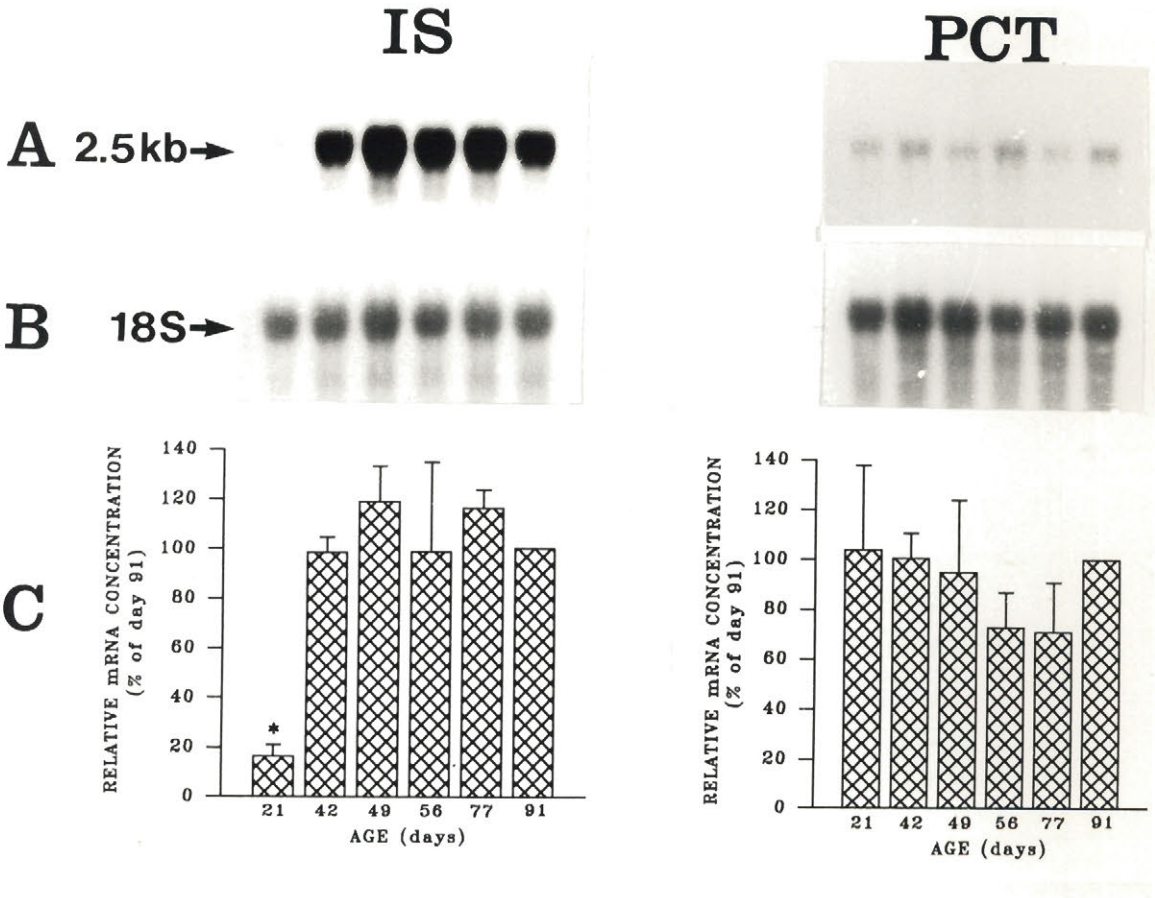
IS PCT DCT CO PCA DCA VAS

Figure 5. 5α-Reductase type 1 mRNA expression during postnatal development in the initial segment and proximal caput epididymidis. Fifteen micrograms of total cellular RNA are present per lane. A, Northern blots indicating the 2.5 kilobase hybridizing 5α-reductase type 1 message. B, Reprobe of the Northern blots with an oligonucleotide hybridizing to the 18S ribosomal RNA. C, Steady state mRNA concentrations, as assessed by laser densitometry. Values are expressed as a percentage of the value in the adult animal (day 91) ± SE (n=3). *, Significantly different from days 42-91 ($P < 0.05$).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RELATIVE mRNA CONCENTRATION (% of day 91)
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AGE (days)
RELATIVE mRNA CONCENTRATION (% of day 91)
140
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20
0
21 42 49 56 77 91
AGE (days)

Connecting Text - Chapter III to Chapter IV

In the preceding two chapters, a combination of tissue distribution, endocrine, and developmental studies were used to characterize the regulation of the 5α-reductase type 1 mRNA in the rat epididymis. At the completion of these studies, the identification of a transcript encoding for a second 5α-reductase isozyme in the rat and human was reported in the literature. Based on its order of identification, this transcript was designated as steroid 5α-reductase type 2 . Immediately after its discovery, interest in the type 2 isozyme gained particular attention due to the abundant expression of its mRNA over the type 1 mRNA in many androgen target tissues. This observation was particularly evident in the rat epididymis. Therefore, to gain insight into the regulation of the 5α-reductase type 2 mRNA in the rat epididymis, and potentially its physiological importance, three complementary experiments were designed to study its expression in the tissue. These experiments are presented in the next chapter.

Chapter IV

The mRNAs for the Steroid 5α-Reductase Isozymes, Types 1 and 2, Are Differentially Regulated in the Rat Epididymis

Robert S. Viger and Bernard Robaire

Abstract

The enzyme steroid 5α-reductase (EC 1.3.1.22) catalyzes the conversion of testosterone to its biologically active form, dihydrotestosterone (DHT), in many androgen-sensitive target tissues. In the epididymis, the 5α-reduced metabolites of testosterone, DHT and 5α-androstan-3α,17β-diol (3α-diol), are considered the primary regulators of epididymal structure and function. Two rat 5α-reductase transcripts, designated types 1 and 2, have been identified. Our laboratory has previously characterized the endocrine and developmental regulation of the 5α-reductase type 1 mRNA in the rat epididymis. However, regulation of the type 2 mRNA has not been investigated. In the present study, three complementary groups of experiments were done to characterize the regulation of epididymal 5α-reductase type 2 mRNA expression. To gain insight into the relative importance of the 5α-reductase isozymes in the rat epididymis, these data have been compared with those previously obtained for the type 1 mRNA and enzyme activity. In the first experiment, Northern blot analysis revealed that 5α-reductase type 2 mRNA was predominantly expressed in the proximal caput epididymidis of the adult rat. This regional distribution pattern differed markedly from the patterns previously described for the type 1 mRNA and enzyme activity. In the second experiment, the expression of 5α-reductase type 2 mRNA in the epididymis was studied as a function of increasing postnatal age. Surprisingly, epididymal 5α-reductase type 2 mRNA expression did not change significantly during postnatal development. Again, this result was in marked contrast to the dramatic developmental changes that were observed for 5α-reductase enzyme activity and for type 1 mRNA. In the final experiment, the effect of unilateral efferent duct ligation revealed that 5α-reductase type 2 mRNA levels increased selectively in the initial segment of the ligated side; this was in marked contrast to the dramatic

decrease (greater than 60%) observed for type 1 mRNA levels. Interestingly, the decline in 5α-reductase enzyme activity after efferent duct ligation was similar to that observed for type 1 mRNA levels. Taken together, these experiments demonstrate that 5α-reductase type 1 and type 2 mRNAs are differentially regulated in the rat epididymis. Furthermore, the dissimilarity between the regulation of the 5α-reductase type 2 mRNA and of the type 1 mRNA and/or enzyme activity strongly supports the suggestion that the type 2 transcript is poorly expressed as an active enzyme in this tissue.

Introduction

The epididymis is a single, highly convoluted tubule that constitutes a major portion of the testicular excurrent duct system in mammals. Spermatozoa are transported from the efferent ducts to the vas deferens via the epididymis; as they traverse this tissue, they are exposed to a complex, changing microenvironment (1). This microenvironment is established by the highly active absorptive and secretory activities of the cells that line the epididymal epithelium (2). Consequently, spermatozoa are rendered mature in the caput-corpus epididymidis and are then stored in the caudal region of the tissue (3). Although the mechanisms underlying the maturation and storage of spermatozoa in the epididymis have yet to be fully identified, the maintenance of these epididymal functions is dependent on the presence of androgens (4-6). Androgenic control of epididymal functions has been suggested to be mediated by the 5α-reduced metabolites of testosterone, 5α-dihydrotestosterone (DHT) and 5α-androstan-3α,17β-diol (3α-diol), and not by testosterone itself (7,8).

In many androgen-sensitive target tissues, androgen action is initiated by the binding of DHT to the androgen receptor (9). The conversion of testosterone to the biologically potent androgen, DHT, is catalyzed by the membrane bound enzyme 4-ene steroid 5α-reductase (EC 1.3.1.22). 5α-Reductase activity is abundantly expressed in the epididymis; this activity is present in both the nuclear and microsomal subcellular fractions (10-12). Direct micropuncture studies have confirmed these in vitro observations by revealing that the predominant androgen found in the epididymal lumen is DHT (13,14). Functionally, the abundance of 5α-reductase enzyme activity and DHT in the epididymis has been supported by studies which have identified DHT as the active androgen present in the nuclei of epididymal cells after injection of radiolabelled testosterone (15).

Two 5α-reductase isozymes have been identified in the rat and human through the molecular cloning of their respective cDNAs (16-19). These 5α-reductase isozymes, which are the products of separate genes (20-22), have been named type 1 and type 2 according to the chronological order of their identification. The isozymes differ with respect to their biochemical properties, pharmacological characterization, and tissue distribution (16-19,23-26). We have previously characterized the endocrine and developmental regulation of the type transcript 1 in the rat epididymis; this regulation is multifactorial and epididymal segment-specific (27,28). Importantly, the control of 5α-reductase type 1 mRNA expression in the epididymis was shown to closely parallel the regulation that we and others have reported for epididymal 5α-reductase enzyme activity (29,30).

In contrast to the 5α-reductase type 1 transcript, regulation of the type 2 mRNA in the epididymis has not yet been investigated. In the human, the type 2 isozyme has come under close scrutiny due to the reported occurrence of natural mutations and/or deletions in the type 2 gene in individuals with 5α-reductase deficiency (18,22,31,32). Further interest in characterizing the regulatory mechanisms of the type 2 gene has been raised by the availability of 5α-reductase blockers that are effective in humans (33,34) and which are apparently potent inhibitors of type 2 isozyme (18,25). In the rat, tissue distribution studies have revealed that the 5α-reductase type 2 is the dominant transcript expressed in the male reproductive tissues (19). Interestingly, 5α-reductase type 2 mRNA expression is higher in the epididymis than in any other rat tissue examined thus far (19). Based on these observations, it was proposed by others that 5α-reductase type 2 was the isozyme expressed in the rat epididymis that was important in terms of androgen action (19,35).

To test this hypothesis, three complementary groups of experiments were

done to characterize the regulation of epididymal 5α-reductase type 2 mRNA expression. Our results clearly indicate that the expression of the 5α-reductase mRNAs, types 1 and 2, are differentially regulated in the rat epididymis. Furthermore, comparison of the present type 2 mRNA data with our previous type 1 mRNA and enzyme activity data strongly suggests that despite its abundant expression in the epididymis, the 5α-reductase type 2 transcript is not efficiently expressed as an active enzyme.

Materials and Methods

Animals

Sprague-Dawley rats were purchased from Charles River Canada (St. Constant, Quebec, Canada). Adult male rats, weighing 300-350 g, were used for the longitudinal distribution and efferent duct ligation studies. For the developmental study, timed-gestation pregnant dams were obtained. One day after birth, female offspring were removed and the remaining males were placed at random with the postpartum females. Animals were weaned on postnatal day 21. Development of the animals was monitored by assessing gains in body weight, paired testicular, paired epididymal, and accessory sex tissue weights. These growth curves were comparable to those previously observed (36). All animals were housed under conditions of controlled temperature, humidity, and lighting (12-h light, 12-h dark lighting); food and water was given *ad libitum.*

Experimental Design

Experiment 1 - Longitudinal Distribution of 5α-Reductase Type 2 mRNA in the Adult Rat Epididymis

Animals were ether anesthetized and killed by decapitation. The

epididymides were dissected free of fat and sectioned into four regions (initial segment, caput, corpus, and cauda) as previously described (27). The ventral prostate and pituitary were also retained for comparison purposes. Tissues samples were immediately frozen in liquid nitrogen and stored at -80 C until used for RNA isolation. A total of ten animals were used to prepare five separate samples, each consisting of tissues pooled from two animals (n=5).

Experiment 2 - The Effect of Postnatal Age on 5α-Reductase Type 2 mRNA Expression in the Rat Epididymis

Developmental changes in epididymal 5α-reductase type 2 mRNA expression were studied at six selected ages during postnatal life (days 21, 42, 49, 56, 77, and 91). The timepoints were chosen to coincide with known developmental events. Postnatal days 21 and 42 reflect the periods before and after the pubertal increase in serum androgens in the male rat (36-38). Specific 5α-reductase enzyme activity is at its peak on day 42 (36). Postnatal days 49 and 56 are marked by the first appearance of spermatozoa in the caput and cauda epididymides, respectively (36). By day 77, specific 5α-reductase enzyme activity has markedly declined (36). Finally, postnatal day 91 was used to represent the adult animal. At each postnatal age, animals were ether anesthetized and killed by cervical dislocation. Epididymides were obtained and sectioned into 6 regions (initial segment, proximal caput, distal caput, corpus, proximal cauda, and distal cauda) as previously described (28). The total number of animals used on days 21, 42, 49, 56, 77, and 91 were 21, 12, 9, 9, 6, and 6, respectively. The number of animals used at age was optimized to obtain sufficient tissue for RNA isolation. Epididymal tissue sections obtained from each postnatal age were pooled and separated into three distinct groups. Therefore, each age was studied in triplicate

(n=3). Tissues were stored at -80 C until used for RNA isolation.

Experiment 3 - The Effect of Efferent Duct Ligation on the Expression of the 5α-Reductase mRNAs in the Adult Rat Epididymis

Adult male rats were unilaterally efferent duct ligated on the right side. The contralateral epididymis was used as an internal control. The efferent ducts were accessed by exposing the testis-epididymis via a scrotal incision under ether anesthesia. Using 4-0 silk (Cyanamid Canada, Montreal, Quebec, Canada), the ligature was carefully positioned so as not to disturb the blood flow entering and exiting the testis. Seven days following the surgery, the rats were ether anesthetized and killed by decapitation. The epididymides were removed and sectioned into four regions (initial segment, caput, corpus, and cauda) as previously described (27). The effectiveness of the ligation was assessed by monitoring epididymal weight from the control and ligated sides of the animals. A total of ten animals were used to prepare five separate groups, each consisting of epididymal tissue sections pooled from two animals (n=5). Tissue samples were stored at -80 C until used for RNA isolation.

RNA Isolation

Total cellular RNA was isolated by the guanidinium thiocyanate-cesium chloride method (39). In brief, frozen epididymal tissues were homogenized in 5 vol (ml/g) guanidinium thiocyanate solution (4.0 M guanidinium thiocyanate, 0.1 M Tris-Cl pH 7.5, 1% ß-mercaptoethanol) using a Brinkman polytron (Rexdale, Ontario, Canada). A volume of 1.2 ml of the homogenate was then layered onto a 2.8 ml cushion of CsCl (5.7 M CsCl, 0.01 M EDTA pH 7.5) in 4 ml Beckman *g*-MAX *k*onical Quick-Seal tubes (Mississauga, Ontario, Canada). The tubes were

RNA Quantitation

Northern blots were quantitated by scanning the resulting autoradiograms with an LKB Ultrascan Laser Densitometer (LKB Biotechnology, Uppsala, Sweden). Variations in RNA loading were corrected by normalizing the integrated peak areas obtained for the 5α-reductase mRNAs to those obtained for the 18S ribosomal RNA. The linear range of the densitometer was previously established by linear regression analysis of quantitated data obtained from a series of Northern blots containing defined amounts of liver RNA. All scanning values (integrated peak areas) were maintained within this linear range.

Statistical Analysis

In the longitudinal and developmental studies, data was analyzed by the nonparametric Kruskal-Wallis analysis of variance. When significance was reported by the analysis of variance, differences between groups were identified using the Mann Whitney U- test. For the ligation study, significant differences between the control and ligated groups were determined using the Student's t-test. For all statistical analyses, $P \leq 0.05$ was considered significant. The analyses were done with aid a computer program (CSS: Statistica, Statsoft, Inc., Tulsa, OK).

Results

Longitudinal Distribution of 5α-Reductase Type 2 mRNA Expression in the Adult Rat Epididymis

The adult rat epididymis was sectioned into four regions in order to define the spatial distribution of the type 2 message for 5α-reductase. Northern blot analysis revealed the presence of single hybridizing 5α-reductase type 2 mRNA species with a molecular size of 3.6 kilobases in all epididymal regions (Fig. 1,

upper panel). The 5α-reductase type 2 mRNA was abundantly expressed in the rat epididymis; adequate signal could be obtained with exposure times of less than 24 hours. In contrast to the pattern that we have described for the 5α-reductase type 1 transcript (27), the most intense type 2 signal was present in the caput epididymidis, followed by the initial segment. Densitometric quantitation revealed that steady state 5α-reductase type 2 mRNA concentrations in the caput epididymidis were approximately 1.4 fold higher than those in the initial segment (Fig. 1, lower panel). A dramatic decrease in 5α-reductase type 2 mRNA expression occurred beyond the caput epididymidis. 5α-Reductase type 2 mRNA levels in the corpus and cauda epididymidis were 10% of those found in the caput epididymidis; these concentrations were comparable to those present in the pituitary. Interestingly, 5α-reductase type 2 mRNA expression in the prostate was barely detectable; quantitation revealed that prostatic type 2 mRNA levels represented a mere 4% of those found in the caput epididymidis (Fig. 1, lower panel).

Longitudinal Distribution of 5α-Reductase Type 2 mRNA in the Rat Epididymis at Different Postnatal Ages

Age-related changes in 5α-reductase type 2 gene expression between epididymal segments were assessed by studying the longitudinal distribution of the type 2 transcript. The regional distribution of 5α-reductase type 2 mRNA expression in the rat epididymis was examined at six selected ages during postnatal development; two representative ages are shown in Fig. 2.

In the immature rat (day 21), 5α-reductase type 2 mRNA expression was most abundant in the initial segment and proximal caput epididymidis (Fig. 2A, left panel). Relative 5α-reductase type 2 mRNA levels in these two epididymal

regions, however, were not significantly different from each other on day 21 ($P > 0.05$) (Fig. 2B, left panel). Within the caput epididymidis, 5α-reductase type 2 mRNA levels were not uniformly expressed. This was revealed by the significant 50% decrease in type 2 mRNA levels between the proximal and distal regions of this epididymal segment. The lowest 5α-reductase type 2 mRNA concentrations were observed in the corpus epididymidis; mRNA levels in this epididymal segment were only 25% of those found in the initial segment and distal caput epididymidis. 5α-Reductase type 2 mRNA concentrations in the proximal and distal portions of the cauda epididymidis were comparable to those found in the distal caput.

A similar longitudinal distribution pattern for the 5α-reductase type 2 mRNA was found in pubertal rats on postnatal day 42 (Fig. 2A, right panel). At this age, however, 5α-reductase type 2 mRNA concentrations in the proximal caput epididymidis were now higher than those present in the initial segment (Fig. 2B, right panel). Comparable regional distribution patterns were also observed in the epididymides of rats at the other timepoints, i.e., on days 49, 56, 77, and 91 (data not shown). The most prominent feature of these distribution patterns was the abundant 5α-reductase type 2 expression in the proximal caput epididymidis. Interestingly, this distribution pattern was markedly different from the one that we have previously reported for the type 1 transcript in the epididymis (27).

Effect of Increasing Postnatal Age on 5α-Reductase Type 2 mRNA Expression Within Each Epididymal Segment

An independent set of Northern blots were prepared to identify potentially important age-related changes in 5α-reductase type 2 mRNA expression within each epididymal segment. Therefore, in contrast to the blots for the longitudinal distribution experiment (i.e., many segments studied, one age at a time), each

Northern blot consisted of RNA from the same epididymal segment but from rats of different ages (i.e., many ages studied, one segment at a time). Surprisingly, no significant age-related changes in 5α-reductase type 2 mRNA expression were found. This finding was significant since it was in marked contrast to our previously reported results for the 5α-reductase type 1 mRNA (27). This important difference in the developmental regulation of the type 1 vs type 2 transcript is highlighted in Fig. 3, which compares the effect of increasing postnatal age on the expression of the 5α-reductase mRNAs in the initial segment of the rat epididymis (the data for type 1 5α-reductase mRNA are adapted from reference 27).

Effect of Unilateral Efferent Duct Ligation on the Expression of the mRNAs for the 5α-Reductase Isozymes in the Adult Rat Epididymis

Unilateral efferent duct ligation was used to determine the importance of direct testicular secretions on 5α-reductase type 2 mRNA expression in the rat epididymis. Since such secretions have been proposed to be critical in the maintenance of 5α-reductase type 1 mRNA levels in the epididymis (11), the expression of the type 1 transcript in the efferent duct ligated animals was also monitored as an effective comparison for the type 2 transcript; the unligated side served as control.

One week efferent duct ligation resulted in a significant decrease in epididymal weight when compared to the control intact side (control: 438 ± 13 mg, ligated: 345 ± 10 mg; $P < 0.05$ by Student's t-Test). The magnitude of this decrease was consistent with the decreases that have been reported when direct testicular input to the epididymis was removed by either efferent duct transection or unilateral orchidectomy (11,41). The effects of unilateral efferent duct ligation on the expression of type 1 and type 2 5α-reductase mRNAs are shown in Fig. 4.

Inspection of the Northern blots revealed two striking features. The first was that the effects of efferent duct ligation were highly epididymal segment-specific, i.e., the initial segment of the epididymis was clearly the region of the tissue that was sensitive to the ligation; no apparent changes in the expression of the 5α-reductase mRNAs were evident in the other epididymal segments (Fig. 4). The second was that the response to efferent duct ligation in the initial segment of the epididymis was different for the two 5α-reductase transcripts (Fig. 4). In the initial segment, densitometric quantitation of the Northern blots showed that efferent duct ligation caused a greater than 60% decrease in 5α-reductase type 1 mRNA levels when compared to control (Fig. 5). In marked contrast, 5α-reductase type 2 mRNA levels increased by nearly 2 fold under the same conditions (Fig. 5). In the other epididymal segments, however, densitometric quantitation revealed no significant changes in the mRNA levels for both 5α-reductase isozymes ($P > 0.05$; Fig. 5).

Discussion

It has been previously suggested by Berman and Russell (35) that the two 5α-reductase isozymes may play distinctive roles in androgen metabolism in the rat. Based on tissue distribution studies, anabolic and catabolic roles in the metabolism of androgens and other steroids were attributed to the type 2 and type 1 5α-reductase isozymes, respectively (19). In those studies, the mRNA for the type 2 isozyme was shown to be primarily expressed in the male reproductive tissues whereas the mRNA for the type 1 isozyme predominated in the peripheral tissues (19). In the epididymis, the importance of the type isozyme 2 was further emphasized by the finding that 5α-reductase type 2 mRNA expression in this tissue was higher than in any other rat tissue studied (19). The results of the studies described above clearly indicate that the 5α-reductase mRNAs are

differentially regulated in the rat epididymis. More importantly, comparison of the present findings with our previous type 1 mRNA and enzyme activity data has allowed us to strongly suggest that despite its abundant expression in the epididymis, the 5α-reductase type 2 transcript is not effectively expressed as an active enzyme in the tissue.

Normington and Russell have reported the 5α-reductase type 2 mRNA is expressed with a positional gradient along the rat epididymis; the highest levels were found in proximal portion of the tissue (19). In the present study, we have extended this initial observation by more precisely defining the distribution of the type 2 mRNA along the adult rat epididymis. The highest 5α-reductase type 2 mRNA levels were localized to the proximal caput epididymidis. Surprisingly, this result was in marked contrast to predominant expression of the type 1 mRNA, type 1 protein, and 5α-reductase enzyme activity that we have previously characterized; the type 1 mRNA, type 1 protein, and 5α-reductase enzyme activity all reside primarily in the initial segment of the epididymis (27,29,41). It is important to note that *in vitro* 5α-reductase enzyme assays do not discriminate between the activities of the different 5α-reductase isozymes since they both recognize the same substrate, i.e., testosterone. Thus, epididymal 5α-reductase enzyme activities that have been previously reported must be regarded as the sum of the activities derived from both isozymes. Taking this into consideration, the disparity between the longitudinal distribution of 5α-reductase enzyme activity and type 2 mRNA expression in the rat epididymis is the first indication that the type 2 transcript may not be effectively expressed as an active enzyme in comparison to the type 1 transcript.

One of the key regulatory features of epididymal 5α-reductase type 1 mRNA expression and enzyme activity is the dramatic increase that occurs during

postnatal development (12,28,36). This developmental increase is of particular functional significance since it occurs just before the first appearance of spermatozoa in the epididymis (36),and thus, is consistent the 5α-reduced androgen-dependent spermatozoal maturation and storage functions of this tissue. Surprisingly, 5α-reductase type 2 mRNA expression did not show any significant developmental changes in the epididymis. Importantly, the discrepancy between the developmental profiles for the 5α-reductase type 2 mRNA on the one hand and the type 1 mRNA and enzyme activity on the other lends further credence to our hypothesis that the type 2 mRNA is ineffectively expressed as an active enzyme in the epididymis.

Previous experiments from our laboratory and those of others have demonstrated that an intact connection between the testis and epididymis is critical for the maintenance of 5α-reductase enzyme activity, type 1 mRNA and protein expression, and the normal morphology of principal cells, particularly in the initial segment of the epididymis (11,27,30,42,43). Hence, a unilateral efferent duct ligation study was designed to determine the effect of removing direct testicular input to the epididymis on the expression of the 5α-reductase type 2 mRNA. Remarkably, this procedure did not result in a decrease, but rather in an increase, in 5α-reductase type 2 mRNA concentrations in the initial segment of the epididymis. The explanation for such an increase is not clear. One possibility is that it may be the result of a dilution effect since many other epididymal messages and/or proteins are known to decrease dramatically in the epididymis after the removal of direct testicular input (11,27,44-47). Thus, an increase in 5α-reductase type 2 mRNA levels under conditions where type 1 mRNA expression and enzyme activity clearly decrease is once again inconsistent with the type 2 transcript being expressed as a major component of the 5α-reductase enzyme activity found in the

rat epididymis.

When the proteins are expressed in mammalian cells, the 5α-reductase isozymes have different pH optima (48). For example, the type 1 isozyme is maximally active near a neutral pH, whereas the type 2 isozyme has a more acidic pH optimum (48). Importantly, when these values are contrasted with the previously described pH profile for epididymal 5α-reductase enzyme activity (49), it is the neutral pH of the type 1 isozyme that is consistent with the actual epididymal activity.

Thus, taken together, these data indicate that the mRNAs for the 5α-reductase isozymes are differentially regulated in the rat epididymis. Interestingly, such a differential regulation of the 5α-reductase transcripts has not been reported in other rat tissues such as the prostate (19), but has been suggested for the human (26).

In conclusion, the experiments presented in the current study have provided strong evidence to suggest that, despite its abundance in the rat epididymis, the 5α-reductase type 2 transcript is poorly expressed as an active enzyme in comparison to the type 1 transcript. Furthermore, the close association between epididymal 5α-reductase enzyme activity and the type 1 mRNA with respect to tissue distribution, regulation, and pH profile strongly suggests that 5α-reductase type 1 is functionally the dominant isozyme expressed in the rat epididymis.

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Figure 1. Distribution of 5α-reductase type 2 mRNA along the adult rat epididymis. Fifteen micrograms of total RNA were subjected to Northern blot analysis using a cDNA probe specific for rat 5α-reductase type 2 (upper panel). The size of the hybridizing message is indicated on the *left*. RNA loading was monitored by reprobing the Northern blot with an oligonucleotide specific to the 18S ribosomal RNA (middle panel). Steady state 5α-reductase 2 mRNA levels were assessed by densitometric quantitation (lower panel). Relative 5α-reductase type 2 levels are expressed as a percentage of the value in the initial segment ± SEM (n=5). IS, initial segment; CT, caput; CO, corpus; CA, cauda; PR, prostate; PIT, pituitary. *, Significantly different from IS; **, Significantly different from IS and CT ($P < 0.05$).

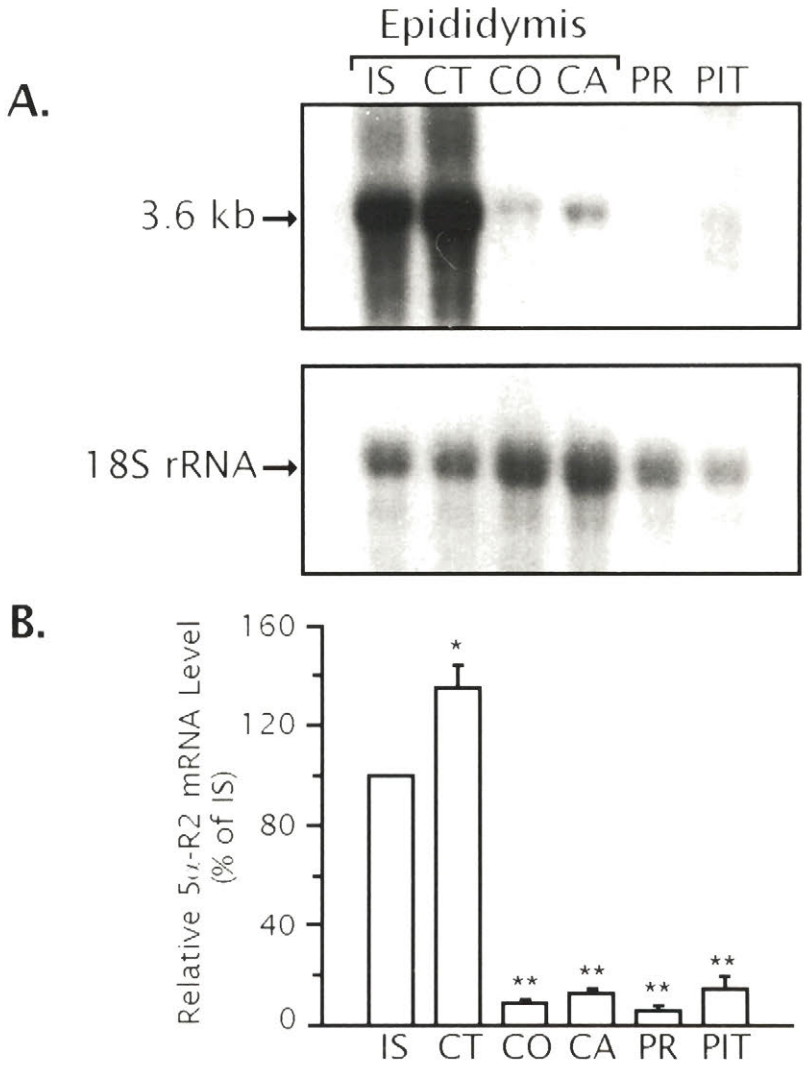

A.
Epididymis
IS CT CO CA PR PIT
3.6 kb
18S rRNA
B.
Relative 5α-R2 mRNA Level (% of IS)
160
120
80
40
0
*
**
**
**
**
IS CT CO CA PR PIT

Figure 2. Regional expression of 5α-reductase type 2 mRNA in the rat epididymis on postnatal days 21 and 42. Fifteen micrograms of total RNA were subjected to Northern blot analysis (upper panel). The size of the hybridizing 5α-reductase type 2 message is indicated on the *left*. RNA loading was monitored by reprobing the Northern blot with an oligonucleotide specific to the 18S ribosomal RNA (middle panel). Steady state 5α-reductase 2 mRNA levels were assessed by densitometric quantitation (lower panel). For each postnatal age, relative 5α-reductase type 2 mRNA levels are expressed as a percentage of their respective initial segment values ± SEM (n=3); the day 42 data were scaled to fit on the same axis range used for the day 21 data. IS, initial segment; PCT, proximal caput; DCT, distal caput; CO, corpus; PCA, proximal cauda; DCA, distal cauda. a, Significantly different from IS; b, significantly different from IS and PCT; c, significantly different from IS, PCT, and DCT ($P < 0.05$).

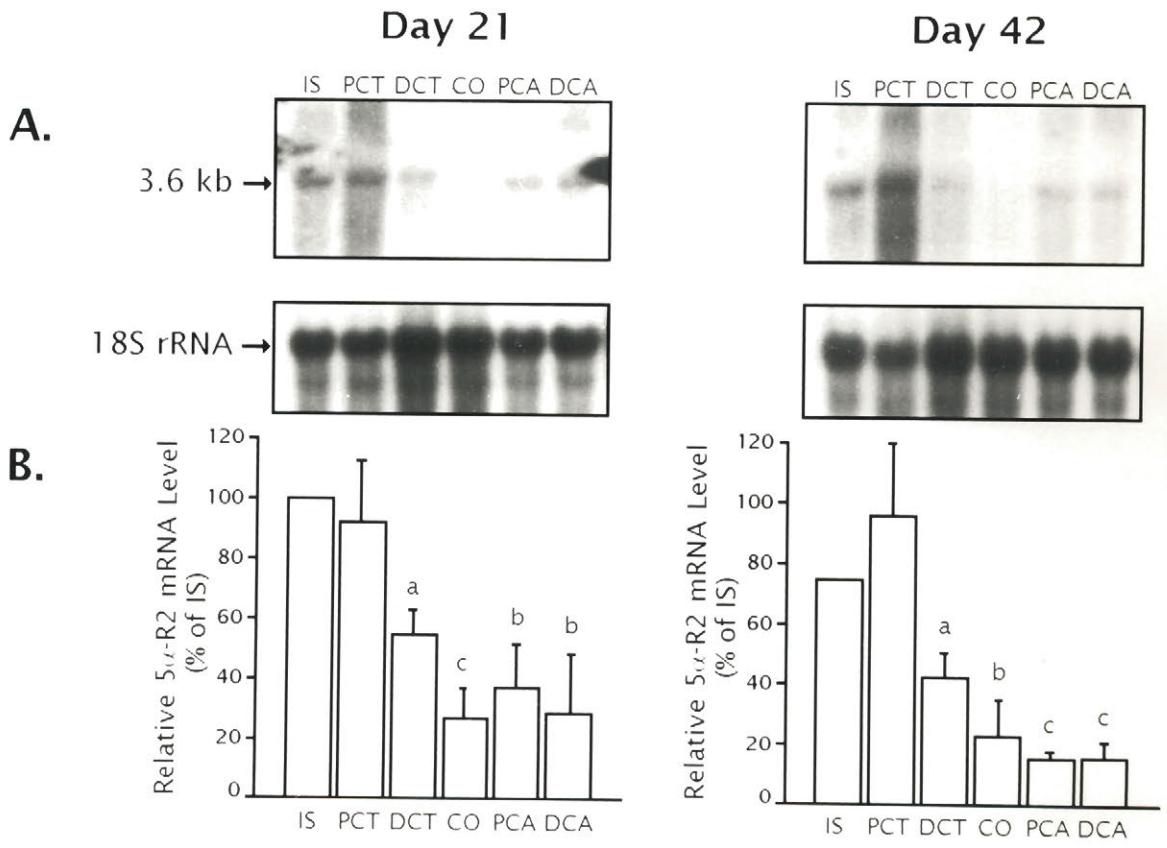

Day 21
Day 42
A.
IS PCT DCT CO PCA DCA
3.6 kb
18S rRNA
B.
Relative 5α-R2 mRNA Level (% of IS)
120
100
80
60
40
20
0
a
b
c
b
IS PCT DCT CO PCA DCA

Figure 3. Developmental regulation of the expression of the 5α-reductase mRNAs in the initial segment of the rat epididymis. Northern blots obtained for the respective 5α-reductase transcripts were quantitated by densitometric scanning as described in *Materials and Methods*. For each 5α-reductase transcript, relative mRNA levels are expressed as a percentage of their respective day 91 values (n=3). *, Significantly different from days 42-91 ($P < 0.05$). †, data adapted from Viger and Robaire (1992), Endocrinology 131:1534-1540.

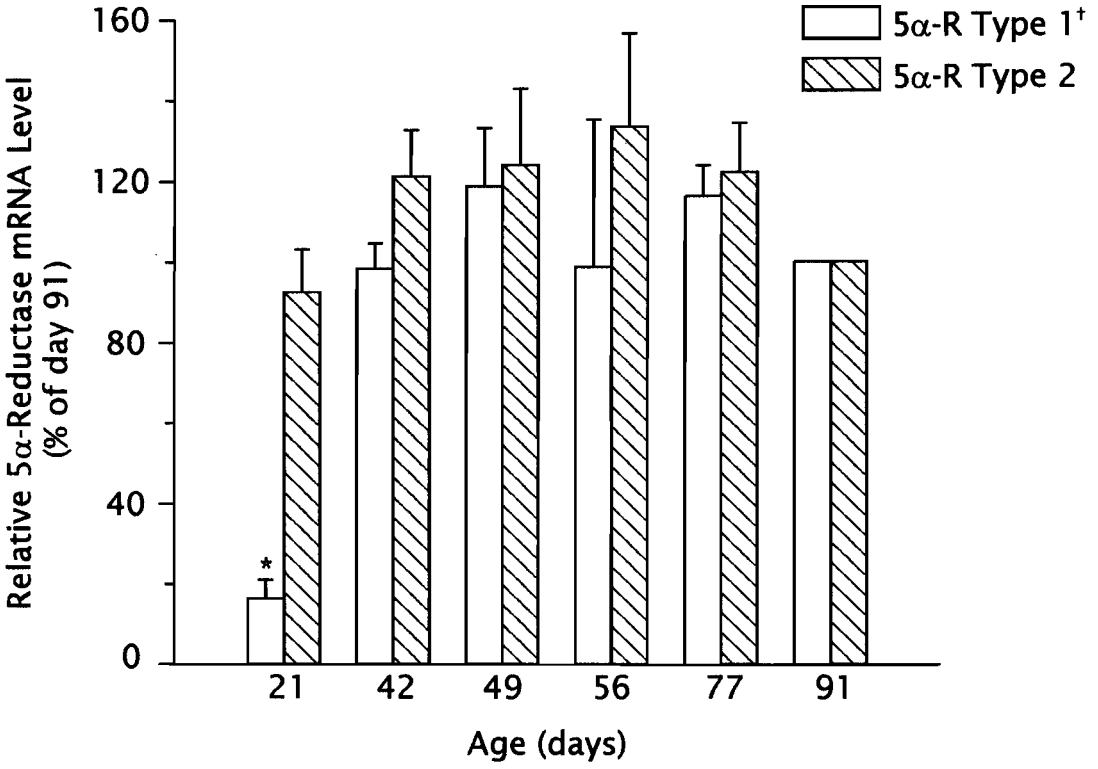

160
120
80
40
0
Relative 5α-Reductase mRNA Level
(% of day 91)
*
21
42
49
56
77
91
Age (days)
5α-R Type 1†
5α-R Type 2

Figure 4. Effect of efferent duct ligation on the expression of the 5α-reductase mRNAs in the different regions of the adult rat epididymis. Adult male rats were unilaterally efferent duct ligated for seven days. The contralateral epididymis served as an internal control. Fifteen micrograms of total RNA were subjected to Northern blot analysis using cDNAs specific for the 5α-reductase mRNAs, types 1 and 2 (upper and middle panels). Exposure of the autoradiograms were optimized separately for each 5α-reductase transcript and epididymal region. RNA loading was monitored by reprobing the Northern blots with an oligonucleotide specific to the 18S ribosomal RNA (lower panel). C, control; L, ligated.

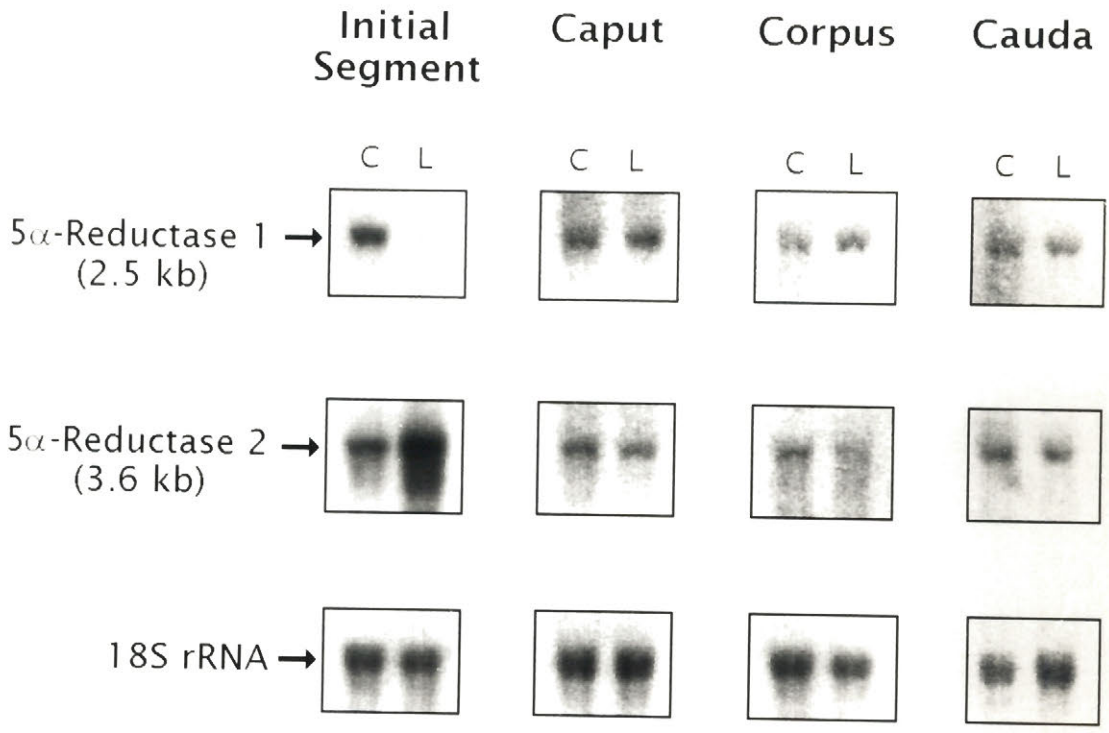
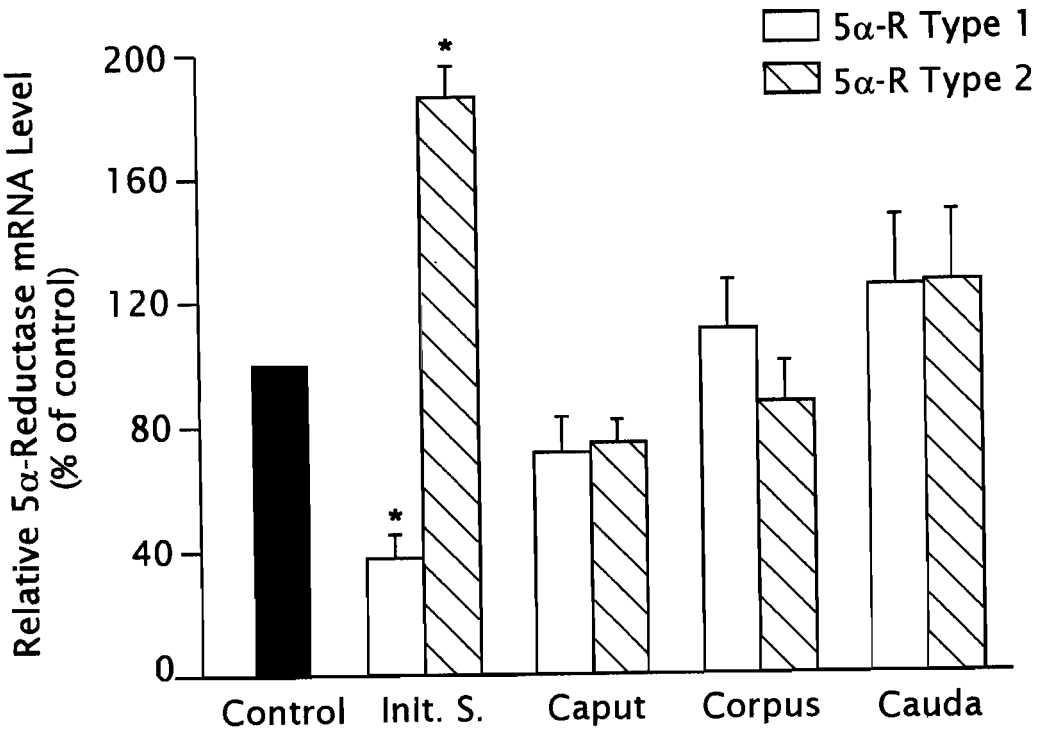

Initial Segment
Caput
Corpus
Cauda
C L
C L
C L
C L
5α-Reductase 1
(2.5 kb)
5α-Reductase 2
(3.6 kb)
18S rRNA

Figure 5. Steady state mRNA levels for the 5α-reductase isozymes in the adult rat epididymis seven days following unilateral efferent duct ligation. Northern blots obtained for the respective 5α-reductase transcripts were quantitated by densitometric scanning as described in *Materials and Methods*. For each 5α-reductase transcript, relative mRNA levels in each epididymal region are expressed as a percentage of their respective control values ± SEM (n=5). *, Significantly different from control ($P < 0.05$).


5α-R Type 1
5α-R Type 2
Relative 5α-Reductase mRNA Level
(% of control)
200
160
120
80
40
0
*
*
Control
Init. S.
Caput
Corpus
Cauda

Connecting Text - Chapter IV to Chapter V

In chapters III and IV, developmental studies were used to characterize the expression of the 5α-reductase mRNAs in the rat epididymis. The postnatal timeperiod studied ranged from shortly after birth to adulthood. These developmental studies proved to be instrumental in defining the regulation and relative importance of the 5α-reductase transcripts in the epididymis. Similar approaches have also been successfully used in the characterization of many other epididymal proteins. Although, many studies have described epididymal functions and their regulation, little is known about how aging affects this tissue. Using a recently developed animal model to study reproductive aging, data has become available in the literature to suggest that aging directly affects the male reproductive tract. Thus, to determine whether the epididymis, which is a major component of the male reproductive tract, is affected by aging, experiments were designed to study gene expression in senescent rats. Since the epididymis is a highly androgen-dependent tissue, particular attention was given to specific markers of androgen action, i.e., the 5α-reductase mRNAs and the androgen receptor. These experiments are presented in the following chapter.

Chapter V

Gene Expression in the Aging Brown Norway Rat Epididymis

Robert S. Viger and Bernard Robaire

Abstract

The mammalian epididymis is the site where spermatozoa are matured and then stored. Though many studies have described epididymal functions and their regulation, little is known about how aging affects this tissue. The Brown Norway rat, which does not show the many age-related pathologies common to other rat strains, was used as a model to study aging of the epididymis. The present study was designed to determine the effect of aging on the mRNA levels for selected markers of epididymal function. Brown Norway rats ranging in age from 6 to 30 months were examined at 6 month intervals; epididymides were sectioned into caput-corpus and cauda regions. Relative mRNA concentrations were assessed using Northern blot analysis and specific cDNAs for the rat 5α-reductase isozymes, types 1 and 2, proenkephalin, the androgen receptor, epididymal proteins B/C and D/E, and sulfated glycoprotein-2 (SGP-2, clusterin). Northern blots were quantitated by densitometric scanning. In the caput-corpus epididymidis, 5α-reductase type 1 and type 2 mRNA levels decreased significantly by 43% and 33%, respectively, between 6 and 12 months and by 64% and 40%, respectively, between 6 and 30 months. No significant change, however, was found in the expression of the 5α-reductase mRNAs in the cauda epididymidis. Interestingly, proenkephalin mRNA was only detected in the caput-corpus epididymidis of 6 month-old rats. In marked contrast to the 5α-reductase isozymes and proenkephalin, no significant age-related changes were observed in the mRNA levels for the androgen receptor, protein B/C, or protein D/E. No age-related changes in mRNA expression for SGP-2 occurred in the caput-corpus epididymidis. However, in the cauda epididymidis, SGP-2 mRNA levels rose by 2 fold between 6 and 18 months, and then decreased sharply by 75% between 18 and 30 months. We conclude that as the epididymis ages, the expression of

genes for certain specific markers of epididymal function is affected in a region-specific manner. Further, the decrease in the concentrations of the mRNAs for the 5α-reductase isozymes and proenkephalin in the epididymis between 6 and 12 months is thus far the earliest marker for aging in the male reproduction tract of the Brown Norway rat.

Introduction

The mammalian epididymis is a single highly convoluted tubule that links the efferent ducts to the vas deferens. Several functions have been ascribed to the epididymal epithelium. Well-established epididymal functions include the absorption, secretion, and synthesis of many micro- and macromolecules, and the metabolism of steroids and other compounds (1). Together, these epididymal processes contribute to the formation of an appropriate luminal microenvironment for the functional maturation, storage, and protection of spermatozoa as they traverse the tissue (2,3). Over the past three decades, the numerous studies which have described the histology and biochemistry of the epididymis have focused primarily on the timeperiod between birth and adulthood (1). As a result, very little information is available on how aging affects this tissue.

In humans and rodents, male reproductive aging is characterized by testicular dysfunction. In both species, aging of the mammalian testis is accompanied by a decrease in testosterone production and atrophy of the seminiferous tubules (germ cell loss) (4-15). Recent studies have demonstrated that the Brown Norway rat, which is not encumbered by the many age-related pathological changes common to other rat strains, is a suitable model to study male reproductive aging (8,10,11). Thus, to gain insight into whether the epididymis, which is a major component of the testicular excurrent duct system, is affected by aging, experiments were designed to study gene expression in the Brown Norway rat epididymis. Since the epididymis is a highly androgen-dependent tissue, particular attention was given to specific markers of androgen action.

Our results indicate that as the epididymis ages, only certain specific markers of epididymal function are affected; further, the nature of this age-related

effect is dependent on the segment of the epididymis being studied.

Materials and Methods

Animals

Brown Norway rats at 6, 12, 18, 24, and 30 months of age were obtained through the National Institute on Aging, Bethesda, MD, and supplied by Charles River. Rats were housed in an aseptic environment and maintained under controlled lighting (14-h light, 10-h dark) and temperature (22 C). Food and water was given *ad libitum*.

Tissue Collection

Rats were killed by decapitation. Epididymal tissues were trimmed of excess fat, immediately frozen in liquid nitrogen, and stored at -80 C. Before RNA isolation, frozen epididymides were sectioned into caput-corpus and cauda regions as previously described (16). No tissues were pooled; total RNA was isolated from epididymal tissue sections obtained from individual rats. A total of five animals were used per age group (n=5).

RNA Isolation

Total cellular RNA was isolated by the guanidinium thiocyanate cesium chloride (CsCl) method (17). In brief, frozen epididymal tissues were homogenized in 5-10 vol (ml/g) guanidinium thiocyanate solution (4.0 M guanidinium thiocyanate, 0.1 M Tris-Cl pH 7.5, 1% ß-mercaptoethanol) . Total RNA was then obtained by centrifuging a 1.2 ml volume of homogenate through a 2.8 ml cushion of CsCl (5.7 M CsCl, 0.01M EDTA pH 7.5) in 4 ml Beckman *g*-MAX *k*onical Quick-Seal tubes (Beckman Canada, Mississauga, Ontario, Canada). The tubes were

centrifuged at 25 C in a Beckman SW 41 rotor for 4 hours at maximum speed (41,000 rpm). Following the centrifugation, the RNA pellet was resuspended in a minimal volume of TE/SDS (0.01 M Tris, 0.001 M EDTA, 0.1% SDS, pH 7.5) and reprecipitated with ethanol. RNA concentration was measured by the absorbance at 260 nm (Beckman DU7 spectrophotometer). Under these conditions, RNA yields were consistently 50 µg RNA/ml homogenate or more, for both the caput-corpus and cauda epididymidis.

Northern Blot Analysis

Fifteen micrograms of total cellular RNA were denatured before electrophoresis by adding formaldehyde, formamide, and borate buffer (0.1 M sodium borate, 0.1 M boric acid, and 0.001 M EDTA, pH 7.6) to final concentrations of 6%, 40%, and 0.02 M, respectively, and heating the mixture for 5 min at 65 C. The denatured RNA was size fractionated in 1% agarose formaldehyde-containing gels. Subsequent Northern blot transfer to nylon membranes was done as previously described (18).

Hybridization Probes

Gene expression in the aging Brown Norway rat epididymis was studied by successively hybridizing, stripping, and reprobing the Northern blots with cDNA probes for the following mRNAs: (i) rat steroid 5α-reductase isozymes, types 1 and 2 (kindly provided by Dr. D. Russell, University of Texas Southwestern Medical Center) (19,20); (ii) a 1082 bp *Eco*R1-*Eco*R1 fragment of the rat androgen receptor cDNA (kindly provided by Dr. E. Wilson, University of North Carolina at Chapel Hill) (21); (iii) a 750 bp *Eco*R1-*Hind*III fragment of the cDNA for rat epididymal protein B/C , a 700 bp *Eco*R1-*Eco*R1 fragment of the cDNA for rat epididymal

protein D/E, and a cDNA encoding rat proenkephalin (kindly provided by Dr. J. Douglass, Vollum Institute, Oregon Health Sciences University) (22); and (iv) a plasmid containing the cDNA encoding for sulfated glycoprotein-2 (kindly provided by Dr. M. Griswold, Washington State University) (23). The procedures for membrane hybridization and stripping were done as previously outlined (18).

To monitor the amount of total RNA loaded in each lane of the Northern blots, all hybridization membranes were finally probed with an oligonucleotide that is specific for the 18S ribosomal RNA (24). The oligonucleotide was end-labelled with ^{32}P-ATP using standard procedures (17). Membranes were autoradiographed for various times by exposure to Kodak X-Omat AR film (Eastman Kodak, Rochester, NY) with intensifying screens (Fisher Biotech, Montreal, Quebec, Canada) at -80 C.

RNA Quantitation

Autoradiograms were quantitated using an LKB Ultrascan XL Laser Densitometer (Pharmacia-LKB Biotechnology, Uppsala, Sweden). The integrated areas obtained for each cDNA probe were normalized to the corresponding areas obtained with the 18S ribosomal RNA probe. Densitometric readings were restricted to the linear range of the densitometer, as previously described (16).

Statistical Analysis

Statistical analysis was done by one-way analysis of variance, followed by a Newman-Keuls test to detect significant differences between means (25). $P < 0.05$ was considered significant.

Results

Effect of Aging on the Expression of RNA Transcripts for the 5α-Reductase Isozymes and the Androgen Receptor in the Brown Norway Rat Epididymis

To assess the effect of aging on androgen action in the Brown Norway rat epididymis, the steady state mRNA levels for the two components involved in the mechanism of androgen action were studied: the 5α-reductase isozymes, which catalyze the synthesis of the major biologically active androgen, 5α-dihydrotestosterone, and the androgen receptor, which is the effector of androgen action at the cellular level.

The results for the first of these markers, the 5α-reductase type 1 isozyme, are presented in Fig. 1. In the Brown Norway rat, a single 2.5 kilobase 5α-reductase type 1 RNA transcript was detected in both epididymal regions studied (Fig. 1, upper panels). This transcript size was identical to the one that has been described previously for several tissues of the adult male Sprague Dawley rat, including the epididymis (18,20). Quantitation of the Northern blots by laser densitometry revealed an age-related effect that was region-specific. In the caput-corpus epididymidis, steady state 5α-reductase type 1 mRNA levels decreased significantly by 43% between 6 and 12 months of age (Fig. 2, bottom left panel). The extent of this decrease was further augmented between 12 and 30 months of age. By 30 months, 5α-reductase type 1 mRNA concentrations had declined by 64% when compared to those in 6 month old rats. The dramatic reduction in type 1 mRNA expression in the caput-corpus epididymidis was in marked contrast to what was found for the cauda epididymidis. In this region of the tissue, no significant age-related changes were found (Fig. 1, right panels).

Changes in the expression of the 5α-reductase type 2 mRNA in the aging Brown Norway rat epididymis, although not as dramatic, were similar to those

found for the type 1 isozyme. The results for the 5α-reductase type 2 transcript are shown in Fig. 2. In the Brown Norway rat, a predominant 3.6 kilobase 5α-reductase type 2 mRNA species was detected in both epididymal regions (Fig. 2, upper panels). This transcript size was identical to the one that has been reported for the Sprague Dawley rat (20). In the caput-corpus epididymidis, 5α-reductase type 2 mRNA levels decreased significantly by 33% between 6 and 12 months and by 40% between the 6 to 30 months timeperiod. As was the case for the type 1 message, in the cauda epididymidis no significant age-related changes in 5α-reductase type 2 mRNA expression were observed (Fig. 2, bottom right panel).

The second facet in understanding the effect of aging on the expression of markers for the components of androgen action in the epididymis was to study the expression of the androgen receptor. The data for the androgen receptor mRNA concentrations in the Brown Norway rat epididymis are presented in Fig. 3. At all ages examined, a single 10 kilobase hybridizing RNA transcript was observed (Fig. 3, top two panels). This size was consistent with the mRNA species that has been previously described with respect to several androgen-sensitive tissues of the adult male Sprague Dawley rat (21). The androgen receptor transcript was abundantly expressed in the caput-corpus epididymidis when compared to the caudal region of this tissue. This finding was clearly evident from the fact that androgen receptor mRNA levels in the caput-corpus epididymidis could be easily quantitated after 3 days of autoradiographic exposure (Fig. 3, left panel second from top), whereas in the cauda epididymidis, 12 days of exposure were required (Fig. 3, upper right panel). Quantitation by densitometric scanning revealed that in contrast to the 5α-reductase isozymes, relative androgen receptor mRNA levels did not change significantly as a function of aging; this was the case for both epididymal regions studied (Fig. 3, bottom panels).

Effect of Aging on the Expression of RNA Transcripts for Androgen-Sensitive Epididymal Proteins and Proteins Controlled by Other Factors

Complementary to the understanding of androgen action in the aging Brown Norway rat epididymis is to study the expression of specific androgen-dependent genes, since it is these genes that are the ultimate targets in the cascade model of androgen action. For this purpose, the steady state mRNA concentrations of three androgen-dependent genes were assessed: protein B/C, protein D/E, and sulfated glycoprotein-2 (SGP-2). The epididymal expression of the first two markers is stimulated by androgens (22), whereas the latter marker, SGP-2, constitutes an androgen-repressed transcript in selective regions of the epididymis (26). We also investigated the expression of epididymal proenkephalin mRNA since this marker has been reported to be tightly regulated in a paracrine manner by androgen-dependent testicular factor(s) (22).

The results for protein B/C and protein D/E are shown in Figs. 4 and 5, respectively. A single RNA transcript of 0.85 kilobases and 1.2 kilobases in size was identified for protein B/C and protein D/E, respectively, at all ages examined (Figs. 4 and 5, upper panels). These transcript sizes were identical to those that have been described for other rat strains (27,28). Consistent with the results reported previously (22), the expression of the protein B/C transcript was region-specific; it was present only in the caput-corpus epididymidis (Fig. 4, left upper panel). Interestingly, however, quantitation by laser densitometry revealed no significant changes in the expression of either transcript in the aging Brown Norway rat epididymis (Figs. 4 and 5, bottom panels).

Although the expression of two androgen-stimulated epididymal genes was not compromised apparently, as result of aging, a very dramatic effect on SGP-2 mRNA expression did occur (Fig. 6). At all ages studied, a single hybridizing

mRNA species of 1.9 kilobases in size was detected in both epididymal regions (Fig. 6, upper panels). This RNA transcript size was in the characteristic range for rat SGP-2 (23,26). Consistent with previous findings (26), SGP-2 mRNA was overwhelmingly abundant in the caput-corpus region of the epididymis, as compared to the cauda epididymidis (Fig. 6, top two panels). This was strikingly evident when the exposure times needed for quantitation between the two epididymal regions are compared: one hour was sufficient for the caput-corpus epididymidis (Fig. 6, left panel second from top) and twenty-four hours for the cauda epididymidis (Fig. 6, right top panel). Densitometric quantitation of the Northern blots indicated that in the caput-corpus epididymidis, SGP-2 mRNA levels did not change significantly as a consequence of aging (Fig. 6, left bottom panel). A strikingly different pattern was observed in the cauda epididymidis, however (Fig. 6, bottom right panel). In this epididymal region, SGP-2 mRNA levels increased significantly by 2 fold between 6 and 18 months of age. This was subsequently followed by an abrupt decline in SGP-2 mRNA levels between 18 and 30 months. At the 30 month timepoint, SGP-2 mRNA expression had decreased by 75% in comparison to the mRNA levels present at 18 months.

Lastly, studies designed to assess epididymal proenkephalin mRNA expression revealed that it was possible to detect this message only in the caput-corpus epididymidis and only in that of 6 month-old Brown Norway rats (Fig. 7).

Discussion

In contrast to other tissues of the male reproductive tract, reports of studies in the literature which have focused on the effects of aging in the epididymis have been scarce. In the few studies that have been done in the rat, the most common feature has been the gradual decline in the concentration of epididymal

spermatozoa with advancing age (29-32). In both humans and rodents, aging of the testis is frequently accompanied with germ cell loss and consequently, a decrease in daily sperm production (9,10,12,31,33,34). Thus, with respect to the epididymis, the decrease in sperm content in aging rats may simply represent a deficit in testicular sperm production. Saksena et al. (29) reported a significant reduction in progeny outcome in rat litters that were sired by aged males. Again, the lower fertility in these older animals may be a reflection of decreased sperm numbers in the male reproductive tract. Alternatively, it is inviting to speculate that epididymal function is directly affected as a result of aging. Unfortunately, the necessary studies to test this hypothesis have not been done. In the present study, however, we report, for the first time, that aging affects the expression of important markers of epididymal function.

In the epididymis, 5α-reductase is the rate limiting enzyme in the synthesis of dihydrotestosterone (DHT) and 5α-androstan-3α,17β-diol (3α-diol). DHT and 3α-diol have been suggested to be the primary androgen(s) responsible for the functional maturation and storage of spermatozoa in the epididymis (35). We have previously shown that the expression and regulation of the 5α-reductase type 1 mRNA is highly correlated with 5α-reductase enzyme activity in the rat epididymis (18). Thus, the decrease in expression of the 5α-reductase isozymes, and in particular the type 1 transcript, in the aging Brown Norway rat epididymis strongly suggests that the ability of the aging tissue to produce 5α-reduced androgens is compromised. The fact that this decrease occurred selectively in the caput-corpus epididymidis is also consistent with segment-specific nature of the regulation that has ascribed to 5α-reductase type 1 mRNA in the rat epididymis (16,18).

It has been recently demonstrated by several groups that serum testosterone levels decline in the aging Brown Norway rat (8,10,11). Two lines of

evidence, however, would suggest that the present decrease in the 5α-reductase mRNAs, particularly the type 1 transcript, in the aging epididymis is independent of the decrease in serum testosterone levels. First, the primary factor regulating 5α-reductase type 1 mRNA expression and enzyme activity in the proximal portion of the epididymis is a testicular factor that is not testosterone (18,36). Secondly, and more importantly, the greatest drop in epididymal 5α-reductase type 1 mRNA levels in the aging Brown Norway rat occurred between 6 and 12 months, whereas in similar animals, a significant decrease in serum testosterone levels was not apparent until the 18-24 month timeperiod (8). Thus, based on these observations, it is inviting to speculate that the decreased expression of the proposed factor controlling epididymal 5α-reductase enzyme activity and type 1 transcript expression is an early marker for aging in the Brown Norway rat testis. Moreover, the fact that proenkephalin mRNA expression, which is known to be regulated in a paracrine manner by a testicular factor (22), was also found to be markedly reduced between 6 and 12 months lends further credence to the hypothesis that the ability of the testis to secrete certain factors must be affected as early as 12 months. It is interesting to note, however, that the histological appearance of the seminiferous epithelium and expression of some Sertoli cell products have been reported previously to be unaltered during this same 6-12 month time period (9).

In contrast to the 5α-reductase mRNAs, androgen receptor mRNA expression did not change significantly in the aging epididymis. This finding suggests that despite possible deficiencies in the production of active androgen in the aging epididymis through decrease 5α-reductase isozyme expression, the potential of the tissue to respond to exogenously administered androgen through its receptor remains intact. The ability of testosterone to restore the

physicochemical characteristics of epididymal sperm in old rats as compared to those of young animals has partly corroborated this proposal (32). Further, functional activity of the androgen receptor in the aging Brown Norway rat epididymis was demonstrated by studying the gene expression of two highly androgen-stimulated epididymal proteins, i.e., proteins B/C and D/E, in the present study. The mRNA concentrations for these proteins, which are also known as epididymal retinoic acid binding protein (eCRABP; 37) and epididymal glycoprotein (AEG; 38) , respectively, were not altered in the aging rat epididymis. Thus, this result confirmed the presence of functional androgen receptors in the aging epididymis. Interestingly, the absence of an age-related decline in androgen receptor mRNA expression in the epididymis is in marked contrast to the decrease that has been reported for other aging rat tissues such as the liver (39,40). In aging hepatic tissue, the disappearance of the androgen receptor transcript is functionally associated with marked androgen insensitivity (39,40).

The expression of the mRNA for sulfated glycoprotein-2 (SGP-2) was studied for two reasons. First, its regulation by androgens in the rat epididymis is well-established (26). Secondly, SGP-2 has proven to be a useful marker for apoptosis and/or cellular injury in many tissues (41-44). In the caput-corpus epididymidis, SGP-2 mRNA levels are androgen insensitive (26). Therefore, the finding that caput-corpus epididymal SGP-2 mRNA concentrations did not change significantly with advancing age, despite a fall in serum testosterone levels, is consistent with the lack of androgenic regulation of SGP-2 in this region of the tissue. In the cauda epididymidis, SGP-2 mRNA levels are repressed by testosterone (26). The dramatic increase in SGP-2 mRNA expression that was observed in 18 month old Brown Norway rats may be a reflection of decreased serum testosterone concentrations. However, serum testosterone levels were not

markedly reduced in the Brown Norway rat until 24 months of age (8). Therefore, as an alternate explanation, the overwhelming increase in SGP-2 mRNA levels observed in 18 month-old rats may be a early marker of cellular damage occurring in this region of the epididymis . Moreover, the fact that this significant increase in SGP-2 mRNA levels at 18 months is temporary, i.e., SGP-2 mRNA levels fell back sharply between 24-30 months, is consistent with the transient increases that have been reported in other cases of tissue injury or cellular damage (45,46).

In conclusion, this is first report to identify markers for aging in the rat epididymis. Age-dependent alterations in gene expression in the Brown Norway rat epididymis were not restricted to a single morphological region of the tissue; changes in both the caput-corpus and cauda epididymidis were observed and were apparent as early as 12 months of age. These findings should pave the way for further studies directed at defining the consequences of aging on epididymal structure and function.

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Figure 1. 5α-Reductase type 1 mRNA expression in the caput-corpus and cauda epididymidis of aging Brown Norway rats. Fifteen micrograms of total RNA are present in each lane. *Upper panel*, Northern blots showing the characteristic 2.5 kilobase 5α-reductase type 1 transcript. *Middle panel*, Reprobe of the Northern blots with an oligonucleotide to the 18S ribosomal RNA. *Lower panel*, Steady state 5α-reductase type 1 mRNA concentrations, as assessed by laser densitometry. In both epididymal regions, values are expressed as a percentage of their respective 6 month levels, as the mean ± SEM (n=5). *, Significantly different from 6 months ($P < 0.05$).

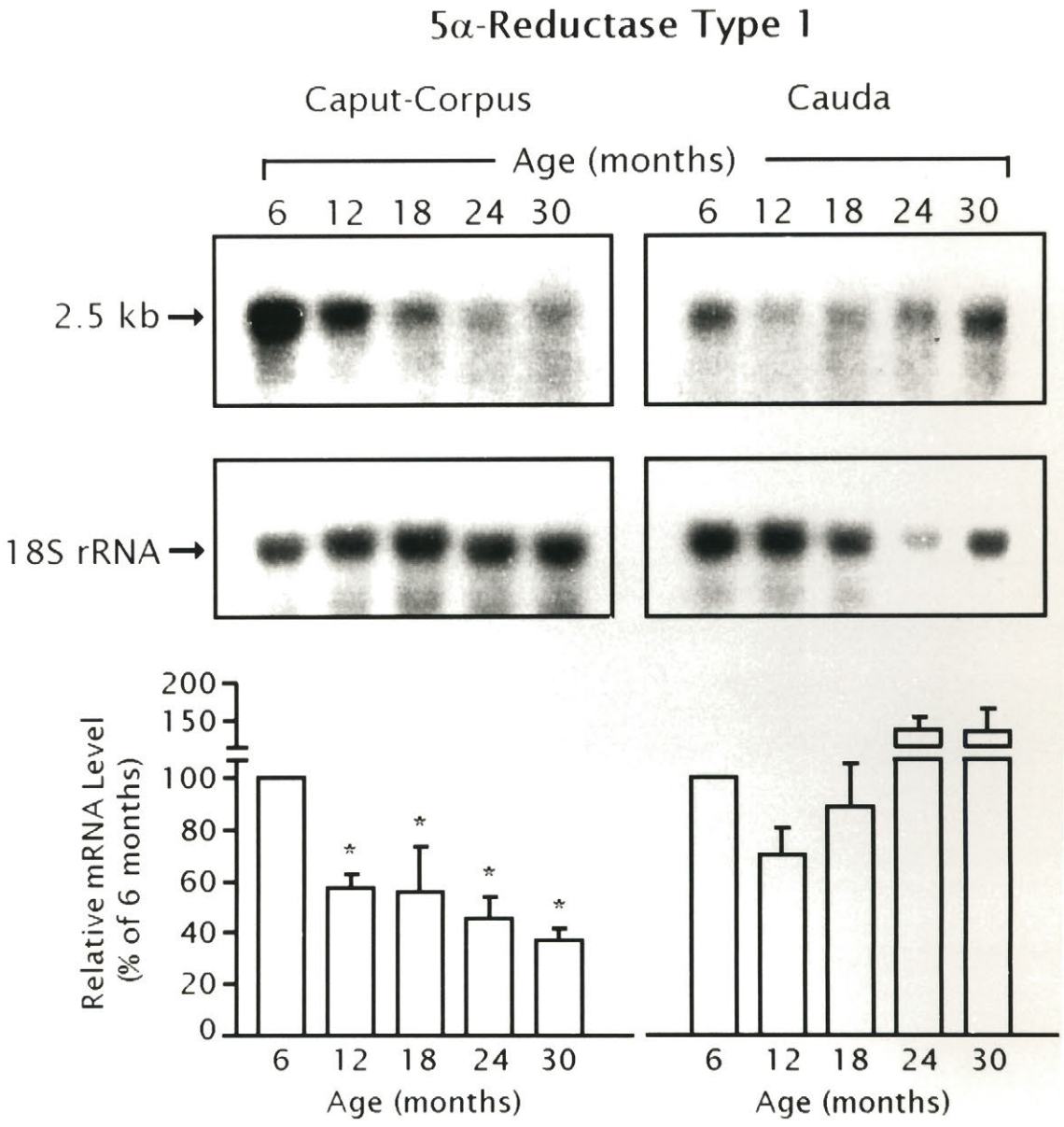

5α-Reductase Type 1
Caput-Corpus
Cauda
Age (months)
6 12 18 24 30
6 12 18 24 30
2.5 kb
18S rRNA
Relative mRNA Level (% of 6 months)
200
150
100
80
60
40
20
0
*
6 12 18 24 30
Age (months)
6 12 18 24 30
Age (months)

Figure 2. 5α-Reductase type 2 mRNA expression in the caput-corpus and cauda epididymidis of aging Brown Norway rats. Fifteen micrograms of total RNA are present in each lane. *Upper panel*, Northern blots showing the characteristic 3.6 kilobase 5α-reductase type 2 transcript. *Middle panel*, Reprobe of the Northern blots with an oligonucleotide to the 18S ribosomal RNA. *Lower panel*, Steady state 5α-reductase type 2 mRNA concentrations, as assessed by laser densitometry. In both epididymal regions, values are expressed as a percentage of their respective 6 month levels, as the mean ± SEM (n=5). *, Significantly different from 6 months ($P < 0.05$).

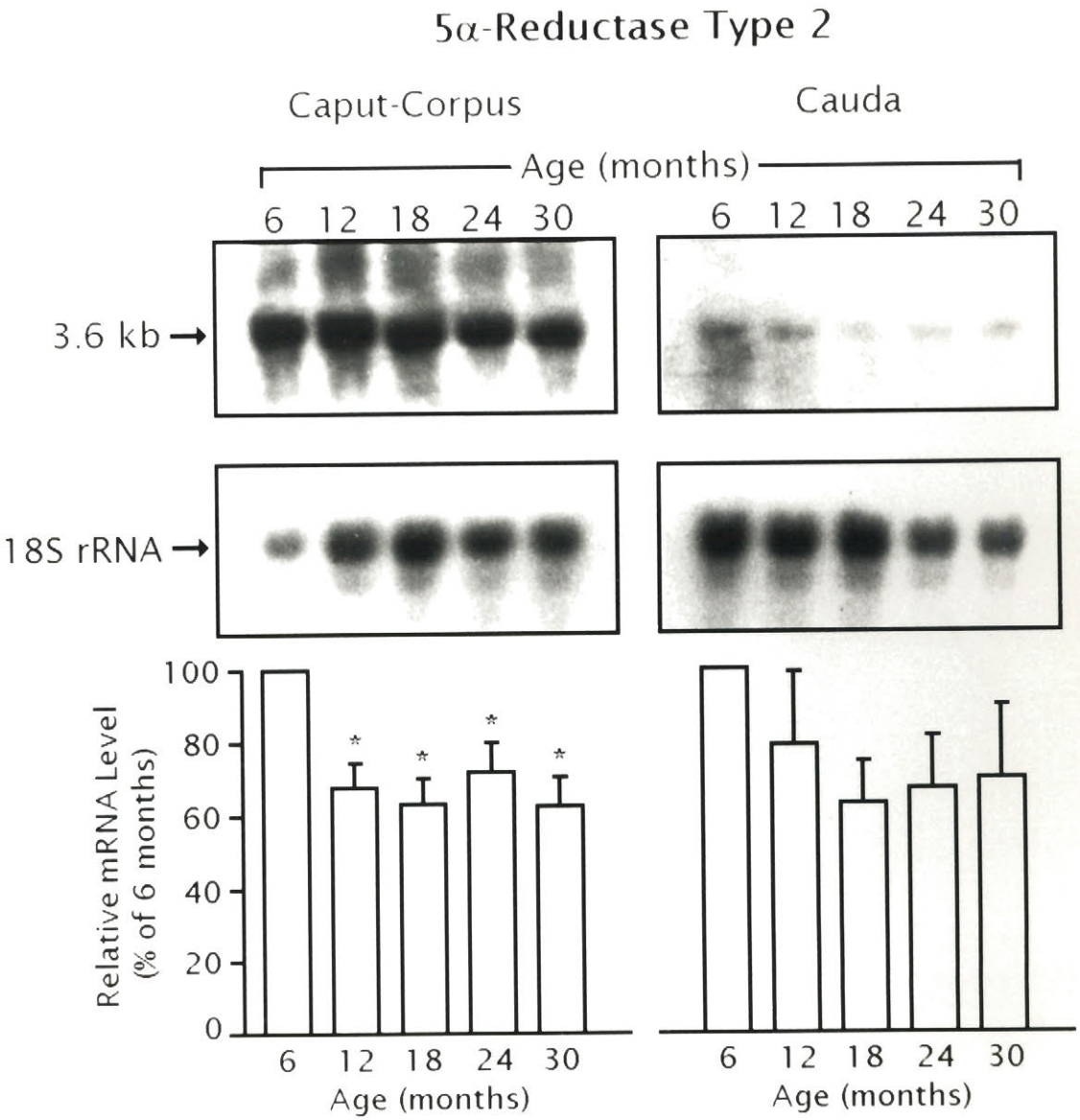

5α-Reductase Type 2
Caput-Corpus
Cauda
Age (months)
6 12 18 24 30
6 12 18 24 30
3.6 kb
18S rRNA
Relative mRNA Level (% of 6 months)
100
80
60
40
20
0
*
*
*
*
6 12 18 24 30
Age (months)
6 12 18 24 30
Age (months)

Figure 3. Androgen receptor mRNA expression in the caput-corpus and cauda epididymidis of aging Brown Norway rats. Fifteen micrograms of total RNA are present in each lane. *Upper panels*, Northern blots indicating the 10 kilobase androgen receptor transcript after 3 and 12 days of autoradiographic exposure. *Middle panel*, Reprobe of the Northern blots with an oligonucleotide to the 18S ribosomal RNA. *Lower panel*, Steady state androgen receptor mRNA concentrations as assessed by laser densitometry. In both epididymal regions, values are expressed as a percentage of their respective 6 month levels, as the mean ± SEM (n=5). All values did not differ significantly from each other ($P > 0.05$).

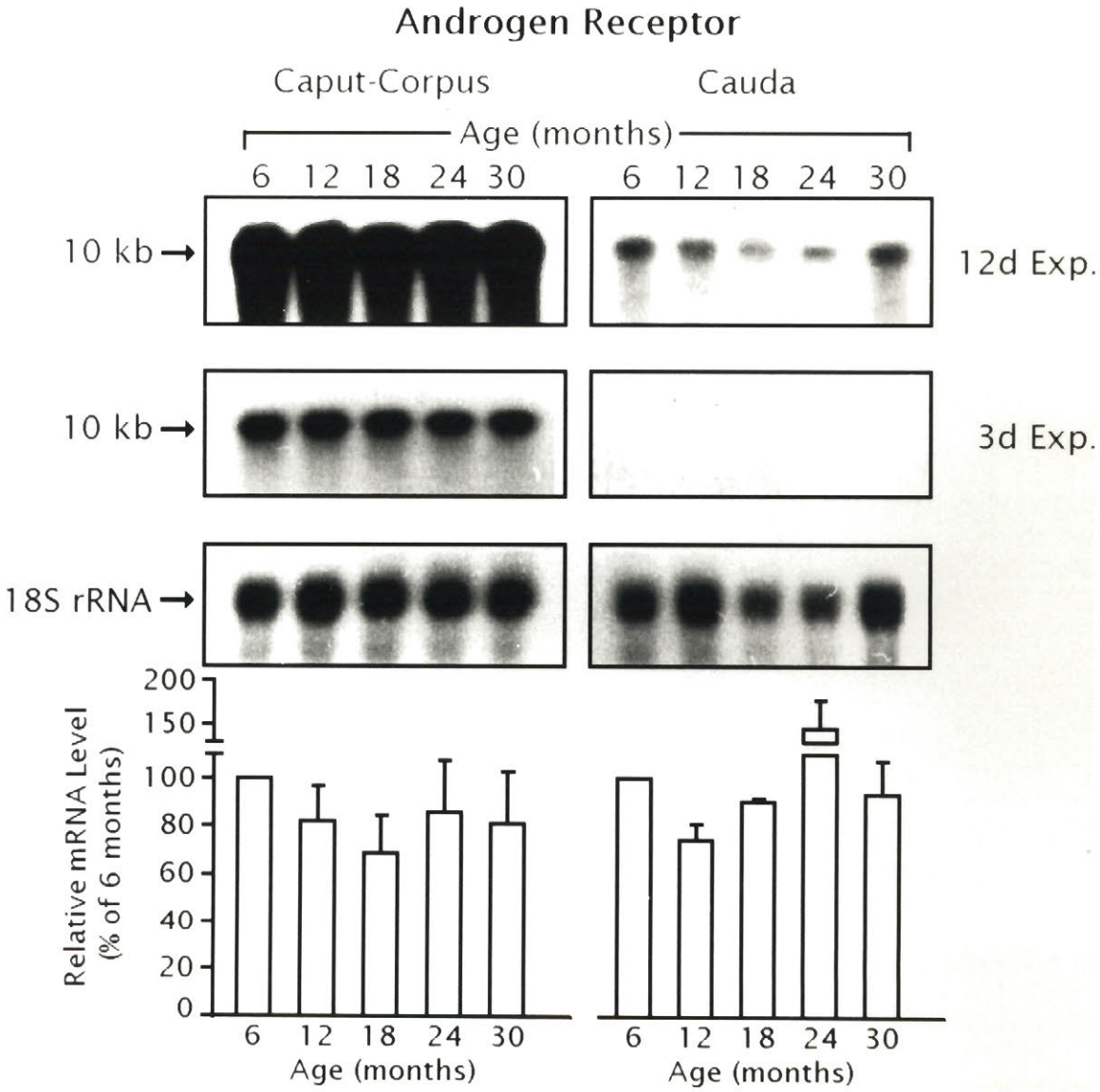

Androgen Receptor
Caput-Corpus
Cauda
Age (months)
6 12 18 24 30
6 12 18 24 30
10 kb
12d Exp.
10 kb
3d Exp.
18S rRNA
Relative mRNA Level
(% of 6 months)
200
150
100
80
60
40
20
0
6 12 18 24 30
Age (months)
6 12 18 24 30
Age (months)

Figure 4. Protein B/C (epididymal cytoplasmic retinoic acid binding protein, eCRABP) mRNA expression in the caput-corpus and cauda epididymidis of aging Brown Norway rats. Fifteen micrograms of total RNA are present in each lane. *Upper panel*, Northern blots indicating the 0.85 kilobase protein B/C transcript. Note that protein B/C mRNA is undetectable in the cauda epididymidis. *Middle panel*, Reprobe of the Northern blots with an oligonucleotide to the 18S ribosomal RNA. *Lower panel*, Steady state protein B/C mRNA concentrations as assessed by laser densitometry (ND, non-detectable). Values in the caput-corpus epididymidis are expressed as percentage of the 6 month level (n=5). All values did not differ significantly from each other ($P > 0.05$).

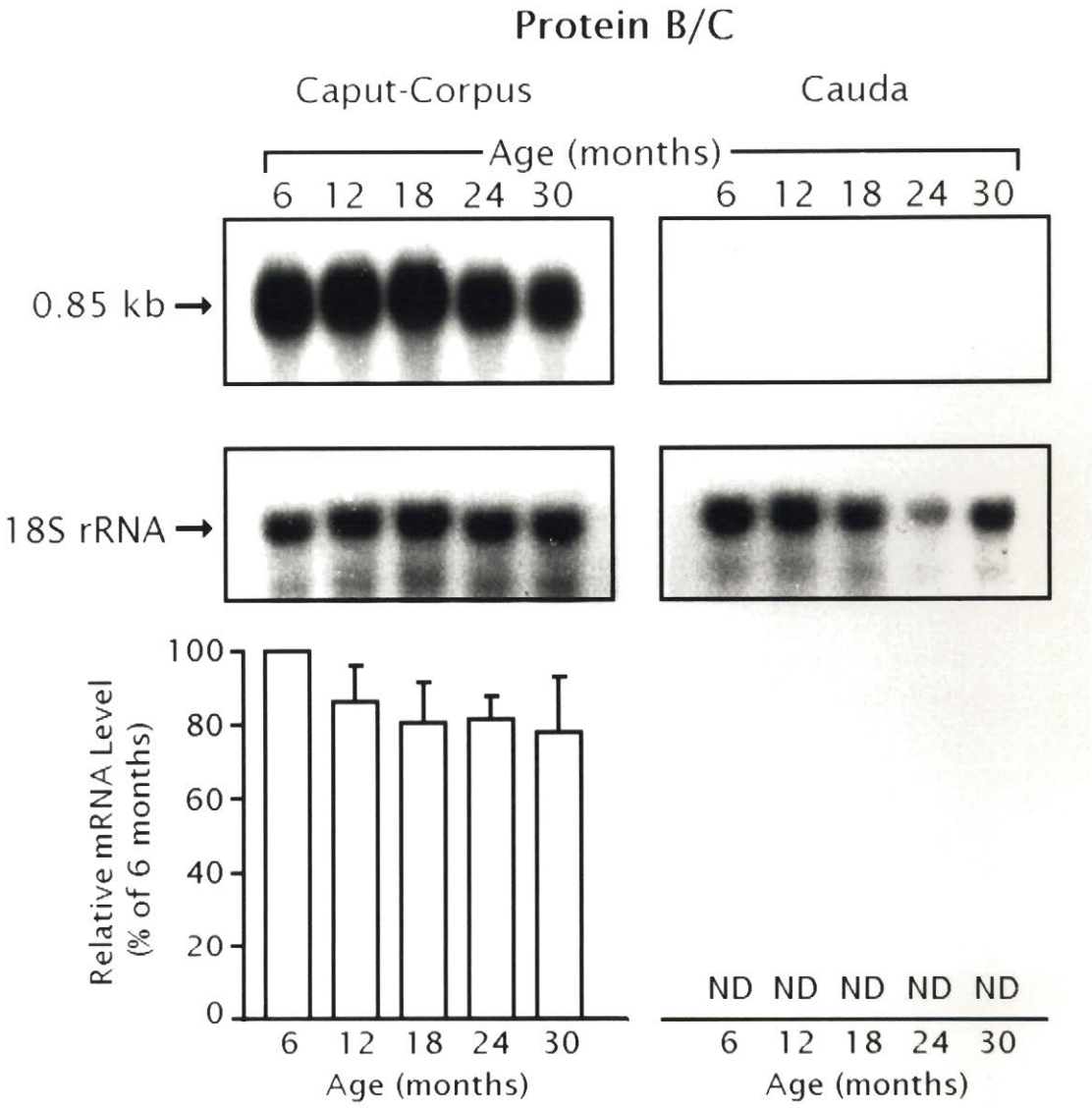

Protein B/C
Caput-Corpus
Cauda
Age (months)
6 12 18 24 30
6 12 18 24 30
0.85 kb
18S rRNA
Relative mRNA Level (% of 6 months)
100
80
60
40
20
0
6 12 18 24 30
Age (months)
ND ND ND ND ND
6 12 18 24 30
Age (months)

Figure 5. Protein D/E (acidic epididymal glycoprotein, AEG) mRNA expression in the caput-corpus and cauda epididymidis of aging Brown Norway rats. Fifteen micrograms of total cellular RNA are present in each lane. *Upper panel*, Northern blots indicating the 1.2 kilobase protein D/E transcript. *Middle panel*, Reprobe of the Northern blots with an oligonucleotide to the 18S ribosomal RNA. *Lower panel*, Steady state protein D/E mRNA concentrations as assessed by laser densitometry. In both epididymal regions, values are expressed as a percentage of their respective six month levels, as the mean ± SEM (n=5). All values did not differ significantly from each other ($P > 0.05$).

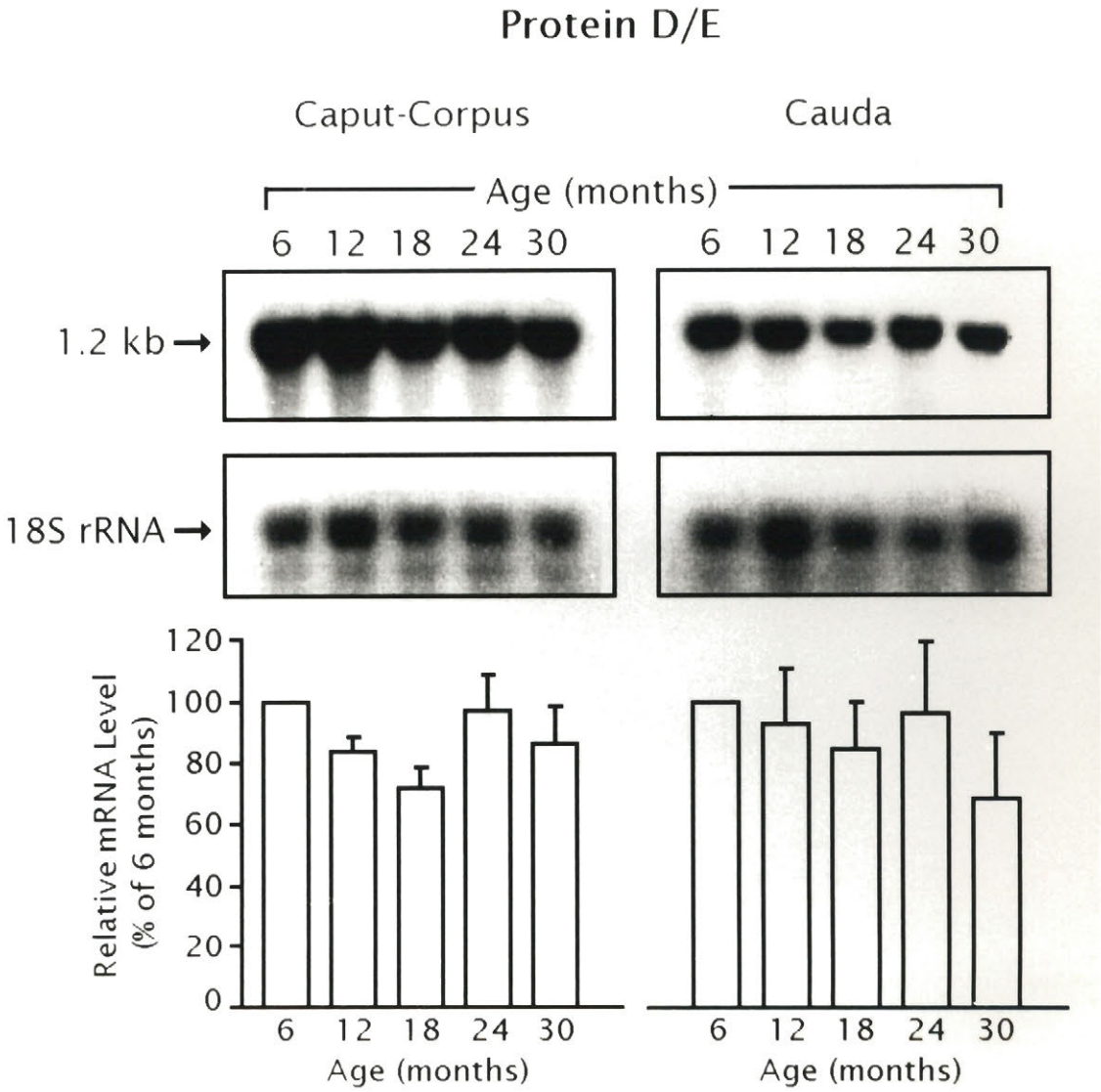

Protein D/E
Caput-Corpus
Cauda
Age (months)
6 12 18 24 30
6 12 18 24 30
1.2 kb
18S rRNA
Relative mRNA Level (% of 6 months)
120
100
80
60
40
20
0
6 12 18 24 30
Age (months)
6 12 18 24 30
Age (months)

Figure 6. Sulfated glycoprotein-2 mRNA expression in the caput-corpus and cauda epididymidis of aging Brown Norway rats. Fifteen micrograms of total cellular RNA are present in each lane. *Upper panels*, Northern blots indicating the 1.9 kilobase sulfated glycoprotein-2 transcript after 1 and 24 hours of autoradiographic exposure. *Middle panel*, Reprobe of the Northern blots with an oligonucleotide to the 18S ribosomal RNA. *Lower panel*, Steady state sulfated glycoprotein-2 mRNA concentrations as assessed by laser densitometry. In both epididymal regions, values are expressed as a percentage of their respective 6 month levels, as the mean ± SEM (n=3-5). *, Significantly different from months 6,12,24, and 30 ($P < 0.05$). **, Significantly different from months 6 and 18 ($P < 0.05$).

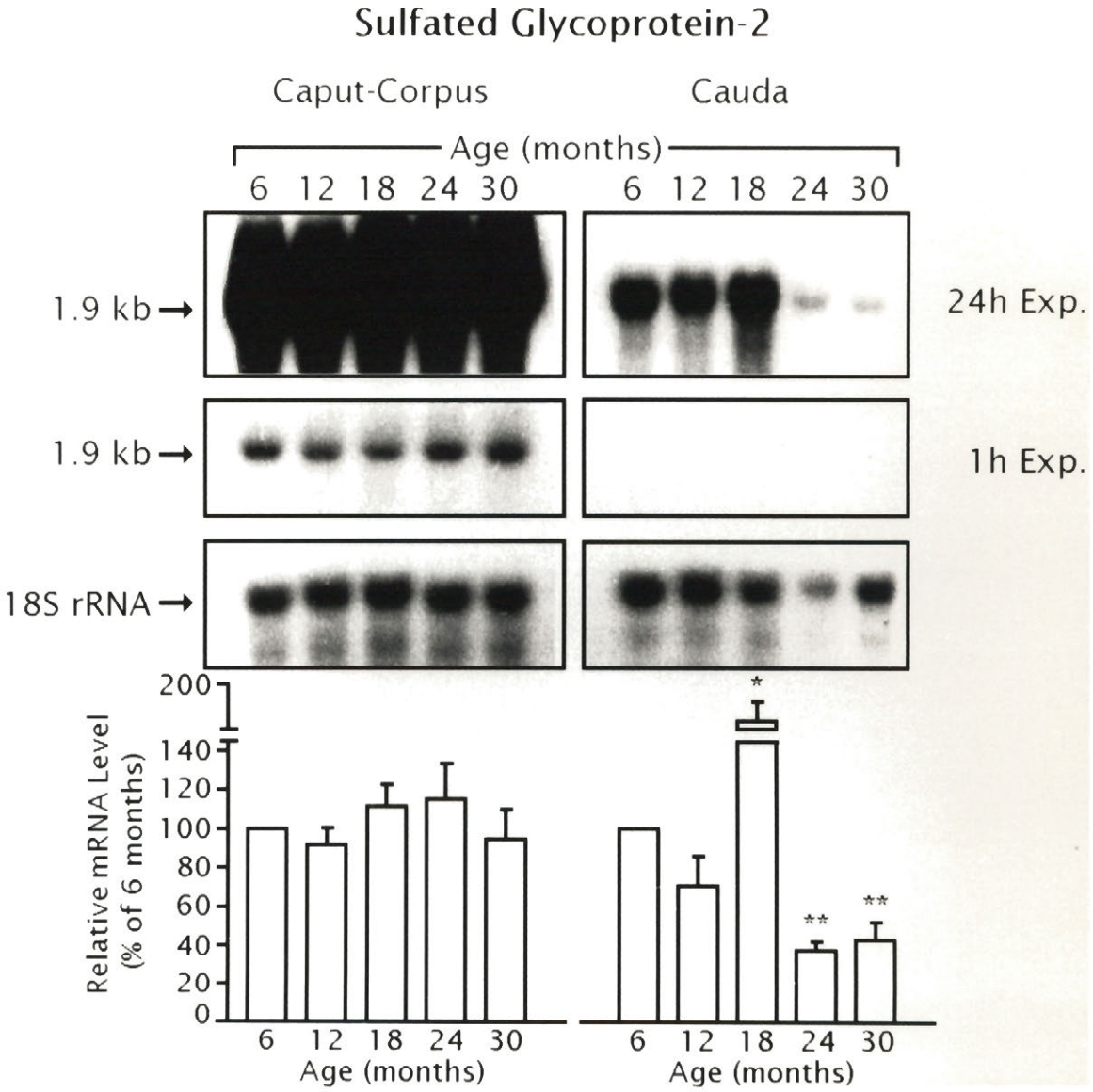
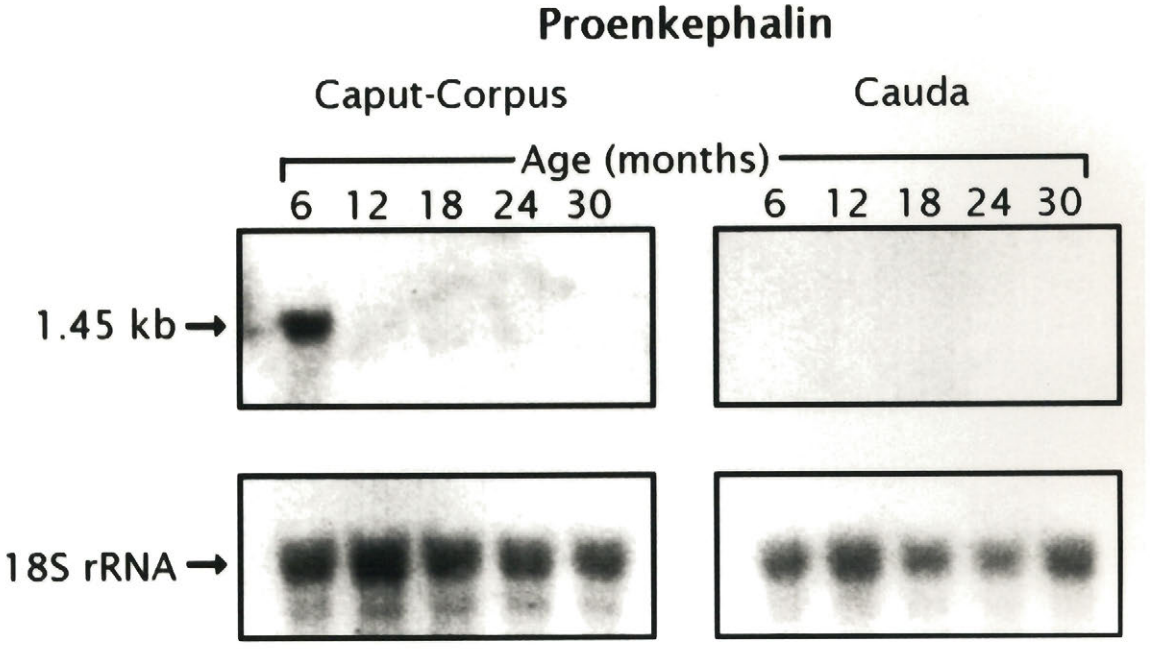

Sulfated Glycoprotein-2
Caput-Corpus
Cauda
Age (months)
6 12 18 24 30
6 12 18 24 30
1.9 kb
24h Exp.
1.9 kb
1h Exp.
18S rRNA
Relative mRNA Level
(% of 6 months)
200
140
120
100
80
60
40
20
0
*
**
**
6 12 18 24 30
Age (months)
6 12 18 24 30
Age (months)

Figure 7. Proenkephalin mRNA expression in the caput-corpus and cauda epididymidis of aging Brown Norway rats. Fifteen micrograms of total RNA are present in each lane. *Upper panel*, Northern blot indicating the 1.45 kilobase proenkephalin transcript. Note that the proenkephalin mRNA is predominantly expressed in the caput-corpus epididymidis of the 6 month-old rat . *Lower panel*, Reprobe of the Northern blots with an oligonucleotide to the 18S ribosomal RNA.


Proenkephalin
Caput-Corpus
Cauda
Age (months)
6 12 18 24 30
6 12 18 24 30
1.45 kb
18S rRNA

Connecting Text - Chapter V to Chapter VI

In the preceding chapters, tissue distribution, endocrine, developmental, and aging studies were used to provide insight on how the mRNAs for the 5α-reductase isozymes in the rat epididymis are regulated. In comparing these data with those previously reported on the regulation of 5α-reductase enzyme activity in the epididymis, it became apparent that another potential regulatory site for the enzyme may reside at the level of the protein itself. Evidence to support this hypothesis was clearly apparent in the developmental studies that were presented in chapter III. For example, the presence of the 5α-reductase type 1 transcript in the developing rat epididymis preceded the appearance of measurable enzyme activity by at least two weeks. Furthermore, 5α-reductase type 1 mRNA expression did not decline with age as the animals approached adulthood, whereas enzyme activity did, after an initial rise and peak. Posttranscriptional regulatory mechanisms have been proposed for some epididymal proteins and other proteins in the male reproductive tract. Thus, a similar mechanism for regulating epididymal 5α-reductase would certainly be possible. However, to study regulation at the protein level, an antibody to 5α-reductase was an absolute prerequisite. For many years, the classical approach of obtaining antibodies via protein purification has remained unsuccessful despite many attempts by several different laboratories. In chapter VI, this problem was circumvented by using the novel approach of synthetic peptides. Since it was shown in chapter IV that the expression and regulation of epididymal 5α-reductase enzyme activity closely paralleled the type 1 transcript, the emphasis was placed on obtaining and characterizing an antiserum directed against the type 1 enzyme. Once obtained, this specific antiserum was used to immunolocalize the 5α-reductase type 1 enzyme in the adult rat epididymis and importantly, under different experimental

conditions. The results of these experiments are presented in the following chapter and its corresponding appendix.

Chapter VI

Immunocytochemical Localization of 4-Ene Steroid 5α-Reductase Type 1 along the Rat Epididymis during Postnatal Development

Robert S. Viger and Bernard Robaire

Abstract

Dihydrotestosterone (DHT), the active androgen in many tissues, is synthesized from testosterone by the enzyme 4-ene steroid 5α-reductase (5α-reductase; EC 1.3.1.22). In the epididymis, the maturation and storage of spermatozoa are dependent on the presence of 5α-reduced androgens. The regulation of epididymal 5α-reductase is complex. To date, the regulation of this enzyme has been studied extensively at the level of enzymatic activity and more recently at the mRNA level. Regulation at the level of the protein, however, remains poorly understood. We have raised rabbit polyclonal antibodies to a 24mer synthetic peptide whose sequence was determined from the predicted amino acid sequence for rat 5α-reductase type 1 to immunolocalize the 5α-reductase type 1 protein in the rat epididymis during postnatal development. Western blot analysis revealed a specific immunoreactive band of 26 kD in male rat liver, epididymis, and prostate; this apparent molecular size is identical to the one that is obtained when the 5α-reductase type 1 cDNA is expressed in mammalian cells. Furthermore, the relative protein levels, liver > epididymis > prostate, were consistent with the mRNA levels for type 1 rat 5α-reductase. Perfusion-fixed paraffin-embedded epididymal tissue sections were used to immunolocalize type 1 5α-reductase. In the adult rat epididymis, the most intense immunoperoxidase reaction was observed in a discrete lobule of the initial segment of the epididymis. A progressive decrease in staining intensity occurred distally along the tissue to the cauda epididymidis. The staining reaction was specific to cytoplasmic elements of epithelial principal cells; no reaction was evident over nuclei. However, specifically in the initial segment, very intense staining was seen in the infranuclear region of the principal cells. In the proximal caput epididymidis, the staining was primarily confined to an oval region above the

nuclei whereas in the remaining epididymal regions, weak staining was seen throughout the cytoplasm. Thus, the intracellular localization of the 5α-reductase type 1 protein changed as one moved down the epididymis. Finally, the pattern of immunolocalization of 5α-reductase type 1 protein was different in the epididymis of rats of different postnatal ages. On day 7 no reactivity was noted; by day 28 a weak apical staining of principal cells was seen throughout the epididymis; by day 47, the adult pattern of staining had been established. Our results revealed that the expression and intracellular localization of the 5α-reductase type 1 protein are both age-dependent and epididymal segment-specific.

Introduction

The mammalian epididymis is a narrow, highly specialized, convoluted tubule that connects the testis to the vas deferens. As spermatozoa traverse the epididymis, they are exposed to a complex microenvironment that is constantly in a state of flux (1,2). Spermatozoa are rendered mature in the caput-corpus region of the tissue and are then stored in the cauda epididymidis (3,4). Although the mechanisms underlying the maturation and storage of spermatozoa in the epididymis have yet to be fully understood, the maintenance of these epididymal processes is directly dependent on the presence of androgens (4-6). Androgenic control of epididymal function has been suggested to be mediated by the 5α-reduced metabolites of testosterone, 5α-dihydrotestosterone (DHT), and 5α-androstan-3α, 17β-diol (3α-diol) and not by testosterone itself (7,8).

Testosterone is the major androgen secreted by the mature testis. Some androgen actions such as the embryonic differentiation of the Wolffian ducts, the promotion of muscle growth, and the maintenance of spermatogenesis are mediated through the action of this testicular hormone (9,10). In many other androgen-sensitive target tissues, such as prostate and epididymis, testosterone is converted to its more potent biologically active form, DHT, by the enzyme 4-ene steroid 5α-reductase (5α-reductase; EC 1.3.1.22). In the epididymis, 5α-reductase enzymatic activity is high in comparison to other rat tissues (11). Functionally, the abundant presence of this enzyme in the epididymis is reflected by the observation that, although this tissue receives mainly testosterone from direct testicular secretions, the major androgen present in the epididymal lumen is DHT (12).

The regulation of rat epididymal 5α-reductase is complex (2,13). At the subcellular level, the enzyme is associated with both the nuclear and microsomal

fractions of epididymal homogenates (14,15). Regulation of the enzyme found in the microsomal fraction has been suggested to be androgen dependent (15). In contrast, the activity of the enzyme associated with the nuclear fraction is critically dependent upon direct testicular secretions (16). This testicular factor is not testosterone itself, but its synthesis has been characterized as androgen dependent (17).

In the rat and human, the 5α-reductase enzyme is present in at least two isozymic forms which differ in their biochemical properties and the tissue distributions of their respective mRNAs (18-21). RNA transcripts encoding the two isozymes have been designated type 1 and type 2 according to their chronological order of identification. Recently, our laboratory has characterized the endocrine and developmental regulation of the type 1 transcript in the rat epididymis (22,23). This regulation is both multifactorial and epididymal segment-specific (22,23).

For many years, the subcellular distribution and cell-type specificity of the steroid 5α-reductase enzyme has been a contentious issue. Until now, this controversy has been unresolved due to the lack of specific markers for the actual protein. In the present study, we have used synthetic peptides to generate a rabbit polyclonal antiserum specific for the rat 5α-reductase type 1 protein. This antiserum was used to immunolocalize the type 1 protein along the epididymis during postnatal development. Our results have revealed that the expression and the intracellular localization of the 5α-reductase type 1 protein in the rat epididymis are both segment-specific and age-dependent.

Materials and Methods

Animals

Timed-gestation pregnant Sprague-Dawley rats were purchased from

Charles River Canada (St. Constant, Québec, Canada). The rats were maintained on a 12-h light, 12-h dark lighting schedule and given food and water *ad libitum*. One day after birth, female offspring were removed and the male littermates were placed at random with the postpartum females. Animals were weaned on postnatal day 21.

Male New Zealand White rabbits, 2.0-2.5 kg were purchased from Charles River Canada. The rabbits were housed in an aseptic environment and were maintained on a 12-h light, 12-h dark lighting schedule. The animals were given a daily ration of food; water was given *ad libitum*.

Antibody Preparation

A specific anti-5α-reductase type 1 serum was obtained by generating polyclonal antibodies directed against synthetic peptides corresponding to portions of the predicted amino acid sequence for the protein (18). Initially, two peptides were synthesized (IAF Biochem International, Montreal, Quebec, Canada). These peptides corresponded to two spans of hydrophilic amino acids, amino acids 161-191 and 238-250, which are encoded in the predicted protein sequence for rat 5α-reductase type 1 (18). Both peptides were conjugated to the carrier protein keyhole limpet hemocyanin (KLH). Polyclonal rabbit antibodies were raised against each peptide.

Before immunization, the animals were bled to obtain preimmune serum. Initially, rabbits were given a subcutaneous injection (interscapular region) containing a 1:1 (vol/vol) mixture of 0.2 mg conjugated peptide in Freund's complete adjuvant (Gibco Canada, Burlington, Ontario, Canada). Subsequent booster injections consisted of a 1:1 mixture of 0.2 mg conjugated peptide in Freund's incomplete adjuvant (Gibco Canada). Two weeks after each injection,

serum was collected and analyzed for the presence of 5α-reductase type 1 antibodies by Western blot analysis. Using this criterion, it was established that of the two peptides initially synthesized, only peptide 169-191 proved successful in providing the desired antiserum against the 5α-reductase type 1 protein. Therefore, only bleeds from this rabbit (peptide 169-191) were pooled. A purified IgG fraction was obtained by affinity chromatography using a protein G Sepharose column (Dimension Laboratories, Mississauga, Ontario, Canada). The titer of IgG fraction was approximately 20 times higher than the whole antiserum. Consequently, routine dilutions used for immunocytochemistry were 1:50 for whole antiserum or 1:1000 for the purified IgG fraction.

Western Blot Analysis

Tissue preparation. Adult rat tissues (liver, prostate, epididymis, and muscle) were homogenized in phosphate-buffered saline (PBS; 10 mM phosphate, 140 mM NaCl, 3mM KCl, pH 7.4) using a Brinkman polytron (Rexdale, Ontario, Canada). The homogenization buffer contained a broad spectrum of protease inhibitors: 1 mM phenylmethylsulfonyl fluoride (Sigma), 2 μg/ml aprotinin (Boehringer Mannheim Canada, Montreal, Quebec, Canada), 1 μg/ml leupeptin and 1 μg/ml pepstatin (Boehringer Mannheim Canada). Microsomal preparations were obtained by centrifuging the tissue homogenates at 4 C for 1 h at 100,000 x g. Microsomal pellets were resuspended in homogenization buffer containing the protease inhibitors. Protein concentrations were measured spectrophotometrically using a Bio-Rad protein assay kit (Bio-Rad Canada); BSA was used as a standard.
Immunoblotting. Aliquots of rat microsomal preparations (50 μg) were denatured before electrophoresis by boiling the samples for 5 min in loading buffer containing 1% sodium dodecyl sulfate (SDS) and 1% β-mercaptoethanol. The denatured

protein samples were separated in a 15% polyacrylamide gel containing 0.1% SDS (wt/vol). The gel was electrophoresed at 200 V for 1 h at 22 C using the Bio-Rad Mini-Protean II electrophoresis apparatus (Bio-Rad Canada). The separated proteins were subsequently transferred to nitrocellulose membrane (Bio-Rad Canada) by electroblotting at 100 mV for 1 hour in transfer buffer (25 mM Tris, 192 mM glycine, 20% methanol (vol/vol), pH 8.4) using the Bio-Rad Mini Trans-Blot apparatus (Bio-Rad Canada).

Immunodetection of the 5α-reductase type 1 protein was achieved using an alkaline phosphatase immunodetection kit (Amersham Canada, Oakville, Ontario, Canada). Nitrocellulose filters were blocked for 2 hours at 22 C in 20 mM Tris-Cl, pH 7.4, 140 mM NaCl (TBS), and 15% powdered skim milk (wt/vol). The filters were then incubated overnight at 4 C in TBS containing 10% powdered skim milk and a 1:100 dilution of either anti-5α-reductase or preimmune serum. Following the incubation, the filters were washed three times in TBS containing 0.1% Tween-20 (Bio-Rad Canada) for 5 min each at 22 C. The filters were subsequently incubated for 20 min at 22 C in TBS containing 10% powdered skim milk and a 1:2000 dilution of a biotinylated donkey anti-rabbit immunoglobulin. Finally, the filters were washed as above and then incubated for 20 min at 22 C in TBS containing 10% powdered skim milk and a 1:3000 dilution of strepavidin-alkaline phosphatase. After thorough washing, immune complexes were visualized using a combination of nitro-blue tetrazolium (NBT) and 5-bromo-4-chloro-3-indolyl phosphate (BCIP) as substrates for the phosphatase reaction.

Developmental Expression of the 5α-Reductase Type 1 Protein as Assessed by Light Microscope Immunocytochemistry

5α-Reductase type 1 protein expression was studied in epididymides

obtained from animals at four ages during postnatal development: 7, 28, 47, and 91 days. Each time point was chosen to coincide with known developmental events. On postnatal day 7, serum androgens and 5α-reductase enzymatic activity are undetectable; the blood-epididymal barrier has not formed (24-28). By day 28, the formation of the blood-epididymal barrier is already complete; epididymal 5α-reductase enzymatic activity can now be detected (24,28). Serum androgens, however, remain low (24-26). Postnatal day 47 is marked by high 5α-reductase enzymatic activity and the presence of rising serum androgen concentrations (24-26). Finally, postnatal day 91 was chosen to represent the adult animal.

Tissue Preparation for Light Microscope Immunocytochemistry

For each of the above described postnatal ages, Sprague-Dawley rats were anesthetized with a single intraperitoneal injection of pentobarbital (1.0 ml/kg; Somnotol®, M.T.C. Pharmaceuticals, Cambridge, Ontario, Canada). The reproductive tracts were fixed for ten minutes with Bouin's fixative by retrograde perfusion through the abdominal aorta. With the exception of the corpus and cauda epididymides from adult animals, retrograde perfusions resulted in good fixation of the epididymis. Prograde perfusions, in other animals, were done in order to obtain optimal fixation of the corpus and cauda regions of the epididymis from adult animals. In a retrograde perfusion, ligatures of the abdominal aorta are placed above the iliac arteries and below the hepatic arteries. Fixative is then forced in against the direction of the circulation by means of a needle inserted in this section of the aorta; excess fixative exits through the nicked renal vein. In a prograde perfusion, however, no ligature of the abdominal aorta is placed above the iliac arteries and the fixative is administered in the direction of the circulation

via a needle inserted into the abdominal aorta which is distal to the ligature below the hepatic arteries.

Following the perfusion, the epididymides were removed and cut along their longitudinal axes so that given sections would include more than one epididymal region, i.e., the initial segment, caput, corpus and cauda, as described by Hermo et al. (29). Epididymal tissues were then postfixed for 24 hours in Bouin's fixative, dehydrated, and embedded in paraffin. Longitudinal tissue sections were cut (5 μm) and mounted on glass slides.

Immunostaining

Prior to immunolabelling, sections were deparaffinized with xylene and rehydrated by a series of 5 min immersions in graded alcohol solutions. During the hydration process, residual picric acid from the Bouin's fixative was inactivated in 70% ethanol containing 1% lithium carbonate. Endogenous peroxidase activity was neutralized in 70% alcohol containing 1% (vol/vol) hydrogen peroxide. After hydration, free aldehydes were blocked by immersing the sections for 5 min in 300 mM glycine.

The 5α-reductase type 1 protein was immunolocalized using the horseradish peroxidase method essentially as outlined by Cyr et al. (30). Epididymal sections were blocked for 15 min with 10% whole goat serum (Dimension Laboratories) in TBS (20 mM Tris-Cl, 140 mM NaCl, pH 7.4) containing 1% bovine serum albumin (BSA). Sections were subsequently incubated with primary antibody (anti-5α-reductase type 1, 1:50 dilution in TBS) for 1.5 h at 37 C. Tissue sections were then washed in TBS containing 0.1% Tween-20 (Bio-Rad Canada, Mississauga, Ontario, Canada), blocked with 10% goat serum and incubated with a goat anti-rabbit peroxidase-linked secondary antibody (Sigma

Chemical Co., St. Louis, Missouri) for 30 min at 37 C (1:250 dilution in TBS). Specific antibody binding was visualized using diaminobenzidine as substrate for the peroxidase reaction (31). Finally, epididymal sections were counterstained with 0.1% methylene blue, dehydrated in a series of graded alcohol solutions, immersed in xylene, and mounted in Permount. Preimmune rabbit serum was used as a negative control (1:50 dilution in TBS).

Results

Western Blot Analysis

The anti-5α-reductase type 1 serum directed against amino acid positions 169-191 detected a specific protein band with an apparent molecular weight of 26 kD in male rat liver, prostate and epididymis (Fig. 1, right panel) which was absent when preimmune serum was used (Fig.1, left panel). Qualitatively, the relative protein amounts were liver > epididymis >> prostate. No specific 5α-reductase type 1 protein was detected in rat muscle. Both the preimmune and anti-5α-reductase sera non-specifically recognized several higher molecular weight protein bands (Fig. 1, left and right panels).

Immunocytochemical Localization of the 5α-Reductase Type 1 Protein Along the Adult Rat Epididymis

Using anti-5α-reductase serum (antipeptide 169-191), the 5α-reductase type 1 protein was found to be remarkably concentrated in a discrete lobule of the initial segment of the adult rat epididymis (Fig. 2), identified as the proximal initial segment (29). Beyond the initial segment, the intensity of the immunostaining decreased gradually along the tubule (Fig. 2). The immunoperoxidase reaction was very weak in the distal regions of the tissue (data not shown).

Higher power micrographs revealed that in all epididymal regions, the immunoperoxidase staining reaction was specific to cytoplasmic elements of epithelial principal cells; no staining was evident over the intertubular space, spermatozoa or the lumen itself (Figs. 3 and 4). In marked contrast to the specific staining seen with the anti-5α-reductase serum, there was a complete absence of reaction over the epididymal epithelium when preimmune serum was used (Fig. 5).

The pattern of immunostaining varied as a function of epididymal region (Figs. 3 and 4). In the proximal initial segment (Fig. 3a), the epithelial principal cells were highly reactive. Some principal cells, however, were more intensely labelled than others. The other major epithelial cells types (basal and halo cells) were clearly unreactive (Fig. 3a). Within the principal cells, the immunoperoxidase reaction product was found throughout the cytoplasm; nuclei were unreactive. Importantly, however, very intense staining in the infranuclear region of the principal cells, which is known to be abundant in rough endoplasmic reticulum, was seen specifically in this segment of the epididymis (Fig. 3a).

In the distal initial segment, the epithelial principal cells were moderately stained (Fig. 3b). Unlike the proximal initial segment, the immunoperoxidase reaction was uniform throughout the cytoplasm; infranuclear staining was less evident. Basal cells and nuclei were unreactive.

The immunostaining pattern observed in the proximal caput epididymis was unique. In this epididymal region, the staining reaction was restricted to an oval region above the nuclei which is known to contain a well-developed Golgi complex as well as lysosomes; the basal compartments of the epithelial principal cells were completely unreactive (Fig. 3c). Similar to the initial segment, no immunoperoxidase reaction was observed over nuclei and the cytoplasm of the other major epithelial cells types (i.e., basal and halo cells).

The unique staining reaction of the principal cells in the proximal caput epididymidis disappeared as one reached the distal caput epididymidis. In this epididymal region, the epithelial principal cells were weakly but uniformly stained; again nuclei were unreactive (Fig. 3d). The other epithelial cells types present (basal, halo, and clear cells) were also unreactive.

Finally, the specificity of the immunoperoxidase reaction for the epithelial principal cells was also evident in the corpus and cauda regions of the epididymis, Figs. 4a and b, respectively. In the corpus and cauda epididymides, the principal cells were weakly stained throughout the cytoplasm; nuclei were unreactive (Figs. 4a and b). The absence of an immunoperoxidase staining reaction for the other epithelial cell types was apparent, especially the clear cells in the cauda epididymidis (Fig. 4b).

Immunocytochemical Localization of the Steroid 5α-Reductase Type 1 Protein in the Epididymis of Rats at Different Postnatal Ages

The immunocytochemical staining pattern for the 5α-reductase type 1 protein in the initial segment-caput region of the epididymis was studied at three discrete ages during postnatal development. At all ages examined, no specific immunoperoxidase reaction product was found over the intertubular connective tissue space and the lumen of the duct (Fig. 6). The epithelium of the epididymis was completely unreactive on postnatal day 7, a representative region of the epididymis is shown in Fig. 6a. On day 28, a weak apical reaction was observed in the principal cells of the initial segment and caput epididymidis (Fig. 6, b and c). Basal cells and the nuclei of principal cells were unreactive. A similar reaction was also present in both the corpus and cauda epididymides (data not shown). By day 47, the staining reaction became more intense. The lobular staining pattern along

the epididymis was characteristic of the adult rat. At this age, the 5α-reductase type 1 protein was abundantly expressed in the proximal initial segment and was moderately expressed in the distal initial segment (Fig. 7). The intensity of the immunoperoxidase reaction decreased progressively along the length of the tubule in a similar fashion to the adult rat (data not shown).

At high magnification, the age-dependent variations in intracellular staining patterns were clearly evident (Fig. 6). On day 47, the intracellular staining pattern took on its characteristic adult appearance (Fig. 6, d and e). In the initial segment, the epithelial principal cells were strongly reactive; basal cells were completely unreactive (Fig. 6d). Although the nuclei of the principal cells were unreactive, an intense staining reaction in the infranuclear region of these same cells was present in this segment of the epididymis (Fig. 6d). In the proximal caput epididymidis, the intracellular localization of the immunoperoxidase reaction product was clearly restricted to an oval region present above the nuclei of the principal cells (Fig. 6e). The staining patterns observed in the distal caput, corpus, and cauda epididymides were identical to those described for the adult rat (data not shown).

Discussion

In the present study, synthetic peptides were used to generate a polyclonal antiserum specific for rat type 1 steroid 5α-reductase. At the light microscope, this antiserum was used to immunocytochemically localize the 5α-reductase type 1 protein along the epididymis and during postnatal epididymal development. Our results conclusively demonstrate that both the intensity of expression and intracellular distribution of the 5α-reductase type 1 protein are epididymal segment-specific and age-dependent.

On immunoblots, the anti-5α-reductase type 1 serum recognized a specific immunoreactive band with an apparent molecular weight of 26 kD. Several lines of evidence confirm that this band corresponds to the 5α-reductase type 1 protein. First, the observed molecular size is identical to the one that has been reported when the rat type 1 5α-reductase cDNA is expressed in mammalian cells (32). Second, the relative intensities of the 26 kD protein found in male rat liver, epididymis, and prostate were consistent with the previously reported mRNA levels for type 1 5α-reductase (22). Finally, the 26 kD protein was undetectable in rat muscle, a tissue in which both 5α-reductase enzymatic activity and type 1 mRNA expression are present at very low levels (11,21).

It has been well established that neither 5α-reductase enzymatic activity nor mRNA levels are uniformly distributed along the epididymis in many species (2,21,22). In the rat, 5α-reductase enzymatic activity is localized primarily in the initial segment of the epididymis (13). The present immunolocalization studies have more precisely defined the longitudinal distribution of the type 1 enzyme in the adult rat epididymis. As suspected from previous mRNA and enzyme activity studies, the 5α-reductase type 1 protein was predominantly expressed in the initial segment of the epididymis. The novel finding, however, was that within the initial segment itself, the type 1 protein was remarkably concentrated in a very discrete lobule. Using the terminology described by Hermo et al. (29), this region was identified as the proximal initial segment. Regional variations in immunostaining within the initial segment have also been reported for other epididymal proteins (29,33,34). These variations occur in the absence of major structural differences between the epithelial principal cells (2). It has been suggested that regional variations in immunostaining are indicators of functional differences among the epithelial cells in the different regions of the initial segment (34). The present

finding that a major epididymal function (the production of 5α-reduced androgens) is predominantly localized in a specific region of the initial segment is consistent with this hypothesis.

The expression of the 5α-reductase type 1 protein is developmentally regulated in the epididymis. The absence of immunocytochemically detectable 5α-reductase type 1 protein on postnatal day 7 is consistent with previous enzymatic activity measurements (24). Interestingly, however, the lack of detectable 5α-reductase enzymatic activity and type 1 protein expression at this age did not correlate with the previously reported type 1 mRNA levels (23). This discrepancy strongly supports the hypothesis that post-transcriptional mechanisms play an inherent role in the regulation of epididymal 5α-reductase (22,23). This may not be surprising since post-transcriptional regulation has also been proposed for several other epididymal proteins (35-39), and other proteins in the male reproductive tract (40,41).

The 5α-reductase type 1 protein was first detected on postnatal day 28. This coincides well with the first appearance of measurable 5α-reductase activity in the epididymis (15,24). Moreover, the weak staining pattern seen throughout the epididymis at this age is similar to the previously established pattern for type 1 5α-reductase mRNA expression (23). By day 47, however, the pattern of 5α-reductase type 1 protein expression became adult in appearance. Importantly, these results indicate that the regulatory factors responsible for the adult pattern of expression are already present by day 47. The dramatic shift in intensity and regional distribution of type 1 protein expression between postnatal days 28 and 47 were similar to those previously reported at the mRNA and enzyme activity levels (15,23,24).

At all postnatal ages studied and in all epididymal regions,

immunocytochemical analysis revealed that the expression of the 5α-reductase type 1 protein is specific to the epithelial cells. This result confirms the observation that expression of the type 1 mRNA is also limited to the epididymal epithelium (42). It has been proposed that the expression of the 5α-reductase enzyme is confined to a particular epithelial cell type (43). Our findings conclusively demonstrate that the expression of the type 1 protein is specific for the principal cells. No 5α-reductase type protein expression is found in the other major epithelial cell types (basal, halo, and clear cells). In the epididymis, principal cell functions (e.g., the synthesis and secretion of glycoproteins) are highly androgen-dependent (13). Therefore, the presence of the 5α-reductase type 1 protein specifically in principal cells is consistent with the site of androgen action. The cell type specificity of the 5α-reductase type 1 protein in the rat epididymis is in marked contrast to the rat prostate. In a study reported by Berman et al. (42), the 5α-reductase type 1 protein was found specifically in basal epithelial cells of the prostate and not in the lumenal epithelial cells, as we have shown for the epididymis (42). The exact reason for this difference is presently unknown, but it suggests that the 5α-reductase type 1 enzyme has disparate functional roles in these two tissues.

In the rat epididymis, 5α-reductase enzymatic activity measurements have suggested two intracellular localizations for the enzyme: nuclear and microsomal (cytoplasmic) (15). In the present study, the immunocytochemical reaction for the 5α-reductase type 1 protein was uniquely cytoplasmic. A similar intracellular localization has been reported when the human 5α-reductase genes are expressed in Chinese hamster ovary cell lines (44). Although the nuclei of the principal cells were not immunolabelled, very intense infranuclear staining was found, specifically in the proximal initial segment of 47 day-old and adult rats.

Importantly, the highest levels of nuclear 5α-reductase enzymatic activity are also found in this same epididymal region (12). Furthermore, nuclear 5α-reductase enzymatic activity, type 1 mRNA expression, and the maintenance of the normal morphological appearance of principal cells are all critically dependent on the presence of direct testicular secretions in the initial segment of the epididymis (12,22,45). Based on these observations, we can speculate that the infranuclear localized form of the 5α-reductase type 1 protein is regulated in a similar manner. This intense infranuclear localization may be required for the activation of highly androgen dependent genes which are vitally important for maintaining epididymal function in the more distal areas of the tissue. It is interesting to note that the present subcellular localization of the type 1 protein is also quite different from that described in a recent report by Hiipakka et al., where the protein was localized specifically to nuclei in all rat tissues studied (46).

In conclusion, the present study demonstrates that in the rat epididymis, the 5α-reductase type 1 protein has distinct intracellular localizations, is cell-type specific, and is expressed in age-dependent and region-specific manner. Furthermore, the generation of a specific antiserum against the steroid 5α-reductase type 1 enzyme should provide an invaluable tool for the immunocytochemical localization of this protein in other rat tissues which, until now, has remained controversial.

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Figure 1. 5α-Reductase type 1 protein expression in adult rat tissues. Total microsomal protein was prepared as described in *Materials and Methods*. Aliquots (50 µg protein) were subjected to immunoblotting using preimmune rabbit serum (*left panel*) and anti-5α-reductase type 1 serum (*right panel*). Both sera were diluted 1:100. Protein molecular size standards are indicated by the *small arrowheads* on the *left*. The migration of the specific 26-kD 5α-reductase type 1 protein is indicated by the *boldface arrow*. L, liver; P, prostate; IS, initial segment of the epididymis; M, muscle. kd, Kilodaltons.

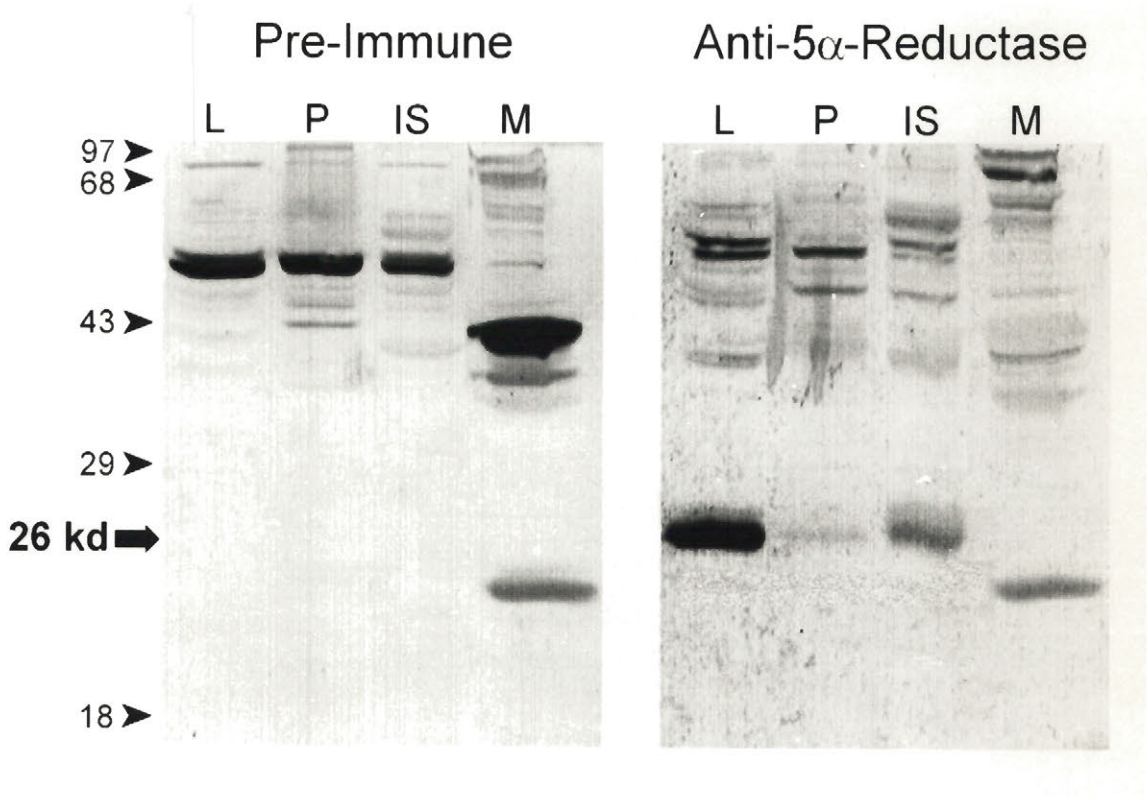

Pre-Immune
Anti-5α-Reductase
L
P
IS
M
L
P
IS
M
97
68
43
29
26 kd
18

Figure 2. Low power light micrograph of the caput-corpus region of the adult rat epididymis immunostained with anti-5α-reductase type 1 serum. The 5α-reductase type 1 protein is abundantly expressed in a discrete lobule of the initial segment (*large arrow*). Magnification, x12.5.

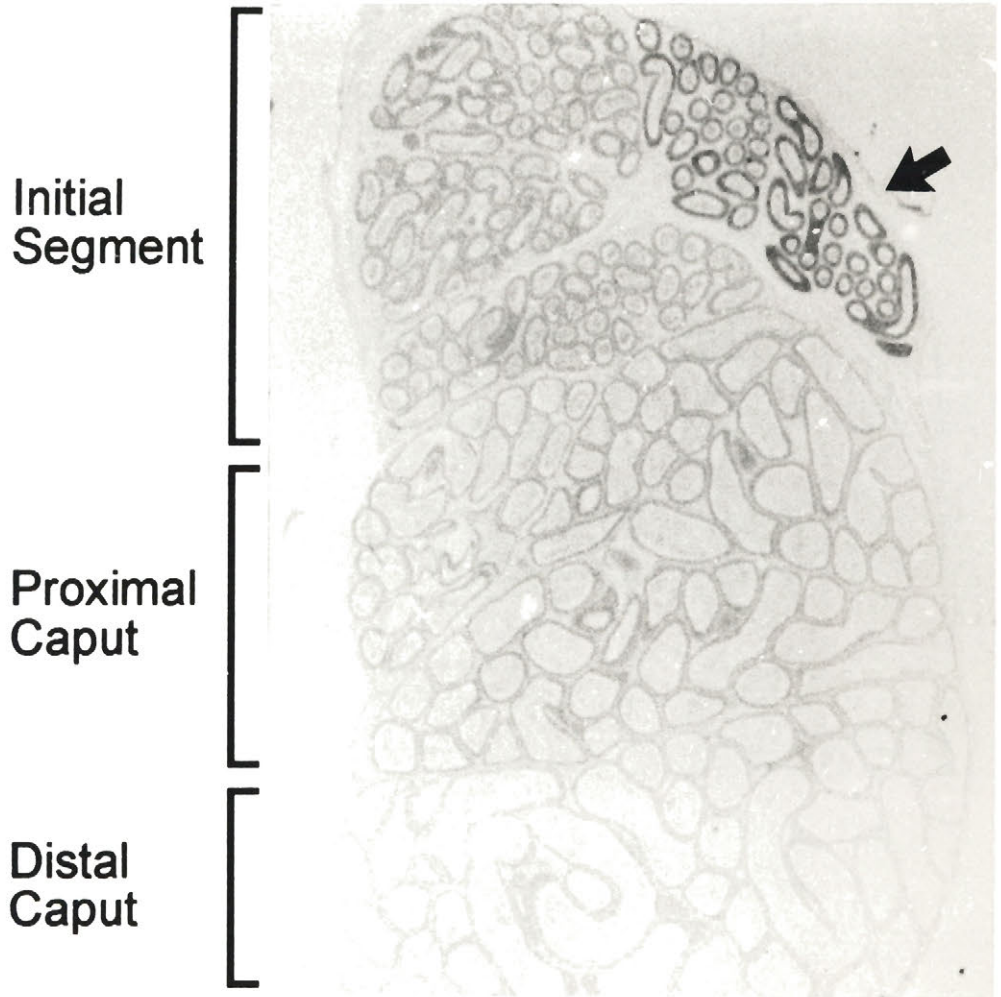

Initial
Segment
Proximal
Caput
Distal
Caput

Figure 3. High power light micrographs of the proximal initial segment (a), distal initial segment (b), proximal caput (c), and distal caput (d) regions of the adult rat epididymis. Epithelial principal cells (P), nuclei of principal cells (n), basal cells (B), clear cells (C), intertubular connective tissue space (IT), lumen of the ducts (Lu), and spermatozoa (S) are shown. In a, staining in the infranuclear region of the principal cells is designated by the *arrowheads*. In c, the immunoperoxidase staining reaction is confined to an oval region present above the nuclei of the principal cells *(large arrows)*. Magnification, x400.

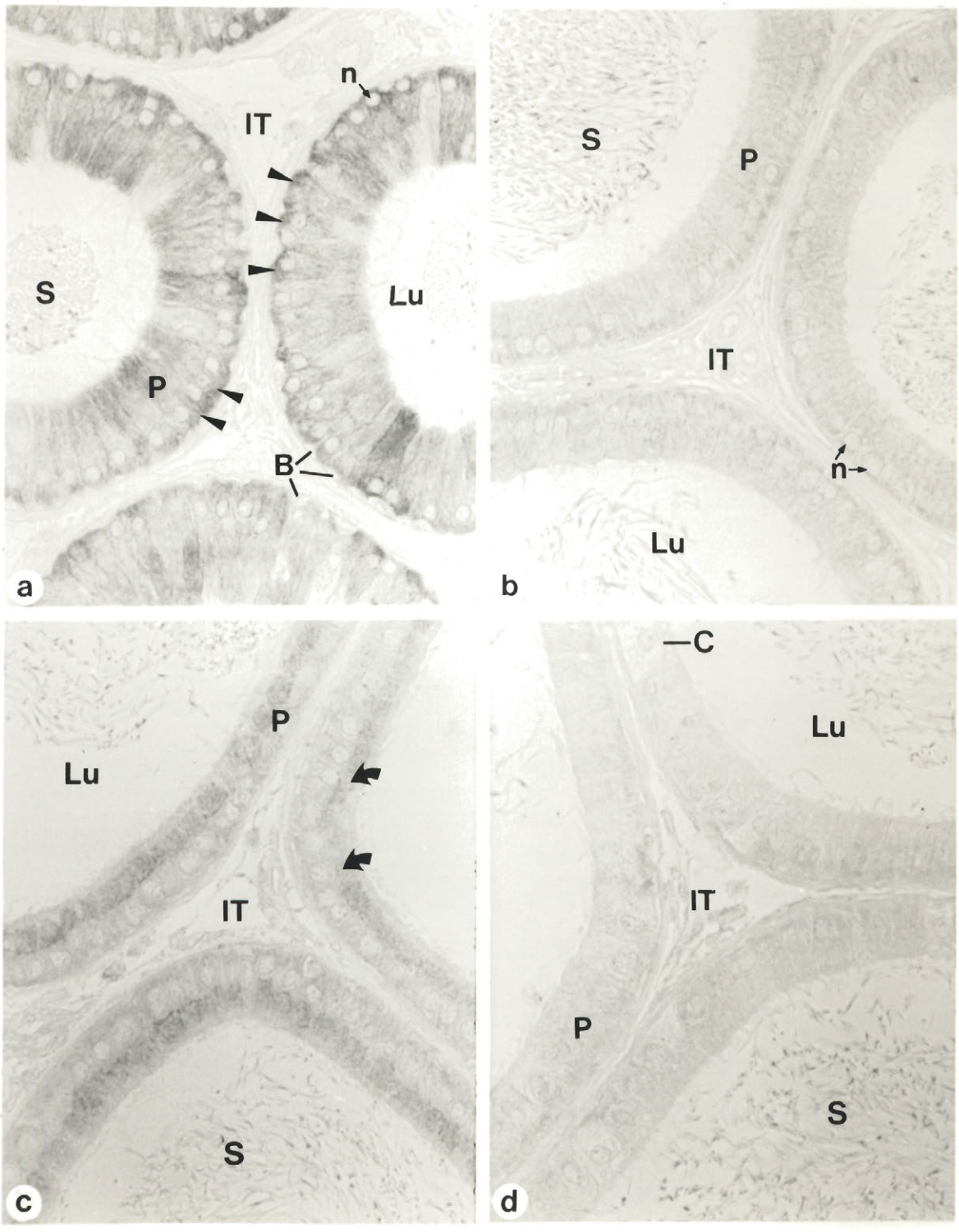

n
IT
S
Lu
P
B
a
S
P
IT
n
Lu
b
P
Lu
IT
S
c
—C
Lu
IT
P
S
d

Figure 4. High power light micrographs of the corpus (a) and cauda (b) regions of the adult rat epididymis. Epithelial principal cells (P), nuclei of principal cells (n), basal cells (B), clear cells (C), intertubular connective tissue space (IT), lumen of the ducts (Lu), and spermatozoa (S) are shown. Magnification, x400.

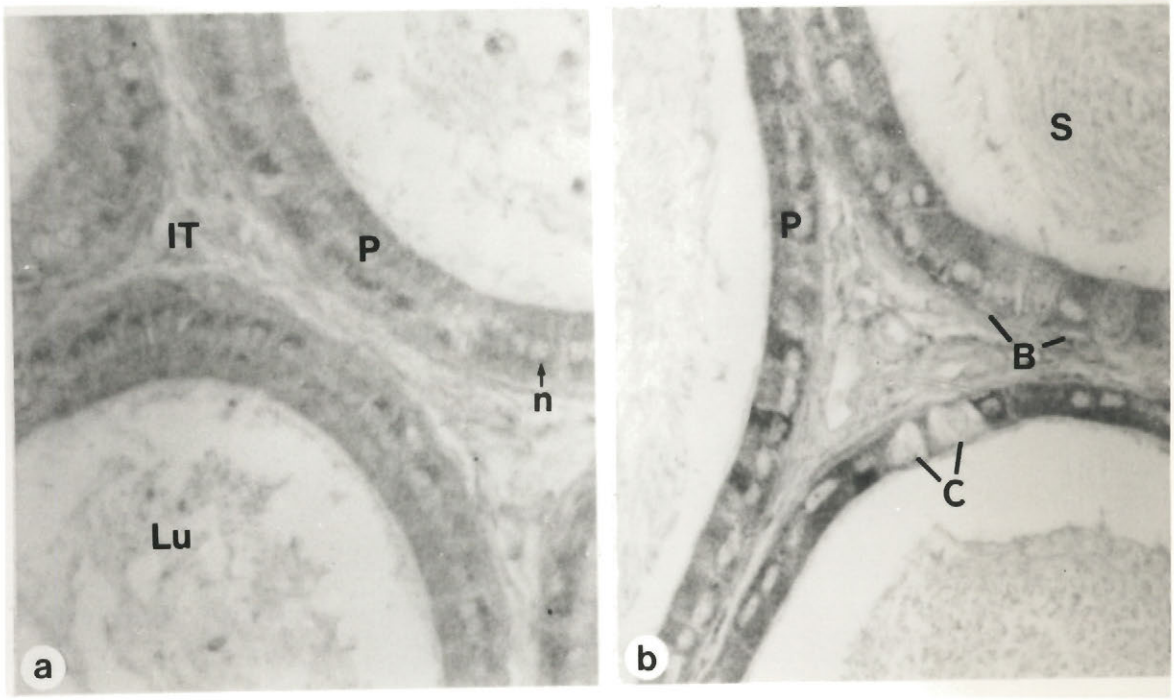

IT
P
n
Lu
a
P
S
B
C
b

Figure 5. High power light micrograph of the proximal initial segment immunostained with preimmune rabbit serum. The epithelium (E), spermatozoa (S), intertubular connective tissue space (IT), and lumen of the ducts (Lu) are shown. Magnification, x290.

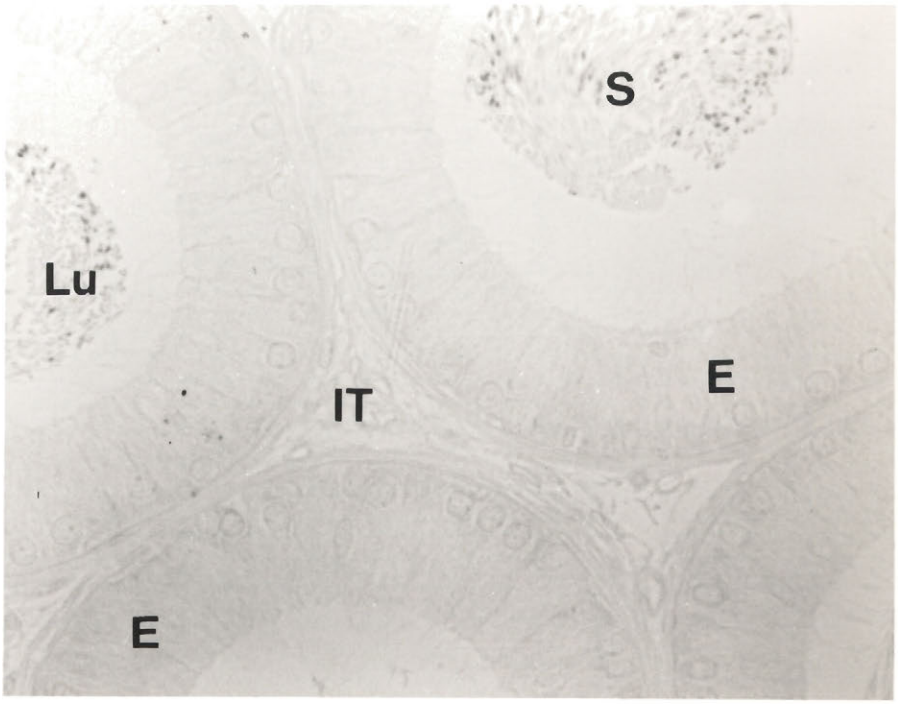

S
Lu
E
IT
E

Figure 6. High power light micrographs of the initial segment-caput (a), proximal initial segment (b and d), and proximal caput (c and e) regions of epididymides from 7 (a)-, 28 (b and c)-, and 47 (d and e)- day-old rats. The epididymal epithelium (E), epithelial principal cells (P), nuclei of principal cells (n), basal cells (B), intertubular connective tissue space (IT), lumen of the ducts (Lu), and spermatozoa (S) are shown. In b and c, the apical staining of the epithelium is indicated by the *arrows*. In d, the intense staining in the infranuclear region of the principal cells is indicated by the *arrowheads*. In e, the unique staining reaction present above the nuclei of the principal cells in the proximal caput epididymidis is indicated by the *curved arrow*. Magnification, x400.

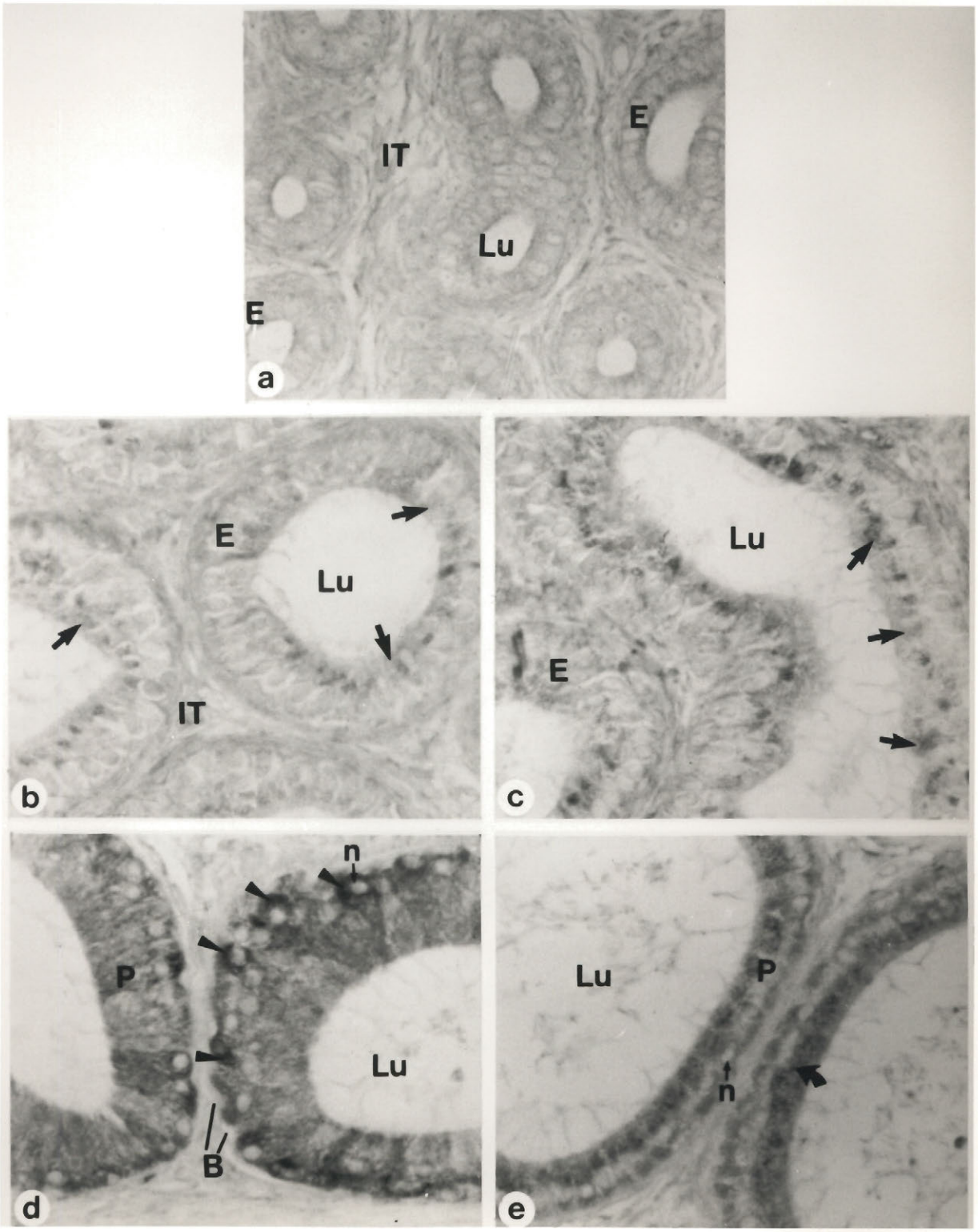
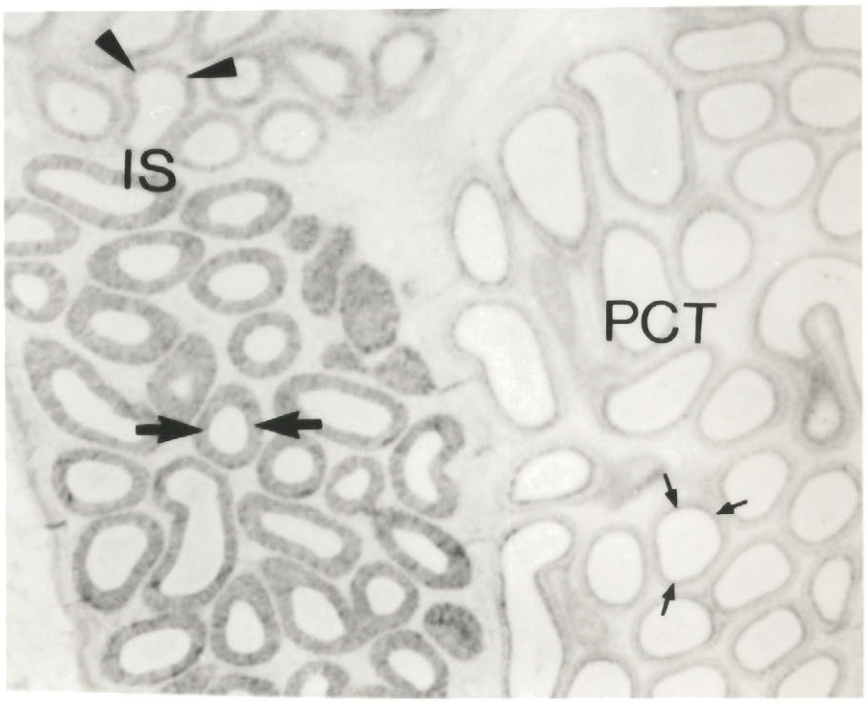

E
IT
Lu
E
a
E
Lu
IT
b
Lu
E
c
n
P
Lu
B
d
Lu
P
n
e

Figure 7. Low power light micrograph of the initial segment-caput region of the epididymis on postnatal day 47. The intense, uniform staining of the epithelium in the proximal initial segment is designated by the *large arrows*, whereas the less intense staining of the distal initial segment is indicated by the *arrowheads*. IS, initial segment; PCT, proximal caput. Magnification, x40.


IS
PCT

Appendix

The Effect of Efferent Duct Ligation on 5α-Reductase Type 1 Protein Expression along the Rat Epididymis

Using subcellular fractionation, previous studies have established that 5α-reductase enzyme activity is associated with both the nuclear and microsomal fractions of epididymal homogenates (1,2). In the previous chapter, however, an important finding was the absence of 5α-reductase type 1 protein immunoreactivity over epididymal cell nuclei. Although intranuclear labelling was not apparent, very intense infranuclear staining was present in epithelial principal cells, specifically in the proximal initial segments of post-pubertal rats. It is important to note that the highest levels of nuclear 5α-reductase enzyme activity are also present in this same anatomical region of the epididymis (3). Moreover, the initial segment of the epididymis is the site where nuclear 5α-reductase enzyme activity, type 1 mRNA expression, and the maintenance of the normal morphological appearance of epididymal principal cells are critically dependent on the presence of direct testicular secretions (4,5,6). Thus, based on these observations, it was proposed that the infranuclear localized form of the 5α-reductase type 1 protein was regulated in a manner consistent with the type 1 mRNA (5) and enzyme activity (3), that is, by a substance that is transported into the epididymis directly via the efferent duct; this substance has been proposed to be most likely of Sertoli cell origin and its synthesis to be under the control of testosterone (3).

To test this hypothesis, the 5α-reductase type 1 protein was immunolocalized in epididymides of efferent duct ligated rats. Immature male Sprague Dawley rats were efferent duct ligated at the age of 15 days postpartum. The effects of the ligation were then assessed by killing the animals when they reached the postnatal ages of 49, 58, and 64 days. At each age, epididymides were fixed with Bouin's fixative by retrograde perfusion through the abdominal aorta. Following the perfusion, the epididymides were removed and cut along their longitudinal axes so that given sections would include more than one epididymal

region, i.e., the initial segment, caput, corpus and cauda. Epididymal tissues were then postfixed for 24 hours in Bouin's fixative, dehydrated, and embedded in paraffin. Longitudinal tissue sections were cut (5 μm) and mounted on glass slides. The 5α-reductase type 1 protein was immunolocalized using the horseradish peroxidase method as described in the previous chapter. The primary antibody (anti-5α-reductase type 1 serum) was used at a dilution of 1:50. Preimmune rabbit serum was used as a negative control (1:50 dilution).

The initial time point (day 49) was chosen since it represents the earliest known age when the adult pattern of 5α-reductase type 1 protein expression (longitudinal and subcellular) is manifested. Thus, under these conditions, the day 15 (immature) epididymides were never exposed to the direct testicular secretions that were hypothesized to be critical for establishing the adult pattern of 5α-reductase type 1 protein expression (chapter VI). The effects of efferent duct ligation on the expression and subcellular localization of the 5α-reductase type 1 protein at day 49 are shown in Figs. 1 and 2.

At low power, efferent duct ligation resulted in a striking decrease in 5α-reductase type 1 protein expression, specifically in the proximal initial segment of the epididymis when compared to control (Fig. 1, a and b). No significant changes in type 1 protein expression, however, were observed in the other regions of the tissue. At high power, the decrease in 5α-reductase type 1 protein expression in the proximal initial segment was accompanied by a dramatic shift in intracellular localization of the protein (Fig. 2). In the proximal initial segment, efferent duct ligation resulted in a complete elimination of the intense infranuclear staining that is normally found in the same epididymal region of a control rat (Fig. 2, a and b). The 5α-reductase type 1 protein immunoreactivity that remained in the proximal initial segment appeared to be restricted to an oval region present above the

nuclei (Fig. 2b). Interestingly, this type of intracellular localization is primarily found in the proximal caput epididymidis of control rats (chapter VI). In the other regions of the epididymis, efferent duct ligation did not affect the subcellular localization of the 5α-reductase type 1 protein when compared to the control rats (the proximal and distal caput from control (c and e) and ligated (d and f) animals are shown in Fig. 2). The effects of efferent duct ligation observed in the epididymis of the day 49 rat were identical to those obtained for the 58 and 64 day-old animals (data not shown). In marked contrast to the specific staining seen with the anti-5α-reductase serum, there was a complete absence of reaction over the epididymal epithelium when preimmune serum was used (data not shown).

Taken together, these results strongly support the hypothesis that the infranuclear localized form of the 5α-reductase type 1 enzyme is regulated in a paracrine manner by a testicular factor which enters the epididymis via the efferent ducts. Thus, in terms of 5α-reductase enzyme activity, it is proposed that the infranuclear localized type 1 protein immunoreactivity represents what has previously been termed as the nuclear fraction enzyme. In contrast to nuclear fraction 5α-reductase, microsomal fraction 5α-reductase enzyme activity is present throughout the epididymis, is expressed at a lower level and is under the control of circulating androgens (2). Since circulating androgen levels are not compromised in an efferent duct ligated animal model (7,8), it is further proposed that the apically localized 5α-reductase type 1 immunoreactivity that remains after efferent duct ligation represents what has previously been termed as the microsomal fraction enzyme.

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3. **Robaire B, Scheer H, Hachey C** 1981 Regulation of epididymal steroid metabolizing enzymes. In: Jagiello G, Vogel HJ (eds) Bioregulators of Reproduction. Academic Press, New York, pp 487-498
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8. **Wang JM, Gu CH, Tao L, Wu XL** 1985 Effect of surgery and efferent duct ligation on testicular blood flow and testicular steroidogenesis in the rat. J Reprod Fertil 73:191-196

Figure 1. Low power light micrographs of the initial segment-proximal caput region of the epididymis from control (a) and efferent duct ligated (b) rats on postnatal day 49. In the control rat epididymis (a), the 5α-reductase type 1 protein is abundantly localized in a discrete lobule of the initial segment which has been defined as the proximal initial segment (PIS). Note that this intense immunoreactivity is eliminated in the efferent duct ligated rat epididymis (b). The other epididymal regions shown are the middle initial segment (MIS), distal initial segment (DIS), and proximal caput epididymidis (PCT). Magnification, x40.

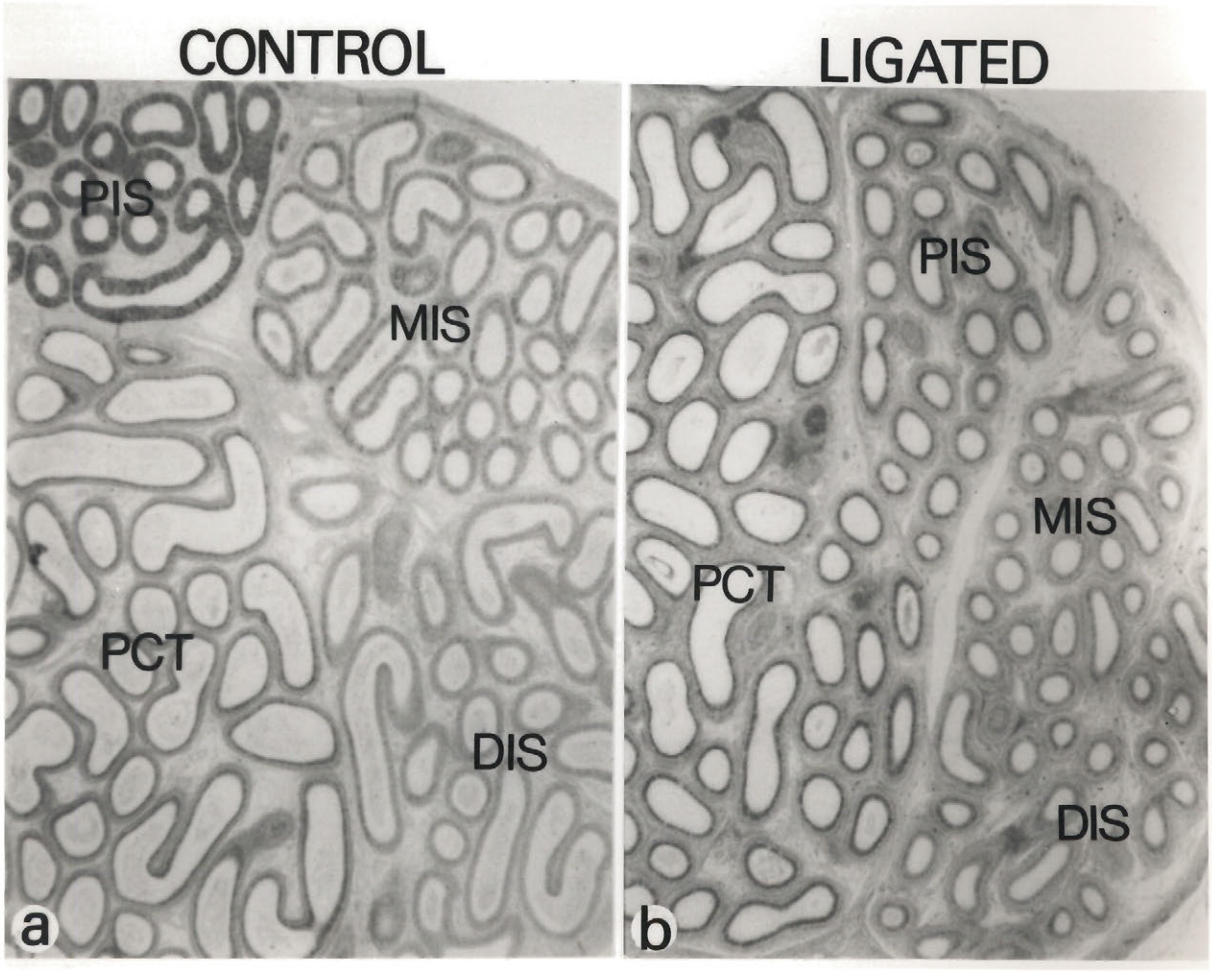
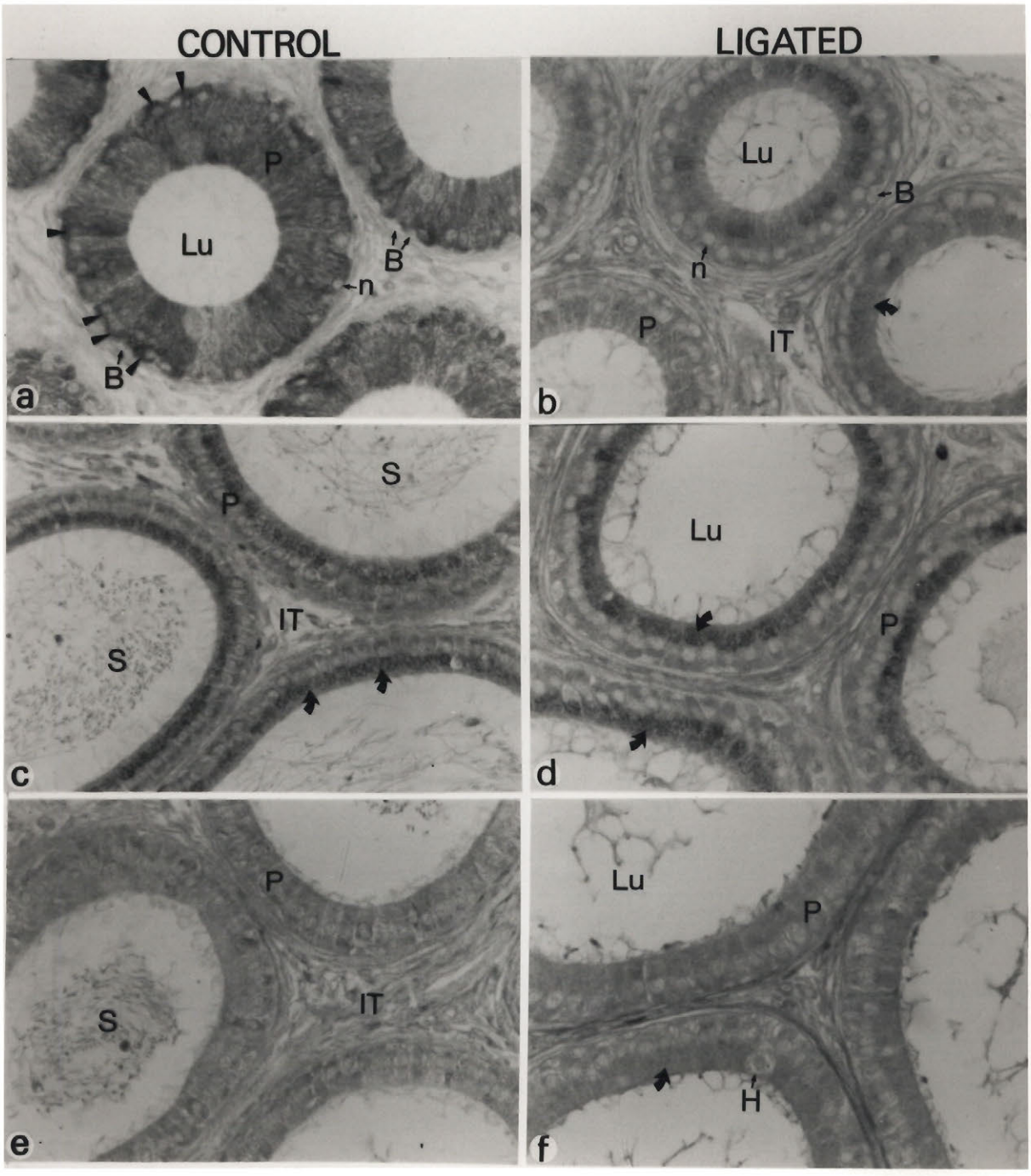

CONTROL
LIGATED
PIS
MIS
PCT
DIS
PIS
MIS
PCT
DIS
a
b

Figure 2. High power light micrographs of the proximal initial segment (a and b), proximal caput (c and d), and distal caput (e and f) regions from control (a, c, and e) and efferent duct ligated (b, d, and f) rats on postnatal day 49. Epithelial principal cells (P), nuclei of principal cells (n), basal cells (B), halo cell (H), intertubular connective tissue space (IT), lumen of the duct (Lu), and spermatozoa (S) are shown. In the proximal initial segment of the control rat (a), intense staining in the infranuclear region of the principal cells is designated by the *arrowheads*. Similar staining is not apparent in the same region of the efferent duct ligated rat (b). In b, c, d, and f, the immunoreactivity is confined to an oval region present above the nuclei of the principal cells (*large arrows*). In b, d, and f, note the absence of spermatozoa in the epididymal lumen of the efferent duct ligated rats. Magnification, x280.


CONTROL
LIGATED
P
Lu
B
n
B
a
Lu
B
n
P
IT
b
S
P
IT
S
c
Lu
P
d
P
Lu
S
IT
e
Lu
P
H
f

Chapter VII

Discussion

Since the early 1970s, the regulation of 5α-reductase enzyme activity in the epididymis and many other androgen-sensitive target tissues has been the subject of numerous studies. In spite of the considerable information that has been accumulated on the enzyme, the road towards defining the molecular mechanisms that control 5α-reductase activity has been decidedly slow. Insight into the regulation of the 5α-reductase gene(s), mRNA(s), and protein(s) has not been possible due to the lack of specific genetic and immunocytochemical probes. Recently, some of these probes, notably the 5α-reductase cDNAs, termed types 1 and 2, have been developed and made available (1-4). In this thesis, these cDNAs were used to characterize, for the first time, the regulation of the 5α-reductase transcripts in the rat epididymis. Further, the predicted amino sequence deduced from the cDNA for rat 5α-reductase type 1 was used to construct synthetic peptides. These peptides were successfully used to generate a novel anti-5α-reductase type 1 serum. Thus, the localization and regulation of 5α-reductase protein expression could be investigated in the epididymis and other rat tissues.

This chapter will be divided into three sections. First, I will discuss the significance of some of the key findings presented in this thesis. The second section will integrate these findings; a scheme for the regulation of epididymal 5α-reductase will be proposed. Based on the present work and that of others, I will conclude by elaborating on future studies that could be used to further our understanding of the regulation of epididymal 5α-reductase.

The Regulation of Epididymal 5α-Reductase: Significance and Implications of the Present Studies

In the adult rat epididymis, several mRNAs and/or proteins are expressed

in a region-specific manner (5-17). The longitudinal distribution patterns found for the 5α-reductase transcripts, type 1 (chapter II) and type 2 (chapter IV), and the type 1 protein (chapter VI) are consistent with this observation. The regulatory mechanisms that control regionalized gene expression in the epididymis are not clear. Based on the changing composition of the luminal fluid along the epididymal duct (18), one could easily surmise that region-specific gene expression is determined by the presence (or absence) of various regulatory factors in the different segments of the tissue. Although this may be true for certain genes, it cannot be viewed as a general mechanism. The latter is quickly discounted by the data available in the literature. A good example is provided by the discordance between the well-established patterns for androgen concentrations in the epididymis (19) and the regional expression of many androgen-dependent genes such as the androgen-stimulated gene, protein D/E (20). If the above hypothesis were true, then the expression of the aforementioned gene in the epididymis should be tightly correlated with androgen levels in the tissue. Comparison of these two patterns reveals that they are clearly not correlated. In the epididymis, luminal androgen concentrations (testosterone +DHT) decrease markedly from the proximal to the distal regions of the tissue (19), whereas protein D/E gene expression follows the exact opposite pattern.

An interesting corollary to the example cited above is the observation that many mRNAs and/or proteins manifest characteristic checkerboard patterns of expression in the epididymis (7,15,21-23). That is, in a given epididymal region, some cells can exhibit strong expression whereas adjacent cells, which are also of the same type, can show a much lower level of expression (e.g., Fig. 3a of chapter 6). Thus, if the presence of a luminal factor(s) in a particular region of the epididymis was the sole determinant of whether a gene (or protein) is turned on,

then all cells in that given region should show similar levels of expression. The prevalence of checkerboard patterns of gene (or protein) expression in the epididymis is clearly contrary to this proposal. Obviously, other mechanisms must account for the regional specificity of gene expression in the epididymis. Further study into this phenomenon should shed some light on this remarkable facet of epididymal physiology.

In chapters II and VI, 5α-reductase type 1 mRNA and protein expression were predominantly localized to the initial segment of the epididymis. Further, they were shown to be regulated primarily by a testicular factor that is secreted directly into the epididymis via the efferent ducts; this paracrine factor was not testosterone. Interestingly, a review of the literature identifies three other genes that exhibit analogous patterns of expression in the epididymis. These genes are the opiod precursor, proenkephalin in the rat (20), and the proto-oncogene A-raf (10) and cystatin-related epididymal specific (CRES) gene (16) in the mouse. Of these three genes, the regulation of proenkephalin and CRES has been described (7,16,20). Comparison of these data with those of the 5α-reductase type 1 mRNA (chapter II) reveals a striking similarity; they are all apparently controlled by a testicular factor that is not testosterone itself but is testosterone dependent. Therefore, it is inviting to speculate that in the initial segment of the epididymis, this same paracrine factor is responsible for regulating the expression of three completely unrelated genes. Since the control of 5α-reductase type 1, proenkephalin, and CRES gene expression were postulated to occur at the transcriptional level, this putative testicular factor may represent a "master regulator" of many genes expressed primarily in the initial segment of the epididymis. In the future, comparison of the 5'-flanking region of these three genes should prove interesting in determining whether they share a common response

element that could possibly bind this regulatory factor.

In chapter V, the common regulatory pathway for 5α-reductase type 1 and proenkephalin gene expression was a critical piece of evidence that led to the novel conclusion that the ability of the testis to synthesize and/or secrete certain factors directly into the epididymis may be affected by aging. Further, the discovery that this change could be observed as early as 12 months of age was by far the earliest reported marker for aging in the male reproductive tract of the Brown Norway rat. In previous studies done by others (24-27), age-related disorders, such as the focal degeneration of the seminiferous epithelium and changes in serum concentrations of testosterone and gonadotropins, were not evident until 18 months of age. Moreover, they only became dramatically apparent by 24 months.

An important complement to regionalized gene expression is the observation that many genes expressed in the epididymis also exhibit region-specific patterns of regulation (5-7, 9-12). Segment-specific regulation of epididymal 5α-reductase was clearly demonstrated in all studies presented in this thesis. Moreover, it occurred at different levels, i.e., at the mRNA (chapters II-V) and protein (chapter VI and its appendix) levels. Previous studies have also demonstrated segment-specific regulation of 5α-reductase at the level of enzyme activity (28). Thus, based on these observations and those of others, it is important to emphasize that the epididymis cannot be regarded as a homogeneous tissue. Rather, it must be studied on a segment by segment basis. Like regionalized gene expression, the mechanisms that underlie the segment-specific nature of regulated gene expression in the epididymis are not clear. However, based on the paracrine regulation of the mRNAs for 5α-reductase type 1 (chapter II), proenkephalin (20), and CRES (16), one can speculate that the different

regions of the epididymis may contain specialized mechanisms for responding to certain hormonal and/or non-hormonal factors. One such mechanism is discussed later when a scheme for the regulation of epididymal 5α-reductase is proposed.

Postnatal development studies have been commonly used to characterize the regulation of gene and protein expression, and the activity of a number of different enzymes in the epididymis (12,20,29-31). Their value in elucidating potential regulatory factors for poorly understood epididymal markers arises from the fact that many key developmental events and/or expression profiles for several epididymal genes and proteins are well-established. In the rat, notable developmental markers include the completion of the blood-epididymis barrier by day 21 (32), increased androgen production by Leydig cells between days 20-25 (33), and the first appearance of spermatozoa in the epididymis at approximately day 49 (34). Without hesitation, the parameter that has been most frequently correlated with the developmental expression of epididymal genes and proteins is androgens.

An excellent example of a truly androgen-dependent pattern of expression in the epididymis is provided by the recent report by Palladino and Hinton (31) on the developmental expression of the γ-glutamyl transpeptidase (GGT) mRNAs. This pattern is characterized by a burst of expression at approximately days 20-25 which is coincidentally the same for increased androgen production by the testis. Other epididymal genes that exhibit a similar pattern include the abundantly expressed and highly androgen-stimulated genes, proteins B/C and D/E (20). This type of pattern, however, was not observed for the 5α-reductase transcripts, types 1 (chapter III) and 2 (chapter IV). Thus, it was concluded that androgens were not the primary regulators of the 5α-reductase transcripts in the epididymis. Interestingly, the developmental pattern observed for the 5α-reductase type 1

transcript (chapter III) was very similar to the one reported for proenkephalin (20). Again, this close association between proenkephalin and 5α-reductase type 1 is yet another indication that the expression of these genes may be similarly regulated in the epididymis.

For both 5α-reductase type 1 (chapter III) and proenkephalin (20), mRNA expression in the proximal epididymis is low until day 28; it increases substantially between days 28-56, and plateaus thereafter. A segment by segment analysis of 5α-reductase type 1(chapter III) revealed that the above pattern was conferred solely by remarkable changes occurring specifically in the initial segment of the epididymis. This pattern was inconsistent with spermatozoa, or a factor carried by them, as the regulator of 5α-reductase type 1 mRNA expression in the initial segment of the epididymis. Spermatozoa do not arrive in the epididymis until approximately day 49 (34), which is long after the rise in 5α-reductase type 1 mRNA concentrations in the initial segment of the epididymis. Rather, comparison of this pattern, with those described for other epididymal markers, points to androgen-binding protein (ABP;35,36) as a candidate factor in the control of 5α-reductase type 1 mRNA expression in the epididymis. Later, I will elaborate on the potential role of ABP when I present a scheme for the regulation of epididymal 5α-reductase.

It is important to stress that the marked increase in 5α-reductase type 1 mRNA expression in the initial segment of the epididymis is also of particular functional importance since it occurs just before the first appearance of spermatozoa in this region of the tissue (34). One can assume that the increase in epididymal 5α-reductase type 1 mRNA (chapter III), and consequently 5α-reductase activity (34,37), is necessary to prime the epididymis with the active androgen DHT and its 3α-metabolized product, 3α-diol, before the arrival of

sperm. Thus, this type of regulatory mechanism would be an efficient way for the tissue to perform its DHT- and 3α-diol-dependent spermatozoal maturation and storage functions.

In chapters II and III, a combination of tissue distribution, endocrine, and developmental studies were used to characterize the multifactorial and segment-specific regulation of 5α-reductase type 1 mRNA in the rat epididymis. At the completion of these studies, the identification of a transcript that encoded a second 5α-reductase isozyme in the rat was reported by Normington and Russell (4). Based on its order of identification, this transcript was designated as steroid 5α-reductase type 2. Immediately after its discovery, interest in the type 2 isozyme gained particular attention due to the abundant expression of its mRNA, relative to that of the type 1 mRNA, in many androgen target tissues (4); this observation was particularly evident for the rat epididymis. Based solely on its tissue distribution and its abundance in the epididymis, Normington and Russell (4) concluded that, functionally, 5α-reductase type 2 was the dominant isozyme expressed in the epididymis. Since both 5α-reductase isozymes recognize the same substrate, i.e., testosterone, then one must consider 5α-reductase enzyme activity as the sum of the activities derived from both isozymes. Hence, if the above hypothesis were correct, then the expression and regulation of the 5α-reductase type 2 transcript in the epididymis should closely parallel the expression and regulation of 5α-reductase enzyme activity. This hypothesis, as proposed by Normington and Russell (4), was tested by a series of studies that were presented in chapter IV.

The studies in chapter IV provide three critical pieces of evidence which discount Normington and Russell's conclusion that 5α-reductase type 2 is functionally the dominant isozyme expressed in the rat epididymis. The first line

of evidence arises from the longitudinal distribution of 5α-reductase type 2 mRNA in the tissue. Both 5α-reductase type 1 mRNA (chapter II) and nuclear 5α-reductase enzyme activity (38) are highly confined in their expression along the epididymis; they are overwhelmingly abundant in the initial segment region of the tissue. In marked contrast, the type 2 mRNA is most prevalent in the caput epididymidis (chapter IV). The second line of evidence comes from a comparison of the developmental profiles for 5α-reductase enzyme activity (34,37) and the mRNAs for the type 1 isozyme (chapter III) with that of the type 2 isozyme (chapter IV). This comparison reveals striking correlations between the 5α-reductase enzyme activity and the type 1 mRNA and not the type 2 mRNA. The final line of evidence, and perhaps the most convincing, is provided by a comparison of the effect of efferent duct ligation on 5α-reductase enzyme activity (39) and the expression of the 5α-reductase mRNAs (chapter IV) in the proximal region of the epididymis. The removal of direct testicular input to this region of the tissue causes a dramatic decrease in both 5α-reductase enzyme activity and type 1 mRNA expression whereas the type 2 mRNA markedly increases. An increase in 5α-reductase type 2 mRNA concentrations under conditions which cause a sharp decline in both type 1 mRNA levels and enzyme activity is inconsistent with the type 2 transcript being expressed as a major component of the 5α-reductase enzyme activity found in the rat epididymis. Therefore, it was concluded that 5α-reductase type 1 was the predominant isozyme expressed in the rat epididymis. This conclusion is further supported by the observations that, in vitro 5α-reductase activities of the expressed type 1 cDNA (40) and the actual epididymal enzyme (41) have similar neutral pH optima. In contrast, the pH optimum of the type 2 isozyme is significantly more acidic (40).

The present finding that the 5α-reductase type 1 isozyme has a more

prominent functional role in androgen versus non-androgen target tissues in the rat is in agreement with a very recent report by Eicheler et al. (42) in the human. In contrast to the conclusions of Thigpen et al. (43), Eicheler et al. showed that 5α-reductase type 2 is not restricted to androgen target tissues but rather, is present in a large number of cells and tissues in both males and females (42). The revelation that one 5α-reductase isozyme is preferentially expressed over the other has potentially significant pharmacological implications. For example, if one had the task of designing a drug to block 5α-reductase activity in a particular tissue, then it would be of paramount importance to know which 5α-reductase isozyme to target. Presently, practical applications of this problem can be found.

Finasteride, the 5α-reductase blocker that has been approved for therapeutic management of benign prostatic hypertrophy (BPH), is apparently a potent competitive inhibitor of the human type 2 isozyme but is a weak inhibitor of the type 1 isozyme (40). In human skin, 5α-reductase type 1 has recently been identified as the predominant isozyme (44). In addition to its function in normal skin physiology, 5α-reductase has also been proposed to be implicated in several androgen-dependent disorders such as acne vulgaris, seborrhea, male pattern baldness, and hirsutism (45-47). Thus, if finasteride (a type 2-specific blocker in the human) were used to treat these skin disorders (where type 1 predominates), the effectiveness of this drug would be expectedly poor. Instead of finasteride, a specific 5α-reductase type 1 blocker would be the drug of choice. In the human, selective 5α-reductase type 1 inhibitors have begun to be developed (48).

From the immunocytochemical studies described in chapter VI, several novel conclusions may be drawn. First, the localization of 5α-reductase type 1 in the initial segment of the epididymis was unique. Previously unknown, the 5α-reductase type 1 protein was abundantly localized in very discrete lobules of the

initial segment, the proximal initial segment. The second finding was that the 5α-reductase type 1 protein was not localized within principal cell nuclei but rather, showed a prominent cytoplasmic localization. Although no intranuclear labelling was found, significant infranuclear staining was apparent, specifically in the initial segment of the epididymis. Interestingly, the area of intense infranuclear staining coincided with the same region where the bulk of epididymal nuclear 5α-reductase enzyme activity is known to reside. Thus, it was concluded that the infranuclear localized form of the 5α-reductase type 1 protein represented the nuclear 5α-reductase enzyme.

This conclusion was substantiated by the results of an experiment which studied the effects of efferent duct ligation on the expression and subcellular localization of the 5α-reductase type 1 protein in the different regions of the adult rat epididymis (appendix to chapter VI). After efferent duct ligation, only one epididymal region was sensitive to this procedure; this region was identified as the proximal initial segment. Consistent with nuclear 5α-reductase enzyme activity and type 1 mRNA expression, type 1 protein expression markedly decreased. More importantly, however, the infranuclear localization of the 5α-reductase type 1 protein in the proximal initial segment completely disappeared. Therefore, this strongly supported the conclusion that the infranuclear localized form of the 5α-reductase type 1 protein is nuclear 5α-reductase. After efferent duct ligation, the 5α-reductase type 1 immunoreactivity that remained in all epididymal regions was supranuclear and cytoplasmic. Since serum testosterone concentrations are not compromised in an efferent duct ligated animal model (49,50), this supranuclear staining was equated with the microsomal 5α-reductase enzyme. The activity of the latter enzyme is found throughout the epididymis, is expressed at a lower level and is under the control of circulating androgens (28).

In the proximal initial segment, the distinct regulatory mechanisms that control the expression of the infranuclear and supranuclear localized forms of the 5α-reductase type 1 protein present an intriguing physiological question. How do principal cells in the proximal initial segment manage to differentially regulate the targeting of proteins which are apparently synthesized from the same transcript? Presently, a definitive answer to this question is not known. A review of the literature would even suggest that this type of phenomenon is unprecedented. Aside from the current data on type 1 5α-reductase, the only other occurrence for this type of regulated intracellular protein targeting in the epididymis is found in the recent study by Cyr et al (51). In their study, the intracellular localization of connexin 43 (Cx43), a gap junctional protein, was found to differ under different hormonal conditions. In normal rats, Cx43 could be found between principal and basal cells, but not between adjacent principal cells. However, in a castrate rat, Cx43 could be found between adjacent principal cells in addition to the principal-basal cell localization; this effect was reversed with exogenous testosterone administration. Thus, these independent findings, for two radically different proteins, would suggest that regulated intracellular protein targeting in the epididymis is a plausible scenario.

A Model for the Regulation of Epididymal 5α-Reductase

The data provided by the studies in this thesis have significantly advanced our understanding of the regulation of epididymal 5α-reductase. Importantly, these new data can be consolidated with those obtained in the past in order to provide an updated model for the regulation of epididymal 5α-reductase. A summary of the different factors controlling 5α-reductase and androgen action in the rat epididymis is presented schematically in Figure 1.

It is proposed that testosterone enters principal cells from both the basal cell surface, where it arrives from the peripheral circulation, and from the lumen, either free or bound to androgen binding protein (ABP). Once in the cell, testosterone is converted to DHT on endoplasmic reticulum membranes closely associated with the nuclear membrane or on membranous elements found in the supranuclear region of the cytoplasm. The conversion of testosterone to DHT in close proximity to the nucleus occurs predominantly in the proximal initial segment of the epididymis. This "nuclear" 5α-reductase activity is represented by the infranuclear localized form of the 5α-reductase type 1 protein. In the proximal initial segment, the infranuclear localized 5α-reductase type 1 protein or "nuclear" 5α-reductase enzyme activity is primarily controlled by a testicular factor that is secreted directly into the epididymis via the efferent ducts (Fig. 1, left and middle panels). Since this factor also controls 5α-reductase type 1 mRNA expression in the initial segment of the epididymis, one can speculate that this factor acts as a transcriptional regulator for the type 1 gene. In contrast to nuclear 5α-reductase, microsomal 5α-reductase enzyme activity, which is represented by the type 1 protein in the supranuclear cytoplasm of the cell, is under the control of circulating androgens (i.e., testosterone).

For many years, the identity of this testicular factor has been proposed to be ABP (38). Several lines of suggestive evidence support this proposal. First, epididymal nuclear 5α-reductase enzyme activity (34,37), type 1 mRNA expression (chapter III) and ABP show similar developmental profiles (35,36). Second, ABP is a Sertoli cell product whose synthesis is under the control of androgens (52); the putative testicular factor controlling epididymal nuclear 5α-reductase enzyme activity is also androgen-dependent (53). Third, ABP is actively endocytosed in the proximal segment of the epididymis through a receptor-

mediated mechanism (54,55), and hence, the epididymal region where nuclear 5α-reductase activity and type 1 mRNA and protein expression are highest and most sensitive to the paracrine regulatory factor. The last line of evidence is provided by a study by Anthony et al. (56) that determined a positive correlation between epididymal ABP and epididymal sperm fertilizing ability. Since the acquisition of sperm fertilizing ability in the epididymis is dependent on 5α-reduced androgens, and hence, the 5α-reductase enzyme, then the above correlation would also be consistent with ABP as a critical regulator of epididymal 5α-reductase.

In the initial segment of the epididymis, the DHT that is produced is concentrated in the nucleus by passive facilitated transport due to the presence of androgen receptors within the nucleus that bind DHT avidly (Fig. 1, middle panel). Androgen action is initiated by binding of DHT to the receptor in a manner similar to that described for other steroid responsive tissues (57). The abundant presence and tight regulation of nuclear 5α-reductase enzyme activity, via the type 1 mRNA and its protein in the initial segment of the epididymis, thus provides an excellent mechanism for optimally converting the massive amount of testosterone that this region receives as a result of direct testicular secretions (18). It is further proposed that, in the initial segment of the epididymis, the large amount of DHT formed, whether it be near the nucleus or in the supranuclear cytoplasm, is released back into the lumen in order to provide the most active androgen to mediate androgen action in the rest of the tissue (Fig. 1, right panel). Since principal cells in these segments of the epididymis do not bear any discernable infranuclear localized 5α-reductase type 1 protein, ABP is not considered a major regulator of the type 1 protein in these regions of the tissue. Rather, the primary regulator of the supranuclear localized form of the 5α-reductase type 1 protein (i.e., microsomal 5α-reductase), is proposed to be testosterone which is provided

by the peripheral circulation.

A notable exclusion from the scheme presented above is the role of 5α-reductase type 2. From both a functional and regulatory perspective, the studies in chapter IV clearly demonstrated that 5α-reductase type 2 must have a minor role in mediating androgen action in the epididymis when compared to the type 1 isozyme. The type 2 isozyme may provide a low, basal level of 5α-reductase activity throughout the epididymis. The reason for which the epididymis expresses so much of this transcript when it is apparently not utilized effectively remains an enigma.

From a physiological standpoint, the mechanism for the regulation of epididymal 5α-reductase, as presented above, raises an important question: Why is 5α-reductase so abundantly expressed and tightly regulated in the initial segment of the epididymis? Although the exact answer to this question cannot be given at this time, I can speculate on two reasons for these observations. First, I propose that, in the initial segment of the epididymis, the presence of the biologically potent androgen, DHT, is required to maximally stimulate androgen-dependent genes whose expression are critical for the maintenance of normal epididymal function. Since testosterone, the substrate for the 5α-reductase enzyme, is primarily bound to ABP as it enters the epididymal lumen (18), then having ABP turn on the 5α-reductase type 1 gene would provide an excellent mechanism for optimally converting the massive amount of testosterone that has to be converted in this very small region of the tissue. Second, the abundant synthesis of DHT specifically in the initial segment would be an efficient strategy for providing the most active androgen to mediate androgen action in the rest of the tissue.

The Regulation of Epididymal 5α-Reductase: Future Directions

Now that the molecular tools to study the steroid 5α-reductase enzymes (genes, cDNAs, antibodies) are at hand, they have paved the way for a more detailed understanding of how androgens and more importantly, the 5α-reduced metabolites of testosterone, mediate their action on the cells that line the epithelium of the epididymis and other tissues. Clearly, the studies in this thesis have provided a solid basis for future studies directed at understanding the regulation of epididymal 5α-reductase. I can foresee two different avenues that could be pursued: (i) the definitive identification of the putative testicular factor controlling nuclear 5α-reductase enzyme activity and type 1 mRNA and protein expression; and (ii) a further understanding of androgen action at the cellular level through electron microscopic localization of the type 1 in epididymal principal cells.

I firmly believe that the necessary tools to conclusively identify the paracrine factor controlling 5α-reductase in the initial segment of the epididymis are now available. The most important tool was provided by the cloning of the 5α-reductase genes (58-60). Based on the studies presented in this thesis, it is highly probable that this factor acts as a transcriptional regulator. If so, then this factor should bind to the 5' upstream promoter region of the rat 5α-reductase type 1 gene. Thus, testicular fluid, or better still rete testis fluid, could be used in the initial screening of potential factors that bind to the 5' region of the 5α-reductase type 1 gene. Moreover, this technique would be an excellent method of directly testing whether ABP can bind and act as a transacting factor for the 5α-reductase type 1 gene in the epididymis.

In this thesis, the development of a specific antiserum to 5α-reductase type 1 was a major leap forward towards understanding the role of this enzyme in mediating androgen action in the epididymis and other androgen target tissues.

In the epididymis, this was begun with the light microscopic localization of the 5α-reductase type 1 protein and would be extended by immunolocalization studies at the level of the electron microscope. The latter could be successfully used to resolve long outstanding issues such as whether the enzyme needs to be present in the nuclear membrane in order to optimally mediate androgen action in certain tissues.

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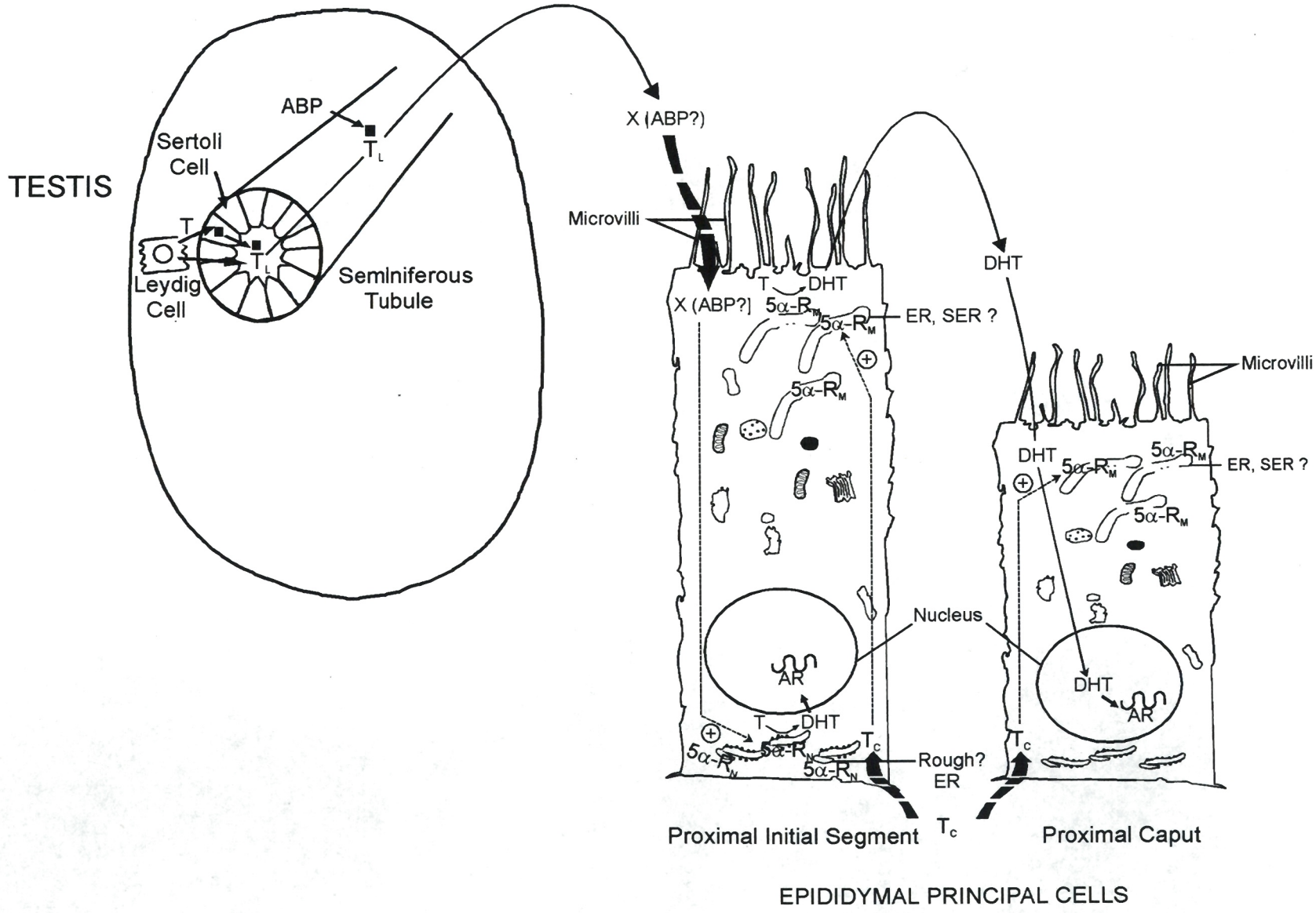
Figure 1. Proposed mechanism for the regulation of 4-ene steroid 5α-reductase and androgen action in the rat epididymis. Leydig cells synthesize testosterone (T) which stimulates the Sertoli cell to secrete ABP into the lumen of the seminiferous tubule. In the initial segment of the epididymis, ABP is proposed to be the paracrine factor regulating the infranuclear localized 5α-reductase enzyme (nuclear 5α-reductase). In contrast, the apically (supranuclear) localized 5α-reductase enzyme (microsomal 5α-reductase), found throughout the epididymis, is under the control of circulating androgens. The synthesis of locally high concentrations of DHT in the initial segment of the epididymis provides an excellent mechanism for stimulating critically important androgen-dependent genes in this region of the epididymis and for providing the most active androgen to mediate androgen action in the rest of the tissue. ABP - androgen-binding protein, 5α-R_N - nuclear 5α-reductase, 5α-R_M - microsomal 5α-reductase, ER - endoplasmic reticulum, SER - smooth endoplasmic reticulum, DHT - dihydrotestosterone, T_c - testosterone in the general circulation, T_L - luminal testosterone.


TESTIS
ABP
Sertoli Cell
T
T_L
Leydig Cell
Seminiferous Tubule
X (ABP?)
Microvilli
T
DHT
X (ABP?]
5α-R_M
ER, SER ?
AR
Nucleus
Rough? ER
T_C
Proximal Initial Segment
DHT
Proximal Caput
EPIDIDYMAL PRINCIPAL CELLS

List of Original Contributions

1. Study of the longitudinal distribution of the 5α-reductase type 1 mRNA reveals that this message is highly regionally localized in its expression along the adult rat epididymis; it is highest in the initial segment of the tissue.

2. Bilateral orchidectomy results in a marked reduction in 5α-reductase type 1 mRNA concentrations in all regions of the epididymis; testosterone replacement can maintain the expression of this message in the caput, corpus, and cauda epididymidis, but not in the initial segment, even when given at very high doses.

3. Unilateral orchidectomy causes a sharp decrease in 5α-reductase type 1 mRNA concentrations in the initial segment of the epididymis; this procedure results in a small decrease in type 1 mRNA levels in the caput epididymidis, but has no effect in the corpus and cauda regions of the tissue. Based on these three studies, I conclude that the endocrine regulation of epididymal 5α-reductase type 1 mRNA is mulitifactorial and segment-specific.

4. Postnatal developmental studies also reveal that epididymal 5α-reductase type 1 expression is age-dependent and segment-specific. A segment by segment analysis demonstrates that this developmental regulation is specific to the initial segment of the epididymis; in this region, 5α-reductase type 1 mRNA levels are found to dramatically increase between postnatal days 21 and 42.

5. The expression of the 5α-reductase type 2 mRNA in the epididymis is established to be region-specific but it predominated in the caput epididymidis while the 5α-reductase type 1 mRNA predominates in the initial segment of the epididymis of the adult rat.

6. In the epididymis, the expression of the 5α-reductase type 1 mRNA is age-dependent, while that of the type 2 transcript remains constant throughout postnatal life.

7. Unilateral efferent duct ligation causes a marked decrease in 5α-reductase type 1 mRNA expression, specifically in the initial segment of the epididymis; in contrast, 5α-reductase type 2 mRNA levels in the initial segment rose significantly after this experimental procedure. Thus, the 5α-reductase mRNAs are differentially regulated in the rat epididymis.

8. The first demonstration of changes in the expression of the mRNAs for the 5α-reductase isozymes, types 1 and 2, proenkephalin, and sulfated glycoprotein-2 (SGP-2; clusterin) are identified in the aging Brown Norway rat epididymis. The novel finding that the mRNAs for 5α-reductase and proenkephalin are altered as early as 12 months of age represents the first index of aging in the male reproductive system found using this model.

9. A novel anti-5α-reductase type 1 serum is developed using synthetic peptides.

10. Using microscopy, the 5α-reductase type 1 protein is demonstrated to be predominantly localized to discrete lobules of the initial segment; the immunoreactivity is specific to cytoplasmic elements, but not nuclei, of epithelial principal cells.

11. The subcellular localization of the 5α-reductase type 1 protein is shown to be epididymal region-specific and age-dependent; immunoreactivity in the infranuclear region of principal cells is unique to the proximal initial segment.

12. After efferent duct ligation, only the infranuclear localization of the 5α-reductase type 1 protein in the proximal initial segment disappears. Thus, the subcellular localization of the type 1 protein is differentially regulated in the proximal initial segment of the epididymis.