The Regulation of Spermatogenesis through the Action of Testosterone

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Biochemistry/Premedicine: Chemistry Minor

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Honors Thesis
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The environment inside the seminiferous tubules, in which germinal cells develop, is entirely controlled by the secretions of the Sertoli cells. Therefore, any substance such as testosterone, an androgen, which is capable of influencing that environment, is able to affect spermatogenesis. This is the reason that the role of androgens in the regulation of Sertoli cells has received such attention in the realm of reproductive biology. It is widely accepted that testosterone is essential to the maintenance of spermiogenesis. In the absence of testosterone, spermatogenesis regresses and eventually stops. Testosterone acts by influencing transcription and metabolic activity of Sertoli cells via androgen binding proteins called receptors. It also stimulates the peritubular myoid cells, surrounding the seminiferous tubules; thereby indirectly affecting Sertoli cells. Despite the vast amount of knowledge that has accrued regarding the action of testosterone in the testis, relatively little is known about its role in regulating spermatogenesis. The purpose of this study is to use gene filters to help clarify testosterone’s contribution to the spermatogenic process. With the advent of gene arrays and new genomic technology it has become possible to simultaneously screen thousands of genes for their expression under a set of given conditions. Ideally, it will become possible to observe which genes are regulated by testosterone and which proteins are produced in response to its presence. In the long run a better understanding of Sertoli cell regulation will enable us to gain a more thorough understanding of testosterone’s role in spermatogenesis.

This honors thesis is organized into two sections. The first half of this paper provides a general synopsis of testosterone’s mode of action in the context of spermatogenesis. Supporting research is discussed and cited when applicable. The latter portion examines gene expression within the testis of ethane dimethane sulfate (EDS) treated rats in the presence and absence of testosterone.
PART I: THE ROLE OF TESTOSTERONE IN SPERMATOGENESIS

Every aspect of male fertility and phenotype is dependent on the production of testosterone (Sharp et al, 1990). Without testosterone, spermatogenesis ceases to take place and in the absence of Leydig cells, which produce testosterone, sperm production can be maintained for up to 10 weeks if intratesticular testosterone levels remain high (Sharp, 1990). Androgens, a general term for testosterone, are produced in Leydig cells located in the interstitial space of the testis. Testosterone is secreted by Leydig cells and diffuses throughout the testis. Once released into the intracellular space within the testis testosterone enters cells and binds to androgen receptors located within the Sertoli cells and myoid cells and stimulates transcription. In this paper we focus on the mechanism of testosterone influence on spermatogenesis.

PHYSIOLOGY OF THE TESTIS AND BLOOD-TESTIS BARRIER IN RATS

The inner portion of the seminiferous tubule is composed of a layer Sertoli cells (Fig. 1). The Sertoli cell consists of an apical and basal section. The apex of each cell stretches into the tubular lumen and houses germinal cells in the later stages of spermatogenesis. The basal portion of the Sertoli cell holds the germinal cells at the earlier stages of spermatogenesis. This section of the Sertoli cells also acts as one of two target sites for androgen action. Each Sertoli cell lies adjacent to five or six other cells in a two-dimensional sheet-like manner, which folds back on itself to form a tube-like structure (Fig. 2). These tubules maintain their composition with an intercellular network of microtubules, intermediate filaments and actin. This network forms a barrier to proteins and substances in the blood, thus creating the second of two barriers in the blood-testis division. The first layer of the
blood-testis barrier is outside the seminiferous tubule and consists of the basal lamina, myoid cells, a glycoprotein coat and the lymphatic endothelium. These outer layers are collectively known as the tunica propria (Fig. 3). The basal lamina (basement membrane) is a homogenous layer of finely filamentous glycoprotein material about 0.15 μ thick. The next layer consists of myoid cells (“interlamellar cells” of Clermont, 1958; “peritubular contractile cells” or Ross, 1967; “myoid cells” of Dym, 1970) that overlap one another to form a continuous coating around the basal lamina. It is this myoid cell portion that serves as the second of two target sites for androgen influence in the testis. The coating of the myoid cells consists of another glycoprotein layer, essentially identical to the basal lamina. The outer surface of the seminiferous tubules is coated with epithelial-like cells called the lymphatic endothelium. The three layers, apart from the myoid cells, help to maintain the structural integrity of the seminiferous tubules and the blood-testis barrier.

The seminiferous tubules are grouped next to one another to form the bulk of the testis. The Interstitial space between the tubules houses the Leydig cells, where testosterone is produced and secreted, blood vessels and various other cells (immune cells, fibroblasts, etc.). The ends of the seminiferous tubules meet at the rete ducts where germ cells are collected and transported, via the efferent ducts, into the epididymis. In the epididymis they mature further and develop motility. Testosterone is also transported from the testis to the epididymis, bound to androgen binding protein (ABP), to maintain the epididymal tissue.
THE ORGANIZATION OF SPERMATOGENESIS AND THE STAGE-SPECIFIC ACTION OF TESTOSTERONE

Spermatogenesis occurs in the seminiferous tubules in a cyclical manner consisting of 14 stages in the rat and seven in humans. For the purpose of this paper we will only be addressing spermatogenesis in rats (Fig. 4). One spermatogenic cycle is finished when all fourteen stages have been completed. A type A-spermatagonium passes through four and a half such cycles before it becomes a mature spermatid and is released into the lumen of the seminiferous tubule. As the germ cell passes through each cycle it migrates from the basal portion of the Sertoli cells towards the apex, where it is finally released into the lumen of the seminiferous tubule at stage VII of the fifth cycle. At any point in time a single Sertoli cell is associated with germ cells of only one stage.

During the first phase of spermatogenesis the spermatagonium divides to produce two daughter cells; one of which enters spermatogenesis while the other remains a stem cell. The cell undergoes a number of mitotic divisions to become a B-spermatagonia and then a preleptotene spermatocyte by stage VII of the second cycle. The second phase of spermatogenesis begins at stage VII of the second cycle and is termed the meiotic phase. During this phase the spermatocyte gives rise to four haploid round spermatids, via meiotic divisions, which enter the first phase of spermiogenesis (the acrosomal phase). The acrosome develops during this time. These three spermatids then enter the nuclear condensation and elongation phase at stage VIII of cycle four. The major structures of the tail form during this time. At the beginning of the fifth cycle of spermatogenesis the spermatid eliminates the majority of its cytoplasm, via endocytosis by the
Sertoli cell, and is released from the apex of the Sertoli cell. The resulting germ cell is referred to as a spermatozoon. In the rat the duration of spermatogenesis is 51.6-53.2 days (Knobil, 1994).

Testosterone becomes essential between stages VI-VIII. When Leydig cells are destroyed by treatment with ethane dimethane sulfonate (EDS) a stage specific degeneration of germ cells is observed. Based on $[^{35}S]$methionine incorporation, it was also shown that there was a marked decrease in total protein secretion in seminiferous tubules isolated at stages VI through VIII. The degradation of stage VI germ cells is the first biological sign of androgen depletion. The remaining stages of the spermiogenic cycle showed no changes in response to the depletion of Leydig cells (Sharpe, 1990, 1992; Kerr, 1993).

A number of observations support the hypothesis that testosterone action is focused on stages VI-VIII. Sharpe et al, using radiolabeled analysis of transcription, discovered 7 androgen regulated proteins (ARPs) which are released specifically during stages VI-VIII, named ARP-1 through ARP-7. These proteins are only secreted when testosterone is present (1992). ABP secretion is also stage dependent. Concentrations of ABP peak in stages VII-XI. The minimal concentrations of ABP occur during stages IV-V (Ritzen, 1982). Since ABP levels in the testis increase in response to rising concentrations of androgens it is unlikely that testosterone is active during other stages. During stages VI-VIII an increase in the volume of the rough endoplasmic reticulum occurs. The diameter of the lumen of the seminiferous tubules increases. This is partially dependent on the presence of elongate spermatids in the seminiferous tubule. Following treatment with EDS the volume of the lumen did not change during stages VI-VIII. The expansion of the lumen coincides with an increase in seminiferous tubule fluid (Knobil, 1994).

MECHANISMS OF ANDROGEN INFLUENCE ON SERTOLI CELL FUNCTION

There are two main mechanisms through which testosterone is able to influence spermatogenesis that have been agreed upon by researchers in reproductive biology. Testosterone can interact directly with the basal portion of the Sertoli cell via specialized proteins. This mechanism consists of a multiple step process involving androgen receptors (AR), chaperone proteins and chromatin acceptor sites. This process is capable of maintaining the minimum concentration of testosterone required for spermatogenesis. Testosterone is also able to stimulate the production of paracrine factors from myoid cells in the tunica propria. One paracrine factor, P-mod-S, stimulates the production of transferrin, androgen binding protein (ABP) and ceruoplasmin by Sertoli cells (Skinner, 1987). In recent years it has also been postulated that testosterone may influence spermatogenesis by binding to ABP released into the seminiferous tubule. This ABP-testosterone
complex would then bind to membrane receptors for ABP located on germ cells. However, at the present time this is only a theory with little sound evidence (Knobil, 1994).

EVIDENCE FOR DIRECT ACTION ON SERTOLI CELLS

Possibly the most compelling evidence for the direct action of androgens on Sertoli cells is the existence of androgen regulated proteins, produced by the Sertoli cells, whose level of secretion is directly related to the concentration of testosterone in the testis. A high-affinity androgen-binding protein was first discovered using a 105,000 x g supernatant fraction of rat epididymis. The binding protein was found in the epididymis with an identical component being observed in the testis supernatant. Using retardation coefficients in gels of differing pore size, the molecular weight was determined to be about 90,000 daltons (Ritzen et al, 1971; Ritzen et al, 1973). It was later shown that the androgen binding protein (ABP) was produced in the rat testis and transported to the efferent ducts in the efferent duct fluid (French et al, 1973). In addition to being highly selective towards androgen binding, ABP levels in the testis are directly related to the concentration of testosterone present in the testis (Louis, 1978; Elkington, 1976; Rommerts, 1977). During periods of increased androgen production ABP levels rose accordingly. Rommerts observed that after the 6th or 7th day of incubation in the presence of testosterone, ABP production from cultured Sertoli cells increased significantly (1977). In 20-day-old rats, administration of testosterone was also shown to illicit a 3 fold increase in ABP levels; half-maximal response occurred at a dose of 4 nm (Louis, 1978). ABP is currently thought to act as a buffering mechanism to changing testosterone levels. Sertoli cells increase their production of ABP in response to rising levels of testosterone production by Leydig cells. ABP then binds to testosterone and transports the steroid to the epididymis where it maintains the epididymal tissue and further sperm development.

Further support for the direct action of testosterone comes from the existence of an androgen receptor within the Sertoli cell cytoplasm. Tindall et al discovered the presence of a protein (MW = 200,000 daltons) within the cytoplasm of Sertoli cells. This protein has a high binding affinity for androgens (1974). Due to its steroid specificity, which is similar to the cytoplasmic receptor in rat ventral prostate, this protein was initially named CR. In more recent years, referring to the protein as 'androgen receptor' has gained wide spread popularity. Both terms will be used interchangeably in this paper. CR has a high affinity for both testosterone and testosterone propionate and is not androgen dependent. It is required for the uptake of testosterone and testosterone propionate. It was been suggested that, in addition to acting as a testosterone transport
mechanism, ABP might also contribute to the uptake of testosterone into the Sertoli cells. Although having an additive effect on androgen uptake, it was determined that ABP was not required for the transfer of androgen into the epididymal nucleus (Tindall, 1974). Tindall et al. used 1,2,6,7-3H-testosterone (3H-T) and 1,2-3H-dihydrotestosterone (3H-DHT) instead of testosterone or TP in order to detect the androgen's location in the testis and determine the mechanism of CR, and possibly ABP, action. Within intact testis, 3H-T was transported into the lumen of the caput epididymis, creating a reservoir of 3H-T (Fig. 4). Following hypophysectomy and the disappearance of ABP from the caput epididymis, testicular 3H-T was no longer concentrated in the lumen (Fig. 6). However, CR binding and transport of 3H-T into the epididymal nuclei was still active.

Cyproterone acetate, which selectively inhibits CR binding without affecting ABP, was employed to further examine androgen uptake. In the epididymis, cyproterone acetate inhibited nuclear uptake of 3H-DHT to the same extent that it inhibited binding to CR; thus supporting the notion that CR is required for androgen uptake and ABP does not participate in the direct action of testosterone on Sertoli cells. Autoradiographic studies were used to confirm the inhibition of CR by cyproterone acetate while ABP action was not influenced (Tindall, 1974) (Fig. 5).

Following androgen binding the androgen-CR complex translocates the to nuclear membrane within 30 minutes of androgen exposure. Androgen-receptor complexes bind an element in the promoter of certain genes (Sanborn et al., 1977). The CR-androgen complex is suspected to stimulate transcription. The addition of testosterone to cultures of Sertoli cells from 25-day-old rats has been shown to result in a 'biphasic preferential stimulation' of RNA polymerase II by 2-
fold within 15 minutes. After 15 minutes the activity marginally declined but soon returned again at 3 and 6 hours (Sanborn et al, 1977).

EVIDENCE FOR INDIRECT ACTION OF ANDROGENS VIA PERITUBULAR MYOID CELLS

In recent years it has been determined that androgens have an additional method of regulating Sertoli cell function via peritubular myoid cells. In the intact testis, Sertoli cells and peritubular cells lie adjacent to one another, separated by a glycoprotein layer called the basal lamina. Along with a number of collagen fibrils, peritubular cells compose the tubular wall surrounding the seminiferous tubules. It has been suggested that both peritubular cells and Sertoli cells are responsible for secreting this tubular wall. Support for this hypothesis comes from the observation that Sertoli cell-enriched aggregates plated on top of a multilayer of peritubular cells undergo a structural reorganization to form complex protrusions that grossly resemble the seminiferous tubules. This structural transformation does not occur when either the Sertoli cells or the peritubular cells are cultured independently (Skinner, 1985). In addition to having a structural influence over Sertoli cells, peritubular cells regulate the metabolic function of Sertoli cells in a manner similar to a combination of follicle-stimulating hormone (FSH), insulin, retinol, and testosterone (FIRT) (Norton, 1989).

In the presence of androgens, peritubular cells secrete paracrine factors which stimulate the production of ABP and transferrin by Sertoli cells. One of these factors is called P-Mod-S (a peritubular protein [P] that modulates [Mod] Sertoli cell [S] function) (Skinner, 1985; 1986; 1988; Norton, 1989, Swinnen, 1989). P-Mod-S exists in two forms: P-Mod-S (A) and P-Mod-S (B). Both P-Mod-S (A) and P-Mod-S (B) are rendered inactive by a trypsin digestion and have molecular weights of 56,000 Daltons and 59,000 Daltons, respectively. The proteins differ significantly in their amino acid content and the relationship between the two is currently unknown. P-Mod-S (B) was found to be both the more hydrophobic and more biologically active than P-Mod-S (A) (Skinner, 1987). Purification studies have shown that both forms of P-Mod-S can account for virtually all of the bioactivity of the proteins secreted by the peritubular cells (Skinner, 1985; 1986; 1988; Swinnen, 1989; Norton, 1989).

Once produced and secreted, P-Mod-S amplifies the androgen stimulation of ABP and transferrin production in Sertoli cells. When Sertoli cells are cultured in minimal essential medium (MEM) the rates of ABP and transferrin production gradually decline over time. The addition of testosterone to the medium retards this decline. The retardation of ABP and transferrin production
was even greater when Sertoli cells were co-cultured with peritubular cells. This response was also elicited in the presence of conditioned medium in which peritubular cells had been previously maintained (Skinner, 1985). Additional studies have resulted in similar findings, thereby adding support to the role of P-Mod-S as a Sertoli cell regulating agent.

Like Sertoli cells, peritubular cells possess an androgen receptor (Verhoeven, 1979; Norton, 1989). Verhoeven et al measured 5α-Reductase and 17β-hydroxysteroid levels in Sertoli cell and peritubular cell fractions (1979). 5α-Reductase converts androgens to estrogens. The presence of 5α-Reductase is an indicator of androgen uptake and metabolism. Likewise, the presence of 17β-hydroxysteroid is further validation of androgen uptake by these cells. In the cell fractions it was observed that the 5α-Reductase activity and 17β-hydroxysteroids in interstitial cells was nearly twice that in Sertoli cells. Peritubular cells have also been shown to respond to androgen levels by secreting high concentrations of P-Mod-S when testosterone is present in large quantities and vice versa. Similar concentrations of other hormones, such as 17β-estradiol do not stimulate P-Mod-S secretions. (Skinner, 1984).

A THIRD MECHANISM FOR TESTOSTERONE INFLUENCE

In 1994 it was suggested that ABP might still play a role in the influence of testosterone on spermatogenesis (Knobil). Knobil noticed that ABP is secreted bidirectionally into the lumen of the seminiferous tubule and the interstitial space. It was also noted that ABP is transcribed from the same gene as sex-hormone-binding globulin (SHBG) (Porto, 1991). Porto et al showed the existence of binding sites on testicular membranes which are similar to SHBG receptors in other tissues (1991). In other tissues these receptors are capable of internalizing the SHBG and stimulate cAMP accumulation (1991; Gerard, 1991). Since germ cells are capable of cAMP accumulation and possess similar sites for SHBG binding, Knobil suggests that it is theoretically possible for an ABP-testosterone complex to be internalized by germ cells in order to stimulate cAMP accumulation. This hypothesis also comes from the observation that, relative to the amount of testosterone that is required to occupy all androgen receptors within the seminiferous tubules, the large amount produced by Leydig cells is not biologically efficient. Therefore, Knobil suggest that it is highly likely that another uptake mechanism exists (1994). This is only a hypothesis and, at this time, there is no sound evidence to prove or disprove this idea.
ANDROGEN INFLUENCE ON SERTOLI CELLS AND TRANSCRIPTION WITHIN THE SEMINIFEROUS TUBULES

Sertoli cells are often referred to as the nurturing cells for developing germinal cells. Every aspect of the environment surrounding germinal cells is in some manner controlled and regulated by Sertoli cells. The blood-testis barrier is created through tight junctions between the basal sections of neighboring Sertoli cells. Seven major glycoproteins, uniquely synthesized and secreted by Sertoli cells, supply some of the basic nutritional requirements of germinal cells. Of these seven two are known to be regulated by the presence of testosterone. One of the most studied glycoproteins, outside of ABP, is an iron transport protein referred to as transferrin. It was discovered by Skinner and Griswold, using sodium dodecyl sulfate-polyacrylamide gels, when it was noticed that a glycoprotein produced by Sertoli cells had a molecular weight similar to rat serum transferrin (1980). Testicular transferrin is also responsive to testosterone levels within the testis. Sertoli cells in culture from 10- and 20-day-old rats increased their secretion of testicular transferrin in the presence of androgens (Skinner, 1982). The relatively low concentration of transferrin observed at any time within the cell suggested that androgens elicit the production of transferrin and not the release of pre-existing transferrin. \[^{35}S\] methionine introduced into the testis along with hormones resulted in an increase in \[^{35}S\] methionine incorporation into testicular transferrin; thus supporting the argument for an increase in transferrin production over simple secretion (Skinner, 1982).

Cyclic protein-2/cathepsin L has been identified as the second of the seven major glycoproteins produced and secreted by Sertoli cells to be influenced by androgen presence. In a similar manner to transferrin cyclic protein-2/cathepsin L levels are directly related to androgen levels within the testis. This effect, however, occurs on a much lower scale than transferrin (Karzai, 1992). Karzai and Wright observed cathepsin L synthesis to increase by 30% in the presence of testosterone (1992). Unfortunately, in their study this amount was just marginally below statistical significance.

In addition to these two glycoproteins testosterone stimulates the transcription of seven androgen regulated proteins (ARPs) from various cells within the testis (McKinnell, 1992). ARP-1 (MW = 9.5 kDa) and ARP-2 (MW = 13-14.5 kDa) are secreted by round spermatids during stages VI-VIII when testosterone response is highest. This finding has significant implications for the role of germ cells during spermatogenesis. This is discussed in the following section. ARP-3 (MW = 27-30 kDa), ARP-4 (MW = 38 kDa) and ARP-5 are transcribed at an increased rate by
Sertoli cells during stages VI-VIII. ARP-6 (MW = 56 kDa) and ARP-7 (MW = 59 kDa) are produced and secreted by the peritubular myoid cells. ARP-6 and ARP-7 are comparable to P-Mod-S (A) and P-Mod-S (B), respectively, in size and isotopic properties (McKinnell, 1992).

THE ROLE OF GERM CELLS IN THE REGULATION OF SPERMATOGENESIS BY TESTOSTERONE

It is logical to assume that germ cells play little role in determining their fate in the spermatogenic process; however, this is not the case. At any point in time, within the seminiferous tubule, there are germ cells in every stage of the spermatogenic process. In this complex process some of the regulatory influence comes directly from the germ cells themselves. Onada and Djakiew (1992) showed that preparations enriched in either pachytene spermatocytes or round spermatids elicited a 3-fold increase in the incorporation of radiolabeled methionine into proteins secreted by Sertoli cells. McKinnell also showed that germ cells in certain stages of the spermatogenic cycle stimulate the transcription and release of specific ARPs from Sertoli cells and peritubular myoid cells (1992). This influence only occurs during stages VI-VIII when testosterone induces the transcription of these proteins (McKinnell, 1992; Sharpe, 1993). Using isolated seminiferous tubules, McKinnell selectively eliminated pachytene spermatocytes by treatment with methoxyaetic acid (MAA). Depletion of both round and elongate spermatids was accomplished as a result of maturation of the tubules (Onada, 1992; Djakiew, 1992).

Upon the removal of pachytene spermatocytes ARP-6 and ARP-7 transcription ceased. ARP-3, ARP-4 and ARP-5 production also marginally decreased. Since ARP-6 and ARP-7 are suspected to be P-Mod-S (A) and P-Mod-S (B), respectively, this finding suggests that pachytene spermatocytes are somehow able to influence the action of peritubular myoid cells (directly or indirectly). With the removal of round spermatids ARP-1 and ARP-2 disappeared from the media. Sharpe showed that following treatment with EDS ARP-1 and ARP-2 secretion significantly decreased despite the presence of round spermatids, showing that the it is unlikely that germ cells produce ARP (1988). ARP-1 and ARP-2 are released into both the seminiferous tubules and the interstitial space; characteristic of Sertoli cell secreted proteins (Sharpe, 1988). The removal of elongate spermatids from the seminiferous tubules did not inhibit the depletion of any particular protein. However, upon its removal McKinnel observed that total methionine incorporation in the testis decreased by 42 percent (1992). These findings indicate that, not only do germ cells influence the action of spermatogenic process, cells at certain stages stages influence transcription at specific stages of spermatogenesis in the seminiferous tubule. Pachytene spermatocytes are
required before peritubular myoid cells are able to secrete P-Mod-S. Likewise, overall transcription in the seminiferous tubules will reach maximal level only in the presence of elongate spermatids, regardless of the presence of testosterone. These findings indicate that germ cells play a larger role in spermatogenesis than previously thought. Their influence appears to be just as important as that of testosterone; so much so that, in their absence, androgen regulation of spermatogenesis no longer occurs.
PART II: CHANGES IN GENE EXPRESSION WITHIN THE TESTIS AS STUDIED USING LEYDIG CELL DESTRUCTION AND TESTOSTERONE PROPIONATE REPLACEMENT

ABSTRACT

This communication investigates androgen action at the genomic level by examining the alterations in gene expression which take place when testosterone is allowed to sustain spermatogenesis in Leydig cell deprived testis. Ten week-old rats were treated with 75 mg of ethane dimethane sulfate (EDS) per kg body weight to eliminate Leydig cells, thereby halting testosterone production. Half of the population received 2 mg of testosterone propionate daily. Prostate and seminiferous vesicle weight was recorded to determine the efficacy of treatment and RNA samples from each group were screened and analyzed using Research Genetics gene filters. Using gene array analyzing software, expressed sequence tag (EST) AA818737, from Rat GeneFilter release 1 GF 300, was observed in the testis of EDS treated rats, following testosterone treatment. The EST was sequenced and a probe prepared for a poly-A enriched northern blot. A GeneBank search for known gene sequences found one gene from a muscle tissue cDNA library.

MATERIALS AND METHODS

EDS Treatment

Twelve 10-week-old rats were treated with 75 mg/mL EDS per kg of body weight. Half of this population also received 2 mg testosterone propionate daily. On day six, the rats were sacrificed. Prostate, testis and seminiferous vesicles were removed and weighed to determine the efficacy of EDS and testosterone treatment (EDS + T). Testis RNA from each group (EDS and EDS + T) was purified and used to screen a gene filter.
Isolation of RNA

RNA was purified using Trizol Reagent (Life Technologies), following the protocol supplied by the manufacturer. Testis from each group (EDS and EDS + T) were homogenized and placed in 1 mL of Trizol reagent. Samples were centrifuged at 12,000 X g for 10 minutes at 8 °C to remove extracellular material. Phase separation was accomplished by adding 0.2 mL of chloroform per 1 mL of Trizol reagent and incubating the sample at 25 °C for 3 min. The resulting colorless upper aqueous phase was transferred to a new tube and the remaining phenol-chloroform phase discarded.

RNA was precipitated by mixing 0.5 mL of isopropyl alcohol per 1 mL Trizol reagent initially used in homogenization. The samples were incubated at 25 °C for 10 min. and centrifuged at 12,000 X g for 15 minutes at 8 °C. The resulting supernatant was removed and the pellet washed once with 1 mL 75% ethanol. Samples were vortexed and centrifuged at 7,500 X g for 5 min. at 8 °C. The resulting supernatant was removed. Resuspension was accomplished by adding 50 µL of DEPC treated H2O and incubating samples at 60 °C for 10 min. Following resuspension, purified RNA was stored at -70 °C for hybridization of GeneFilter.

Preparation of GeneFilter Probe

Rat GeneFilter microarray release 1 GF 300 was supplied by Research Genetics (Huntsville, Alabama). GeneFilters were washed in boiling 0.5% SDS and agitated for 5 min. to remove residuals. GeneFilter membranes were placed in hybridization roller tubes and saturated with 5.0 mL MicroHyb hybridization solution (Research Genetics). The GeneFilters were prehybridized at 42 °C for 2 hours with denatured 5.0 µL Cot-1 DNA (Life Technologies) and 5.0 µL Poly dA (Research Genetics).

Primer solutions were prepared in a 1.5 mL centrifuge tube. Each solution consisted of 1 µg of RNA from one test group (EDS or EDS + T), 2.0 µL Oligo dT (Research Genetics) and DEPC treated H2O in a total volume of 10.0 µL. Priming solutions were denatured in 70 °C for 10 min. From the denatured RNA priming solutions 33P probes were constructed. The elongation mixture consisted of 6.0 µL 5X First Strand Buffer (Life Technologies), 1.0 µL DTT, 1.5 µL dNTP, 1.5 µL Reverse Transcriptase, and 10 µL 33P dATP. The solution was incubated for 90 min. at 37 °C.
The solution was brought up to 100 mL and loaded into a Bio-Spin 6 Chromatography Column (Bio-Rad) to purify the resulting probe. To ensure successful labeling of the probe counts per minute (cpm) of the probe were measured. The resulting purified probe was denatured in a boiling waterbath for 3 min. The denatured probe solution was then pipetted into the roller tube containing the GeneFilter. The GeneFilter microarrays were incubated overnight for 12 to 18 hours at 42 °C in a roller over with a rotation of 9 rpm.

Following hybridization, the GeneFilters were washed twice with 2X SSC and 1% SDS at 50 °C for 20 min. and once with 0.5X SSC and 1% SDS at 25 °C for 15 min. GeneFilters were wrapped in Saran wrap to prevent drying out and analyzed using a Molecular Dynamics Phosphorimager.

**Gene Array Analysis**

Both **Pathways** (Research Genetics inc.) and **ScanAlyze2** (Stanford University) Gene filter analysis software were used to analyze the filters. In the **Pathways** program a default grid setting was chosen to analyze the filter. A direct comparison using the green-red color scale was used to detect differences between gene expression and intensity on the filters. An intensity limit of 1050 was set up as a minimum and the lower ratio limit was set at 4.50. Any spots scoring lower than either of these values were not considered significant. Two trials were completed to ensure reproducibility. No minimum limits were established in **ScanAlyze2**. Intensities were calculated for every spot on the filters. However, only those spots which received ratios of 5.00 and above in the **Pathways** software were examined. The difference in intensities and the background values of each filter were calculated.

**Plasmid Culture and Amplification**

cDNA clone (ID# UI-R-A0-AY-G-12-0-UI, Research Genetics Inc.) and streaked onto 12 LB ampicillin agar plates and incubated overnight. LB medium was prepared using 10g of bacto tryptone, 5g of bacto-yeast extract, 10g of NaCl, ampicillin added to the final concentration of 1 μg/mL, and 15g agar per liter. The pH was adjusted to 7.4 using 5N NaOH (~0.2 mL), the solution autoclaved for 20 minutes and poured into 100 mL culture plates.

Twelve more LB ampicillin plates were streaked with a single colony from each of the first twelve and incubated overnight. One colony from each plate was then used to prepare a miniprep from which a probe was to be prepared.
PCR Screening

To determine which colony had the cDNA insert we used polymerase chain reaction (PCR) screening to search for the Plasmid. Each colony was then subjected to PCR using a standard protocol. The resulting solutions from each colony were electrophoresed on a 1% agarose gel to detect the presence of the cDNA insert. Those solutions in which the cDNA insert was detected were consolidated and used in the preparation of a $^{32}$P probe.

Preparation of Probe

A probe was constructed from the plasmid containing the cDNA insert for future northern blot analysis of gene expression within the different organs. EcoRI and Hind III were used to remove the cDNA insert from the bacterial plasmid. The digested plasmid was electrophoresed on a 1% Tris base, acetic acid, EDTA (TAE) agarose gel to determine if the digestion occurred. Samples were then consolidated, electrophoresed on a 0.8% low melting point agarose gel and extracted using Qiagen gel purification kit (protocol specified by Qiagen).

The $^{32}$P radioactive probe was constructed using the *Life Technologies RadPrime Labeling System*. The protocol used for the preparation of the probe was supplied by the manufacturer.

Affinity (poly-A enriched) Chromatography

Rat organ RNA was isolated from two 20-day-old male rats and two 60-day-old female rats using Trizol reagents and the protocol specified by the manufacturer. The organs which were removed are listed in appendix 1. RNA was resuspended in 24 μL of DEPC treated H$_2$O and stored at -70 °C. The chromatography loading buffer consisted of 500 mM NaCl, 10 mM Tris (pH 7.4), 0.1% SDS and 1 mM EDTA. A 400 μg sample of RNA from each organ was suspended in 500 μL of DEPC treated H$_2$O. The loading buffer and RNA solution were combined to make the loading solution and poured over the column.

The column, consisting of oligo-dT cellulose, was treated with 75% NaOH along with loading buffer before the beginning of the procedure and before the addition of each new RNA sample. The loading solution (RNA + buffer) was passed through the column twice and the eluent allowed to run completely off the column. The column was washed with 1 to 2 mL of a wash buffer, consisting of 250 mM NaCl, 10 mM Tris (pH 7.4), 0.1% SDS, and 1 mM EDTA. This was
immediately followed by DEPC treated H₂O. Finally, the elution buffer was heated to 70 °C and 1 mL was run through the column and collected. The elution buffer was composed of 10 mM Tris (pH 7.4) and 1 mM EDTA. This process was completed for each of the 12 RNA samples.

A 1 μL aliquot of glycogen was added to each sample of poly-A enriched RNA along with 1/10 the volume of NaOAc. The resulting solution was transferred to 1.5 mL Eppendorf tubes (700 μL/tube) and precipitated with 350 μL of isopropyl alcohol. The mixture was centrifuged at 15,000 x g for 45 min. The resulting pellet was washed with 75% EtOH and resuspended in 40 μL of DEPC treated H₂O. This was stored at -70 °C to be used in the northern blot.

Northern Blot Analysis of EST

Briefly, a northern blot of the rat organ samples was performed using standard protocols. RNA from the 12 rat organ samples were combined with a loading buffer and heated to 70°C for 10 min. and loaded onto a 1% agarose formaldehyde gel. The gel consisted of 50% formamide, 10% 10X MOPS, 37% formaldehyde, 5% saturated bromophenol blue, 5% glycerol, and 13% H₂O and 1g agarose. The gel was electrophoresed at 150 volts and the nucleic acids transferred onto a Hybond-N nylon membrane (Amersham Pharmacia Biotech.). Nucleic acids were cross-linked onto the membrane by ultra-violet radiation (1600 kJ). The membrane was incubated with the ³²P radioactive probe at 42 °C overnight in a 50% formaldehyde, 5X SSC, 4X Dehhardt’s, 0.5% SDS solution containing 100 μg/mL salmon sperm DNA. Following hybridization, the membrane was washed twice with 0.1 X SSC, 0.1% SDS at 65 °C and once at room temperature (25 °C). The membrane was wrapped in Saran wrap and placed on a phosphor screen. Phosphor screens were scanned with a Molecular Dynamics phosphorimager.

Cycle Sequencing of EST

A 1 μg sample of the testosterone regulated cDNA plasmid was sequenced using 3.37 μL of 0.65g/L. (3.2 picomoles) T7-1 primer and 1.23 μL of 1.68g/L (3.2 picomoles) T3-1 primer, 4.0 μL of BigDye mix and ddH₂O in a total volume of 10 μL. The standard PCR protocol was supplied by BigDye Terminator Cycle Sequencing Reaction kit. The sequence data was analyzed using a Perkin Elmer Applied Biosystems 377 Automated DNA Sequencer.
RESULTS

EDS Treatment

EDS treatment was determined to be successful. The weight of the prostate and seminiferous
tubules of the EDS alone treated rats were determined to be $0.10 \pm 0.02 \text{ g}$ and $0.29 \pm 0.06 \text{ g}$
respectively. The prostate and testis of the EDS + T treated rats weighted $0.33 \pm 0.14 \text{ g}$ and $1.41 \pm
0.14 \text{ g}$, respectively.

Gene Array Analysis

One expressed sequence tag (EST) was found in both trials to be upregulated in the presence of
testosterone. Using Pathways' software, EST
AA818737 (cDNA ID# UI-R-A0-AY-G-12-0-U1) scored an intensity
difference of 3669.44 between the rats which were given EDS alone and
the rats which were also administered testosterone propionate on a daily basis (Fig. 7). The EST
was upregulated in the presence of testosterone propionate. This EST also scored a ratio of 5.41
between the two groups. ScanAlyze2 calculated a pixel intensity of 42 for the rats which did not receive testosterone propionate and an intensity of 3074.5 for the rats which did receive testosterone propionate daily. The background pixel intensity for the two groups was approximately 52,000, differing slightly for each filter.

Northern Blot

Due to the unpredictable nature of scientific research, the northern blot data was not available by
the time this Honors Thesis was prepared. The first trial was corrupted due to the presence of
RNAsse in the samples. This caused the degradation of the organ RNA on the blot so that only a
large smear was seen when the northern was screened with the probe. In the next attempt, as outlined above, we are using affinity chromatography to select out only the messenger RNA. This is achieved through the use of an oligo-dT cellulose column which binds to the poly-A tails on messenger RNA (mRNA) when the loading solution is passed through it. Once the mRNA has bound to the column it is washed and removed into an 1.5 mL eppendorf tube. The resulting solution almost completely consists of mRNA. In a typical northern blot all types of RNA (tRNA, mRNA, and rRNA) would be present. The northern using poly-A enriched RNA will consist primarily of mRNA. We are also employing the use of reverse transcription PCR (RT-PCR) to screen for the testosterone regulated mRNA. RT-PCR is more sensitive than northern blotting, thus we have another technique to screen for the T regulated message. The northern blot and an analysis and discussion of the results will appear in a supplement at the end of this thesis.

EST Sequence

0 11
 21 31 41
GNAGNNAGNN NGCGGC GGNN  
31 41
NCNNTCCCN AACCNTNTGA ACGCCC CACC

51 61
NNTTTNGGTA NNTTTNCCC TTTCTGAT A AATGATAC T TTATTATAAT

101 111
TTTTAAAAA ACAGGACTCG TCTAAAGCT C TCTCTCTCT CTCTCTCTCG

151 161
CTCTCTCACA CACACACACA CACACACACA CACACACACA CACACACACA

201 211
CGTGCGCACA CTGATTTTCT GTGCTGATTT TCATACCCCT CAATTGGCT

251 261
AACAAGTTAT GCCTCAGA AG TTTTCCAGT TCAACTCATA CTTCAACTTG

301 311
AATACCAGGG ATCATCAGTC TTGTTTTGTG ATACGTCAGT GATGACAGCT

351 361
GGTGTA CATT TGCTGAAGAA ATCATTTTCTG CTGGTCTCAG C ACCCCTGAC

401 411
TG TGGGC ACT GTGTGTGGNG GGCTAGAATA TGGATCTCGA TGCCACTCAC

451 461
TACCCTGCTG GTATGCTCTC CCTCTCTCTC ACTGCTCTCT TGAATCTCTA

501 511
CAGGGGT TGG GTAAACA CACT GTAGC AAAAA GGTATC AACT GGTAGCGG

551 561
TG CCTTAGAC CAGTAC AAT GGTACTG AAGTC ACTAG AAGAG C GCCCTAACT

601 611
GNGGGCTTAC AA TTA GAAT CAA CCAAAGTAAG C AATGGGCGAG

651 661
CCTTGGGT G TATGA AAAAA AGCTTG GATC CGAATT TTTGA AAGAG C TTCGAAATC

701 711
CACGGGGGAC TTT TGTGCTC ACCAATCCAGAATCGAAT CGAATCTTT AAAAA GGCGCTCGAG

751 761
GGC CAAAATG CCTAT ANGGG AGNCGG GATA AATCCGNA
DISCUSSION

Gene Array Software

Due to the relatively recent development of gene arrays there are no set standards for their analysis. There are also no established guidelines for the design of analysis software. Consequently, different software companies may use completely different strategies for establishing grids and calculating intensities and ratios. In this study we circumvented the possible problems this may have caused by using two different types of software when analyzing our gene arrays. Pathways, a trademark of Research Genetics, was an obvious choice since our gene filters were also developed by the same company. The Pathways software is a very user friendly automated software. The researcher controls the positioning of the grid on the filter via 16 control points located at the outer edges of the filter. The user places a set of crosshairs on top of each control point and the program then sets up the grid using those points as guidelines. At the end of this process the user is given a choice between the calculated grid and a default grid.

When calculating the results of the analysis, Pathways allows the user to set up a minimum intensity difference and a minimum ratio between the gene filters. Any spot which has a value above both the specified ratio and intensity is shown on a report form which indicates the Gene Bank accession number and cDNA number of each gene. The report also shows the location of the spot on the filter and whether the identification of the gene is known. This final report can then be printed out or saved as an Microsoft Excel file.

The ScanAlyze2 software, developed at Stanford University, allows for a much more in-depth control of the grid at the loss of user friendliness. This program is available free of charge via the internet at Stanford’s homepage and is not designed for any particular type of filter. As a result, the user must specify every aspect of the filter they are using (# of spot per column/row, size of spots, etc.). However, this program also allows for the researcher to manipulate every aspect of the grid, even to the point of shifting individual spots to ensure the program is analyzing the correct region. Once the grid is established it can be saved as a file and loaded at a later date (a feature not available with Pathways).

As with the gridding process, the results section in ScanAlyze2 presents much more information to the user than does Pathways, but at a cost. The results in this program are automatically saved as a data file which must be viewed separately from the program itself.
Microsoft Notepad or Wordpad to open the file the researcher is given a variety of information in a format similar to a spreadsheet. All the spots are listed vertically along with their location, intensities, three different ratio calculations, background intensities, and four different statistical analyses. All these calculations are labeled with abbreviations which can be read about in the manual.

In our study we used these two programs to compliment and check one another. The *Pathways* program was used initially to locate the spots which scored ratios above 5.00. The ratio of 5.00 was chosen after discussing the matter with other researchers in the field. We concluded a ratio of 5.0 or greater was due to a result of different intensities and not due to background noise. Once we determined that EST AA818737 satisfied our criteria we reanalyzed the filter using *ScanAlyze2*. After discovering that other scientists were having problems with reproducing their results with *Pathways* we decided this step was necessary. Indeed, the results did vary somewhat each time we reanalyzed the filters using *Pathways*. As a means of checking our data from *Pathways* we employed the use of *ScanAlyze2* to examine the spots which were considered significant by *Pathways*. *ScanAlyze2* offers a much more in-depth calculation of statistical significance and probability of error in its analysis. By using both programs we were able to ensure the reproducibility of our results and ensure that any results we obtained were not affected by faults inherent in the software itself.

**Gene Array Analysis**

Using the *Pathways* software it was shown that EST AA818737 was up-regulated in the presence of testosterone. This observation was also supported when the filters were analyzed using *ScanAlyze2*. This implies that this particular gene is either involved in the role of testosterone in spermatogenesis or is part of a system that is androgen dependent. We do know that it is highly unlikely that this gene is involved in androgen production since all the Leydig cells were destroyed by treatment with EDS. It is also possible that this particular gene is one of the upregulated genes observed by Sanborn et al (1977). None of the upregulated genes were sequenced at that time, so it is not possible to determine whether this is the case. It is very plausible that EST 818737 codes for one of the androgen regulated proteins isolated by McKinnell (1992). An estimation of the molecular weight and possible amino acid structures of the proteins coded for by this gene will be pursued to look into this possibility. The computer software program, GCG, will be employed to research further into this possibility. Since the effects testosterone are restricted to stages VI to VIII of the spermatogenic cycle, EST 818737 expression is also likely to be restricted to this
period. A method to analyze this possibility is to isolate and examine a portion of the seminiferous

tubules which exists solely in stages VI - VIII. By comparing the expression of EST 818737 in

these tubules to that in tubules at other stages of the spermatogenic cycle for confirmation.

The next step in this study is to run an organ northern blot to observe where in the body this
gene is expressed besides the testis. In addition to this, a northern blot of the different cell types
within the testis would indicate where this gene is being produced. This is indeed our next course
of action, along with the estimation of the transcription products, and will be attached to this thesis
upon its completion. In addition, cell specific expression could be confirmed by in situ
hybridization.

**Sequencing**

The EST was sequenced and cloned as shown in the results section. Sequencing the EST allowed
us to develop a probe and primer for cloning and running the northern blot. The sequence
information allowed us to run a database search on GeneBank to determine if this EST had been
sequenced previously and whether its function was known. The search indicated that a similar
version of this gene (only 372 bp long) was expressed in the muscle tissue of rats. This is not
surprising since muscle tissue is highly responsive to the levels of testosterone in the body.
Unfortunately, this is the only gene in GeneBank that is significantly similar to EST AA818737.
The next step in this process is to use the computer software program GCG to determine possible
start sites and exon/intron information. Possible protein sequence and secondary structure
information can also be determined using GCG.

Since this Honors Thesis is being written before the completion of this research, a portion of
the findings and discussion will appear as a supplement. Thus far it has been shown that EST
AA818737 is transcribed at an increased rate in the testis when testosterone propionate is present.
There exists a similar gene in the muscle tissue which is also responsive to the presence of
androgens. The inference that we can make from this discovery is that this gene is somehow
involved in testosterone’s role in spermatogenesis. How exactly this gene is involved has yet to be
determined.

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Figure 6. Tindall D, Hansson M, Stumpf W, French F, Nayfah S 1974 Further Studies on the Accumulation and Binding of Androgen in Rat Epididymis. Endocrinology 95: 1119
APPENDIX

RNA was obtained from the two 10-week-old male rats from the following organs.
- muscle
- lung
- spleen
- testis

RNA was obtained from two 60-day-old female rats from the following organs.
- pancreas
- ovaries
- uterus
- mammary glands