Prospects for Monitoring Spermatogenesis

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Contents

Declaration

The experiments described in this thesis were the unaided work of the author except where the acknowledgement is made by reference. No part of this work has been previously accepted for any other degree, nor is any part of it being submitted concurrently in candidature for another degree.

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Abstract

The objective of the studies in this thesis is to use the rat as a model of human spermatogenesis to evaluate whether proteins produced by the testis can gain access to blood and provide a method for monitoring spermatogenesis. At present there are no means of assessing the normality or otherwise of the various phases of germ cell development non-invasively i.e. by the measurement of a marker in peripheral blood. The regulation of germ cell development involves the secretion of proteins by both the Sertoli cells and germ cells, and there is evidence which suggests that some of these proteins may gain access to the peripheral circulation in normal physiology and in increased amounts when the seminiferous tubules are damaged. The studies described in this thesis have used the rat model of spermatogenesis to, firstly, evaluate creatine as a marker of testicular damage. Secondly, to ascertain whether seminiferous tubule derived proteins can be detected in any of the following biological fluids: testicular interstitial fluid (IF), spermatic vein (SV) blood, testicular vein (TV) blood or peripheral (PV) blood. Thirdly, to assess whether screening a testis cDNA expression library provides a useful strategy for cloning cDNAs encoding testis secreted proteins.

The testis was found to contain high levels of creatine, approximately half of that measured in skeletal muscle. Measurement of creatine in seminiferous tubule homogenates showed that levels were not dependent on the stage of the spermatogenic cycle and were not acutely affected by testosterone withdrawal. Levels were significantly decreased following depletion of germ cells using methoxyacetic acid, especially at those time points when pachytene spermatocytes were degenerating and when round spermatids were absent. In addition, measurement of creatine in rete testis fluid and IF suggested that it is secreted bidirectionally; levels in IF decreased during sexual maturation showing a similar profile to that shown for Sertoli cell secreted proteins. These results would suggest that creatine is present in both the Sertoli and germ cells. Damage was induced to specific cell types within the testis, either to the Sertoli cells, germ cells or to the Leydig cells. Acute damage significantly increased creatine levels in IF with all treatments but changes in plasma levels were more variable. These results suggested that the level of creatine in blood is not an informative marker of acute testicular damage.

An antiserum was raised against adult rat seminiferous tubule conditioned medium (STCM) and used to detect testicular proteins in samples of IF, SV, TV and PV plasma from adult rats by Western blotting. This technique detected proteins secreted by seminiferous tubules in IF but not in any of the plasma samples. The antiserum specifically detected three proteins in IF from control rats with molecular weights of 24, 16 and 14 kDa respectively. Following, the disruption of spermatogenesis by short-term testicular heat treatment 24 hours earlier (43°C for 30 min), the abundance of the three

proteins in IF was increased and induced the appearance of several other less abundant proteins, all with molecular masses below 25 kDa. The 24 kDa protein present in IF was identified as the germ cell secretory product, phosphatidylethanolamine binding protein (PEBP) and the 14 kDa protein was putatively identified as a germ cell protein regulated by androgen protein, ARP-2. These results demonstrate that germ cell proteins can gain access to the interstitium under both normal physiological conditions and more easily after testicular damage. The inability to detect testicular proteins in plasma may be due to the insensitivity of the techniques used.

Eight individual cDNA clones were identified by screening a testis library using the STCM antiserum. Northern blot analysis of the cDNA clones identified that one cDNA, 813B4, recognised two abundant mRNA transcripts specifically expressed in adult testis and epididymis. Further library screening was performed to obtain a clone encoding the entire cDNA sequence. Sequence analysis revealed that the cDNA had significant homology to four expressed sequence tags but no homology to any sequences with a reported function. mRNA expression was investigated using both Northern blots and non-radioactive *in situ* hybridisation. This confirmed that 813B4 mRNA was expressed in germ cells, the mRNA was first detectable in the cytoplasm of stage VII pachytene spermatocytes and thereafter in all germ cells until step 15 spermatids. Southern blot hybridisation demonstrated that the gene for this germ cell specific cDNA (GCS1) was conserved in several species including humans and monkeys.

In conclusion, this work has identified several proteins and one cDNA which may have potential use as markers of germ cells. Although these studies were unable to detect testicular proteins in blood they have demonstrated the necessity of developing immunoassays to specific proteins for an unequivocal assessment of whether secreted proteins can be used to provide information on the normality of spermatogenesis.

Abbreviations

ABP	Androgen binding protein
ADP	Adenosine diphosphate
AP	Alkaline phosphatase
ARP-2	Androgen regulated protein-2
ATP	Adenosine triphosphate
BCIP	5-Bromo-4-chloro-3-indolyl-phosphate
BOS	Buthionine sulfoximine
bp	base pairs
BSA	Bovine serum albumin
cAMP	Cyclic adenosine monophosphate
cDNA	Complementary DNA
CP-2	Cyclic protein-2
cRNA	Complementary RNA
CTP	Cytosine triphosphate
Dig	Digoxigenin
dNTP	Deoxyribonucleotide triphosphate
DNA	Deoxyribonucleic acid
mDNB	meta Dinitrobenzene
DTT	Dithiothreitol
EDS	Ethane dimethane sulphonate
EDTA	Ethylenediaminetetraacetic acid
EGME	Ethylene glycol monomethyl ether
EPIP	Endometrial progesterone induced protein
FSH	Follicle stimulating hormone
GCS1	Germ cell specific cDNA 1
GnRH	Gonadotrophin releasing hormone
GTP	Guanosine triphosphate
hCG	Human chorionic gonadotropin
i.p.	Intra-peritoneal
IF	Interstitial fluid
IPTG	Isopropyl β-D-thiogalactopyranoside
Kb	Kilobase pairs
kDa	Kilo Dalton
LDH-C4	Lactate dehydrogenase-C4
LH	Luteinizing hormone
MAA	Methoxyacetic acid
2-ME	2-Methoxyethanol

mRNA	messenger RNA
NAD	Nicotinamide adenine dinucleotide
NADPH	Nicotinamide adenine dinucleotide phosphate
NB	Nitrobenzene
NBT	Nitroblue tetrazolium
ORF	Open reading frame
PAGE	Polyacrylamide gel electrophoresis
PBS	Phosphate buffered saline
PCR	Polymerase chain reaction
PEBP	Phoshatidylethanolamine binding protein
PEG	Polyethylene glycol
PV	Peripheral venous
PVDF	Polyvinylidene difluoride
RIA	Radioimmunoassay
RNA	Ribonucleic acid
rNTP	Ribonucleotide triphosphate
RTF	Rete testis fluid
SDS	Sodium dodecyl sulphate
SGP-1	Sulphated glycoprotein-1
SGP-2	Sulphated glycoprotein-2
SHBG	Sex hormone binding globulin
ST	Seminiferous tubule
StAR	Steroidogenic acute regulatory protein
STCM	Seminiferous tubule conditioned medium
STF	Seminiferous tubule fluid
SV	Spermatic venous
T	Testosterone
TBE	Tris buffered EDTA
TBS	Tris buffered saline
TEA	Triethanolamine
TEMED	N,N,N',N'-Tetramethylethylenediamine
TRIS	Tris(hydroxymethyl)aminomethane
TTP	Thymidine triphosphate
TV	Testicular venous
UTP	Uridine triphosphate

Chapter 1. Introduction

There is growing concern over several reports which suggest that there is an increasing incidence in problems associated with male reproductive health. The statistical analysis of 61 publications on semen quality world-wide during the period 1938-1990 showed a significant decrease in mean sperm count of normal men by approximately 40% (Carlsen *et al.*, 1992). This study has attracted some criticism of its statistical validity (Farrow, 1994; Bromwich *et al.*, 1994). However, a recent study has evaluated semen quality in 1351 fertile men who donated sperm to one centre in Paris during the past 20 years, and was able to demonstrate a significant decline in sperm counts and also in sperm motility and morphology (Auger *et al.*, 1995). This fall in sperm quality appears to be related to the donor's year of birth (Auger *et al.*, 1995; Irvine, 1994). During this same time period the incidence in various countries of testicular cancer and of genito-urinary tract abnormalities such as hypospadias and cryptorchidisms also appears to be on the increase (Giwercman & Skakkebaek, 1992). Approximately 15% of all couples will have infertility problems, with 'male factors' thought to be responsible in half of these cases (Skakkebaek *et al.*, 1994). The fact that the underlying cause of these effects on the male reproductive tract is unknown serves to highlight the lack of understanding of the aetiology of male infertility. One hypothesis has suggested that environmental factors acting in fetal and early neonatal life may be responsible for the deterioration in semen quality, testicular cancer, cryptorchidism and hypospadias (Sharpe & Skakkebaek, 1993; Sharpe, 1994a). Since similar abnormalities of the male reproductive tract were induced in the sons of women exposed to diethylstilbestrol during pregnancy and can be induced in pregnant animals exposed to exogenous oestrogen, changes in lifestyle associated with an increased exposure to oestrogens have been implicated.

The decline in sperm counts, morphology and motility indicates that spermatogenesis is impaired. However, the complex cellular interactions which are responsible for spermatogenesis and the factors which regulate this process are poorly understood, resulting in a lack of understanding of the basic mechanisms involved in causing a deterioration in semen quality and an inability to treat male infertility. The aim of the work in this thesis was to explore possible approaches for monitoring spermatogenesis. At present, there are no markers which can be used to assess the normality of the various steps in spermatogenesis (Sharpe, 1992). If suitable assays could be developed they would prove invaluable in the assessment of male infertility both in the clinical situation and in screening for adverse effects of drugs and environmental chemicals on male reproductive ability. It is generally accepted that proteins secreted by both the Sertoli cells and the germ cells are instrumental in controlling spermatogenesis; this dialogue between the various cells in the seminiferous epithelium is thought to change in accordance with the stage of development of the germ cells (Sharpe, 1993). The ideal

solution to monitoring spermatogenesis would involve measuring key proteins whose role in the regulation of spermatogenesis is well characterised and which can be detected non-invasively, preferably in a sample of peripheral blood (Sharpe, 1992).

The general aim of these studies was to use a rat model of spermatogenesis to assess the feasibility of identifying ST secreted proteins and their potential to be used to inform on spermatogenesis by non-invasive means. It is hoped that these studies will provide the necessary background that will allow characterisation of human ST secreted proteins which can then be evaluated as to their usefulness in the clinical management of male infertility and also as markers of testicular damage in toxicity studies.

Thesis objectives:

1. To evaluate the feasibility of using creatine as a non-invasive marker of acute testicular damage in the rat. (Chapter 4)

2. To ascertain, using Western blot analysis, whether proteins derived from the seminiferous tubules in the adult rat testis are able to gain access to testicular interstitial fluid and/or blood in the normal physiological situation, and whether this is altered by the induction of damage to spermatogenesis. (Chapter 5)

3. To assess a new strategy (expression screening) for identifying novel testicular cDNAs and their encoded proteins which might be of use in the monitoring of spermatogenesis. (Chapters 6 and 7)

Chapter 2. Review of the Literature

2.1. Introduction

The testis has two major functions, the production of spermatozoa by the seminiferous tubules and the synthesis of androgens by the Leydig cells in the interstitium. Both of these processes are closely interrelated; spermatogenesis can only be maintained in the presence of adequate levels of testosterone. Androgens are also extremely important for the development of secondary sexual characteristics and therefore for determining masculinity. The studies described in this thesis are aimed at assessing the feasibility of using testicular proteins to monitor spermatogenesis. To provide a background for this work this chapter will review the structural organisation of the testis, its physiology and the importance of endocrine and paracrine factors in the regulation of spermatogenesis. Emphasis is placed on proteins secreted by the cells of the seminiferous epithelium and factors which determine their feasibility as potential markers of spermatogenesis. The final section reviews the use of testicular toxicants as 'biochemical tools' to investigate the cellular interactions which comprise spermatogenesis, since this is a key approach to the studies undertaken in this thesis.

2.2. Functional organisation of the testis

The testes, in both man and rat, are located within the scrotal sac external to the body and are maintained at a temperature of 32-34°C. The testis has a highly complex structural organisation specialised for the development of the germ cells and the endocrine support of this process. The bulk of the testis is composed of seminiferous tubules, which are connected at both ends to a complicated network of channels known as the rete testis. The efferent duct system connects the testis to the epididymis, which is the site of maturation and storage of the spermatozoa (Fig. 1). Surrounding the tubules is the interstitial tissue which is comprised of blood vessels, lymphatics, nerve fibres, Leydig cells, macrophages and connective tissue. The Leydig cells occur as groups associated with blood vessels and the seminiferous tubules. A multilayered wall composed of basement membrane, peritubular myoid cells and connective tissue separates the seminiferous tubules from the interstitium. The seminiferous tubules contain Sertoli cells which surround and support the developing germ cells. The presence of inter-Sertoli cell tight junctions, a major constituent of the blood-testis barrier, divides the area within a seminiferous tubule into basal and adluminal compartments (Fig. 2). The basal compartment contains the spermatogonia and the adluminal compartment contains the meiotic (except for the preleptotene spermatocytes) and post-meiotic germ cells.

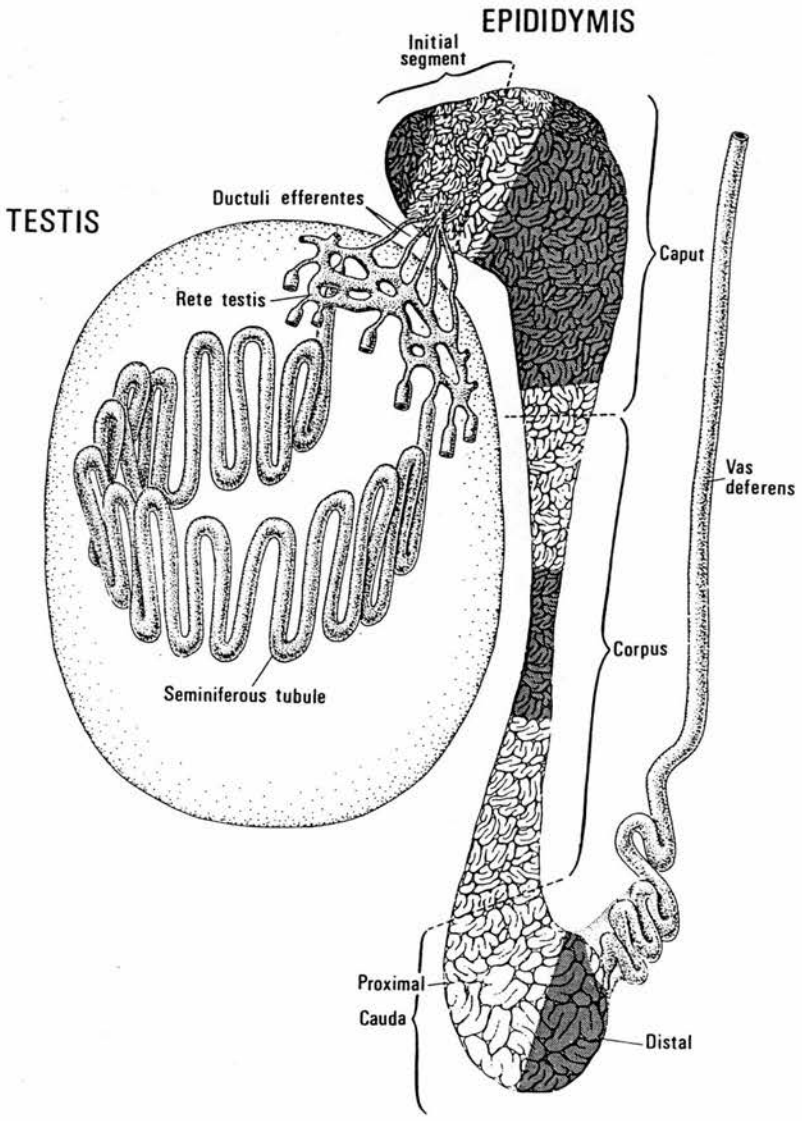


Figure 1. Diagrammatic representation of the testis and epididymis, showing a seminiferous tubule connecting to the rete testis which leads into the efferent ducts, epididymis and vas deferens. The different regions of the epididymis are indicated. *Adapted from Robaire & Hermo, 1988.*

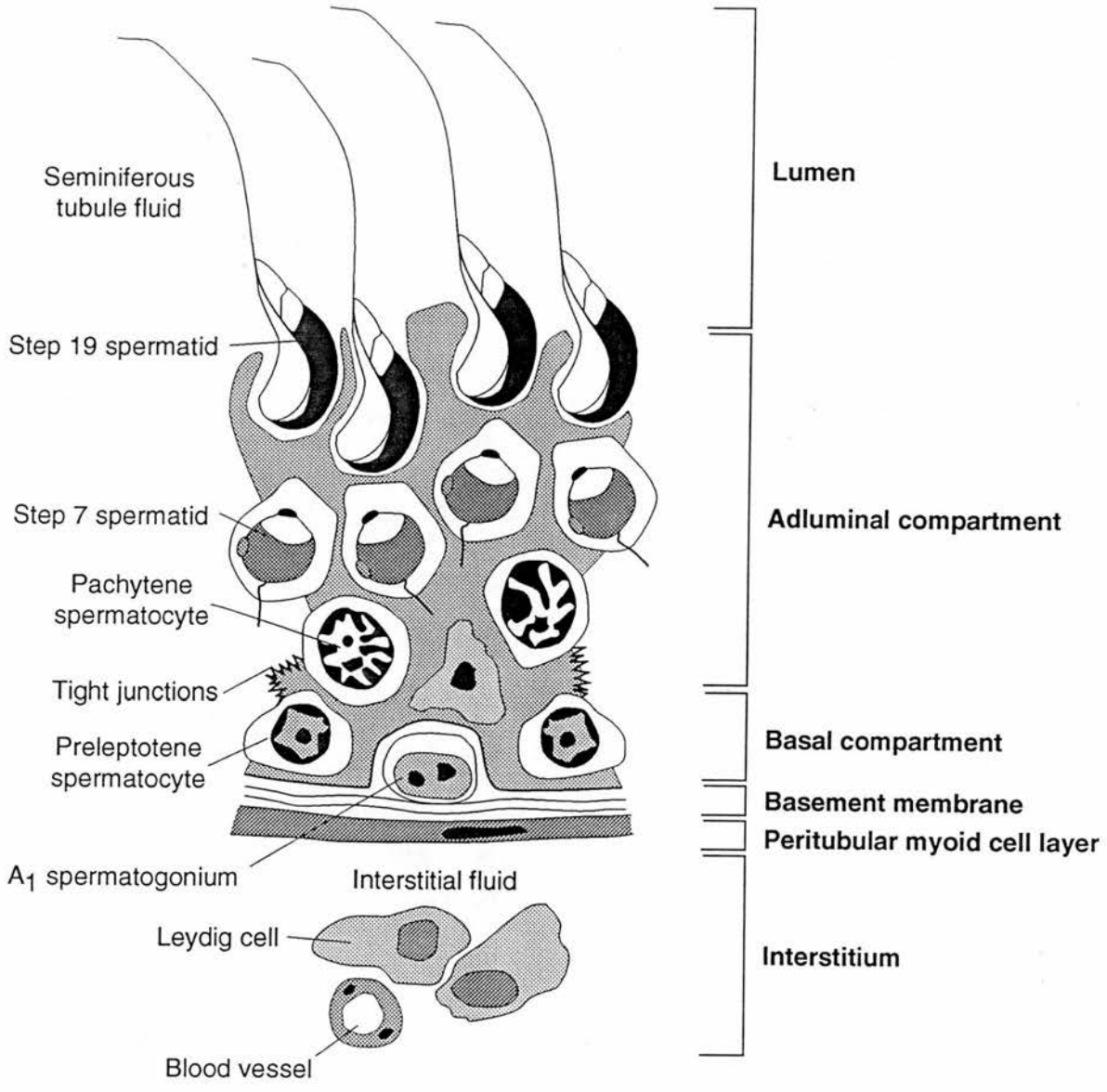


Figure 2. A schematic illustration of a Sertoli cell and its associated germ cells at stage VII of the spermatogenic cycle. It depicts the division of the seminiferous epithelium into basal and adluminal compartments by the inter-Sertoli cell tight junctions. *Adapted from Sharpe, 1994b.*

2.2.1. Seminiferous Tubules

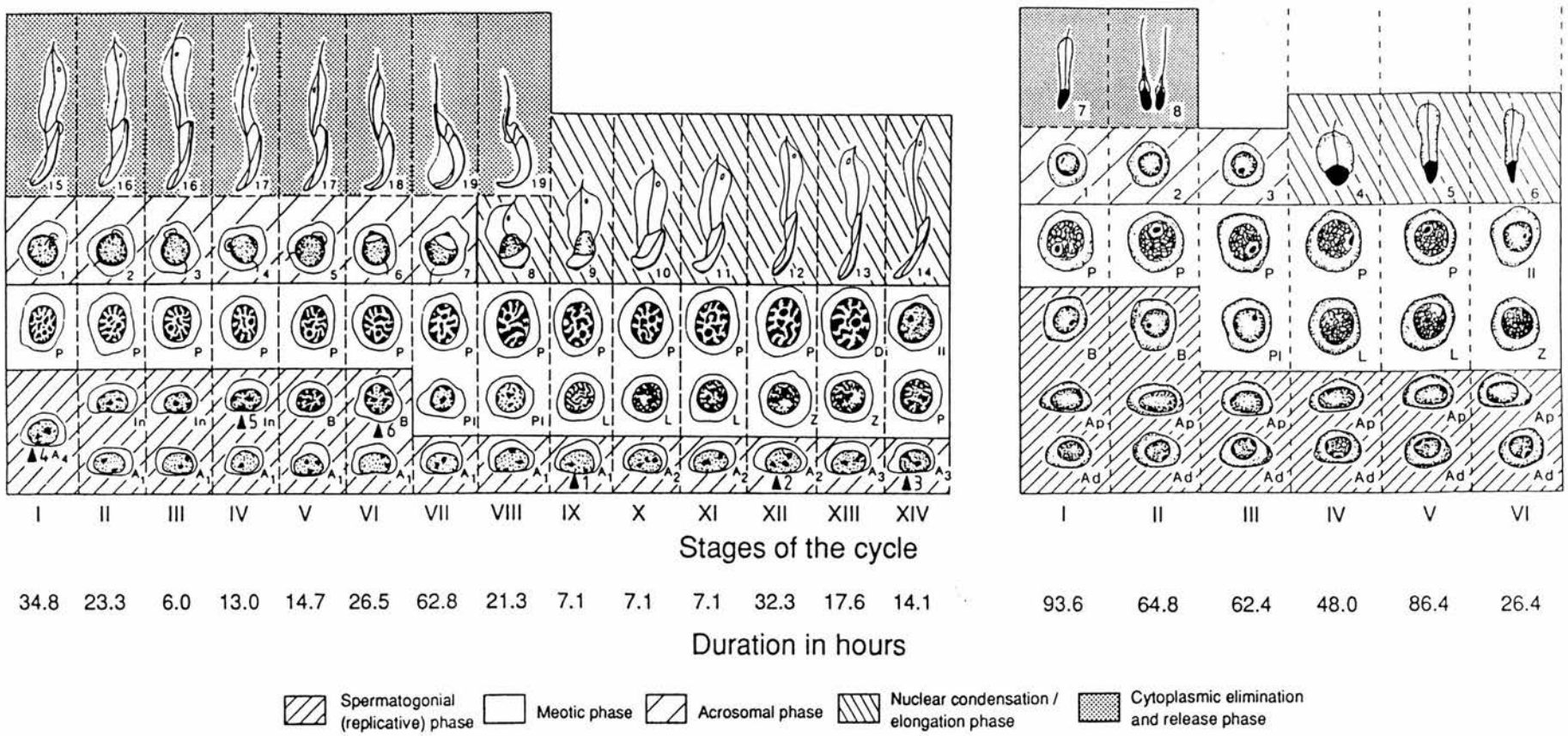
2.2.1.1 Spermatogenesis

Spermatogenesis is the process whereby type A spermatogonial stem cells divide and differentiate within the seminiferous epithelium to produce morphologically mature spermatozoa (Sharpe, 1994b). The general organisation of spermatogenesis appears to be conserved among mammals (for references see Sharpe, 1994b), such that it can be divided into stages with each stage having a defined complement of germ cells associated with the Sertoli cells. Each stage lasts a fixed period of time, and the time taken for development to progress through a complete succession of stages is defined as the cycle. In the rat, the spermatogenic cycle is divided into 14 stages based on morphological changes to the spermatids (Leblond & Clermont, 1952); more advanced histological techniques have improved classification of the different stages (Hess, 1990) (Fig. 3). Each germ cell passes through the 14 stages of the spermatogenic cycle 4.5 times, taking about 52 days (Clermont, 1972). The wave of the seminiferous epithelium is the distance between two identical stages along the length of the seminiferous tubule (Perey *et al.*, 1961); this can be visualised by transillumination of freshly isolated seminiferous tubules (Parvinen & Vanha-Perttula, 1972). When the tubules are viewed under a stereo-microscope they show distinct light absorption patterns which are associated with the degree of condensation of the nuclei of the late spermatids and their organisation and position within the epithelium. The study of isolated stages of the spermatogenic cycle became possible when transillumination was coupled with microdissection (Parvinen & Ruokonen, 1982).

The spermatogenic cycle in humans has a more complex arrangement making it less amenable for study than the cycle in the rat. Only six stages have been defined and generally three stages can be observed in a single tubule cross-section (Clermont, 1963) (Fig. 3). This is because the stages are organised in a helical arrangement rather than segmentally as seen in many other species (Schulze & Rehder, 1984; Schulze *et al.*, 1986). Investigation of the arrangement of stages along the length of tubules and number of atypical cell types within a stage in men with low, medium or high daily sperm production rates has revealed that the pattern of organisation of the stages is related to the efficiency of spermatogenesis (Chaturvedi & Johnson, 1993; Sharpe, 1994b). A germ cell passes through the spermatogenic cycle 5.5 times and spermatogenesis has a duration of approximately 74 days in man (Heller & Clermont, 1964).

Spermatogenesis can be divided into essentially three phases; proliferation, meiosis and spermiogenesis (Fig. 3). During the first phase, an initial spermatogonial stem cell division generates two types of cells, one enters spermatogenesis and the other remains a stem cell. In the rat, the type Ao stem cell spermatogonia then undergo six

Figure 3. The spermatogenic cycle in the rat and man. These figures represent the fixed complement of germ cells associated with Sertoli cells at the different stages of the spermatogenic cycle, there are 14 stages in the rat but only 6 in man. Each stage lasts for a fixed period of time which is shown below each stage number. The different shaded regions on the figures represent the various phases of spermatogenesis. A_1 to A_4, In and B represent the A, intermediate and B type spermatogonia respectively. In the human spermatogenic cycle there are only two types of spermatogonia A dark (Ad) and A pale (Ap). During the spermatogonial phase, sequential mitoses occur which are marked by arrowheads. The B-spermatogonia enter the meiotic phase as preleptotene spermatocytes (Pl) and then develop through leptotene (L), zygotene (Z) and pachytene (P) primary spermatocytes stages before the final meiotic division (II, secondary spermatocyte), during which each spermatocyte gives rise to four haploid round (step 1) spermatids. In the rat, steps 1-19 spermatids or steps 1-8 spermatids in the human, go through the process of spermiogenesis. This can be subdivided into three phases, the acrosomal phase, the nuclear condensation and elongation phase which is followed by the cytoplasmic elimination and release phase. *Adapted from Sharpe, 1994b.*


I II III IV V VI VII VIII IX X XI XII XIII XIV
Stages of the cycle
34.8 23.3 6.0 13.0 14.7 26.5 62.8 21.3 7.1 7.1 7.1 32.3 17.6 14.1
93.6 64.8 62.4 48.0 86.4 26.4
Duration in hours
Spermatogonial (replicative) phase
Meotic phase
Acrosomal phase
Nuclear condensation / elongation phase
Cytoplasmic elimination and release phase

mitotic divisions at precise times during the spermatogenic cycle to give rise to A_1, A_2, A_3, A_4, intermediate and finally B type spermatogonia (Clermont, 1962) (Fig. 4). During proliferation, the progeny of each division remain connected by cytoplasmic bridges; this is observed in the spermatogonia, spermatocytes and spermatids (Dym & Fawcett, 1971). In man, the stem cell spermatogonia, termed type A dark (Ad) because of their appearance, undergo an initial division giving rise to an Ad and an A pale (Ap) spermatogonium and the Ap type undergoes a further mitotic division to form type B spermatogonia (Clermont, 1972; Paniagua *et al.*, 1987) (Fig. 4). The reduced number of spermatogonial divisions in humans compared to rats may be another reason for the reduced efficiency of spermatogenesis found in man (Sharpe, 1994b). The type B spermatogonia divide further to form preleptotene spermatocytes which enter a lengthy meiotic phase. The preleptotene spermatocytes replicate their DNA from the end of stage VII through to VIII after which meiosis is initiated (Parvinen *et al.*, 1991). The primary spermatocytes are subdivided according to the stage of meiosis consisting of preleptotene, leptotene, zygotene, pachytene and diplotene spermatocytes consecutively. Genetic recombination occurs in the early pachytene stage. The mid to late pachytene stages are responsible for abundant RNA synthesis, especially the production of long-lived messages necessary for spermiogenesis (Monesi *et al.*, 1978; Soderstrom & Parvinen, 1976). RNA synthesis gradually decreases towards the end of the pachytene stage and then resumes in secondary spermatocytes and continues at a low rate in round spermatids (Monesi *et al.*, 1978). At stage XIV, the spermatocytes undergo two meiotic divisions resulting in the formation of the haploid spermatid.

Spermiogenesis describes the structural re-modelling of the haploid spermatid to form a spermatozoon, and can be divided into three phases. Firstly, the acrosome develops during stages I-VII which continues to change in shape and position throughout the rest of the spermatogenic cycle (Leblond & Clermont, 1952). RNA synthesis ceases in the step 8 spermatids as nuclear condensation and elongation begins (Monesi *et al.*, 1978). These alterations are achieved by changes in nuclear DNA binding proteins. The histones present in early spermatids (steps 1-8) are first replaced by transition proteins during steps 13-15 and then by protamines (Mali *et al.*, 1989; Hecht, 1990). This brings about a change in DNA packaging from nucleosomal to linear arrays (Ward & Coffey, 1991). Concurrent with the nuclear changes is the development of the flagellum and its associated structures. The final phase of development is the elimination of cytoplasm. This is achieved by a reduction in cytoplasmic volume due to the loss of water from the cell by membrane pumps (Sprando & Russell, 1987), and the formation of tubulobulbar complexes between the Sertoli cells and the head region of late spermatids (Russell, 1979). At release, also known as spermiation, the mature spermatid becomes detached from the Sertoli cell and is released into the lumen; the eliminated cytoplasm is now

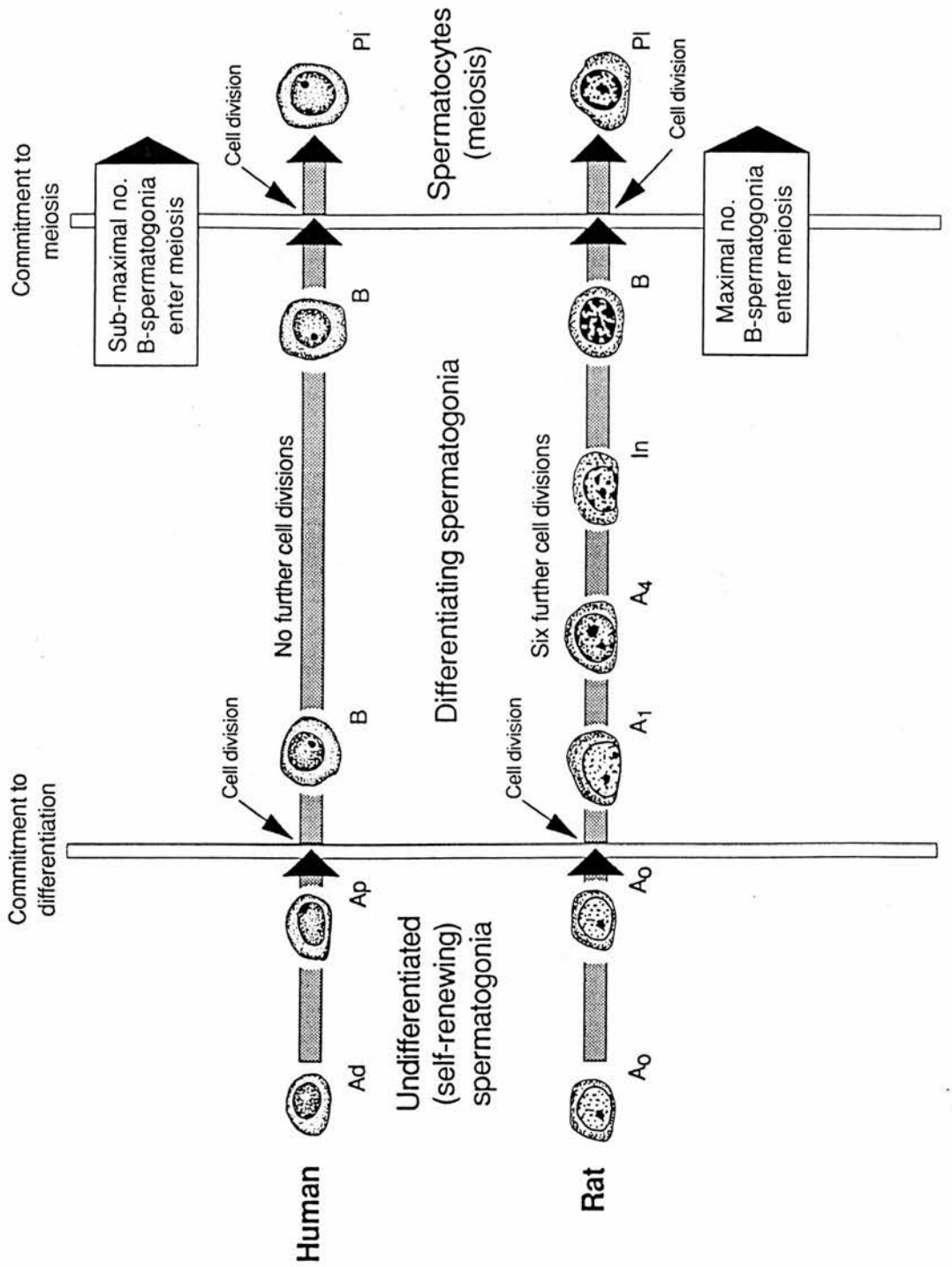


Figure 4. Spermatogonial divisions in the rat and human. The reduced number of divisions in humans in comparison to rats means that fewer numbers of B-spermatogonia enter meiosis. *Adapted from Sharpe, 1994b.*

termed a residual body and is endocytosed by the Sertoli cell (Russell, 1993).

The rat is the most widely studied model of spermatogenesis and is therefore the best understood. The main advantage to studying the rat testis is that the seminiferous tubules can be classified into the 14 stages of spermatogenesis both *in vivo* and *in vitro*. This allows investigation of how the different stages are regulated. *In situ* hybridisation and immunocytochemistry can be used to localise mRNA and protein expression, respectively, on testis sections which shows whether expression is stage regulated or not. This is important for determining whether particular gene products might have a specific function in spermatogenesis. Similar studies can be performed *in vitro* using stage dissected seminiferous tubules to investigate mRNA expression and protein secretion as well as their regulation by other factors. These type of approaches together with detailed morphological analysis of the seminiferous epithelium during the spermatogenic cycle have demonstrated that both the morphology and function of the Sertoli cell varies through the cycle reflecting the changing germ cell composition of the seminiferous epithelium (see sections 2.2.1.3. and 2.2.1.4.). Hormonal control of spermatogenesis is also stage dependent. FSH and testosterone have been shown to act during specific stages of the spermatogenic cycle (see section 2.3). The early stages of spermatogenesis are thought to be predominantly under the control of FSH. Receptor numbers and FSH binding is maximal at stage I, whilst FSH stimulated cAMP production occurs during stages I-V (Parvinen, 1993). In contrast, testosterone preferentially acts at stages VII and VIII. Following androgen withdrawal, germ cell degeneration is first observed at these stages (Sharpe, 1994b). Immunocytochemistry has shown that the expression of the androgen receptor protein is maximal at stages VII-VIII (Bremner *et al.*, 1994; Vornberger *et al.*, 1994). Protein secretion by staged seminiferous tubules is increased at stages VII-VIII in comparison to the other stages and this increase has been shown to be androgen dependent (Sharpe *et al.*, 1992). The roles of FSH and testosterone in the maintenance of adult spermatogenesis still remains to be elucidated. Another example of a protein which acts at stages VII-VIII is plasminogen activator which is secreted by Sertoli cells (Lacroix *et al.*, 1981). This enzyme is thought to be involved in the release of spermatids from the seminiferous epithelium and also the movement of primary spermatocytes from the basal compartment, through the inter-Sertoli cell tight junctions into the adluminal compartment. Both of these events occur at stage VIII. Another advantage of using the rat testis to study spermatogenesis is that several methods of disrupting this process are available, such as the toxicants ethane dimethane sulphonate (EDS) and methoxyacetic acid(MAA). This approach to the study of spermatogenesis is discussed in detail in section 2.6. Determining when something is expressed or where a factor acts during the spermatogenic cycle helps to give some indication of its possible function, but because of the complexity of spermatogenesis this is not an easy task.

2.2.1.2. The blood-testis barrier

The existence of a blood-testis barrier has been demonstrated both morphologically and physiologically. The penetration of dyes (Kormano, 1967; 1968) and electron-opaque markers (Dym & Fawcett, 1970) have shown that the most effective component of the barrier is formed by tight junctions between adjacent Sertoli cells. To a lesser extent the peritubular myoid cells act as a barrier but there are open junctions between some of the myoid cells allowing penetration of lanthanum as far as the inter-Sertoli cell tight junctions (Dym & Fawcett, 1970). The endothelial linings of the blood vessels and the lymphatics are also thought to provide a partial barrier (Ploen & Setchell, 1992; Holash *et al.*, 1993). Physiological observations based on comparison of the fluids inside the seminiferous tubules and rete testis with testicular interstitial fluid and blood plasma have shown major differences in composition (reviewed by Setchell *et al.*, 1994). Studies of the ability of various marker substances injected into the circulation to appear in the seminiferous tubule and rete testis fluid has demonstrated that the barrier allows rapid entry of some substances but it excludes others indicating that it is selectively permeable (Setchell, 1980; Setchell *et al.*, 1994).

The blood-testis barrier develops at the initiation of puberty, between days 15 to 18 of postnatal life in the rat. This has been shown morphologically by penetration with dyes (Kormano, 1967), lanthanum (Vitale *et al.*, 1973) and hypertonic fixatives (Russell *et al.*, 1989). In contrast, physiological studies indicate that barrier formation is more gradual since a barrier to the penetration of water-soluble chromium-EDTA was not complete by day 25 (Setchell *et al.*, 1988). As the formation of inter-Sertoli cell tight junctions coincides with onset of puberty, it has been suggested that it may be controlled by hormones and/or germ cells. Both inhibition of gonadotrophin release (Vitale *et al.*, 1973) and the depletion of germ cells by *in utero* irradiation (Tindall *et al.*, 1975) were found to only delay rather than prevent formation, suggesting that the regulation of occluding junction formation between adjacent Sertoli cells is more complex.

The presence of the barrier compartmentalises the seminiferous epithelium such that the meiotic cells are sequestered adluminally in a unique environment (Setchell, 1980; Setchell *et al.*, 1994). This environment is produced by the regulation of movement of substances into and out of the tubule and Sertoli cell secretions. The Sertoli cell junctions allow a concentration gradient to operate across the barrier as well as maintaining a fluid filled lumen necessary for the transport of sperm to the epididymis. The barrier is also thought to sequester germ cell antigens from immunoglobulins or lymphocytes and thus prevent an immune response (Setchell, 1980). It has now been demonstrated that autoantigenic germ cells exist outside the barrier (Yule *et al.*, 1988), which suggests that the barrier is not the only mechanism which prevents an autoimmune reaction and there must be other immunoregulatory mechanisms operating in the interstitium (Mahi-Brown

et al., 1988). This is supported by the fact that the testis is an immunologically privileged site as shown by the ability of tissue grafts to survive when transplanted into the interstitial region of the rat testis (Maddocks & Setchell, 1990).

2.2.1.3. Structure and function of the Sertoli cell

Sertoli cells are the only somatic component of the seminiferous epithelium and their main function is the co-ordination of spermatogenesis. The Sertoli cells provide both physical and nutritive support for the differentiating germ cells. The presence of Sertoli cell tight junctions sequesters the meiotic germ cells within the adluminal compartment; so that they are totally dependent on factors produced or transported by the Sertoli cells. The secretory aspect of Sertoli cell function is discussed in more detail in section 2.2.1.4. The Sertoli cell also plays a crucial role in spermiation and their endocytic activity is responsible for the degradation of residual bodies (Russell, 1993; Morales & Clermont, 1993). The mechanisms by which the Sertoli cells regulate the complex process of spermatogenesis remain to be elucidated.

A Sertoli cell possesses an extremely complex morphology with a large irregular shaped nucleus at its base and fine cytoplasmic processes which extend towards the lumen enveloping the germ cells (de Kretser & Kerr, 1994; Russell, 1993). The cytoplasm contains numerous mitochondria and cytoskeletal elements which is consistent with the Sertoli cell's ability to change shape to accommodate the mobility and morphology of the developing germ cells. Smooth endoplasmic reticulum predominates over the rough type; this together with the lack of secretory granules in association with the Golgi or vesicles indicates that the Sertoli cell has little of the organelles usually associated with transport and secretion of proteins. Other cytoplasmic components include membrane bound granules, ribosomes, glycogen, lipid, lysosomes and vacuoles.

During the cycle of the seminiferous epithelium corresponding changes in Sertoli cell ultrastructure have been demonstrated (Morales & Clermont, 1993; Ye *et al.*, 1993). The number of Sertoli cells per unit length of the tubules remains constant (Wing & Christensen, 1982). Some studies have described marked cyclical changes in the volume of Sertoli cell cytoplasm such that their absolute volume is smallest during stages VII-VIII and largest during stages XII-XIV (Kerr, 1988a; Morales & Clermont, 1993). Re-investigation using different morphometric analysis suggested there was an increase in volume density from stage IX although no significant cyclical variation could be demonstrated (Ye *et al.*, 1993). Rough and smooth endoplasmic reticulum show opposite changes in volume and surface area at different points of the cycle such that rough endoplasmic reticulum is maximal at stage VII and smooth endoplasmic reticulum at stages XIII-XIV (Kerr, 1988b). The cyclical changes of rough endoplasmic reticulum but not smooth endoplasmic reticulum have been confirmed in another study (Ye *et al.*,

1993). The Golgi was found to change its location within the cytoplasm from the basal region to the apical cytoplasm at stages VII-VIII (Ueno & Mori, 1990). Further investigation found that this change failed to occur in the absence of the step 19 spermatids (Ueno *et al.*, 1991). This localisation of the Golgi to the apical region of the Sertoli cell also coincides with an androgen dependent increase in protein secretion by isolated staged seminiferous tubules in culture (Sharpe *et al.*, 1992). Lysosomes also show cyclical changes in location within the cytoplasm and number (Morales *et al.*, 1986). Following spermiation at stage VIII, the residual bodies are endocytosed by the Sertoli cell resulting in the formation of phagosomes. At stage IX, the phagosomes migrate from the apex of the cell towards the base. Simultaneously, the lysosomes, which have been localised next to the basement membrane, move upwards to the centre of the cell where they fuse with the phagosomes. It has also been shown that the volume of lysosomes increases during stages X-XIV (Morales *et al.*, 1986; Kerr, 1988b). Estimation of mitochondrial volume during the cycle suggests that volume is maximal at stages XIII-XIV, though cyclic changes are not as marked as seen for other organelles (Kerr, 1988b; Morales & Clermont, 1993). Several studies have demonstrated that lipid droplet content within Sertoli cells is maximal at stages IX-II (Kerr *et al.*, 1984, Ueno & Mori, 1990; Morales & Clermont, 1993, Ye *et al.*, 1993). It is thought that the accumulation of lipid droplets is a result of digestion of the residual bodies. These observations of cyclic ultrastructural changes in the Sertoli cell reflect changes in function necessary for the regulation of the spermatogenic cycle.

2.2.1.4. Secretory function of the Sertoli cell

This aspect of Sertoli cell function is responsible for the production of seminiferous tubule fluid as well as the secretion of proteins, growth factors and energy metabolites which provide the necessary environment for spermatogenesis. Sertoli cells are also able to produce oestradiol by the aromatisation of testosterone secreted from the Leydig cells (Dorrington & Khan, 1993). The aromatase enzyme is only expressed during fetal/neonatal life whilst the Sertoli cells are multiplying. The physiological role of oestradiol within the testis during this period has not been established but it maybe involved in the regulation of Sertoli cell proliferation.

Production of seminiferous tubule fluid (STF)

This fluid is thought to have several important functions in the testis. It is involved in the nutrition of the germ cells and spermatozoa, the transport of substances to the germ cells and lumen, it enables cell-cell communication within the seminiferous epithelium and is responsible for the transport of spermatozoa to the epididymis. STF has a distinctive composition in comparison to other fluids (Setchell *et al.*, 1994). The potassium

concentration is much higher than in blood whereas the levels of sodium, chloride and calcium are present in similar amounts to that found in blood (Jenkins *et al.*, 1980). The total protein concentration is much lower than in interstitial fluid and plasma. Carbohydrates, amino acids and steroids also differ from plasma reflecting the selective permeability of the blood-testis barrier.

The Sertoli cell was suggested to be responsible for STF production when it was found that rats in which germ cells had been depleted by treatment with busulphan *in utero*, were still able to produce STF (Setchell, 1969). The mechanism of STF production is not clear. One theory is based on the presence of a Na-K translocating ATPase pump on the basal membrane of the Sertoli cell (Waites & Gladwell, 1982; Hinton & Setchell, 1993). Sodium ions would diffuse into the cell and then be pumped back into the interstitium in exchange for potassium ions which would then diffuse into the lumen. Since carbonic anhydrase inhibition decreases secretion of STF bicarbonate ions are also thought to be essential for secretion. The movement of ions would establish an osmotic gradient enabling water to be drawn into the tubules. The actual physiological situation is likely to be more complex since the immunolocalisation of sodium-potassium ATPase indicated that this enzyme is distributed on the apical and lateral membranes of the Sertoli cell, and may also be associated with the inter-Sertoli cell junctions (Byers & Graham, 1990). Recent evidence demonstrates that microtubules are also involved in the secretion of seminiferous tubule fluid (Richburg *et al.*, 1994). Secretion was quantitated by the rate of transport of micro-injected oil droplets in the lumen of isolated rat seminiferous tubules. The rate of secretion was significantly decreased when animals were treated *in vivo* with colchicine and also when protein secretion was disrupted using brefeldin A, which affects intracellular membrane trafficking.

The onset of STF production was proposed to begin around the time of lumen and blood-testis barrier formation (Vitale *et al.*, 1973). The use of efferent duct ligation established that this was correct as fluid production could not be measured before day 15 postnatally but increased rapidly after day 20 (Jégou *et al.*, 1982). A similar result was gained in another study by Russell *et al.* (1989). The technique of efferent duct ligation was suggested to be a more accurate reflection of seminiferous tubule flow rather than production; fluid flow began around day 20 but fluid production may begin earlier. The regulation of STF production is discussed in section 2.4.1.

Secreted proteins

The analysis of both cell and seminiferous tubule culture medium using two-dimensional electrophoresis has allowed identification and characterisation of some of the most abundant Sertoli cell secreted proteins (Kissinger *et al.*, 1982; Wright *et al.*, 1983). Transferrin, ceruloplasmin, sulphated glycoprotein-1(SGP-1) and sulphated

glycoprotein-2 (SGP-2) comprise 80% of total protein secreted by Sertoli cells, and SGP-2 alone constitutes 50% (Griswold, 1988). Sertoli cell secreted proteins show several characteristics. Many are secreted in a distinct pattern which changes according to the stage of the seminiferous epithelium (Parvinen, 1993). In addition, several proteins are secreted bidirectionally such that they are secreted via the apex of the Sertoli cell into seminiferous tubule fluid and also via the base into the interstitial fluid (Sharpe, 1988). Sertoli cells are also capable of secreting proteins similar to those found in serum (Wright *et al.*, 1981). These characteristics will be discussed further in the context of some of the secreted proteins described below; where possible their putative roles in the support of spermatogenesis will be discussed.

Androgen binding protein (ABP)

Androgen binding protein (ABP) has high affinity binding sites for both dihydrotestosterone and testosterone, it is secreted by Sertoli cells into seminiferous tubule fluid and transported to the epididymis (French & Ritzén, 1973). Immunocytochemical localisation of ABP indicated that it was endocytosed in the efferent ducts and the proximal region of the caput epididymis (Attramadal *et al.*, 1981; Pelliniemi *et al.*, 1981); this has since been shown to be receptor mediated (Guéant *et al.*, 1991). These results suggested that ABP was involved in the transport of testosterone to the epididymis. ABP is a glycoprotein with a native molecular weight of 85-90 kDa, it has two identical subunits with molecular weights of 47 and 41 kDa whose heterogeneity is due to differences in post-translational processing (Joseph *et al.*, 1987). ABP and human sex hormone-binding globulin, which is produced by the liver, are encoded by the same gene (Hammond *et al.*, 1989) and show 68% homology at the amino acid level (Joseph *et al.*, 1987).

ABP secretion is bidirectional. It has been calculated that in the adult rat 67% of ABP secretion is apical and 33% basal (Bardin *et al.*, 1994). This pattern of secretion is developmentally regulated. ABP can be detected in plasma both immunologically and by steroid binding from days 15-20 postnatally, levels are maximal around days 20-25 and then decline rapidly until day 40 (Danzo & Eller, 1985; Nazian, 1986; Sharpe & Bartlett, 1987). In contrast, intratesticular and epididymal levels of ABP increase into adulthood, although ABP cannot be detected in the epididymis until around day 20 (Tindall *et al.*, 1975; Danzo & Eller, 1985). Experimental disruption of spermatogenesis affects the pattern of bidirectional secretion (Sharpe, 1988). Busulphan, ethane dimethane sulphonate (EDS), heat and cryptorchidism all increase secretion of ABP into the blood (Morris *et al.*, 1987; Morris *et al.*, 1988; Sharpe & Bartlett, 1987). From these and other studies, it has been suggested that secretory changes are regulated by the germ cells (Pineau *et al.*, 1989; Sharpe, 1993).

ABP secretion is also stage dependent. Ritzén *et al.* (1982) showed that secretion by isolated seminiferous tubules was maximal at stage VIII and minimal at stages II-V; this was confirmed when a similar pattern of expression of ABP mRNA was described (Linder *et al.*, 1991). This supports other evidence that stages VII-VIII of the rat spermatogenic cycle are the most responsive to testosterone (Sharpe, 1994b). The precise role of ABP is unclear but it is thought that it might be involved in the regulation of testosterone action on spermatogenesis. A high affinity receptor for ABP has been identified on rat germ cells (Felden *et al.*, 1992), and it has been demonstrated that monkey germ cells are capable of endocytosing human sex steroid binding protein (Gérard *et al.*, 1991). Recently, rat germ cells have been shown to have the ability to internalise ABP by receptor mediated endocytosis from the fluid phase and from Sertoli cell cytoplasm (Gérard *et al.*, 1994). This suggests that steroid binding proteins may act as transporters during spermatogenesis. ABP is also thought to play a role in the maintenance of androgen levels within the testis and epididymis.

Transferrin

Transferrin is a 75 kDa glycoprotein involved in the transport of iron, which is an essential nutrient for cellular development. It is synthesised by the liver (serum transferrin) and also by the Sertoli cells (testicular transferrin). Testicular transferrin was first identified as a major secretory product of Sertoli cells in culture based on its iron binding ability, electrophoretic properties and its cross-reaction with an antibody raised against serum transferrin (Skinner & Griswold, 1980). Subsequent characterisation of testicular transferrin showed that the peptide sequences for serum and testicular transferrin were identical but the proteins were associated with different amounts of carbohydrate (Skinner *et al.*, 1984). Both transferrin mRNA expression and secretion are stage dependent. During stages I-VIII the level of mRNA expression is relatively constant, it decreases at stage IX and then increases until levels are maximal at stages XIII-XIV (Morales *et al.*, 1987a). A similar pattern of protein secretion was observed in isolated staged tubules (Mather *et al.*, 1983). Transferrin is produced at all stages of the cycle although secretion is highest during the time at which meiotic divisions occur, but this may be a reflection of the increased numbers of germ cells which are present in the seminiferous epithelium at this time.

Analysis of the regulation of expression of the mRNA for transferrin has been complicated by the discovery of hemiferrin (Stallard *et al.*, 1991). Northern blot analysis using a probe against transferrin was found to hybridise to two bands in total testis RNA, the normal 2.4kb transferrin mRNA as well as a more abundant 0.9kb transcript. This transcript was cloned and sequenced, and found to show homology to the C-terminal 216 amino acids of transferrin. This transcript was shown to be expressed by round

spermatids. Sequence analysis suggests that hemiferrin may be able to bind iron but its involvement in iron transport is unknown.

A model was proposed to explain the function of Transferrin in the testis (Huggenvik *et al.*, 1984; Sylvester & Griswold, 1994). Initially serum diferric transferrin binds to receptors on the basal surface of the Sertoli cell which is followed by receptor mediated endocytosis. Acidification within a subcellular compartment releases the bound iron which can now bind to testicular transferrin allowing its transport to the developing germ cells, or the iron can be stored temporarily in ferritin present in Sertoli cells. Following this the transferrin-receptor complex is returned to the plasma membrane and serum transferrin is released back into the interstitium. Several pieces of experimental evidence have substantiated this hypothesis. Receptor mediated endocytosis of transferrin by the basal membrane of the Sertoli cell has been demonstrated *in vitro* (Morales & Clermont, 1986) and *in vivo* (Morales *et al.*, 1987b). In the latter study, transferrin bound with radiolabelled iron was shown to be associated with pachytene spermatocytes and round spermatids. This is in agreement with previous findings showing the presence of transferrin receptors on these germ cells (Holmes *et al.*, 1983; Sylvester & Griswold, 1984; Brown, 1985). The uptake of iron bound transferrin by germ cells *in vitro* (Toebosch *et al.*, 1987), and the internalisation of transferrin by receptor mediated endocytosis in germ cells has also been demonstrated (Petrie & Morales, 1992). Toebosch *et al.* (1987) also showed that most of the iron that enters Sertoli cells and round spermatids is incorporated into ferritin. Once the iron has been released inside the germ cells the transferrin is thought to be recycled into STF.

Ceruloplasmin

Ceruloplasmin is 130 kDa protein which is the major copper transport protein in serum and is also thought to be involved in the oxidation of iron during its binding to transferrin (Sylvester, 1993). This protein is also secreted by Sertoli cells in culture as shown using immunoprecipitation with an antibody raised against serum ceruloplasmin (Skinner & Griswold, 1983). This protein has not been studied extensively so little is known about its function in the testis.

Sulphated glycoprotein-1 (SGP-1)

SGP-1 is a heavily glycosylated protein of 70 kDa. Both immunolocalisation and *in situ* hybridisation on testis sections indicated that SGP-1 was a product of the Sertoli cells (Collard *et al.*, 1988). Further investigation of the distribution showed that SGP-1 staining in Sertoli cells was consistent in all 14 stages of the cycle and at the ultrastructural level the antibody reacted with secondary lysosomes and to residual bodies which had fused with lysosomes (Sylvester *et al.*, 1989). SGP-1 was also detected in

STF, rete testis fluid, the epididymis and at much lower levels in serum and interstitial fluid. Immunocytochemical evidence suggests that SGP-1 associated with sperm tails is endocytosed in the rete testis and efferent ducts, although SGP-1 is synthesised by the epididymis but is localised to the lysosomes and does not seem to be secreted (Hermo *et al.*, 1992). Analysis of the cDNA sequence showed homology to a human sphingolipid activator protein (prosaposin) (Collard *et al.*, 1988). Prosaposin is a precursor and partial proteolysis gives rise to four small glycoproteins known as saposins which are involved in the lysosomal hydrolysis of sphingolipids (O'Brien & Kishimoto, 1991). The presence of SGP-1 in lysosomes fused with residual bodies suggests that it might be involved in the degradation of glycolipids in residual bodies (Sylvester *et al.*, 1989). Igdoura *et al.* (1993) used Western blot analysis to show the presence of saposins in the lysosomes of the nonciliated cells of the efferent ducts. Recently, it has been shown that SGP-1 is immunolocalised to Sertoli cell lysosomes and to phagosomes at stages IX and X of the cycle which are responsible for the degradation of residual bodies (Igdoura & Morales, 1995). The same authors also demonstrated that the Sertoli cell lysosomes contained a 65 kDa form of SGP-1 as well as the 15 kDa saposins. This led them to propose that lysosomal Sertoli cell SGP-1 is delivered to the phagocytosed residual bodies where it is responsible for the hydrolysis of membrane glycolipids. The Sertoli cell secreted protein named testibumin, based on cross-reaction of an antibody raised to the protein with albumin (Cheng & Bardin, 1986), has now been reported to be identical to SGP-1 (Mathur *et al.*, 1994). A function for secreted SGP-1 in the reproductive tract is unclear; its presence in STF and RTF would suggest it is involved in the modification of glycolipids in Sertoli cell and germ cell membranes and also the surface glycolipids of elongate spermatids and spermatozoa (Collard *et al.*, 1988).

Sulphated glycoprotein-2 (SGP-2)

SGP-2 is synthesised as a 73 kDa protein which is cleaved to form disulphide-linked subunits of 47 and 34 kDa (Collard & Griswold, 1987). It has been immunolocalised to Sertoli cells, the epididymis, mature spermatids and spermatozoa (Sylvester *et al.*, 1984). Western blot analysis demonstrated that SGP-2 associated with epididymal sperm and in epididymal fluid is composed of monomers of 40 and 29 kDa. The size difference between testicular and epididymal forms of SGP-2 was shown to be due to tissue specific glycosylation (Sylvester *et al.*, 1991). This change was explored further and it was found that testicular SGP-2 is removed from the sperm in the rete testis and efferent ducts where it is endocytosed and is then replaced in the epididymis (Hermo *et al.*, 1991).

Secretion of SGP-2 is stage dependent, with levels maximal at stages VII-VIII in the culture medium of isolated tubules (Kangasniemi *et al.*, 1992), in agreement with analysis of mRNA expression using *in situ* hybridisation (Morales *et al.*, 1987a).

Although SGP-2 is secreted in a cyclical manner it does not seem to be regulated by germ cells or hormones. Expression of SGP-2 mRNA in adult rats was unaffected by hypophysectomy or germ cell depletion following testosterone withdrawal (Roberts *et al.*, 1991; 1992). It has also been shown *in vitro* that the secretion of SGP-2 by isolated staged seminiferous tubules is not altered by EDS treatment or germ cell depletion using MAA (Sharpe *et al.*, 1992; 1993; McKinnell & Sharpe, 1995). This indicates that SGP-2 is constitutively secreted by Sertoli cells.

SGP-2 has been identified in many other tissues and species; various functions have been assigned based on its location or the method of its characterisation (Jenne & Tschopp, 1992; Fritz & Murphy, 1993). Based on sequence analysis, SGP-2 shows homology to human apolipoprotein A-1 and apolipoprotein J and has been found associated with high density lipoproteins suggesting, it may be involved in lipid transport (Collard & Griswold, 1987; de Silva *et al.*, 1990; Jenne & Tschopp, 1992). Other investigators have demonstrated that SGP-2 is identical to clusterin, a protein isolated from ram rete testis fluid which induces aggregation of cells (Fritz *et al.*, 1983; Tsuruta *et al.*, 1990), and human serum protein 40,40 which is a soluble terminal component of the complement cascade which inhibits complement cytolysis (Kirszbaum *et al.*, 1989; Jenne & Tschopp, 1989). The presence of high levels of SGP-2 in the brain suggests it may also play a role in neuronal function (Fritz & Murphy, 1993). SGP-2 has been immunolocalised in various regions of the male reproductive tract, including the testis, epididymis, ventral prostate and seminal vesicles (Sylvester *et al.*, 1984; Sensibar *et al.*, 1993). Expression of SGP-2 mRNA in the prostate was first identified as a testosterone-repressed prostate gene (TRPM-2), the expression of which was induced by cells undergoing programmed death, suggesting that SGP-2 might be involved in apoptosis (Buttyan *et al.*, 1989). The protein has also been characterised in seminal plasma (O'Bryan *et al.*, 1990). Biochemical analysis of the forms of SGP-2 from the testis, epididymis, ventral prostate and seminal vesicles have demonstrated that there is a high degree of variation in the carbohydrate structure between the individual forms (Sensibar *et al.*, 1993). This suggests that the function of SGP-2 may be dependent on the site of its production and its molecular structure.

The function of SGP-2 in the male reproductive tract is unclear. Both testicular and epididymal SGP-2 have the ability to inhibit complement activity, although whether SGP-2 does protect spermatozoa from complement attack remains to be determined (Law & Griswold, 1994). Investigation of the association of SGP-2 with epididymal sperm membranes has shown that the majority of SGP-2 is loosely associated where as a smaller amount can only be removed when the lipid bilayer is disrupted (Law & Griswold, 1994). Analysis of the amino acid sequence of SGP-2 has predicted four regions capable of forming amphipathic helices and several heparin binding sites which

would provide potential lipid and protein binding domains (de Silva *et al.*, 1990; Tsuruta *et al.*, 1990). The significance of the association of SGP-2 with spermatid and sperm membranes is unknown, but it may be important for membrane remodelling during development and maturation, membrane stabilisation, protection or even repair (Sylvester *et al.*, 1991; Fritz & Murphy, 1993; Law & Griswold, 1994). Law and Griswold (1994) also investigated the ability of SGP-2 present in seminiferous tubule fluid, epididymal fluid and blood plasma to form complexes with proteins and lipids. They found that a significant amount of the SGP-2 did aggregate either to itself or to other proteins and lipids but it was not associated with lipoprotein or apolipoprotein A1. This suggests that the fluid form of SGP-2 may be aggregated (Law & Griswold, 1994). Characterisation of SGP-2 in human seminal plasma has led to suggestions that it may be a marker of abnormal spermatozoa and that it may also be involved in the protection of sperm from complement attack within the female genital tract (O'Bryan *et al.*, 1990; 1994a,b).

Cyclic protein-2 (CP-2)

CP-2 is another major secretory product of Sertoli cells and was identified based on its pronounced stage dependent secretory pattern (Wright *et al.*, 1983). Stage dissected seminiferous tubules were cultured with ^{35}S-methionine and the secreted proteins were analysed using two dimensional gel electrophoresis; quantification showed that secretion of CP-2 was 30-fold greater at stage VI in comparison to stages XII-XIV. Synthesis, determined by immunoprecipitation of radiolabelled CP-2 from the cytosol of staged tubules, was found to show the same pattern of cyclicity as secretion (Wright *et al.*, 1989). CP-2 is maximally synthesised and secreted at stages VI-VII. This was supported by immunocytochemical evidence which was also able to show changes in distribution of CP-2 within the Sertoli cell cytoplasm (Zabludoff *et al.*, 1990a). At stage V, CP-2 was localised to the basal portion of the Sertoli cell adjacent to the step 17 spermatids, by stage VI the immunostaining had extended upwards surrounding the step 18 spermatids and at stage VII all of the Sertoli cell cytoplasm was stained. At stage VIII, CP-2 was again localised basally. In the same study, CP-2 could not be detected in the epididymis but was localised in the kidney, brain and posterior pituitary. Synthesis was found to increase during testicular maturation, with the greatest increase occurring between 35-45 days and stage specificity was apparent by 38 days (Zabludoff *et al.*, 1990b). The sequencing of a partial cDNA revealed that CP-2 was the proenzyme form of the cysteine protease cathepsin L (Erickson-Lawrence *et al.*, 1991). Based on the localisation of CP-2 within Sertoli cells and the knowledge that CP-2 was a protease, they proposed that CP-2 could be involved in the disruption of cell-cell adhesive bonds allowing the elongate spermatids to move to the apex of the Sertoli cell in preparation for spermiation. It has recently been shown in adult rats in which specific germ cell types

have been depleted using MAA, that expression of CP-2 mRNA is positively regulated by elongate spermatids and may also be negatively regulated by round spermatids to a lesser degree (Maguire *et al.*, 1993). These results might suggest that the spermatids are capable of regulating their own movement within the seminiferous epithelium by modulating Sertoli cell gene expression of CP-2.

Inhibin

Inhibin is a dimeric glycoprotein which suppresses FSH secretion by the pituitary gland (de Jong, 1988; de Kretser & Robertson, 1989). It is composed of two subunits linked by disulphide bonds, an α-subunit and a smaller β-subunit. There are two types of β-subunit based on sequence differences, βA and βB, giving rise to forms of dimeric inhibin termed A and B. Sequencing of the inhibin subunits has shown significant homology to proteins belonging to the transforming growth factor β gene family. Immunocytochemistry and *in situ* hybridisation suggest that Sertoli cells express the α and βB subunits but not βA (Roberts *et al.*, 1989; Shaha *et al.*, 1989). However, Meunier *et al.* (1988) reported that they could detect the βA subunit in the testis using S-1 nuclease analysis to quantitate mRNA levels. They found that in the adult testis, in comparison to the α-subunit, βA and βB were expressed at 50 and 15 times lower levels, respectively. Purification of inhibin from immature rat Sertoli cell conditioned medium and subsequent analysis demonstrated the presence of an α and βB subunit but not a βA subunit (Grootenhuis *et al.*, 1990).

Inhibin is another Sertoli cell secreted glycoprotein which is secreted bidirectionally and, like ABP, this pattern of secretion is also developmentally regulated (Maddocks & Sharpe, 1990a). Levels of inhibin-α in interstitial fluid and plasma were maximal in immature rats (28 days) but declined with age although this may be a dilution effect rather than a decrease in production of inhibin by the Sertoli cell. In immature rats it was calculated that most of the α-inhibin measured in blood was derived from basal secretion whereas in adults the opposite was found, the majority of α-inhibin was secreted via the apex of the Sertoli cell. This maturational change in the route of secretion is unaffected by the absence of germ cells so it is likely to be an effect of the functional maturation of the blood-testis barrier (Maddocks *et al.*, 1992). In adult rats, most of the apically secreted inhibin is reabsorbed in the rete testis, as shown by an increase in α-inhibin in spermatic venous blood collected after passage through the mediastinal venous plexus which overlies the rete testis (Maddocks & Sharpe, 1989a). Endocytosis of α-inhibin remaining in STF by the proximal parts of the excurrent duct system is thought to occur in the ram (Veeramachaneni *et al.*, 1989).

Bioassays and immunoassays have demonstrated that Sertoli cells in culture are capable of producing inhibin (McLachlan *et al.*, 1988). FSH stimulation of cultured

immature rat Sertoli cells causes a dose dependent increase in inhibin production, which is further stimulated by the addition of adenyl cyclase activators, phosphodiesterase inhibitors and by the addition of dibutyryl cAMP (Bicsak *et al.*, 1987), indicating that FSH stimulates inhibin production by a cAMP dependent mechanism. Several studies have shown that the inhibin subunits are differentially regulated. In immature rat Sertoli cells *in vitro* expression of the mRNA for the α-subunit and immunoreactive inhibin were stimulated by FSH, whereas expression of the βB-subunit was unaffected by FSH but decreased when levels of protein kinase C and intracellular calcium were altered (Toebosch *et al.*, 1989; Klaij *et al.*, 1992). It has also been demonstrated in hypophysectomised immature and adult rats that FSH regulates α- but not βB-subunit mRNA expression (Krummen *et al.*, 1989). These results suggest that the level of βB-subunit determines the production of bioactive inhibin. Immunocytochemical localisation of α–inhibin in the adult rat testis and the secretion of inhibin by isolated staged tubules suggests that it is expressed stage dependently (Merchenthaler *et al.*, 1987; Gonzales *et al*, 1988). This has been confirmed by the analysis of mRNA expression of the α and βB-subunits, such that maximal levels of both subunits are expressed at stages XIII-I but are lowest at stages VII-VIII (Bhasin *et al.*, 1989; Kaipa *et al.*, 1991; Klaij *et al.*, 1994). This distribution follows a similar pattern described for FSH binding and FSH stimulated cyclic AMP production (Parvinen, 1993). It has been demonstrated that the depletion of elongate spermatids from the adult testis using MAA causes a decrease in the secretion of α-inhibin by seminiferous tubules *in vitro* and *in vivo* (Allenby *et al.*, 1991a). In contrast, depletion of pachytene spermatocytes and round spermatids by γ-irradiation was found to cause an increase in α-inhibin mRNA levels (Kaipa *et al.*, 1991).

The endocrine role of inhibin in regulating FSH secretion seems to be more important in the prepubertal rat, since passive immunisation against the α-subunit only caused an increase in FSH levels in immature rats which was lost early in puberty (Culler & Negro-Villar, 1988; Rivier *et al.*, 1988). However, hypophysectomy results in a significant decrease in α-subunit mRNA expression in both immature and adult rats although the decrease is larger in the immature animals; the level of expression was restored by the administration of FSH (Krummen *et al.*, 1989). Moreover, in adult rats specific depletion of elongate spermatids results in reduced secretion of immunoactive inhibin by seminiferous tubules, leading to reduced levels of inhibin in blood and an associated increase in FSH levels (Allenby *et al.*, 1991a). Other evidence suggests that inhibin may have a paracrine role in the testis. The injection of inhibin purified from Sertoli cell conditioned medium was found to decrease the numbers of intermediate and B1 spermatogonia in Chinese hamsters (van Dissel-Emiliani *et al.*, 1989). It has been shown *in vitro* that spermatogonia, spermatocytes and spermatids all have the ability to bind inhibin-A (Woodruff *et al.*, 1992). Krummen *et al.* (1994) have localised specific

binding of inhibin-A *in situ* to rat Leydig cells throughout development and in the adult. The study of the function of inhibin has been complicated by the problems associated with the specific measurement of the various inhibin subunits and the sites of their expression. This is now beginning to be resolved with the production of antisera specific for the bioactive forms of inhibin (Groome & O'Brien, 1993; Groome *et al.*, 1994); hopefully this will help to elucidate the various actions of inhibin.

2.2.1.5. Germ cell secreted proteins

The study of paracrine interactions between Sertoli cells and germ cells has demonstrated that pachytene spermatocytes and round spermatids are capable of secreting proteins which are able to influence Sertoli cell function *in vitro* (Djakiew & Dym, 1988; Le Magueresse & Jégou, 1988; Han *et al.*, 1993; Onoda & Djakiew, 1993; Pineau *et al.*, 1993). Recently, it has been demonstrated that some of the proteins secreted by isolated seminiferous tubules are germ cell in origin, and that their secretion is also androgen regulated (McKinnell & Sharpe, 1995). Currently, there is very little information regarding the identities of germ cell secreted proteins; in contrast the number of gene transcripts shown to be expressed in germ cells is rapidly increasing (Willison & Ashworth, 1987; Erickson, 1990; Wolgemuth & Watrin, 1991; Eddy *et al.*, 1993). One germ cell secreted protein which has been identified is discussed below.

Phosphatidylethanolamine binding protein (PEBP) is a major secretory product of round spermatids. It has a molecular weight of 24 kDa, and was identified by microsequencing (Saunders *et al.*, 1995). *In situ* hybridisation confirmed that this protein was not synthesised by Sertoli cells; the mRNA was first expressed in pachytene spermatocytes at stage VII and was present in all subsequent germ cell stages until step 14 spermatids. The secretory pattern of this protein was determined by culturing staged seminiferous tubules isolated from adult rats in the presence of radiolabelled methionine, secretion of PEBP was found to be maximal at stages I-V and minimal at stages IX-XII. Androgen withdrawal, by the depletion of Leydig cells using EDS, reduced secretion of PEBP by isolated seminiferous tubules but had no effect on mRNA expression. This protein had previously been identified in rat (Grandy *et al.*, 1990) and bovine brains (Schoentgen *et al.*, 1987), rat sperm plasma membranes (Jones & Hall, 1991) as well as in the mouse testis and epididymis (Araki *et al.*, 1992). Western blot analysis indicated that PEBP is a major component of rete testis fluid and cauda epididymal fluid but it is also present in the cytosols of a wide range of tissues including the prostate, liver, kidney, spleen, muscle, mammary gland but not milk or blood serum (Jones & Brown, 1987). Immunolocalisation of PEBP in the mouse showed that in the testis the protein was present in the cytoplasm of elongate spermatids and residual bodies; in the epididymis, PEBP was localised in the epithelium of the distal caput to the distal cauda

(Rankin *et al.*, 1992; Vierula *et al.*, 1992). The immunoreactivity observed in the epididymis originates from synthesis rather than endocytosis of the testicular protein since PEBP was still present in sperm-free epididymides (Vierula *et al.*, 1992).

PEBP seems to have an unusual distribution as it is a major component of the cytosol in many tissues from both male and female rats but has only been detected in the secretions of the male reproductive tract. Sequence analysis has indicated that cloned rat and monkey epididymal cDNAs lack a signal peptide, which is a characteristic of secreted proteins (Perry *et al.*, 1994). This conflicting information suggests that this protein has an unusual secretory mechanism. The function of PEBP is unresolved but it could be involved in membrane remodelling during sperm maturation and might have a similar role in organising membrane components during spermatogenesis (Saunders *et al.*, 1995).

2.2.2. Interstitium

2.2.2.1 Leydig cells

Leydig cells are characterised by a round or ovoid nucleus, prominent mitochondria and a vast network of smooth endoplasmic reticulum which extends throughout the entire cytoplasm with patches of Golgi apparatus and rough endoplasmic reticulum, as well as lysosomes (de Kretser & Kerr, 1994). These structures are all indicative of an actively synthetic cell. Leydig cells are designed for their principal function which is the biosynthesis and secretion of testosterone, and it has been demonstrated that testosterone production is related directly to the amount of smooth endoplasmic reticulum (Ewing & Zirkin, 1983).

The synthesis of testosterone from cholesterol by Leydig cells is regulated by the pituitary hormone LH (discussed in section 2.3.2.). Cholesterol is obtained by *de novo* synthesis from acetate or is transported into the cell by lipoprotein and is then transported to the outer mitochondrial membrane (Saez, 1994). The mechanism of delivery of cholesterol to the mitochondria is not fully understood, but is thought to involve the cytoskeleton and a sterol carrier protein (SCP2) (Saez, 1994; Stocco & Clark, 1994). Recently, a 30 kDa phosphorylated protein has been characterised which rapidly appears in mitochondria following stimulation of steroidogenic cells (Clark *et al.*, 1994; Sugawara *et al.*, 1995). This protein, named steroidogenic acute regulatory protein (StAR), is thought to be involved in the rapid transfer of cholesterol from the outer to the inner mitochondrial membrane and is important in the acute regulation of steroid production (Clark *et al.*, 1994). Recently, it has been found that in three individuals with congenital lipoid adrenal hyperplasia, which is characterised by impaired steroidogenesis in the adrenals and gonads, the StAR gene is mutated and non-functional (Lin *et al.*, 1995). Once in the mitochondrion, the cholesterol is then converted to pregnenolone by a side-

chain cleavage enzyme which is comprised of three proteins, adrenodoxin, adrenodoxin reductase and cytochrome P450. This is the rate-limiting step of steroidogenesis. Pregnenolone is converted to testosterone in the smooth endoplasmic reticulum by one of two separate pathways, depending on the species. The rat utilises the Δ^4-pathway via progesterone, 17-hydroxyprogesterone and androstenedione, whilst in man the major route is the Δ^5-pathway via 17-hydroxypregnenolone, dehydroepiandrosterone and androstenediol (Gower, 1988) (Fig. 5).

Testosterone production is also likely to be regulated by local factors produced within the testis (Sharpe 1990; 1993). It was first demonstrated by Aoki and Fawcett (1978) that localised damage to seminiferous tubules, due to the implantation of anti-androgens, resulted in hyperplasia of the Leydig cells and hypertrophy of the smooth endoplasmic reticulum in the vicinity of the damaged regions. These authors suggested that the seminiferous tubules may be able to influence Leydig cell function. Disruption of spermatogenesis by irradiation *in utero*, vitamin A deficiency, hydroxyurea treatment or cryptorchidism were also shown to result in enlarged Leydig cells and an increase in the organelles associated with steroid biosynthesis but decreased responsiveness to hCG *in vivo* (Rich *et al.*, 1979; Kerr *et al.*, 1979). Since serum LH levels were elevated, Leydig cell dysfunction was thought to be a result of long-term stimulation by LH. It was later demonstrated that hypertrophy of Leydig cells was induced only in the abdominal testis in unilaterally cryptorchid rats even though both testes were exposed to the same LH levels and that the Leydig cell structural changes occurred before a rise in serum LH levels (Risbridger *et al.*, 1981), supporting the existence of local control mechanisms within the testis.

Morphometric analysis has been used to assess if Leydig cell size is regulated by the stage of the seminiferous tubule in rats, since isolated Leydig cells are heterogeneous. Bergh (1982; 1983) found that Leydig cells adjacent to tubules at stages VII-VIII were larger and this size difference was not evident in cryptorchid testes which had no spermatogenic cycle. The stage-dependent regulation of Leydig cell size only became evident at 47 days of age, coincident with completion of the first wave of spermatogenesis (Bergh, 1985a). This increase in cell size associated with stages VII-VIII was not observed in a study by Fouquet (1987), although, he did find that the smooth endoplasmic reticulum content was increased at these stages. It has been shown that an increase in volume of smooth endoplasmic reticulum, rather than in cell size, is associated with increased steroid biosynthesis (Ewing & Zirkin, 1983). These results support other evidence that stages VII-VIII are the main site of androgen action in the rat (Sharpe, 1994b).

Modulation of Leydig cell structure and function by seminiferous tubules requires the factors to be secreted into the interstitial fluid. Several groups have shown the

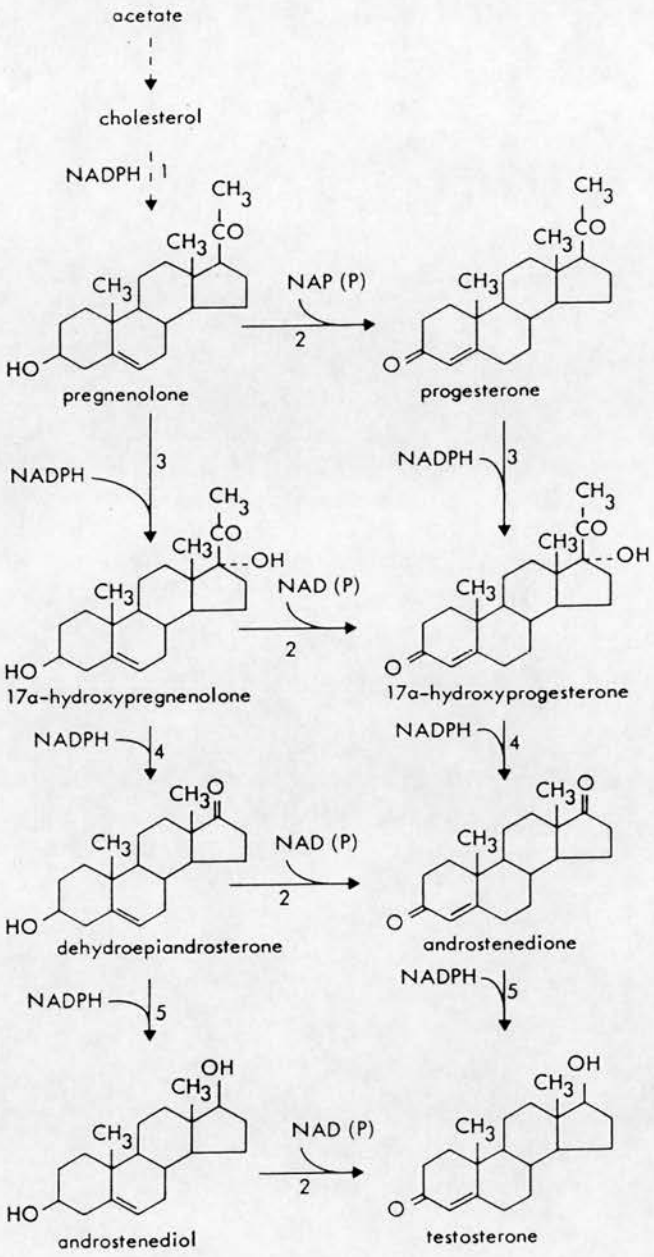


Figure 5. Pathways involved in the biosynthesis of testosterone in Leydig cells. In man, the major route of testosterone synthesis is via the Δ^5-pathway which includes 17α-hydroxypregnenolone, dehydroepiandrosterone and androstenediol. In the rat, the Δ4 pathway via progesterone is utilised which includes 17α-hyroxyprogesterone and androstenedione. The enzymes involved are: cholesterol side-chain cleavage complex (1) which is located in mitochondria; 3β-hydroxysteroid dehydrogenase (2); 17α-hydroxylase (3); steroid $C_{17,20}$-lyase (4); 17β-hydroxysteroid dehydrogenase (5). Enzymes 2-5 are all located in the endoplasmic reticulum of Leydig cells. *Adapted from van der Molen and Rommerts, 1981.*

ability of steroid-free interstitial fluid to increase testosterone production by LH/hCG stimulated Leydig cells *in vitro*, effects which were not due to LH and which could not be replicated by serum (Sharpe & Cooper, 1984; Hedger *et al.*, 1990; Jansz *et al.*, 1990). Induction of seminiferous tubule damage by cryptorchidism, heat or EDS-treatment increased the levels of the steroidogenic stimulatory factor(s) in IF (Sharpe & Cooper, 1984; Bartlett & Sharpe, 1987; Ishida *et al.*, 1987; Drummond *et al.*, 1988). The factor(s) is likely to be a glycoprotein of 57-75 kDa but has not yet been purified (Jansz *et al.*, 1990). In addition there have been many *in vitro* studies on the effects of Sertoli cell and seminiferous tubule conditioned medium, as well as the co-culture of Leydig cells with Sertoli cells on Leydig cell steroidogenesis (Sharpe, 1993; Saez, 1994). Verhoeven and Cailleau (1986; 1987) have shown the presence of a stimulatory factor in both rat Sertoli cell conditioned medium and in human seminiferous tubule conditioned medium which has a molecular weight of 10-30 kDa and is stimulated by FSH and dibutyryl cAMP. Papadopoulos (1991) has described the purification of a 80 kDa human Sertoli cell secreted protein (hSCSP-80) which stimulates steroidogenesis in human and rat Leydig cells as well as in the MA-10 mouse Leydig cell tumour line. Recently, a 70 kDa protein complex has been isolated from immature Sertoli cells which stimulates steroidogenesis (Boujrad *et al.*, 1995). The complex is comprised of 28 and 38 kDa proteins. The 28 kDa protein is FSH induced and is responsible for bioactivity, whilst the 38 kDa protein is important for stabilising or facilitating the effect of the 28 kDa protein. Sequence analysis of the two proteins identified the 28 kDa protein as the tissue inhibitor of metalloproteinase-1 (TIMP-1) and the 38 kDa protein as CP-2 (see above). A 21 kDa protein has been purified from adult rat seminiferous tubule culture medium which inhibits Leydig cell steroidogenesis and is thought to affect the activity of the steroidogenic enzymes, cytochrome P-450 side chain cleavage and cytochrome P-450 17α-hydroxylase/17,20-lyase (Zwain & Cheng, 1994). There is overwhelming evidence that the seminiferous tubules secrete factors that can regulate testosterone production by Leydig cells, but these factors need to purified and shown to have an effect *in vivo* before it can be concluded that they have a physiological role in the testis.

2.2.2.2 Macrophages

Macrophages comprise 20% of all cells in the interstitial tissue and in the rat there are approximately four Leydig cells to one macrophage (Bergh, 1985b; Niemi *et al.*, 1986). Their cytoplasm contains a single indented nucleus, Golgi complex, rough endoplasmic reticulum, coated vesicles and numerous lysosomal vacuoles (Miller *et al.*, 1983). Macrophages are usually situated in close contact with Leydig cells, such that Leydig cell processes are inserted into coated membrane invaginations on the macrophage cell surface, suggestive of functional coupling of Leydig cells and macrophages (Miller *et al.*,

1983). On the cell surfaces not adjacent to Leydig cells, numerous filopodia and lamellopodia extend from the macrophage surface into the surrounding interstitial fluid. Miller *et al.* (1983) also demonstrated that macrophages were endocytically active and possessed receptors for the Fc portions of immunoglobulin G on their surface. Bergh (1985b; 1987) has shown a correlation between changes in cell size and mass of both Leydig cells and macrophages in response to unilateral cryptorchidism or hCG treatment. Macrophages can be depleted from the testis by the injection of liposome entrapped dichloromethylene diphosphate (Cl_2MDP) (Bergh *et al.*, 1993a). Unilateral depletion resulted in a gradual decline in intratesticular testosterone levels but with no effect on testis weight or seminiferous tubule morphology, whilst in the contralateral testes a compensatory increase in Leydig cell size and testosterone levels was observed. The effects of macrophages on Leydig cell testosterone production has been investigated *in vitro* by culture of Leydig cells with macrophages or macrophage conditioned medium (Sun *et al.*, 1993). Both macrophages cultured with Leydig cells, in a 4:1 ratio as seen *in vivo*, and with conditioned medium inhibited LH stimulated testosterone biosynthesis. Binding of hCG to Leydig cell LH-receptors was unaffected by macrophage treatment, indicating that the effect was at a site downstream of receptor binding. Further investigation demonstrated that neither the addition of cAMP or cholera toxin could prevent the inhibition of LH stimulated testosterone production, but the addition of cholesterol, pregnenolone, dehydroepiandrosterone or androstenedione overcame the inhibitory effect (Sun *et al.*, 1994), suggesting that macrophages can somehow affect the transport or availability of cholesterol in Leydig cells. Although the effects on testosterone production observed *in vivo* contradict those found *in vitro* to some extent, the results are indicative of macrophages being involved in the local regulation of Leydig cell steroidogenesis. The presence of macrophages is also important for the differentiation and proliferation of Leydig cells in the adult rat testis since depletion of macrophages in the EDS treated rat prevented the normal regeneration of Leydig cells over the next few weeks (Gaytan *et al.*, 1994a,b). Macrophages are also likely to be involved in immunoregulation since the inflammatory response to hCG is enhanced in macrophage depleted testes (Bergh *et al.*, 1993b) and a similar response was seen 3 days after EDS treatment when macrophages had been removed (Gaytan *et al.*, 1994a).

2.2.2.3 Testicular circulation

Testicular blood flow is the main route for transport of nutrients, secretory products and oxygen to the testis as well as the removal of waste products and testicular secretory products. The control of testicular blood flow is important since the concentration of oxygen in the seminiferous tubules is so low that they are on the brink of hypoxia and Leydig cell function is critically dependent on oxygen. It is thought that modulation of

testicular blood flow and vascular permeability can influence testicular function, discussed in section 2.4.1. The testicular circulation is briefly described below based on information from Setchell *et al.* (1994).

The testicular artery is the principal artery supplying the testis; it is coiled extensively inside the spermatic cord until it reaches the testicular capsule. The artery penetrates the capsule and runs down the epididymal margin and then winds back up the opposite side to enter the parenchyma near the rete before beginning to branch to form the capillary network throughout the interstitium. The testicular capillary network is atypical since the capillaries are covered by a continuous unfenestrated endothelium, which is impermeable to dyes that rapidly penetrate other vascular beds but is still more highly permeable to albumin and other macromolecules (Setchell *et al.*, 1994). The relationship of these characteristics to testicular function is not understood. Blood flow within the capillaries shows rhythmical variations of high and low flow known as vasomotion which are generated by contractions of the precapillary arterioles (Bergh & Damber, 1993). The physiological role of vasomotion is not really understood but some of the factors known to influence vasomotion are discussed in section 2.4.1. The veins draining the testicular parenchyma converge and then form a plexus over the rete testis known as the mediastinal venous plexus. After this plexus, the veins converge and enter the spermatic cord where they form a further venous plexus which envelops the testicular artery to form the pampiniform plexus. This structure performs several functions. It allows countercurrent exchange of heat between the arterial and venous blood enabling the testis to be maintained at a temperature below that of the abdomen, which is crucial for spermatogenesis. Arterio-venous anastomoses are also present in the pampiniform plexus and it has been shown that the concentration of testosterone in testicular venous blood is reduced by 40% after it has passed through this plexus, due to the dilution of outgoing venous blood by incoming arterial blood which is shunted across to the veins from the artery via the anastomoses (Maddocks & Sharpe, 1989b). At present it is unknown whether shunting of arterial blood can be regulated or if it is important for testicular function but it could aid countercurrent heat exchange and reduce pulsatility and pressure of incoming arterial blood to the testis.

2.2.2.4 Testicular lymphatics

The organisation of interstitial tissue is related to the structure of the lymph vessels (Fawcett *et al.*, 1973). In the rat, the lymph vessels are sinusoids rather than capillaries. The endothelium forms a continuous covering over the surfaces of adjacent seminiferous tubules but is discontinuous in areas between three or more adjacent tubules (Clark, 1975). This means that the cells of the interstitial tissue are directly exposed to the lymph; therefore for substances to gain access to the seminiferous tubule they must enter the

interstitial fluid. The interstitial fluid drains into several collecting vessels which emerge from the surface of the testis in three groups (Perez-Clavier & Harrison, 1978). Vessels from the upper pole and mediastinum surround the rete testis and efferent ducts then join the main testicular lymphatic trunk which runs along the spermatic cord. Lymph vessels from the lower part of the testis pass over the epididymis before joining the main testicular lymphatic trunk. The lymph drains into the blood circulation via the thoracic duct.

Interstitial fluid is formed by the filtration of blood plasma at the arterial ends of the capillaries, where in most circumstances the capillary hydrostatic pressure exceeds colloid osmotic pressure. However, in the testis the capillaries have a high vascular permeability and there is no obvious restriction of the passage of proteins into interstitial fluid (Setchell *et al.*, 1994). This results in the protein concentration being almost the same in testicular interstitial fluid as in blood plasma, with the result that there is no significant osmotic pressure gradient between the two fluids. The main factor which determines fluid filtration is the hydrostatic pressure differences between the capillaries and interstitial tissue. These could arise through changes in blood flow or vascular permeability (Setchell *et al.*, 1994). This means that the regulation of capillary pressure will have important consequences for interstitial fluid formation (Sweeney *et al.*, 1991). The formation of interstitial fluid from blood plasma has to be in equilibrium with its resorption into the venules and lymphatic drainage. This is thought to be influenced by vasomotion, with interstitial fluid being generated during periods of high blood flow and reabsorbed during periods of low blood flow (Bergh & Damber, 1993). Regulation of interstitial fluid production will be discussed in section 2.4.1.

2.2.3. Rete Testis

The rete testis is a complex network of interconnecting channels which in the rat is located just under the capsule close to the testicular artery and is covered by the mediastinal venous plexus (Setchell *et al.*, 1994). In many species, including man, the rete testis is located centrally in the testis. At each end of the seminiferous tubules where they open into the rete testis there is a short transitional zone lined with tall columnar cells, capable of endocytosis, which abruptly changes to low cuboidal cells in the rete testis (Hermo & Dworkin, 1988). The cell cytoplasm contains a large nucleus, mitochondria, Golgi and some rough endoplasmic reticulum but has very few of the organelles associated with secretion (Dym, 1976). Dym concluded, based on the morphology of the cells and their inability to incorporate an electron-opaque tracer, that the rete testis did not have a secretory or absorptive function. In contrast to these conclusions, Morales *et al.* (1984) were able to demonstrate endocytosis by the epithelial cells. Various electron dense

markers, which were able to distinguish between fluid phase and adsorptive endocytosis, were injected into the rete testis. They found that substances taken up by fluid phase endocytosis were transported to the lysosomes, but the tracers taken up by adsorptive endocytosis were transported either to the lysosomes or to the lateral and basal surfaces of the cells where they were released into the extracellular spaces, indicating that some proteins endocytosed by the rete testis can end up in the blood or lymph. Rete testis fluid has a composition different from blood plasma, interstitial fluid and seminiferous tubule fluid (Setchell *et al.*, 1994). One of the major differences is in protein composition, such that protein content drops from 6mg/ml in seminiferous tubule fluid to 1mg/ml in rete testis fluid, suggesting that the rete testis is an important site of protein resorption (Hinton & Keefer, 1983). The analysis of fluids collected by micropuncture using gel electrophoresis have also demonstrated that many of the proteins present in seminiferous tubule fluid are not present in fluid collected from the initial part of the efferent ducts (Olson & Hinton, 1985). Since the rete testis is covered by the mediastinal venous plexus it may facilitate the resorption of proteins into the venous blood. Resorption of specific proteins will be discussed in section 2.4.2.

2.2.4. Efferent Ducts

The rat has several efferent ducts originating from the rete testis and there is a sharp delineation between the two structures as the epithelium increases in height to be composed of tall columnar cells (Robaire & Hermo, 1988). The efferent ducts are lined predominantly by non-ciliated cells with the occasional ciliated cell; this is the only region of the male reproductive tract that is lined with a ciliated epithelium (Ilio & Hess, 1994). Initially the efferent ducts are only slightly convoluted but this increases as they progress towards the epididymis and they anastomose with one another (Ilio & Hess, 1994). As they approach the head of the epididymis they become thinner, less coiled and form a single tubule that changes abruptly into the initial segment of the epididymis.

The two cell types of the efferent ducts each have a characteristic morphology. The structure of non-ciliated cells is specialised for endocytosis (Hermo & Morales, 1984; Robaire & Hermo, 1988; Ilio & Hess, 1994). The apical cytoplasm is characterised by a brush border of microvilli in between which are tubular coated pits which give rise to apical tubules within the cell cytoplasm. There is an abundance of endosomes, multivesicular bodies and lysosomes but very little rough endoplasmic reticulum, mitochondria and microtubules. The Golgi shows no evidence of secretory granule formation and is thought to be responsible for the formation of lysosomal enzymes. The supranuclear region contains numerous lysosomes and peroxisomes, the function of which is unknown. The nucleus is present in the basal cytoplasm together

with lipid droplets. Fusion of lysosomes with lipid droplets suggests that the lipid may be formed by the breakdown of endocytosed material. The non-ciliated cells are capable of both fluid-phase and adsorptive endocytosis but there is no evidence to support transport of macromolecules to the lateral and basal surfaces, all endocytosed substances ending up in the lysosomes, unlike the rete testis (Hermo & Morales, 1984). The ciliated cells possess numerous cilia and a few microvilli. The nucleus is in the upper half of the cell and the cytoplasm contains reduced amounts of the structures involved in endocytosis (Robaire & Hermo, 1988; Ilio & Hess, 1994). Hermo *et al.* (1985) demonstrated that these cells were still able to endocytose electron-dense tracers by both fluid-phase and adsorptive endocytosis, however the non-ciliated cells were able to internalise much larger volumes of substances than were the ciliated cells.

The efferent ducts have three main functions, the first being to move non-motile sperm and fluid to the epididymis, the second to concentrate the sperm by the resorption of a large volume of fluid and thirdly the modification of the composition of luminal fluid. It was originally thought that cilia motility would propel spermatozoa along the efferent ducts but further investigation showed that ciliary beat was not exclusively in one direction and therefore tends to stir the fluid rather than direct sperm movement (Ilio & Hess, 1994). The main regulator of fluid flow is thought to be smooth muscle contractions. The efferent ducts are thought to be the main site of fluid absorption; fluid phase endocytosis may be partly responsible but it is also thought to be coupled to active transport of sodium (Ilio & Hess, 1994). Modification of fluid composition is achieved by endocytosis of proteins, specific examples of which are discussed in section 2.4.2. The efferent ducts may have a secretory function though there is no direct evidence of this. Several morphological studies, but not in the rat, have reported the appearance of cytoplasmic extrusions suggestive of apocrine secretion but whether this is real or artefact is subject to debate (Ilio & Hess, 1994). Androgen and oestrogen receptors have been identified in the efferent ducts of the mouse, monkey and human, although the expression of these receptors in the rat has not been investigated (Ilio & Hess, 1994). The functional significance of these receptors is unknown but their presence raises the possibility that fluid resorption and endocytosis could be hormonally regulated.

2.2.5. Epididymis

The epididymis is responsible for sperm maturation as they are transported along its length to the cauda which is the main storage site of spermatozoa (Robaire & Hermo, 1988). During maturation, the spermatozoa acquire the capacity for motility which is incompletely understood but is thought to be brought about by changes in the plasma membrane, acquistion of proteins and alterations in biochemical systems (Yanagimachi,

1994). Spermatozoa also acquire the ability to interact and fertilise eggs which is achieved by remodelling of the lipids and proteins of the plasma membrane to form functional domains (Jones, 1989; Eddy & O'Brien, 1994). Sperm maturation is associated with a luminal environment that is modified in the different regions of the epididymis by secretion and endocytosis (Robaire & Hermo, 1988). Along with the efferent ducts, the proximal caput is a major site of fluid resorption (Setchell *et al.*, 1994). It is well established that the epididymis is capable of secretion and endocytosis of proteins (Hermo *et al.*, 1994).

2.3. Endocrine control of the testis

Testis function is controlled by secretion of the gonadotrophins, follicle stimulating hormone (FSH) and luteinizing hormone (LH), from the anterior pituitary. The secretion of these hormones is regulated by the pulsatile release of gonadotrophin releasing hormone (GnRH) from the hypothalamus. FSH and LH are members of a family of glycoprotein hormones which includes thyroid stimulating hormone (TSH) and placentally derived human chorionic gonadotrophin (hCG). They have a heterodimeric structure consisting of a common α-subunit and a β-subunit which is hormone specific (Bousfield *et al.*, 1994). Both subunits are glycosylated at specific residues; the carbohydrate is responsible for determining the circulatory half-life of the hormone and modulates its biological activity (Bousfield *et al.*, 1994). FSH and LH are produced in gonadotroph cells in the anterior pituitary; their release in response to GnRH is not fully understood. Binding of GnRH to its receptor on the plasma membrane induces an influx of calcium ions and their binding by calmodulin; it also stimulates inositol phospholipid hydrolysis by a G-protein activated phospholipase C which in turn activates protein kinase C (Conn, 1994). Protein kinase C levels and inositol phosphate production are not closely coupled to gonadotrophin release, so they are thought to be involved in chronic regulation of gonadotroph responsiveness (Conn, 1994). The gonadotrophins are released into the blood where they are transported to the testis. The hypothalamo-pituitary-testis axis is summarised in figure 6.

2.3.1. FSH

The effects of FSH on Sertoli cells changes during development since it has been shown that FSH has multiple roles important for the initiation and maintenance of spermatogenesis. FSH binds to receptors present on Sertoli cells which results in the stimulation of adenylate cyclase activity and production of cAMP and subsequent protein

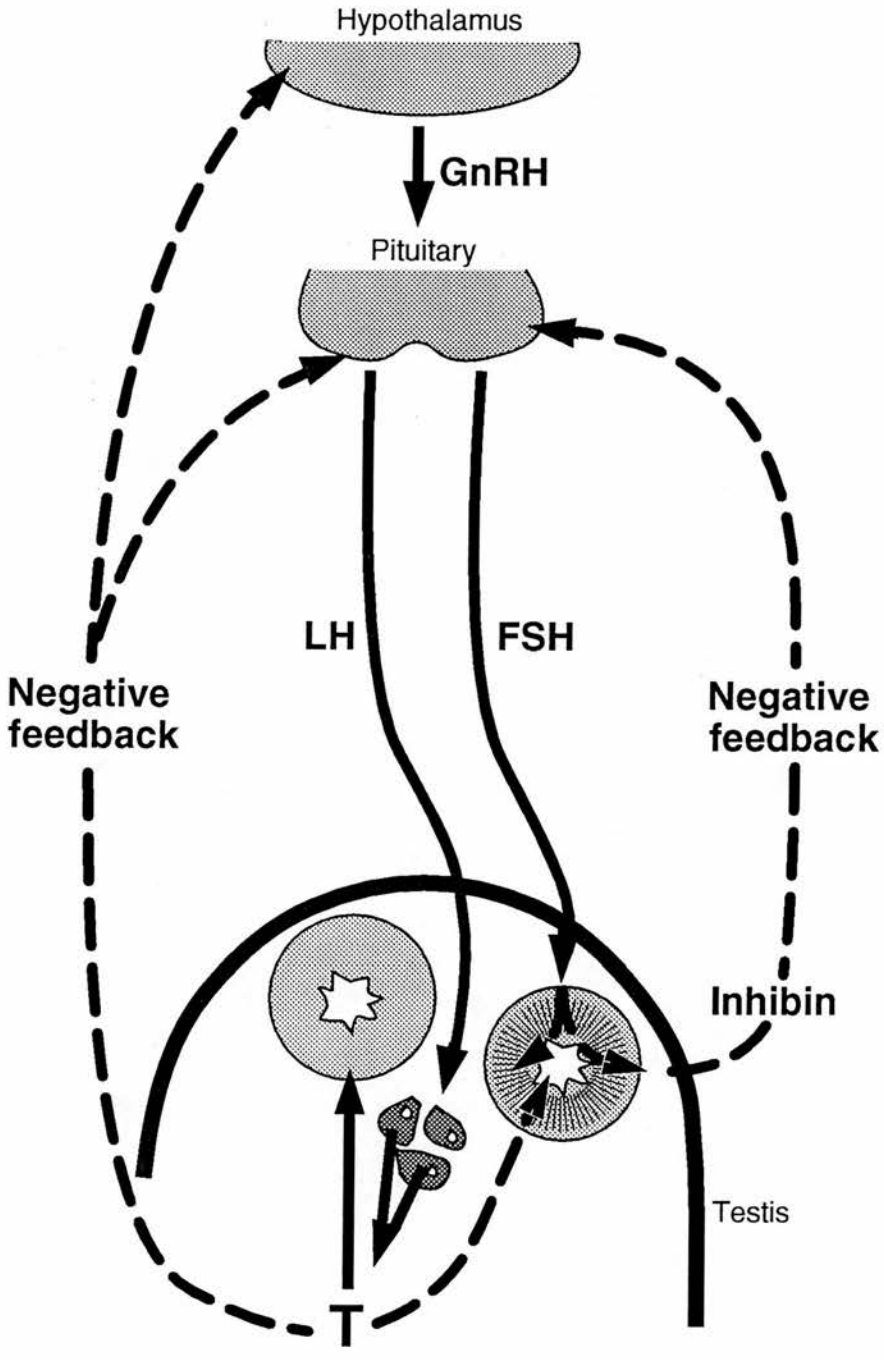


Figure 6. Schematic diagram summarising endocrine control of the testis. Testicular function is modulated by the secretion of gonadotrophins, luteinizing hormone (LH) and follicle stimulating hormone (FSH), from the pituitary gland in response to gonadotrophin releasing hormone (GnRH) produced by the hypothalamus. LH acts on the Leydig cells in the interstitium to stimulate testosterone (T) biosynthesis. FSH acts on the Sertoli cells within the seminiferous tubules which stimulates the secretion of inhibin. Hormone production by the hypothalamus and pituitary is controlled by negative feedback of testosterone and inhibin.

kinase activity (Griswold, 1993). This results in a wide variety of biochemical changes since FSH is able to modulate DNA, RNA and protein synthesis.

FSH is important for regulating proliferation of Sertoli calls in the fetal and neonatal rat testis. At day 18 of gestation both decapitation *in utero* or the administration of FSH antiserum resulted in a dramatic reduction in uptake of tritiated thymidine by Sertoli cell nuclei (Orth, 1984). The multiplication of Sertoli cells continues postnatally but ceases at around day 15 when the adult complement of Sertoli cells is present (Orth, 1982). FSH is important in determining sperm output since Sertoli cells support a finite number of germ cells through development (Russell & Peterson, 1984). Therefore inhibition of Sertoli cell proliferation either in fetal or neonatal life will result in reduced sperm output and testis weight in adulthood (Orth *et al.*, 1988). Several studies have demonstrated the continuing importance of FSH during this period. Rats injected with a potent GnRH antagonist during the first two weeks of postnatal life permanently damaged testis function including effects on both the seminiferous tubules and Leydig cells (Huhtaniemi *et al.*, 1986). In neonatal rats the suppression of FSH by testosterone propionate resulted in a reduction in mitotic activity of germ cells and Sertoli cells whereas the co-administration of FSH and testosterone propionate stimulated proliferation of these cells (Almiron & Chemes, 1988). The administration of an FSH antiserum to 20 day old rats for two weeks reduced testis weight, tubule diameter as well as the numbers of spermatids and spermatocytes, without altering serum testosterone levels (Raj & Dym, 1976). Treatment from birth with an FSH antiserum demonstrated that the numbers of type A spermatogonia were normal but preleptotene spermatocytes and step 7-8 spermatids were reduced, suggesting that FSH is required for the differentiation of type A spermatogonia into preleptotene spermatocytes (Chemes *et al.*, 1979). It is not clear how the effects on the germ cells are mediated by the Sertoli cells. These observations indicate that FSH plays an important role in the initiation of spermatogenesis although the mechanisms by which this occurs have not been elucidated.

During sexual maturation there is a decrease in the responsiveness of isolated Sertoli cells to FSH stimulation, as measured by the production of cAMP, with the most significant change occurring between 18 and 36 days of age (Steinberger *et al.*, 1978). This cannot be explained by a decline in FSH receptor numbers since they have been shown to increase with age in the whole testis (Ketelslegers *et al.*, 1978; Bortolussi *et al.*, 1990). The ability of inhibin to regulate FSH secretion declines with age suggesting that it is more important during early postnatal development. Immunoneutralisation of inhibin using an antiserum raised against the α-subunit was able to increase plasma FSH levels until 20 days of age but this ability was lost at puberty (Culler & Negro-Vilar, 1988; Rivier *et al.*, 1988). During puberty there is a change in hormonal control of the testis from being FSH to testosterone dependent (Sharpe, 1994b). Russell *et al.* (1987)

demonstrated that both FSH and LH were necessary for germ cell survival during spermatogenesis in the pubertal testis. It has also been observed that FSH is involved in the maturation of Leydig cells (Kerr & Sharpe, 1985; Sharpe, 1993).

Elucidating a role for FSH in adult spermatogenesis has proved more difficult to establish in rats (Sharpe, 1994b; Zirkin *et al.*, 1994, McLachlan *et al.*, 1995). Studies in primates have shown that, initially, active immunisation with FSH severely impairs spermatogenesis but after 4.5 years of treatment spermatogenesis recovered qualitatively but not quantitatively since germ cell numbers were reduced (Wickings & Nieschlag, 1980; Srinath *et al.*, 1983). It has since been demonstrated that the administration of FSH to adult monkeys caused a specific increase in Ap spermatogonia which resulted in an increase in the numbers of B spermatogonia, spermatocytes and spermatids over the following weeks of treatment (van Alphen *et al.*, 1988). In GnRH antagonist treated monkeys, FSH was able to maintain the numbers of renewing stem cells; the Ap spermatogonia (Weinbauer *et al.*, 1991). In contrast to these results, treatment with FSH in hypophysectomised testosterone-replaced monkeys significantly increased the numbers of differentiated B spermatogonia but had no effect on Ad or Ap spermatogonia (Marshall *et al.*, 1995). It would seem that further studies are necessary to elucidate the role of FSH in regulating spermatogonial numbers in non-human primates. In humans, the suppression of FSH by hCG resulted in a decline in sperm counts which was reversed by the replacement of FSH but not testosterone (Matsumoto *et al.*, 1986). These results suggest that FSH levels are important for the maintenance of quantitatively normal spermatogenesis in primates and humans. The situation in the rat is more confusing. Immunoneutralisation of FSH in the adult rat was found to have relatively little effect on spermatogenesis (Dym *et al.*, 1979). This can be explained by the difference in the regulation of spermatogonial proliferation in the rat in comparison to monkeys (de Rooij *et al.*, 1989). In rats the number of early differentiating spermatogonia is controlled by density dependent degeneration.

Several studies have investigated the ability of testosterone alone to maintain spermatogenesis in various model systems (Sharpe, 1989a; Sharpe, 1994b; Zirkin *et al.*, 1994). In the hypophysectomised rat testosterone supplementation is only capable of maintaining qualitatively complete spermatogenesis i.e. there is continued production of all spermatogenic cells but subnormal numbers (Bartlett *et al.*, 1989; Santulli *et al.*, 1990). In rats treated with low dose testosterone-oestradiol implants the administration of a higher dose of testosterone failed to restore the germ cell populations to normal (McLachlan *et al.*, 1994), which is similar to the situation seen in humans and primates. However, high levels of testosterone have been shown to support quantitative spermatogenesis in EDS-treated intact rats (Sharpe *et al.*, 1988a) and in rats actively immunised against GnRH (Awoniyi *et al.*, 1992).

There is increasing evidence to support synergism between FSH and testosterone to maintain normal spermatogenesis. Investigation of the effects of FSH and testosterone on the maintenance of spermatogenesis in hypophysectomised rats found that FSH treatment could only maintain the development of preleptotene spermatocytes up until round spermatids and that the co-administration of FSH and low levels of testosterone was able to maintain spermatogenesis almost qualitatively (Bartlett *et al.*, 1989). These observations were confirmed in another study using hypophysectomised rats which were treated with EDS (Kerr *et al.*, 1992). In FSH-treated rats, spermatid development was restricted to stages I-VI of the spermatogenic cycle indicating that other factors besides FSH are necessary for spermiogenesis. Testosterone was able to maintain all 14 stages with the presence of step 1-19 spermatids. Combined administration of the two hormones was able to stimulate spermatogenesis to a greater extent than expected by an additive response suggesting that FSH is able to enhance the response to testosterone. This idea is supported by the ability of FSH to increase both androgen receptor protein and mRNA concentration in immature Sertoli cells (Verhoeven & Cailleau, 1988; Blok *et al.*, 1989; 1992). It has been suggested that in the adult rat, FSH in the presence of testosterone modulates stages I-VI of spermatogenesis and the development of spermatids up to step 6, this may involve regulating the entry of cells into the following androgen-dependent phase (stages VII-VIII) (Sharpe, 1994b). FSH receptors and FSH-stimulated cAMP production are also highest at stages I-VI (Parvinen, 1993). Although, high levels of testosterone alone can maintain quantitatively normal spermatogenesis it remains to be established whether this can also be achieved with much lower levels of androgen co-administered with FSH.

More recent evidence has demonstrated that recombinant FSH does have biological effects in adult rats. Russell *et al.* (1993) showed that the short-term administration of FSH to hypophysectomised rats could inhibit the usual degeneration of germ cells. FSH replacement to GnRH antagonist treated rats is able to delay regression of spermatogenesis and supports the development of spermatogonia through to the preleptotene stage (Sinha Hikim & Swerdloff, 1995). The lack of further development shows a requirement for testosterone and supports the hypothesis that the maintenance of spermatogenesis requires the synergistic action of both hormones. FSH has the capacity to influence binding of the elongate spermatids to the Sertoli cell (Muffly *et al.*, 1994; Cameron & Muffly, 1991). It was found that in testosterone treated hypophysectomised rats daily sperm production is reduced; morphological investigation of Sertoli cell-spermatid interactions showed abnormalities in immunostaining distribution for F-actin and vinculin, cytoskeletal proteins related to adhesion junctions, and in the ectoplasmic specialisations of step 8 spermatids (Muffly *et al.*, 1994). Treatment with FSH for three weeks was able to restore the structure of the ectoplasmic specialisations and distribution

of F-actin and vinculin resulting in normal daily sperm production. This provides evidence that FSH is important for the attachment of spermatids to the seminiferous epithelium.

2.3.2. LH

LH is responsible for stimulating testicular steroidogenesis by the Leydig cells and measurement of LH and testosterone in blood samples has demonstrated that LH is secreted in defined pulses which are followed by episodes of testosterone secretion (Ellis & Desjardins, 1982). The elucidation of how LH stimulates steroidogenesis is complicated by the fact that several signal transduction systems are stimulated by the interaction of LH with its receptors on the Leydig cell (Rommerts & Cooke, 1988; Cooke, 1990). The production of cAMP, an increase in intracellular calcium, the release of arachidonic acid and its metabolites from phospholipids have all been implicated in the regulation of Leydig cell function.

LH is necessary for the maintenance of Leydig cell structure and steroidogenic function. Immunoneutralisation of LH in immature rats results in marked reductions in testis weight, Leydig cell size, tubule diameter, numbers of germ cells and testosterone production (Raj & Dym, 1976). Short-term deprivation of LH in adult rats using testosterone-oestrogen implants or hypophysectomy causes a coincident reduction in the volume of smooth endoplasmic reticulum in the Leydig cells and in the ability of the *in vitro* perfused testis to secrete testosterone (Ewing & Zirkin, 1983). Both of these changes could be prevented by supplementation with LH. Further investigation showed that LH withdrawal was responsible for a reduction in the enzymatic conversion of pregnenolone to testosterone and diminished activity of P_{450} C_{17}-hydroxylase/$C_{17,20}$-lyase (Wing *et al.*, 1984; Klinefelter *et al.*, 1987). Long term LH deprivation in mature rats by hypophysectomy causes Leydig cell regression but not destruction; steroidogenic function can be restored to nearly normal levels by 2 days of hCG treatment although normal morphology took a week to recover (Stocco *et al.*, 1990).

LH is also involved in the regulation of proliferation and differentiation of Leydig cells (Teerds *et al.*, 1994). Leydig cells can be stimulated to proliferate in immature hypophysectomised rats (Teerds *et al.*, 1989a). Studies *in vitro* have shown that whilst LH can induce the differentiation of precursor cells into Leydig cells, proliferation of immature type Leydig cells is dependent on the presence of IGF-1 and TGF-α as well as LH (Teerds *et al.*, 1994). The role of LH in the maintenance of the Leydig cell population in adult rats is less clear. Leydig cells in adult rats form a stable population which is renewed slowly (Teerds *et al.*, 1989b). Proliferation of immature and adult type Leydig cells in the adult testis can be stimulated by supraphysiological doses of hCG

(Teerds *et al.*, 1988). LH is not thought to be required for maintenance of Leydig cell numbers since this is not affected by long term deprivation of LH (Keeney *et al.*, 1988; Stocco *et al.*, 1990). LH may have mitogenic activity since it was observed to increase tritiated thymidine incorporation into a sub-population of Leydig cells although there was no effect on overall cell numbers (Keeney *et al.*, 1990).

2.3.3. Testosterone

It is well established that high intratesticular levels of testosterone are necessary to support spermatogenesis but exactly how testosterone exerts its effects is still unclear (Sharpe, 1994b). Androgen receptors have been immunolocalised to Sertoli cells, peritubular myoid cells, Leydig cells and the muscular layer of arterioles (Bergh & Damber, 1992; Bremner *et al.*, 1994; Vornberger *et al.*, 1994). Androgen receptor immunostaining of the Sertoli cell nucleus is stage dependent in the rat, increasing from stage II to a maximum intensity at stage VII then rapidly declining to become barely detectable during stages IX-XIII (Bremner *et al.*, 1994; Vornberger *et al.*, 1994). Staining in the other cell types was unrelated to the stage of adjacent tubules and the pattern of androgen receptor expression was not affected by specific germ cell depletion using MAA (Bremner *et al.*, 1994). The total number of androgen receptors in the rat testis increases between 25 and 90 days of age, coincident with increasing numbers of Leydig cells and testosterone production (Buzek & Sanborn, 1988).

Withdrawal of testosterone by hypophysectomy, EDS or GnRH antagonist treatment have all demonstrated the stage specific degeneration of germ cells at stage VII of the spermatogenic cycle 3 or 4 days later (Russell & Clermont, 1977; Bartlett *et al.*, 1986; Sharpe *et al.*, 1990; Sinha-Hikim & Swerdloff, 1993). Studies in the EDS treated rat have shown that degeneration can be prevented or reversed by testosterone supplementation depending on time of administration after EDS treatment (Sharpe *et al.*, 1990; Kerr *et al.*, 1993a). Degenerating germ cells are present at stages IX and X-XI 4 days after EDS treatment, but this is presumed to be a consequence of the lack of androgen action as these cells had passed through stage VII (Kerr *et al.*, 1993a). Testosterone withdrawal also induces the appearance of vacuoles in the basal cytoplasm of Sertoli cells at stages VII-VIII (Kerr *et al.*, 1993a). Further investigation indicated that the vacuoles were multiple dilatations of the intercellular spaces associated with tight junctions, suggesting that testosterone withdrawal has affected the direction of secretion of seminiferous tubule fluid (Kerr *et al.*, 1993b). Other morphological studies have shown that testosterone is involved in the conversion of round to elongated spermatids and specifically promotes the conversion of round spermatids between stages VII and VIII (Sun *et al.*, 1990; McLachlan *et al.*, 1994; O'Donnell *et al.*, 1994). All these

observations indicate that germ cells at stage VII must be exposed to testosterone or to testosterone-regulated factors so that they can continue their normal pattern of development.

Testosterone is also involved in the regulation of protein secretion by isolated seminiferous tubules. Incorporation of ^{35}S-methionine into secreted proteins at stages VI-VIII is twice that at stages II-V or IX-XII and this difference can be ablated by testosterone withdrawal (Sharpe *et al.*, 1992); it has been shown that this decrease in protein secretion is not the result of changes in seminiferous tubule fluid production (Sharpe *et al.*, 1994). The androgen-dependent changes in protein secretion by seminiferous tubules at stages VI-VIII were found to be influenced by the germ cell complement (McKinnell & Sharpe, 1992; Sharpe *et al.*, 1993). The depletion of pachytene spermatocytes, round or elongating spermatids using MAA treatment all significantly reduced methionine incorporation into secreted proteins at stages VI-VIII although not quite to the same extent as seen at 4 days after EDS treatment. Further investigation suggests that testosterone is affecting protein secretion by both Sertoli cell and germ cells but the effect is not at the level of protein synthesis (McKinnell & Sharpe, 1995). Testosterone withdrawal was shown to have no effect on total protein synthesis by stage VI-VIII seminiferous tubules or on the constitutive secretion of the major Sertoli cell proteins (SGP-1 and SGP-2) but instead reduced secretion by regulated secretory pathways. This suggests that testosterone seems to have a role in modulating protein processing within the cell. As well as the effects described, testosterone is also involved in regulating blood flow, vasomotion, interstitial fluid volume and STF production, all of which will be discussed in the next section. Clearly, testosterone has many roles all of importance for the normal functioning of the testis.

2.4. Fluid dynamics within the testis

There are three main fluid compartments in the testis: blood, interstitial fluid (which eventually leaves the testis as lymph) and seminiferous tubule fluid which are responsible for the transport of nutrients, hormones and other messengers. They are inter-dependent so their regulation is of fundamental importance in the maintenance of normal testicular function. The existence of several fluid compartments means that secretory products of the seminiferous tubules may have several destinations; both of these topics are discussed in the next two sections.

2.4.1. Regulation of the fluid compartments

There is a strong correlation between testicular blood flow and the output of testosterone into the spermatic vein, indicating that factors which influence blood flow will affect testicular function (Damber & Janson, 1978). There is also a close correlation between testis weight and blood flow, since disruption of spermatogenesis causes both a reduction in testis weight and blood flow (Setchell *et al.*, 1994). This suggests that the seminiferous tubules may be an important regulator of blood flow. Testosterone is one of the main regulators of blood flow; adult rats treated with EDS resulted in a nearly 50% decrease in blood flow a week later which could be restored by the replacement of testosterone (Damber *et al.*, 1992). The mechanism by which testosterone exerts its effects is unknown but it could interact with androgen receptors present on the arterioles or it may act indirectly by inducing the release of vasoactive factors from other androgen responsive cells (Bergh & Damber, 1992; Damber *et al.*, 1992). Blood flow is not affected by temperature unless the testes are heated to an unphysiological temperature i.e. 43°C for 30 minutes (Galil & Setchell, 1987a; Setchell *et al.*, 1995).

No direct relationship exists between vasomotion and total testicular blood flow as changes in blood flow do not necessarily effect vasomotion (Bergh & Damber, 1993), although, like blood flow, vasomotion is regulated by testosterone (Damber *et al.*, 1992; Collin *et al.*, 1993). Vasomotion is inhibited in adult rats which are made cryptorchid whereas maturation depletion of germ cells by irradiation resulted in an irregular pattern of blood flow but not inhibition (Collin *et al.*, 1993). Both heating and cooling the testis to temperatures which did not change total testicular blood flow were able to affect vasomotion (Setchell *et al.*, 1995). This suggests that vasomotion can be regulated independently from blood flow and is controlled by other factors besides testosterone. The physiological role of vasomotion is not completely clear but it almost certainly plays an important role in regulating interstitial fluid (IF) production.

IF is a filtrate of blood plasma, therefore changes in vascular permeability, blood and lymph flow may affect its composition and volume (Setchell *et al.*, 1994). This interdependence is demonstrated by the effects of testosterone withdrawal using EDS-treatment (Maddocks & Sharpe, 1990b). IF volume was reduced by 50% between 6 and 9 days after treatment, a similar level of reduction as seen with blood flow (Damber *et al.*, 1992). Supplementation of testosterone in the absence of Leydig cells could prevent the decline in IF volume and if administered 9 days after EDS-treatment was also able to restore the volume to control levels (Maddocks & Sharpe, 1990b). These observations indicated that testosterone was required for the maintenance of IF volume and it was proposed that the effects of androgen may be mediated by the seminiferous tubules. This theory was investigated using MAA and heat-treatment to deplete the germ cell population

in the seminiferous tubules to assess whether specific germ cell types could regulate IF volume (Sharpe *et al.*, 1991). Both treatments resulted in a significant increase in IF volume when elongate spermatids, but not other germ cell types, were depleted.

Seminiferous tubule fluid (STF) is secreted by Sertoli cells which presumably take up fluid from the interstitium and then secrete it adluminally. Therefore STF production will be dependent indirectly on interstitial fluid formation and blood flow. As expected, STF production in the adult rat is also regulated by testosterone. The decline in STF production induced by hypophysectomy can be prevented or restored by testosterone (Jégou *et al.*, 1983; Au *et al.*, 1986). In contrast, a study using immature rats found that STF production was FSH regulated (Jégou *et al.*, 1982). This is in agreement with the endocrine control of spermatogenesis; FSH is the main regulator of events in the immature rat but during puberty there is a change to androgen dependence (Sharpe, 1994b). In EDS-treated adult rats, the volume of STF is reduced to 50% at days 6 and 8 after treatment, the same order of magnitude as seen for effects on interstitial fluid and blood flow at this time point (Sharpe *et al.*, 1994). Measurement of lumen area of seminiferous tubules has shown that this is significantly greater at stages VII-VIII in comparison to stages I-VI and IX-XIV and lumen area is thought to be a reflection of STF production (Wing & Christensen, 1982; Sharpe, 1989b; Sharpe *et al.*, 1994). This difference in lumen area is abolished 6 days after EDS-treatment but the effects on both lumen area and STF volume can be prevented by the administration of testosterone (Sharpe *et al.*, 1994). Elongate spermatids also seem to play a role in the regulation of tubule fluid production, since it is reduced significantly when mature spermatids are depleted in adult rats by heat treatment (Jégou *et al.*, 1984) and lumen diameter at stage VII is reduced when elongate spermatids are depleted using MAA treatment (Sharpe, 1989b). This provides further evidence that a complex inter-relationship exists between testosterone, the vasculature and the seminiferous tubules which is important in the regulation of blood flow to the testis and the formation of IF and STF.

2.4.2. Routes of secretion and resorption of seminiferous tubule proteins

Understanding the possible fate of proteins derived from the seminiferous tubules is of importance in considering such proteins as a possible means of monitoring spermatogenesis. All of the available information is based on Sertoli cell-derived proteins since very little is known about germ cell-derived products. Most Sertoli cell proteins which are secreted are released in a bidirectional manner, although in the adult rat more protein is secreted apically rather than basally (Sharpe, 1988; Sharpe, 1992). So proteins

can be secreted apically into seminiferous tubule fluid or basally into interstitial fluid; the fate of proteins in both of these compartments is discussed below.

Proteins secreted apically into seminiferous tubule fluid can progress along the male reproductive tract and appear in semen, be resorbed into the blood circulation or degraded. Based on an increase in α-inhibin levels in spermatic venous blood compared to testicular venous blood in the rat, it was suggested that inhibin in seminiferous tubule fluid is resorbed from the rete testis into the bloodstream (Maddocks & Sharpe, 1989a). In support of this, observations in the ram are indicative of inhibin being endocytosed in the rete testis, efferent ducts and epididymis (Veeramachaneni *et al.*, 1989). Resorption of ABP by the epididymis was first suggested when it was found that in efferent duct ligated testes ABP disappeared from epididymal fluid (French & Ritzén, 1973). Immunocytochemistry also indicated that ABP was endocytosed by the distal portion of the efferent ducts and the proximal caput epididymis (Attramandal *et al.*, 1981; Pelliniemi *et al.*, 1981). Endocytosis of ABP by the rat caput epididymis has been shown *in vitro* (Guéant *et al.*, 1991), whilst endocytosis by the efferent ducts and epididymis has been demonstrated *in vivo* in the ram (Veeramachaneni & Amann, 1991). The *in vivo* endocytosis of transferrin by the rete testis, proximal caput and corpus of the epididymis has been demonstrated in the rat, although in the rete testis transferrin is recycled back to the cell surface indicating than the iron shuttle is operating in these cells (Djakiew *et al.*, 1986; Morales & Hermo, 1986). Both SGP-1 and SGP-2 are endocytosed in the rete testis and efferent ducts (Hermo *et al.*, 1991; 1992). In the ram, SGP-2 is predominantly endocytosed by the efferent ducts in comparison to the epididymis (Veeramachaneni & Amann, 1991). It is unknown whether CP-2 can be endocytosed but this protein seems to be degraded as it passes through the epididymis, since it decreases in concentration in epididymal fluid and proteolytic cleavage occurs (Zabludoff *et al.*, 1990a).

Most studies have not attempted to quantify the level of endocytosis in the different regions of the excurrent duct system, the exception being studies in the ram. It was demonstrated that the major site of endocytosis of rete testis fluid proteins as well as ABP, transferrin and SGP-2 was the efferent ducts rather than the regions of the epididymis; however, they did not investigate the role of the rete testis in these studies (Veeramachaneni *et al.*, 1990; Veeramachaneni & Amann, 1991). It seems probable that a similar situation exists in the rat with the efferent ducts and maybe the rete testis being the major sites of endocytic activity. The transport of endocytosed macromolecules to the basal membrane and thus into extracellular fluid has only been demonstrated in the rete testis region of the excurrent duct system (Morales *et al.*, 1984; Hermo *et al.*, 1994). This suggests that proteins endocytosed in the rete testis can be resorbed into the bloodstream, as proposed for inhibin, and that proteins endocytosed at other sites of the

reproductive tract are more likely to be degraded by lysosomes as appears to be the case with ABP, SGP-1 and SGP-2.

The measurement of proteins in semen is complicated by the fact that the epididymis secretes homologues of testicular proteins. The epididymis is thought to be responsible for secreting many proteins involved in sperm maturation. SGP-2 is an example of a protein which is also secreted by the epididymis but the epididymal protein has a different glycosylation pattern in comparison to the testicular protein (Hermo *et al.*, 1991; Sylvester *et al.*, 1991). Therefore some proteins which can be measured in semen will be more useful for studying the process of sperm maturation rather than spermatogenesis. Even if proteins are not completely endocytosed in the excurrent duct system their levels in semen will be reduced further by dilution with secretions from the prostate and seminal vesicles.

Proteins secreted basally into IF can either enter the bloodstream within the testis or pass out with the lymph which will eventually feed into the peripheral circulation in the thorax. Limited information suggests that a partitioning system operates in the interstitium such that proteins enter the lymph and steroids enter the blood (Maddocks & Setchell, 1988). Levels of iodinated human serum albumin and tritiated testosterone were measured in thoracic duct lymph and blood after intratesticular injection in rats. Albumin was preferentially cleared in lymph and testosterone in blood; this also showed that testosterone, even when combined with a binding protein, can be separated from that protein and be resorbed into the bloodstream. Inhibin concentration is higher in testicular lymph than in testicular venous plasma, suggesting that like albumin basally secreted inhibin is cleared in the lymph (Maddocks & Sharpe, 1989a). This suggests that proteins secreted into IF may not pass into the bloodstream within the testis but will instead enter the lymph and leave the testis by this route.

2.5. Endpoints for monitoring spermatogenesis

An understanding of the causes of male infertility is extremely limited and this is reflected in the difficulties associated with diagnosing male infertility and the lack of treatments for this condition. The main problem stems from the poor understanding of spermatogenesis which is compounded by the fact that this process is extremely difficult to monitor non-invasively. Clinical diagnoses are based on semen analysis in conjunction with tests of sperm function. Sperm counts are one of the most utilised semen parameters; men with less than 20 million spermatozoa /ml are considered subfertile and counts of less than 5 million/ml are associated with infertility. This is not the most reliable parameter due to its high variability within individuals which may be linked to transient factors such as abstinence, illness or stress (Baker *et al.*, 1981; Mallidis *et al.*, 1991). The other

limitation of semen analysis is that the absence of sperm from the ejaculate or the presence of defective sperm does not usually provide any indication of when, where or what has gone wrong during the process of sperm production. Although, semen analysis can indicate abnormalities in various parameters such as concentration, motility or functional defects as determined by penetration of hamster oocytes which provides useful guidance for clinical management, there is no understanding of the mechanisms in spermatogenesis which are at fault and are responsible for these disorders.

In addition to semen analysis, serum levels of the hormones FSH, LH and testosterone are measured to allow evaluation of testicular function (Wang & Swerdloff, 1992). Hormone levels can indicate whether there is a problem at the level of the hypothalamus/pituitary or the testis. Elevated FSH levels in conjunction with normal testosterone and LH levels together with azoospermia or severe oligozoospermia is an indication of severe damage to the seminiferous epithelium. The measurement of FSH can be used as a guide to distinguish azoospermic men who have an obstruction in the excurrent duct system since FSH levels should be in normal range. However, endocrine measurements like semen analysis is also subject to limitations such a high degree of variability in hormone levels amongst normal fertile men. FSH levels allow an evaluation of the normality of Sertoli cell function but do not provide specific information on the mechanisms responsible for the defect.

A more immediate method of investigating the normality of spermatogenesis is by testicular biopsy. This enables histological evaluation and it has now been demonstrated that DNA flow cytometry of fine needle aspirates can be used to quantify spermatogenesis (Giwercman *et al.*, 1994). This analysis is based on the DNA content of the different cells of the seminiferous epithelium. An increase or decrease in the relative proportions of cell types is indicative of an abnormality in spermatogenesis. Testicular biopsies are used to determine whether spermatogenesis is normal in azoospermic men whom are thought to have an obstruction in the efferent ducts (de Kretser, 1982). It is considered too invasive and impractical for the general assessment of spermatogenesis in most infertile men as, at best it provides only a morphological description of whether or not there is a problem in spermatogenesis; it does not indicate the cause of the defect.

Another method of assessing the normality or otherwise of germ cell development which may prove useful is the measurement of testicular proteins in semen or blood (Sharpe, 1992). This is based on the fact that development of the germ cells within the seminiferous tubules is controlled by the secretion of paracrine factors by the Sertoli cells and the germ cells themselves, the pattern of secretion of which will change in accordance with the stage of development (Sharpe, 1993). These proteins will end up in STF and IF and secretion by either of these routes may allow access to the blood circulation (Fig. 7). Alternatively, proteins released into STF may remain in the male reproductive tract and

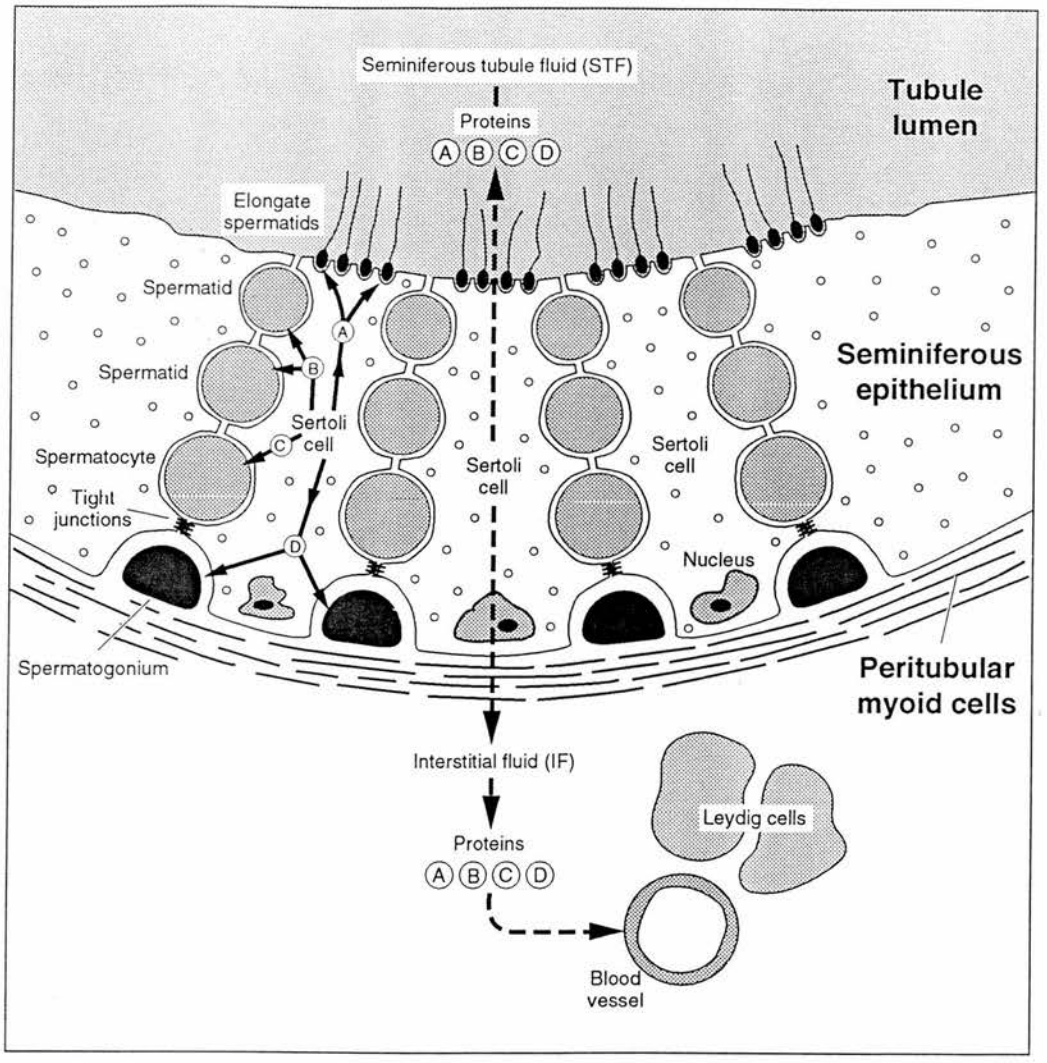


Figure 7. The various stages of germ cell development are regulated by the Sertoli cell by the secretion of specific proteins A to D (in reality there are probably several hundred) which act selectively on the germ cells. These proteins will be secreted into the seminiferous tubule fluid (STF) which surrounds the germ cells and fills the lumen of the seminiferous tubules. STF transports the proteins to the rete testis and epididymis where they may be resorbed into the bloodstream and/or pass into semen with sperm. Some proteins might also be secreted via the base of the Sertoli cell into interstitial fluid (IF). Where they might gain direct access to testicular blood vessels or remain in IF and pass out of the testis in lymph which eventually feeds into the peripheral circulation.

end up in semen. The next sections will review studies evaluating the usefulness of measuring testicular proteins in semen and blood in man as a means of monitoring spermatogenesis.

2.5.1. Evaluation of testicular proteins in seminal plasma

The majority of studies have concentrated on the Sertoli cell secreted proteins, transferrin, and SGP-2, but another protein which has been the source of interest is a cytoplasmic enzyme specific to meiotic and post-meiotic germ cells, lactate dehydrogenase-C4 (LDH-C4).

Several studies have shown that approximately 80% of the transferrin in seminal plasma derives from the testis based on comparison of transferrin levels in seminal plasma from normal and vasectomised men (Holmes *et al.*, 1982; Orlando *et al.*, 1985). However, since transferrin can be detected in the seminal plasma from vasectomised men and in men with agenesis of the seminal vesicles and vas deferens, it suggests that transferrin is also secreted by the prostate and seminal vesicles (Orlando *et al.*, 1985; Chan *et al.*, 1986; Liu *et al.*, 1986). All studies have shown a positive correlation between transferrin levels and sperm concentration and count although it is not understood why there is such a strong association between the two parameters (Holmes *et al.*, 1982; Sueldo *et al.*, 1984; Orlando *et al.*, 1985; Chan *et al.*, 1986; Foresta *et al.*, 1985; Liu *et al.*, 1986; Barthelemy *et al.*, 1988; Ber *et al.*, 1990). Only one study has been able to demonstrate a positive correlation with sperm motility (Ber *et al.*, 1990) and two studies have shown a negative correlation with serum FSH levels (Holmes *et al.*, 1982; Barthelemy *et al.*, 1988). No relationship to sperm morphology (Chan *et al.*, 1986; Barthelemy *et al.*, 1988; Ber *et al.*, 1990) or to fertilising ability based on hamster oocyte penetration test has been shown (Sueldo *et al.*, 1984; Chan *et al.*, 1986), but seminal fluid samples with reduced levels of transferrin showed a decreased capacity to fertilise human oocytes *in vitro* (Sueldo *et al.*, 1984). Since seminal plasma levels of transferrin cannot be used to distinguish between men who are azoospermic due to obstruction of the reproductive tract or seminiferous tubule failure, this suggests that transferrin is not an informative marker of Sertoli cell or seminiferous tubule dysfunction (Chan *et al.*, 1986, Liu *et al.*, 1986). This is further supported by the fact that transferrin levels in normal semen show great variability and some fertile men have values similar to those men with obstructions or spermatogenic failure (Liu *et al.*, 1986).

Ceruloplasmin levels in seminal plasma from men who were fertile, azoospermic or had undergone vasectomy were measured using a solid-phase chemiluminescent method but no difference was observed between the groups, indicating that ceruloplasmin has no use as a marker of seminiferous tubule function (Orlando *et al.*, 1985).

SGP-2 has been demonstrated to be a more informative marker of sperm fertilising ability rather than a marker of spermatogenesis. Localisation of SGP-2 in the human testis showed there was no relationship between pattern and intensity of staining and disruption of spermatogenesis (O'Bryan *et al.*, 1994a). High levels of SGP-2 were expressed at all stages of spermatogenesis in normal and abnormal testicular biopsies including Sertoli cell only testes indicating that SGP-2 secretion is not influenced by germ cells or hormones, a finding which concurs with data from the rat both *in vivo* and *in vitro* (Roberts *et al.*, 1992; Sharpe *et al.*, 1993). These results coupled with the fact that testicular SGP-2 is resorbed in the efferent ducts and epididymis show that SGP-2 has no value as a marker of spermatogenesis. However, the measurement of SGP-2 levels in seminal plasma may have some value for predicting sperm fertilising ability. In 25 semen samples used for *in vitro* fertilisation SGP-2 concentration was found to be significantly predictive of successful fertilisation *in vitro* together with sperm motility and morphology (O'Bryan *et al.*, 1990). Characterisation of SGP-2 bound to human spermatozoa using two monoclonal antibodies, indicated that there are two biochemically distinct forms of the protein (O'Bryan *et al.*, 1994b). Morphologically abnormal sperm are coated with the normal 80 kDa dimeric SGP-2 but this form of the protein is undetectable on normal spermatozoa, which instead show immunostaining within the acrosomal cap using an antibody raised against the α-chain. These observations indicate that SGP-2 may have a role in sperm maturation and therefore prove a useful marker of sperm fertilising ability.

LDH-C4 is a cytosolic enzyme expressed in spermatocytes, spermatids and spermatozoa and is responsible for catalysing the NAD-dependent interconversion of lactate and pyruvate which provides an important source of energy for germ cell metabolism. Transcription of LDH-C4 can be detected in preleptotene spermatocytes (Alcivar *et al.*, 1991) but the protein is not expressed until mid-pachytene spermatocytes (Hintz & Goldberg, 1977; Meistrich *et al.*, 1977). The role of LDH-C4 in male infertility has been the subject of a recent review (Virji & Naz, 1995). Several studies have shown a positive correlation between LDH-C4 levels in seminal plasma or semen and sperm count (Gerez de Burgos *et al.*, 1979, Eliasson & Virji, 1985; Virji, 1985; Orlando *et al.*, 1988; Velasco *et al.*, 1993). It has been demonstrated that LDH-C4 in seminal plasma is not due to release or leakage by sperm, since there is no increase in enzyme levels in semen incubated at 37°C for up to 6 hours and levels remained constant in the same individual when subsequent semen samples were provided at short time intervals (Virji, 1985). This indicated that LDH-C4 originates from the testis and may be a marker of germ cell degeneration in the seminiferous epithelium. Studies of seminal plasma from fertile and infertile men have shown that when LDH-C4 activity is expressed relative to the number of spermatozoa it appears to relate to the degree of germ cell degeneration (Eliasson & Virji, 1985). Activity was especially high in men taking sulphasalazine, an

inhibitor of prostaglandin metabolism which often causes testicular dysfunction, and in infertile men who took daily hot baths. LDH-C4 is undetectable in men with severe oligozoospermia or with azoospermia due to seminiferous tubule lesions or vasectomy, indicating that measurement of LDH-C4 cannot diagnose individuals where germ cell degeneration is not the cause of a low sperm count (Orlando *et al.*, 1988).

There is no straightforward correlation of seminal plasma LDH-C4 levels with sperm motility, morphology or FSH levels (Virji, 1985; Orlando *et al.*, 1988). However, it was found that semen samples with a high LDH-C4/sperm ratio had low nuclear chromatin stability and decreased resistance to lipid peroxidation suggesting that functional properties of the spermatozoa may be related to seminiferous tubule status (Virji & Eliasson, 1985). Several studies have shown that LDH-C4 activity is significantly increased in oligozoospermic in comparison to normozoospermic men (Casano *et al.*, 1991; Velasco *et al.*, 1993; Orlando *et al.*, 1994). The explanation for this difference is unknown but it has been suggested that it could be a reflection of increased numbers of immature germ cells or the presence of residual cytoplasm on immature spermatozoa (Casano *et al.*, 1991). A recent study suggests that LDH-C4 activity may not be the most suitable marker to discriminate between oligozoospermic and normozoospermic men (Orlando *et al.*, 1994). Further investigation is necessary to ascertain whether measurement of LDH-C4 in seminal plasma is a suitable marker of the functional status of the seminiferous epithelium.

2.5.2. Evaluation of testicular proteins in blood

This has proved to be problematic for Sertoli cell proteins due to specificity. Many Sertoli cell proteins share homology with proteins produced at other sites in the body which are also present in blood. Transferrin levels in seminiferous tubule fluid and rete testis fluid are much lower than the levels present in serum, indicating that the majority of circulating transferrin originates from sources other than the testis (Sylvester & Griswold, 1984). This is supported by the fact that there are no differences in serum transferrin levels between normal, oligozoospermic and azoospermic men (Orlando *et al.*, 1985; Ber *et al.*, 1990). Testicular ceruloplasmin shares homology with the serum form of the protein since it can be immunoprecipitated with an antiserum raised against serum ceruloplasmin (Skinner & Griswold, 1983). As with transferrin, no difference in serum ceruloplasmin levels could be demonstrated between normal and azoospermic men (Orlando *et al.*, 1985). ABP shares immunodeterminants with human sex hormone-binding globulin (SHBG) which is secreted by the liver (Cheng *et al.*, 1984). The amino acid sequence of both proteins also shows considerable homology at the amino terminus (Joseph *et al.*, 1987) and it has now been demonstrated that both proteins are encoded by

the same gene (Hammond *et al.*, 1989). Biochemical analysis of human ABP has shown that there are two forms in the human testis which can be distinguished by their ability to bind to concanavalin A due to differences in their peptide sequence and carbohydrate structure (Cheng *et al.*, 1985). Comparison with serum SHBG suggested that while one form was different to SHBG the other was very similar but did show a difference in lectin binding ability, suggesting that it might be possible to distinguish both forms of ABP from SHBG. Another Sertoli cell secreted protein called testibumin was first identified in rats based on its immunological similarity to albumin (Cheng & Bardin, 1986); this protein has now been shown to be identical to SGP-1 based on sequence analysis (Mathur *et al.*, 1994). The antiserum raised against testibumin cross-reacts with a protein in human serum and testicular cytosol but since the antibody only recognises albumin when it is denatured this protein must be distinct from albumin (Cheng *et al.*, 1987). The measurement of SGP-2 in blood is complicated by the fact that this glycoprotein is expressed in many tissues including brain, pituitary, liver, kidney and prostate (Jenne & Tschopp, 1992). In humans there is a complement cytolysis inhibitor present in blood which is identical to SGP-2 (Jenne & Tschopp, 1989). There is some evidence that SGP-2 shows tissue specific glycosylation patterns which may affect its function. Serum SGP-2 has a lower molecular weight than the testicular form but they have not been distinguished immunologically (Cheng *et al.*, 1988). However, serum SGP-2, unlike testicular SGP-2, is unable to induce cell aggregation but when the testicular protein is deglycosylated this ability is lost (Cheng *et al.*, 1988). Analysis of the terminal carbohydrate structure of SGP-2 in the testis, epididymis, ventral prostate and seminal vesicle has also demonstrated molecular heterogeneity (Sensibar *et al.*, 1993). At present, assays are unable to discriminate between proteins secreted by Sertoli cells and those in the circulation derived from other sites in the body, but biochemical analysis of some of these proteins suggests that there are differences either in the amino acid sequence or in carbohydrate structure. This might enable specific antisera to be developed which could used to provide an unequivocal answer as to whether Sertoli cell function can be monitored in blood. The other problem is that the function of most of these proteins in relation to spermatogenesis is not clear, which limits their ability to inform on specific interactions necessary for germ cell development.

The main candidate for a marker of testicular function which can be measured in blood is inhibin. It has proved very difficult to establish that inhibin in adult men is involved in the regulation of pituitary FSH secretion. Serum inhibin levels have been measured in normal men and men with testicular disorders resulting in infertility, but no significant difference was observed between the groups and there was no correlation between serum concentrations of inhibin and FSH (de Kretser *et al.*, 1989). A similar situation was found when inhibin levels were measured in normal men and men with

varicoceles and elevated FSH levels (Plymate *et al.*, 1992). This raised questions concerning what the levels of inhibin in the peripheral circulation reflects. Investigation of the expression of the inhibin subunits in various tissues of the rat has shown that inhibin is produced by brain, pituitary, bone marrow, kidney and spinal cord (Meunier *et al.*, 1988). It has been reported that rat Leydig cells in culture produce immuno and bioactive inhibin (Risbridger *et al.*, 1989). However, other evidence suggests that inhibin from this source does not provide a significant contribution to the peripheral circulation (Maddocks & Sharpe, 1989c) and another study failed to demonstrate the secretion of bioactive inhibin, only the α-subunit was expressed, by normal Leydig cells (de Winter *et al.*, 1992). Inhibin in the circulation of men is thought to be of testicular origin since immunoactive inhibin levels are higher in spermatic venous than in peripheral venous blood (Ishida *et al.*, 1990; Maddocks *et al.*, 1993) and castration results in a rapid and significant decline in serum inhibin levels (Ishida *et al.*, 1990). The major problem in the measurement of circulating inhibin is the specificity of the assay used. It is now recognised that inhibin can exist in several forms in human plasma (Robertson *et al.*, 1995). The inhibin-α subunit is synthesised as a pre-pro-protein (pro-αN/αC) and the β-subunits as pro-proteins; these are cleaved to give free α-subunits and inhibin αβ dimers but only the dimeric form is bioactive. It has now been established that most of the existing studies on inhibin in men have used an assay unable to distinguish between dimeric inhibin and the free α-subunit (Schneyer *et al.*, 1989; Robertson *et al.*, 1989). Therefore to assess whether inhibin is a useful marker of seminiferous tubule function requires an immunoassay which is specific for bioactive inhibin. A two site dimer specific immunoassay has been developed (Groome & O'Brien, 1993). When this assay was used to investigate inhibin levels in men it was found to be almost undetectable and no change in serum concentration was observed in men who were GnRH deficient or had Klinefelter's syndrome (Lambert-Messerlian *et al.*, 1994). This assay has been adapted to discriminate between inhibin-A and inhibin-B, and it has been shown that during the menstrual cycle of women the forms of inhibin exhibit different secretion patterns (Groome *et al.*, 1994). It has been shown that in humans the expression of α-inhibin mRNA but not inhibin βB mRNA can be upregulated by FSH, indicating that inhibin subunits are differentially hormonally regulated (Namiki *et al.*, 1993). This suggests that in order to gain a clearer picture of the role of inhibin in the male both forms of dimeric inhibin need to be measured. At present there is no clear demonstration that inhibin can be used to monitor spermatogenesis, but now that the problem of assay specificity is being addressed an unequivocal evaluation of inhibin seems attainable.

The problems encountered in measuring Sertoli cell proteins in blood suggest that germ cell proteins may provide a better source of suitable markers of spermatogenesis, if it can be demonstrated that these proteins can gain access to the circulation (Sharpe,

1992). There is interesting evidence in rats which have shown that the induction of germ cell degeneration by the administration of testicular toxicants resulted in elevated levels of LDH-C4 activity in peripheral blood for two weeks after treatment, and these changes preceded histological damage to the testis (Reader *et al.*, 1991). This suggests that LDH-C4 could be a marker of actively degenerating germ cells and it would be of interest to evaluate the ability of this protein to indicate ongoing germ cell damage in humans.

2.5.3. Future prospects

At present no protein has been identified, for which the measurement in either seminal plasma or blood is of practical use for monitoring the process of spermatogenesis. This is partly because information on the function and relevance to spermatogenesis of the testicular proteins characterised so far is very limited, and it is unclear if any of these proteins are involved in a specific step of spermatogenesis. This suggests that a different approach may be necessary involving the identification of proteins which play key roles in spermatogenesis in both experimental animals and man (McKinnell *et al.*, 1995). Sensitive assays would have to be developed to assess whether these proteins can be measured in semen or blood and to evaluate their ability to provide meaningful information on spermatogenesis when it is disrupted. If this approach yields positive results then it would indicate the suitability of applying similar studies to men. Based on the information reviewed in the previous two sections, germ cell proteins rather than Sertoli cell secreted proteins may have more potential to be useful markers of spermatogenesis.

2.6. Testicular damage

The induction of damage to specific cell types within the testis is an important *in vivo* approach to elucidate the cellular interactions necessary for normal testis function. Testicular toxicants with specific sites of action can be used as biochemical tools with which to dissect the normal Sertoli cell-germ cell interactions which comprise spermatogenesis and to investigate Leydig cell-seminiferous tubule interactions. Several toxicants have been identified which induce damage to specific cell types within the testis, these include methoxyacetic acid (MAA), ethane dimethane sulphonate (EDS), *meta* dinitrobenzene (mDNB) and nitrobenzene (NB). In addition, short term scrotal heating (43°C for 30 min) has also been demonstrated to induce specific testicular damage. The sections below discuss these methods of inducing damage to the Sertoli cells, germ cells and Leydig cells. The cellular site(s) of damage, what is known about the mechanism of

induction and the potential uses of these methods in the delineation of interactions necessary for the maintenance of spermatogenesis are reviewed.

These methods of testicular perturbation are used as tools within the studies described in this thesis to aid the identification of markers of spermatogenesis. A non-invasive marker of spermatogenesis being defined as a measurable biochemical substance the levels of which in blood change in response to disruption of the process of spermatogenesis. Ideally, the marker would be associated with a particular cell type within the seminiferous epithelium and be able to provide information on the point of impairment of spermatogenesis. The aim of the studies described in chapter 4 is to evaluate the feasibility of using creatine as a non-invasive marker of acute testicular damage in the rat, since other studies have demonstrated that levels of urinary creatine are increased following testicular damage. The induction of damage to the Sertoli cells, germ cells or Leydig cells in rats by different approaches provides a way of assessing whether testicular damage does induce significantly increased plasma creatine levels in a reproducible manner. At the same time, it allows evaluation of whether creatine is a marker of damage to a specific cell type. Chapter 5 describes the studies carried out to ascertain, using Western blot analysis, whether proteins derived from the seminiferous tubules are able to gain access to testicular interstitial fluid and/or blood in the normal physiological situation, and whether this is altered by the induction of damage to spermatogenesis. For this work, local testicular heating was chosen to induce testicular damage since this is a well established method of inducing major disruption of spermatogenesis by non-chemical means. In addition, heat-treatment has been shown to alter the pattern of secretion of Sertoli cell proteins such that increased levels of ABP and inhibin can be measured in interstitial fluid.

2.6.1. Induction of damage to Sertoli cells

A Sertoli cell toxicant is described as a toxicant which induces damage *in vivo* to Sertoli cells before any others signs of disruption to the testis can be observed. Sertoli cell toxicants also induce degeneration and loss of germ cells but this is a secondary effect caused by the alteration of Sertoli cell function. Therefore, it is important to identify accurately the earliest changes induced by a toxicant within the testis to ascertain its specific site of action. The two toxicants discussed in this section are the nitroaromatics, *meta* or 1,3 dinitro benzene (mDNB) and nitrobenzene (NB). Other Sertoli cell toxicants have been described such as the γ-diketones and phthalates but these are outside the scope of this literature review (reviewed by Boekelheide, 1993).

mDNB

mDNB is widely used by the chemical industry as an intermediate in the manufacture of dyes and explosives (Allenby, 1990). The most common result of human exposure to mDNB is methaemoglobinaemia and anaemia. Several studies have investigated the histopathology of the rat testis following administration mDNB and shown that the primary lesion observed is Sertoli cell damage (Blackburn *et al.*, 1988; Hess *et al.*, 1988; Allenby, 1990). A single oral dose of 25mg/kg resulted in vacuolation and retraction of Sertoli cell cytoplasm in the region of the primary spermatocytes in seminiferous tubules at stages VIII to XI within 12-24 hours following administration (Blackburn *et al.*, 1988). This initial Sertoli cell damage was followed by the degeneration and exfoliation of pachytene and secondary spermatocytes leading to a reduction in the number of round spermatids within the seminiferous epithelium. Similar changes were observed in rats given a single oral dose of 48mg/kg mDNB but this study also reported changes in chromatin conformation in the spermatids and the retention of step 19 spermatids (Hess *et al.*, 1988). Further support for mDNB being a Sertoli cell toxicant comes from *in vitro* studies (Foster *et al.*, 1986; 1987a; Cave & Foster, 1990). The addition of mDNB to Sertoli-germ cell co-cultures resulted in Sertoli cell vacuolation, phagocytosis of degenerating spermatocytes and the dose-dependent exfoliation of germ cells from the Sertoli cell monolayer was observed (Foster *et al.*, 1986; 1987a). The addition of mDNB to Sertoli cells in culture for 24 hours was found to cause a dose-dependent and simultaneous increase in the secretion of lactate and pyruvate in comparison to controls (Williams & Foster, 1988). The production of lactate and pyruvate by the Sertoli cell is thought to act as the main supply of energy for the germ cells, since germ cells *in vitro* cannot be fully supported by glucose but are able to utilise lactate and pyruvate to maintain their ATP levels (Jutte *et al.*, 1981; 1982; Grootegoed *et al.*, 1984). This provides further evidence that the Sertoli cells are the site of mDNB action.

Investigation of the metabolism of mDNB *in vitro* by Sertoli cells and Sertoli-germ cell co-cultures has been used to elucidate the possible mechanism of action of this toxicant. It was found that mDNB undergoes nitroreduction to form two major metabolites, 3-nitroaniline and 3-nitroacetanilide (Foster *et al.*, 1986). However, the administration of either of these metabolites to cultures at relevant concentrations gave no evidence of toxicity. These results suggested than an intermediate metabolite formed during nitro reduction was responsible for toxicity. Cave and Foster (1990) proposed a scheme for mDNB reduction via a variety of intermediates, which included a nitroxyl anion radical, 3-nitrosonitrobenzene and 3-nitrophenylhydroxylamine. Using germ cell exfoliation from Sertoli cell monolayers as a marker of toxicity, 3-nitrosonitrobenzene was found to be a more potent toxicant than mDNB (Cave & Foster, 1990). These authors also demonstrated that toxicity was increased by the addition of diethyl maleate

and decreased in the presence of ascorbate or cysteamine, which suggests that toxicity is a result of a free radical mediated mechanism. It has been proposed that the mechanism of Sertoli cell damage is due to redox cycling of the mDNB metabolites, 3-nitrosonitrobenzene and 3-nitrophenylhydroxylamine. This causes the depletion of the Sertoli cell's reducing agents, NADPH and glutathione, which ultimately results in cell death (Foster *et al.*, 1989; Ellis & Foster, 1992).

NB

NB is an important chemical employed in industry for many purposes. These include the production of dyes, solvents, propellants, several aromatic nitro- and amino-compounds as well as being used as an inexpensive oxidising agent (Allenby, 1990). Exposure to NB results in toxic effects to the blood, central nervous system, liver and reproductive organs (Allenby *et al.*, 1990). Histological analysis of rat testes following a single oral dose of 300mg/kg NB was found to induce a similar pattern of damage as observed with mDNB (Allenby, 1990). Within 24 hours of exposure to NB vacuolation of Sertoli cells and the seminiferous epithelium had occurred. Stages VII to XII were particularly affected showing increased vacuolation and numerous degenerating pachytene spermatocytes in comparison to the other stages of the spermatogenic cycle. At later time points, germ cell degeneration and exfoliation became more apparent and the chromatin in the nuclei of round spermatids became more diffuse, indicative of fragmentation. The similarity of testicular histopathology induced by NB in comparison to mDNB suggests that NB is also a Sertoli cell toxicant but this hypothesis was further investigated using *in vitro* studies in which mDNB was also included for comparison of effects observed with the two toxicants. The treatment of Sertoli cell and Sertoli-germ cell cocultures with NB induced Sertoli cell vacuolation and dose-dependent germ cell exfoliation (Allenby *et al.*, 1990). NB was also found to stimulate lactate and pyruvate secretion by Sertoli cells. In this study another parameter of Sertoli cell function, inhibin secretion, was also measured and NB was found to have a biphasic effect such that low and high doses increased secretion in both culture systems. From these results it was concluded that NB is probably a Sertoli cell toxicant, although it is far less toxic than mDNB at equivalent concentrations *in vitro*. The cellular mechanism of NB action on the Sertoli cell has yet to be resolved, but since NB and mDNB have related structures and induce similar effects on Sertoli cell function, it is probable that NB induces toxicity via a similar mechanism to mDNB.

Application to the study of spermatogenesis

Sertoli cell toxicants provide a means of investigating Sertoli cell functions which are important for germ cell viability. The administration of either mDNB or NB *in vivo*

results in the initiation of germ cell degeneration within 24 hours of treatment, so the use of either of these toxicants provides a model system which can be used to investigate the acute changes in Sertoli cell function which result in germ cell death. This would help to delineate the Sertoli-germ cell interactions which occur when spermatogenesis is not disrupted. This could involve assessing the effects of these toxicants on the secretory and structural functions of the Sertoli cell. It is likely that germ cell degeneration is the result of many alterations in Sertoli cell function such as the secretion of proteins and metabolic substrates, cytoskeletal organisation, sites of attachment between germ cells and Sertoli cells and the blood testis-barrier. Investigation of the effects of toxicants on Sertoli cell secretion may also help to elucidate potential markers of altered cell function which would be useful in evaluating the effects of other chemicals and drugs on Sertoli cell function in toxicity studies.

2.6.2. Induction of damage to germ cells

In this section, two methods of inducing damage to specific classes of germ cells are described. The first method is the oral administration of MAA and the second method involves the exposure of the testes to local heating (43°C for 30 min). The cellular site of the lesion induced by these treatments and the mechanism of how they induce toxicity to the germ cells is discussed below.

MAA

MAA is the major metabolite of ethylene glycol monomethyl ether (EGME), a volatile, water miscible organic solvent and emulsifier which is used extensively in the manufacture of surface coatings (paints, lacquers, varnishes etc.), printing inks, textile dyes, hydraulic brake fluids and liquid cleaning products. The major targets of glycol ether toxicity are the central nervous, renal and haemopoietic systems in both animals and man (Allenby, 1990). Two studies have demonstrated a decreased average sperm count in workers exposed to 2-methoxyethanol (2-ME; MAA being the metabolite of this compound) in comparison to controls indicating that the testis is also affected (Ratcliffe *et al.*, 1989; Welch *et al.*, 1988). Administration of either MAA or EGME to rats has been shown to specifically deplete the seminiferous epithelium of pachytene primary spermatocytes (Foster *et al.*, 1983; 1984; Creasy *et al.*, 1985). Foster *et al.* (1983) were the first to report the localisation of the primary site of cellular damage within the testis. Following a single oral dose of 100mg/kg EGME 24 hours earlier, the degeneration of pachytene spermatocytes was observed. The administration of a higher dose (500mg/kg) also showed damage restricted to the spermatocytes evident at 16 hours, and further examination of these cells showed swelling and disruption of mitochondria, cytoplasmic

vacuolation and early condensation of nuclear chromatin. The morphology of all other germ cells and of the Sertoli and Leydig cells was unaffected. These authors also described similar testicular histopathology when rats were administered corresponding doses of MAA suggesting that this metabolite is the active compound responsible for testicular damage. Quantitative analysis of spermatocyte damage was performed following a single oral dose of 250 mg/kg MAA (Creasy *et al.*, 1985). This study showed that there was a sharp transition in germ cell susceptibility to MAA. Pachytene spermatocytes at stage I were affected but not zygotene spermatocytes at stage XIV, and a similar change was seen between dividing spermatocytes at stage XIV and step 1 spermatids which were also not affected by this treatment. In addition, pachytene spermatocytes at stage VII were found to be resistant to toxicity.

Bartlett *et al.* (1988) investigated the consequence of removal of the pachytene spermatocytes on the kinetics of spermatogenesis and on hormone levels for up to 70 days after a single oral dose of 650mg/kg MAA. This study confirmed previous findings on the selectivity of MAA damage to the seminiferous epithelium. After the loss of pachytene spermatocytes, spermatogenesis was found to progress normally in unaffected seminiferous tubules and in those tubules lacking, or with a reduced complement of germ cells. The initial depletion of pachytene spermatocytes meant that 14 days later, round spermatids were depleted from the seminiferous epithelium and elongated spermatids at 21 days. By 70 days testis morphology was found to be qualitatively normal. In addition to a morphological analysis of testicular damage this study also measured testosterone, LH, FSH and ABP levels. Serum FSH and interstitial fluid ABP levels were increased at 3 and 21 days post-treatment indicating that Sertoli cell function was altered. These decreases corresponded with the times of greatest decrease in testicular weight. Testosterone and LH levels remained similar to those measured in the controls.

The effect of selective depletion of pachytene spermatocytes on sperm output and fertility in rats has also been assessed (Ratnasooriya & Sharpe, 1989). Motile sperm output was found to be reduced by approximately 85% at 5-6 weeks after MAA treatment (650mg/kg), however there was no evidence of infertility in the treated male rats during a serial mating trial. These results confirm that spermatogenesis can still proceed even when its normal organisation has been disrupted by the selective depletion of pachytene spermatocytes.

The mechanism of MAA-induced degeneration of pachytene spermatocytes has been investigated *in vitro*. Beattie *et al.* (1984) added MAA to Sertoli cell cultures to ascertain whether lactate production was altered, since pachytene spermatocytes are dependent on the production of lactate by Sertoli cells for viability (Jutte *et al.*, 1982). MAA-treatment was found to significantly reduce lactate levels during the 12 hour culture period, suggesting that inhibition of lactate production by MAA might be a possible

explanation for its toxicity. However, the addition of lactate to Sertoli-germ cell co-cultures treated with MAA failed to prevent germ cell degeneration, indicating that the mechanism of MAA-induced toxicity is via another route (Gray *et al.*, 1985).

It now seems more likely that MAA is affecting RNA synthesis by pachytene spermatocytes. Mebus *et al.* (1989) demonstrated that the concurrent administration of serine to rats treated with 2-methoxyethanol was found to prevent germ cell degeneration. Acetate, sarcosine and glycine were able to significantly reduce degeneration but not eliminate it completely. These compounds are linked to metabolic pathways involving one-carbon units, and these are needed for purine and pyrimidine base synthesis. Mebus *et al.* (1989) have proposed that MAA could be affecting the availability of one-carbon units which results in decreased synthesis of the bases necessary for DNA and RNA synthesis. In comparison to the other germ cell types, mid-to late stage pachytene spermatocytes show the greatest capacity for RNA synthesis (Monesi *et al.*, 1978). So these cells might be more susceptible to alterations in the supply of nucleic acid precursors.

Local testicular heating

The effects of local testicular heating on the seminiferous epithelium in the rat are described briefly in this section. This method of disrupting spermatogenesis is discussed in more detail in the introduction to chapter 5. It is well established that elevation of testicular temperature results in impaired spermatogenesis, the severity of damage depending on the degree of elevation of temperature and its duration. Quantitative analysis of the seminiferous epithelium of rat testes which had been previously exposed to 43°C for 15 minutes has demonstrated that this treatment induces selective germ cell degeneration (Chowdhury and Steinberger, 1964; 1970). Within 48 hours following treatment, with the exception of pachytene spermatocytes at stages V and VI, spermatocytes from the leptotene stage onwards to dividing spermatocytes at stage XIV and step 1 and 2 round spermatids were all depleted from the seminiferous epithelium. Examination of testicular morphology at much earlier time points following heat-treatment showed that 1 hour later, alterations could be observed in spermatocytes at stages IX to XIV and step 1 spermatids. This indicates that these germ cell types are the most susceptible to heat stress. More recently, the damage induced by exposure of the scrotum to 43°C for 30 minutes has been investigated (Bartlett and Sharpe, 1987; Sharpe & Bartlett, 1987; McLaren *et al.*, 1994). Pachytene spermatocyte degeneration was found to be more widespread but still stage specific at 24 hours after heat-treatment. In addition, at stages VI to VIII the step 19 spermatids were observed to be embedded deep within the seminiferous epithelium rather than in the normal position surrounding the edge of the tubule lumen. Vacuoles were evident in some of the seminiferous tubules.

Changes in cellular metabolism are thought to be responsible for mediating heat induced damage to the seminiferous epithelium. A recent study has shown that at 4 hours after scrotal heating (43°C for 30 minutes) there is a significant increase in the incorporation of radiolabelled methionine into both secreted and intracellular proteins at stages VI-VIII but not in seminiferous tubules at stages II-V or IX-XII (McLaren *et al.*, 1994). In contrast, at 24 hours after treatment there was a significant reduction in protein secretion at stages VI-VIII and also in methionine incorporation into intracellular proteins within all three stage groupings of cultured seminiferous tubules. Administration of testosterone was unable to prevent the latter decrease in protein secretion. Measurement of oxygen uptake in rats maintained at 41°C for 90 minutes showed that testicular oxygen consumption doubled in comparison to controls and was sustained for at least 60 minutes (Main & Waites, 1977). There is limited evidence from cryptorchid rats that the activity of enzymes involved in glycolysis is affected by heat (Ewing & Schanbacher, 1969). These authors found a significant decrease in phosphofructokinase activity within 8 hours of exposure to abdominal temperature which preceded alterations in other enzyme activities and morphological evidence of germ cell damage. Phosphofructokinase is the most important enzyme in the control of the rate of glycolysis, so that a decrease in the activity of this enzyme would result in decreased production of ATP. All of these observations suggest that heating induces a rapid upregulation of cellular metabolism which is supported initially by increased oxygen and energy consumption, however, this is not accompanied by an increase in blood flow so the testis enters a state of self induced hypoxia.

Application to the study of spermatogenesis

Both MAA treatment and testicular heating provide methods of specifically depleting germ cells from the seminiferous epithelium which allows the investigation of germ cell modulation of Sertoli cell function. However, MAA provides the most useful tool for manipulating Sertoli-germ cell interactions since the damage it induces to the seminiferous epithelium is more selective than observed following heat treatment. A single dose of 650mg/kg MAA results in degeneration of one type of germ cell, namely, the pachytene spermatocytes with the exception of those at early to mid stage VII. Following treatment, spermatogenesis proceeds with normal kinetics such that the absence of other germ cells from the seminiferous epithelium at later time points is due to maturation depletion i.e. the absence of more mature germ cells is the result of the initial degeneration of the pachytene spermatocytes. Local testicular heating (43°C for 30 minutes) induces more widespread damage, with both primary spermatocytes and early spermatids initially affected. Spermatogenesis does continue following this damage but the loss of germ cells is too

extensive to permit precise conclusions as to the modulatory effects of individual germ cell classes, as is possible with MAA.

2.6.3. Induction of damage to Leydig cells

The selective destruction of Leydig cells in the rat testis using EDS is a well characterised model system for investigating the role of Leydig cells and testosterone in the regulation of spermatogenesis. Examination of testicular morphology using both light and electron microscopy has demonstrated that a single intraperitoneal injection of EDS (75mg/kg) results in complete destruction of all Leydig cells within 1-3 days (Kerr *et al.*, 1985; 1986; Molenaar *et al.*, 1985). Analysis using light microscopy at 24 hours after EDS treatment, showed that the Leydig cells were fragmented to varying degrees and the presence of extensive networks of fibrous tissue within the interstitium (Kerr *et al.*, 1985). However, by 3 days later the Leydig cells and fibrous material had disappeared completely from the testis. At 7 days after treatment Leydig cells remained absent from the interstitium but degenerating germ cells were seen in some stage VII and VIII seminiferous tubules. By 28 days fetal type Leydig cells could be observed. A morphological study has described the presence of spindle-shaped interstitial cells in association with blood vessels and seminiferous tubules 3 weeks after EDS treatment and fetal type Leydig cells were observed in the same location (Kerr *et al.*, 1987). These elongated interstitial cells were proposed as possible precursors of the regenerating Leydig cell population. A pulse-chase experiment has demonstrated that mesenchymal-like interstitial cells rather than macrophage, endothelial or myoid cell populations differentiate into adult type Leydig cells (Hardy *et al.*, 1989). Kerr *et al.* (1986) then studied the induction of Leydig cell damage at the ultrastructural and hormonal level during the 3 days following EDS administration . Alterations to the morphology of smooth endoplasmic reticulum and Golgi apparatus were apparent as early as 6 hours after treatment but changes in nuclear structure were not visible until 12 hours. By 24 hours the Leydig cells were in an advanced state of necrosis and many of these cells were being phagocytosed by macrophages. The structural changes between 6-24 hours were reflected in significant reduction in serum testosterone which became undetectable by day 3. During the same time period, levels of serum LH and FSH became significantly elevated, indicative of diminished negative feedback by testosterone on gonadotrophin secretion. Morphological examination of the seminiferous epithelium at various time points following EDS treatment has shown there is progressive disruption of spermatogenesis between 3 and 24 days (Bartlett *et al.*, 1986). Maximum disruption of spermatogenesis was observed at 2 weeks, at which time the seminiferous tubules either lacked one or more germ cell generations or degenerating germ cells and extracellular

spaces were evident. Spermatogenesis recovered at 3-4 weeks in parallel with Leydig cell regeneration. Qualitatively normal spermatogenesis was observed in the majority of seminiferous tubules at 10 weeks after treatment.

The mechanism by which EDS, an alkylating agent, induces Leydig cell cytotoxicity is unclear but two studies suggest that glutathione is involved. Kelce and Zirkin (1993) found that reducing intracellular levels of glutathione with buthionine sulfoximine (BOS), an inhibitor of glutathione synthesis, allowed Leydig cells to maintain LH stimulated testosterone production and incorporation of radiolabelled methionine during a 3 hour incubation with EDS *in vitro*. Pretreatment with BOS also reduced the extent to which radiolabelled EDS could alkylate Leydig cell proteins. In contrast, the protective effect of BOS was abolished when intracellular levels of glutathione were restored by the addition of glutathione ethyl ester. These results led the authors to propose that glutathione might be involved in regulating the ability of EDS to induce toxicity by the alkylation of Leydig cell proteins. This was followed by a second study, the aim of which was to establish whether BOS could prevent EDS-induced Leydig cell death *in vitro* (Kelce, 1994). Leydig cells were preincubated with BOS or medium for 12 hours, the cells were then exposed to EDS for 3 hours and the cytotoxic effect of this toxicant was assessed 24 hours later. Leydig cell death was assessed using a cytotoxicity assay, LH-stimulated testosterone production and methionine incorporation. Preincubation with BOS was found to protect Leydig cells from EDS-induced death, whilst the addition of glutathione ethyl ester in the presence of BOS restored the ability of EDS to induce cell death. These results suggest that glutathione is involved in the mechanism of EDS-induced Leydig cell death *in vitro*. It remains to be determined how increased levels of glutathione are related to alkylation and whether this proposed mechanism is operating *in vivo*.

Application to the study of spermatogenesis

EDS-treatment is a widely used model for investigating the roles of testosterone and Leydig cells in normal spermatogenesis and testicular function, and also the factors regulating Leydig cell proliferation and maturation. It has proved especially useful since EDS induced cytotoxicity is specific to Leydig cells but to no other cell types within the testis. This model has helped to establish the critical importance of testosterone for the maintenance of spermatogenesis, and is one of the approaches being used to elucidate the mechanism of testosterone's action on this process.

Chapter 3. General Materials and Methods

3.1. Animals

Animals used for these studies were mainly male Wistar rats (adult, aged 65-90 days or immature/pubertal, aged 7-44 days) bred in the MRC Reproductive Biology Unit in Edinburgh. Adult female Wistar rats were used to provide tissues for RNA extraction or *in situ* hybridisation. Rats were maintained under standard conditions of 12 h light:12 h dark and an ambient temperature of 21°C. Food and water were available *ad libitium*. Animals were killed by asphyxiation with CO_2 followed by cervical dislocation.

3.2. Treatments

3.2.1. Scrotal heating

Scrotal heating was performed with the help of Dr. Tanya McLaren and Mr. Denis Doogan. Short term scrotal exposure to heat has been shown to severely disrupt spermatogenesis, specifically inducing the loss of pachytene spermatocytes and early spermatids (Chowdhury & Steinberger, 1964, 1970; Bartlett & Sharpe, 1987; McLaren *et al.*, 1994).

Rats were anaesthetized with an i.p. injection of 3.3ml/kg of a combination of Hypnorm (Janssen Animal Health, Wantage, UK) and Hypnovel (Roche Products Ltd., Welwyn Garden City, UK). The anaesthetic was made up as follows, the Hypnorm (10mg/ml) was diluted with an equal volume of sterile water as was the Hypnovel (2mg/ml), then both were mixed together. When anaesthetized, the lower half of the rat's body, including the scrotum, was immersed for 30 min in a thermostatically controlled water bath maintained at 43°C, while the animals were strapped with velcro ties to a supporting frame. At 24 h after treatment the animals were killed, a sample of peripheral venous plasma was taken and the testes removed for collection of IF as described in section 3.3.1. Heat treatment was performed on two separate occasions and each treatment group contained four adult rats. IF and PV samples were also collected from four sham-treated (anaesthetized but not heat exposed) control rats from the same litter or of the same age.

3.2.2. Ethane dimethane sulphonate (EDS)

Injections of EDS were administered by Dr. Richard Sharpe. This treatment completely destroys Leydig cells within 36 hours with the result that testosterone levels become undetectable (Bartlett *et al.*, 1986; Sharpe *et al.*, 1990).

Rats received a single intraperitoneal injection of 75mg/kg of EDS in dimethylsulphoxide:water (1: 3; v/v). In some EDS-treated rats, 25mg testosterone esters

(Sustanon: Organon Laboratories, Cambridge, UK) were administered subcutaneously in 0.1ml arachis oil every 3 days, beginning on day 0. This treatment has been shown to prevent the adverse changes in spermatogenesis induced by testosterone withdrawal (Sharpe *et al.*, 1988a,b; 1990). Rats were killed by inhalation of CO_2 followed by cervical dislocation at either 4 or 6 days after initial treatment. Rats killed 4 days after EDS-treatment were used in studies investigating the usefulness of creatine as a marker of testicular damage (see chapter 4). Testes were removed for seminiferous tubule isolation (see sections 3.4 and 4.2) and IF collection (see sections 3.3 and 4.2); peripheral blood samples were also collected from some rats (see section 3.3). The testes were removed from rats killed 6 days after EDS-treatment for RNA extraction (see section 3.14.) and for fixation and processing (section 3.11).

3.2.3. Methoxyacetic acid (MAA)

MAA (Aldrich Chemical Co. Ltd., Dorset, UK) was freshly prepared before administration as follows: 10.8mls of MAA (pH 0.5) were adjusted to pH 7.0-7.4 with concentrated sodium hydroxide and the volume made up to 45mls by the addition of normal saline (0.9% w/v sodium chloride). Young adults rats aged approximately 75 days were administered MAA by oral gavage as a single dose of 650mg/kg. Control animals were given an equivalent volume of saline.

Administration of MAA at this dose level has been shown to result in selective depletion of 80-100% of pachytene and later spermatocytes at all stages of the spermatogenic cycle except early to mid stage VII (Bartlett *et al.*, 1988). Spermatogenesis then proceeds with normal kinetics such that at selected time points after MAA-treatment, round and then elongate spermatids are selectively absent due to maturation depletion (Bartlett *et al.*, 1988; Allenby, 1990; Maguire, 1994).

At the following time points after MAA treatment, specifically 3, 7, 14, 21 and 28 days, animals were killed by inhalation of CO_2 followed by cervical dislocation and testes were removed for RNA extraction (see section 3.14.) and fixation and processing for *in situ* hybridisation (section 3.11). Animal were also killed at different time points for the isolation of seminiferous tubules which were used in studies to evaluate the relationship of testicular creatine to spermatogenesis (see chapter 4).

3.2.4. Nitrobenzene (NB)

Dosing was performed with the help of Dr. Tanya McLaren and Mr. Denis Doogan. NB, shown to be 99% pure by gas chromatography and mass spectrometry, was obtained from Sigma (Poole, Dorset, UK). Young adult rats aged approximately 70 days were administered a single oral dose of 300mg/kg of NB made up in corn oil (1ml/kg) by oral gavage. This dose of NB has been shown previously to initially cause Sertoli cell

damage which induces degeneration of germ cells as a secondary event (Allenby, 1990; McLaren, 1993). At 24 hours after treatment blood plasma and testicular IF were collected (see section 3.3).

3.2.5. meta-Dinitrobenzene (mDNB)

Dosing was performed with the help of Dr. Tanya McLaren and Mr. Denis Doogan. mDNB with a greater than 99% purity was obtained from Aldrich Chemical Co. (Gillingham, Dorset, UK). Young adult rats aged approximately 70 days were administered a single oral dose of 50mg/kg mDNB in 1ml/kg polyethylene glycol 600 (PEG 600; Sigma) by oral gavage. This dose of mDNB has been shown previously to cause Sertoli cell damage which induces degeneration of germ cells as a secondary event (Blackburn *et al.*, 1988; McLaren, 1993). At 24 hours after treatment blood plasma and testicular IF were collected (see section 3.3).

3.2.6. Castration

In a single experiment performed by Dr. Richard Sharpe, one adult rat was castrated under anaesthesia and then injected every 3 days with 1mg testosterone esters (Sustanon; Organon) to maintain approximately normal PV blood levels of this steroid. Two weeks after operation, the animal was killed and PV blood collected as described below.

3.3. Collection of samples

Collection of seminiferous tubule fluid, rete testis fluid and the various plasma samples were performed with the help of Dr. Richard Sharpe. The following samples were collected to evaluate whether seminiferous tubule secreted proteins could be detected in these samples and used to monitor spermatogenesis.

3.3.1. Interstitial fluid (IF)

The 'drip' method of collection of testicular IF overnight at 4°C was used (Sharpe & Cooper, 1983), with one important modification. Just prior to isolation of the testes the efferent ducts were ligated with silk thread to prevent any leakage of rete testis fluid (RTF) into the collecting IF. Rats were killed rapidly by inhalation of CO_2 followed by cervical dislocation. Immediately after removal of the testis, it was blotted to remove excess blood, and an incision was made at the caudal end of the testicular capsule taking care not to damage the underlying seminiferous tubules. The testis was then placed upright in a pre-weighed plastic tube so that the testis was suspended 1-2cm above the tube bottom and re-weighed to determine testicular weight. Fluid was allowed to drain from the testis over the next 16 h at 4°C. The testis was then removed and the tubes

centrifuged for 5 min at 1000g to precipitate any contaminating erythrocytes and the interstitial fluid volume was measured by aspiration. The aspirated fluid was stored at -40°C.

3.3.2. Rete testis fluid (RTF)

To collect RTF, normal adult rats were anaesthetized with ether and the efferent ducts of the right testis ligated with silk thread. Sixteen hours later the animals were killed by inhalation of CO_2 followed by cervical dislocation. RTF was then collected into haematocrit tubes from the swollen efferent ducts just anterior to the ligature by puncturing them carefully with a 28 gauge needle; care was taken to ensure that the RTF was not contaminated by blood. The collected RTF was centrifuged at 1000g for 5 min at 4°C and the supernatant stored at -20°C.

3.3.3. Plasma samples

For the collection of plasma samples, animals were anaesthetized with ether and heparinized by injection of 125IU heparin (Leo Laboratories Ltd., Princes Risborough, Bucks, UK) into a femoral vein. The testis was then exposed via a scrotal incision and testicular venous (TV) blood collected into heparinized haematocrit tubes by puncturing the major surface testicular vein (see Fig. 1) with a 28 gauge needle at a point just before its division into the mediastinal venous plexus at the anterior pole of the rete (Maddocks & Sharpe, 1989b). For the collection of spermatic venous (SV) blood an abdominal incision was made and the right spermatic cord exposed and dissected sufficiently to permit a small piece of tinfoil to be inserted below the cord onto which blood could collect. SV blood was then collected from the spermatic vein towards the top of the pampiniform plexus (see Fig. 1) by carefully incising one of the veins in the spermatic cord. When sufficient (0.5-0.8 ml) SV blood had been collected it was transferred into a heparinized tube. Peripheral blood was collected from the posterior vena cava using a heparinized syringe and needle, and the animal then killed by cervical dislocation. Blood samples were centrifuged at 1000g for 30 min at 4°C and the resultant plasma stored at -20°C.

3.4. Preparation of seminiferous tubule conditioned medium (STCM)

Seminiferous tubules (ST) were isolated and cultured from control adult rats using similar methods as detailed previously (Sharpe *et al.*, 1992) but with slight modifications. Testes were kept on ice until dissection, which always occurred within 2 h of death. The testis was decapsulated and placed in a small plastic tissue culture dish containing ice-cold

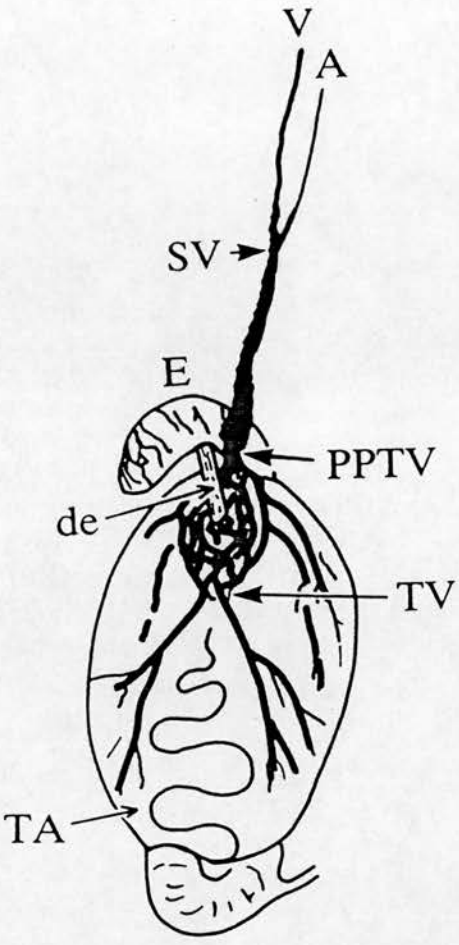


Figure 1. Diagrammatic representations of the vasculature of the rat testis. Showing the spermatic artery (A), the testicular artery (TA), the testicular veins on the surface of the testis (TV) and at the dorsal end of the spermatic cord (PPTV), the spermatic veins at the proximal end of the spermatic cord (SV), and the internal spermatic vein (V). The venous drainage is highlighted and shows the mediastinal venous plexus at the caudal pole of the testis (between TV and PPTV) surrounding the efferent ducts (de) which connect the rete testis to the epididymis (E). Sites of collection of TV and SV blood are indicated by arrows. *Adapted from Maddocks & Sharpe, 1989b.*

Dulbecco's phosphate-buffered saline (PBS; Flow labs, Irvine, Scotland) on a transparent perspex stage fitted to a dissecting microscope, through which ice-cold water was pumped continuously. Subsequent dissection took place on this stage with illumination from below. The central portion of the testis was teased apart gently with watchmakers forceps, and long ST segments (1-5cm in length) were isolated. Long lengths of tubules were isolated since it has been shown previously that their function in culture, based on the secretion of immunoactive inhibin and its response to stimulation with FSH or dibutyryl cyclic AMP, is an accurate reflection of their function *in vivo* (Allenby *et al.*, 1991b). Once sufficient tubules had been isolated they were transferred to PBS in a separate dish and any segments damaged or stretched were excluded. By reference to a transparent grid, a total of 50cm unstaged tubules were transferred to the well of a 6-well plastic culture plate containing 2ml ST culture medium consisting of M199 containing Earle's salts and sodium bicarbonate (Flow Labs) with 4mM L-glutamine (Sigma, Poole, Dorset, UK), 100 IU penicillin/ml and 100mg/ml streptomycin (Flow Labs), 25mM Hepes (Gibco, Paisley, Scotland) and 0.1% w/v polyvinyl alcohol (Sigma). ST were cultured for 24 h at 32°C under an atmosphere of 5% carbon dioxide and 95% air. After incubation, the STCM was aspirated into Eppendorf tubes containing a protease inhibitor (Aprotinin; Sigma) at 0.1% w/v final concentration, and the medium then centrifuged at 1000g for 5 min and the aspirate stored at -40°C.

3.5. Protein estimation

The protein content of test samples was estimated using a protein assay reagent (Bio-Rad) based on a dye colour change in response to protein binding (Bradford, 1976). Either a BSA or IgG standard was used depending on which was more appropriate. The reagent was diluted 1:5 with deionized distilled water (dd water) and then filtered. The standard was diluted to give a standard curve (covering the range 1500µg/ml to 75 µg/ml) using a sample volume of 50µl. Each sample was diluted to give four different dilutions, the protein concentrations of which would fall within those used for the standard curve; a sample volume of 50µl was used. Blank samples contained 50µl dd water. To all of the samples 2.5ml of protein assay reagent was added, vortexed and absorbance then measured in a spectrophotometer at 595nm using disposable cuvettes (Alpha Laboratories Ltd., Eastleigh, UK). The protein content was estimated based on the standard curve.

3.6. Production of antisera

3.6.1. Production of antiserum to STCM

A polyvalent antiserum to STCM was generated as follows. STCM was pooled from 4 rats (total =32mls) and lyophilised, then reconstituted in water and emulsified with Freund's complete adjuvant. For immunisation, two rabbits were each injected with a total of 3.5mg immunogen at 5 different intradermal sites. The animals received booster injections 9 weeks after immunisation using 2mg of immunogen in Freund's incomplete adjuvant and blood was collected 3 weeks later. The blood was allowed to clot and then centrifuged at 1000g for 30 min at 4°C and the resultant serum stored at -40°C.

3.6.2. Production of antiserum to Phosphatidylethanolamine binding protein (PEBP)

PEBP is a protein secreted within the testis by round spermatids (Saunders *et al.*, 1995). A peptide antiserum was raised to PEBP by synthesising a peptide-carrier protein conjugate (Affiniti Research Products Ltd, Nottingham, UK) and using this to immunise two rabbits. The 20 peptide sequence used was N-K-S-G-D-H-R-G-K-F-K-V-A-S-F-R-K-K-Y-C, which corresponds to amino acids 141-159 of the deduced amino acid sequences of rat and monkey epididymal PEBP (Perry *et al.*, 1994). Each rabbit received 0.17mg peptide in 5ml of a 1:1 emulsion of PBS and Freund's complete adjuvant, injected at 5 intradermal sites. Two booster immunisations were administered at 5 and 10 weeks after the primary immunisation, using 0.17mg peptide in 5ml of a 1:1 mixture PBS and Freund's incomplete adjuvant. Blood samples were collected 2 weeks after the booster injections, allowed to clot and then centrifuged at 1000g for 30 min at 4°C and the resultant serum stored at -40°C.

3.7. Protein A affinity chromatography

The IgG fraction was prepared from a portion of the above antisera by Protein A affinity chromatography. A disposable plastic column (Bio-Rad) was packed with 1ml protein A crosslinked to 4% w/v beaded agarose (Sigma) and equilibrated with 0.02M phosphate buffer (pH 7.2). For the purification of IgG, the equipment (all from Bio-Rad) consisted of the column connected to an EP-1 Econo pump, EM-1 Econo UV monitor, model 1325 Econo chart recorder and a model 2110 fraction collector. Before purification, 10mls serum was dialysed overnight at 4°C against 5 litres 0.02M phosphate buffer (pH 7.2). The protein A column was loaded with 2ml dialysed serum and the IgG containing fractions were pooled after each run. Once the serum sample had run into the gel the phosphate buffer was pumped through the column until all of the protein, except for the

IgG which binds to the column, had washed through. The UV monitor and chart recorder were used to monitor the elution of protein from the column. Once the baseline had returned to zero, the IgG was eluted from the column using 0.58% acetic acid (BDH) in 0.15M NaCl (pH 2-2.5) (Sigma). Fractions of 1ml were collected into tubes containing 20µl 3M Tris (pH 11-12) (Sigma) to neutralise the eluted IgG solution. Those fractions containing the highest amounts of IgG, as judged by the chart recorder, were pooled and kept on ice until purification was complete. The IgG was desalted by dialysis overnight at 4°C against 0.1M ammonium acetate (pH 7.2) (BDH). Aliquots of IgG were then lyophilised and stored at -20°C. One aliquot was assayed for protein content using an IgG standard.

3.8. One dimensional SDS-PAGE

Electrophoresis was performed using a Protean II electrophoresis system and a model 3000xi power supply (Bio-Rad Laboratories, Hemel Hempstead, UK) based on the technique described by Laemmli (1970). The apparatus was cooled to 10°C using a chiller/heater circulator unit (Betta-Tech Controls, Newport Pagnell, Bucks, UK). Sample separation was carried out on 7 to 15% w/v acrylamide gradient gels. Each gel had a total volume of 32mls which was composed of 16ml of 7% w/v and 15% w/v gel mixtures. The 7% gel consisted of 4.1ml of premixed 30% acrylamide/BIS (NBL) in 4.0ml 1.5M Tris-HCl (pH 8.8) and 7.8ml dd water. The 15% gel mixture consisted of 8.6ml acrylamide in 4.0ml 1.5M Tris-HCl (pH 8.8) and 3.4ml dd water. The gel mixtures were degassed for 10 min before addition of polymerising agents. Gels were cast using a gradient pouring system which consisted of a gradient pourer sitting on a stirring plate connected to a peristaltic pump (Minipuls 2; Anachem, Luton, UK), the tubing of which was fitted with a 22 gauge needle inserted between the glass plates of the casting apparatus. Polymerisation was initiated by the addition of 4µl TEMED (N,N,N,N'-tetramethylethylenediamine) and 100µl 10% w/v ammonium persulphate (both from Sigma) to each gel mixture. After casting, the gel was overlaid with water-saturated isobutanol (Aldrich Chemical Co.) for 1 h, then washed with dd water and overlaid with 0.375M Tris-HCl (pH 8.8). The gel was stored at 4°C overnight to ensure complete polymerisation before use.

Once the gradient gel had come to room temperature it was rinsed with distilled water and the stacking gel (consisting of 2.9ml acrylamide in 5ml 0.5M Tris-HCl (pH 6.8) and 12.1ml dd water) was cast. A fifteen well comb was inserted into the gel which was left to polymerise for 30 min. Samples were mixed with an equal amount of SDS sample buffer. The sample buffer was made as follows: 1.5% w/v Tris was dissolved in dd water which was adjusted to pH 6.75 with hydrochloric acid. Then 4% w/v SDS, 2%

w/v dithiothreitol (DTT) and 0.05% w/v bromophenol blue were added and the sample buffer was made up to 20ml with dd water. Samples were boiled for 5 min, centrifuged at 1000g for 3 min and then loaded into the wells of the stacking gel. Molecular weight markers (phosphorylase b, 97kDa; albumin, 67kDa; carbonic anhydrase, 43kDa; trypsin inhibitor, 20.1kDa; α-lactalbumin, 14.4kDa; Pharmacia, Milton Keynes, UK) were also loaded onto each gel. The electrolyte buffer used contained 0.3% w/v Tris base, 1.44% w/v glycine and 0.15% w/v SDS (all from Sigma) in dd water. Electrophoresis was performed at 38mA per gel for approximately 3.5 h. After electrophoresis the gel was either silver stained (section 3.9.3) or electroblotted for Western blot analysis (section 3.10).

3.9. Two dimensional SDS-PAGE

3.9.1. Isoelectric focusing (first dimension separation)

The method used has been detailed previously (Sharpe *et al.*, 1992) and is based on the technique described by O'Farrell (1975). All procedures used a Protean II electrophoresis system and a model 3000xi power supply from Bio-Rad Laboratories. The apparatus was cooled to 10°C using a chiller/heater circulator unit (Betta-Tech Controls, Newport Pagnell, Bucks, UK). Tube gels for isoelectric focusing consisted of 5.5g urea (Sigma), 1.33ml premixed 30% acrylamide/BIS (NBL), 2ml 10% w/v nonidet P-40 (BDH, Poole, Dorset, UK), 0.6 ml ampholines (0.25ml pH 5-7, 0.25ml pH 7-9, 0.1ml pH 3-10) (Bio-Rad) and 2.1ml dd water. After degassing for 2 min, polymerisation was initiated by 10µl TEMED and 20µl 10% w/v ammonium persulphate. First dimension isoelectric focusing (IEF) gels were cast to a height of 120mm in 2.5mm internal diameter glass capillary tubes. Gels were then overlaid with 50µl dd water and left for 2 h until polymerised. At this time the overlay was replaced with 20mM NaOH and further polymerised for 1 h. Before placing on the electrophoresis unit, the NaOH on top of the gels was replaced with degassed NaOH. The gels were prefocused for 15 min at 200V, followed by 30 min at 300V and 30 min at 400V, using 6mM phosphoric acid as the anolyte and freshly degassed 20mM NaOH as the catholyte.

Samples were prepared for loading by the addition of an equal volume of isoelectric focusing sample buffer (9.5M urea, 2% w/v nonidet P-40, 2% w/v ampholines and 1% w/v DTT). The samples were incubated at room temperature for 15 min and then centrifuged at 13,000rpm for 3 min and the supernatant loaded onto the tube gels. Each tube gel was then loaded with either 30µg (STCM) or 500µg (IF or various blood samples) protein. To allow measurement of the pH gradient, two tube gels were loaded only with 60µl IEF sample buffer. Gels were focused for 14 h at 400V followed by 2 h at 800V. Sample gels were then extruded using a needle and syringe filled with water

and placed into a storage tube containing 0.5ml sample buffer made up of 10ml 0.5M Tris-HCl (pH 6.8), 20ml 10% w/v SDS, 4ml 0.05% w/v bromophenol blue (Bio-Rad) and 36ml dd water. They were incubated at room temperature for 3 min before being frozen rapidly in ethanol and dry ice and stored at -40°C. Blank tube gels were sectioned into 5mm segments, which were immersed in 1.4ml dd water and allowed to stand at room temperature for 4 h before measurement of the pH.

3.9.2. Second dimension separation

Second dimension separations utilised 7-15% w/v gradient acrylamide gels. Each gel had a total volume of 40mls which was composed of 20ml of 7% and 15% gel mixtures. The 7% v/v gel consisted of 5.1ml acrylamide in 5.0ml 1.5M Tris-HCl (pH 8.8) and 9.8ml dd water. The 15% v/v gel mixture consisted of 10.7ml acrylamide in 5.0ml 1.5M Tris-HCl (pH 8.8) and 4.2ml dd water. After degassing for 10 min, polymerisation was initiated by adding 125µl 10% w/v ammonium persulphate and 5µl TEMED to each gel mixture. Gels were cast using a gradient pouring system as described earlier (section 3.8.). Once cast, the gels were overlaid with water-saturated isobutanol and left to polymerise for approximately 1 h, then washed with dd water and overlaid with 0.375M Tris-HCl (pH 8.8) and stored at 4°C until required. Frozen isoelectric focusing gels were thawed at room temperature and transferred carefully to the top of the slab gel to ensure that no air bubbles were trapped. A plug of gel containing molecular weight markers (Pharmacia) was also loaded. The electrolyte buffer used contained 0.3% w/v Tris base, 1.44% w/v glycine and 0.15% w/v SDS in dd water. Electrophoresis was performed at 38mA per gel for approximately 3.5 h. After which gels were either silver stained (section 3.9.3) or electroblotted for Western blotting (section 3.10).

3.9.3. Silver staining

Silver staining was performed using a kit supplied by Bio-Rad following the manufacturers instructions. Gels were fixed in 40% methanol: 10% acetic acid v/v for a minimum of 30 min, followed by 10% ethanol: 5% acetic acid v/v for a further 30 min. Then oxidised for 5 min using stock reagent diluted 1: 10 in distilled water on the day of use. This was followed by two washes for 30 min with distilled water. The gels were then incubated in silver stain reagent diluted 1: 10 in distilled water for 20 min then rinsed in distilled water. Gels were rinsed in developer (32g/L) until a smoky precipitate appeared. This was then replaced with fresh developer which was changed again after 5 min. Gels were developed until staining was optimal, then the reaction was stopped by the addition of acetic acid crystals (BDH). After staining was completed the gels were placed in distilled water and photographed.

3.10. Western blot analysis

Samples were resolved on 1-D and 2-D gels and then subjected to Western blot analysis using the IgG fraction of the polyvalent antiserum raised in rabbits against STCM or the antiserum raised to PEBP, based on the technique described by Towbin *et al.* (1979). Following electrophoresis, second dimension gels were electroblotted by semi-dry transfer onto a PVDF membrane (Immobilon-P, Millipore, Watford, UK), using a Novablot apparatus (LKB Pharmacia, Uppsala, Sweden). The transfer buffer contained 0.06% w/v Tris base, 0.288% w/v glycine, 0.02% w/v SDS and 20% w/v methanol (BDH). After blotting the lane containing molecular weight markers was cut off and stained with coomassie blue (0.1% w/v coomassie blue in 50% w/v methanol and 7.5% w/v acetic acid) for 30 min and then destained using 50% methanol: 5% acetic acid v/v until staining of the marker proteins was optimal. The membranes were first blocked for 2 h at room temperature with gentle shaking in TBS-Tween (Tris-buffered saline pH 7.4, containing 50mM Tris-HCl, 150mM NaCl and 0.05% v/v Tween 20) to which was added 5% v/v normal sheep serum (SAPU Laboratories, Carluke, Scotland). After blocking, the membranes were incubated overnight at 4°C in primary antibody diluted 1:500 in blocking buffer to reduce non-specific binding. For the control Western blots, the membranes were incubated overnight at 4°C with normal rabbit serum (Dako, High Wycombe, UK) diluted to an equivalent protein concentration as the antiserum. They were then washed three times for 10 min in TBS-Tween and incubated for 45 min in secondary antibody (sheep anti-rabbit IgG, Serotec, Oxford, UK) diluted 1:500 in TBS. This and all subsequent steps were performed at room temperature with gentle shaking. After a further three washes for 10 min in TBS-Tween, membranes were incubated for 45 min in rabbit alkaline phosphatase anti-alkaline phosphatase conjugate (Serotec) diluted 1:2000 in TBS. Membranes were washed twice for 10 min in TBS-Tween, once for 10 min in TBS alone and then equilibrated for 2 min in buffer containing 100mM Tris (pH 9.5), 100mM NaCl and 50mM $MgCl_2$. Blots were then developed immediately in 25ml equilibration buffer to which was added 0.3mg/ml nitroblue tetrazolium (NBT), 0.175mg/ml 5-bromo-4-chloro-3-indolyl-phosphate (BCIP) and 0.24mg/ml levamisole (all from Sigma). Developing was carried out in the dark until staining was optimal.

Samples of STCM and IF were also Western blotted using an antiserum raised against a PEBP peptide, to confirm the identity of one of the proteins. This antiserum was used at a 1:500 dilution following the same protocol as described above.

3.11. Tissue Fixation and processing

3.11.1. Tissue fixation

Fixation of the tissue was performed by Dr. Richard Sharpe as follows. Animals were perfusion fixed with Bouins' fluid via the dorsal aorta. Bouins' fluid containing 500ml 40% v/v formaldehyde, 100ml acetic acid and 2 litres saturated picric acid was filtered before use. The rats were anaesthetised with diethyl ether (May and Baker, Dagenham, UK) and the abdominal cavity exposed. A catheter was inserted into the dorsal aorta and normal saline perfused until the testicular blood vessels were seen to clear (approximately 3 min). Bouins' fluid was then perfused for a period of 45 min. Testes were removed, decapsulated and weighed. The tissue was cut into 2-3mm transverse slices, immersion fixed in Bouins' fluid for 5 h and transferred to 70% ethanol for storage, before processing for *in situ* hybridisation and immunohistochemistry.

3.11.2. Tissue processing and sectioning

Tissue was processed through a graded series of alcohols in an automatic 2LE Processor (Shandon Scientific Limited, Cheshire, England) using a standard 20 hour cycle and embedded in paraffin wax. Tissue processing was performed by Mr. Mike Millar.

Glass microscope slides to be used for *in situ* hybridisation were washed, dried and baked at 300°C for 8 h. Slides were dipped twice in a 4% v/v solution of 3-aminopropyl triethoxysilane (TESPA, Sigma) in acetone (BDH) then rinsed in acetone followed by dd water and dried. The slides were coated to enhance the adherence of tissue sections.

Paraffin wax embedded tissue was sectioned to a thickness of 5μm using a hand operated microtome (Jung RM2035; Leica) and a D-profile knife. Sections were floated onto RNase-free water, transferred onto the treated slides and dried overnight before use. Sections for immunostaining were sectioned as above but were floated onto distilled water.

3.12. Immunohistochemistry

Tissue sections were cleared in histoclear for approximately 10 min and hydrated in decreasing concentrations of ethanol. Slides were then washed in TBS (pH 7.4) for 5 min and the tissue blocked with normal swine serum diluted 1:5 in TBS for 30 min at room temperature.

The purified IgG fraction of the antiserum raised against STCM was used at a dilution of 1:500 (diluted in 1:5 normal swine serum: TBS). The sections were incubated with the antibody overnight at 4°C in a moist box and preimmune rabbit serum at a

dilution of 1:500 in TBS was used as a negative control. Excess antibody was removed by two washes for 10 min with TBS.

An alkaline phosphatase (AP) detection system was used and the secondary antibody was swine anti-rabbit immunoglobulins biotinylated (SARB, Dako). SARB was diluted 1:500 in 1:5 normal swine serum:TBS and incubated on the sections for 30 min. This was followed by two washes for 5 min with TBS. Sections were then incubated for 30 min with ABC/AP complex (Dako) made up in 0.05M Tris followed by two washes for 10 min in TBS. Sections were equilibrated for 2 min in buffer containing 100mM Tris pH 9.5, 100mM NaCl and 50mM $MgCl_2$. The colour development solution containing substrates for alkaline phosphatase (NBT and BCIP) was prepared as described earlier (section 3.10.). The tissue sections were incubated with the development solution beneath coverslips prepared from gel bond film (Flowgen Instruments Ltd.) in a dark humidified chamber at room temperature until staining was optimal (usually after 1 h). After development of the colour reaction, sections were washed in water, dehydrated and mounted with coverslips.

3.13. Plasmid preparation and analysis

The methods described in this section and those which follow all relate to molecular techniques and not all of the chemicals and suppliers are stated in the text. Molecular grade chemicals were obtained from Sigma and IBI, Cambridge, UK. Radiolabelled nucleotides were obtained from Amersham, Lewes, UK or Du Pont, Stevenage, UK. Enzymes were purchased from Boehringer Mannheim or Promega. Phenol/chloroform was bought from CAMLAB, Cambridge, UK and was pre-buffered with Tris, pH 8.0. Autoradiography products were obtained from Kodak supplied by IBI.

3.13.1. Growth of bacterial cultures

Plasmids containing cDNA inserts were prepared by *in vivo* excision from phagemid pools obtained from library screening; the methods are described in chapter 6. Bacteria containing plasmids were cultured in Luria Bertani (LB) broth (appendix) containing 50µg/ml ampicillin. The broth was inoculated with a bacterial colony from an agar plate or from a glycerol stock and incubated overnight at 37°C with shaking at 225 revs.

3.13.2. Plasmid preparation from bacterial cultures

Plasmid DNA was isolated from 10ml bacterial cultures by the alkaline lysis method using the Wizard™ DNA purification system from Promega. Briefly, suspensions were centrifuged at 1600g and the supernatant discarded, cells resuspended in buffer containing 50mM Tris/HCl (pH7.5), 10mM EDTA (Sigma) and 100µg/ml RNase

(Sigma) and lysed with an equal volume of 0.2M NaOH and 1% SDS. The suspension was neutralised with a solution containing 1.32M potassium acetate and 6.4% glacial acetic acid (pH 4.8) then centrifuged for 5 min at 12,000g resulting in sedimentation of bacterial genomic DNA. The supernatant (approximately 600µl) was removed, mixed with 1ml DNA purification resin and passed down a miniprep column which retarded only the plasmid DNA. The column was washed with an ethanol based solution containing 200mM NaCl, 20mM Tris/HCl, pH 7.5, 5mM EDTA and 47.5% v/v ethanol. The DNA was eluted from the column by the addition of 100µl dd water which had been heated to 70°C followed by centrifugation at 12,000g for 1 min.

3.13.3. Analysis of plasmid DNA purity

Purity of plasmid DNA was determined by analysis on a 0.8% agarose minigel. This was prepared using Seakem agarose (Flowgen, Sittingbourne, UK) dissolved in 1x TBE buffer (containing 0.089M Tris base, 0.089M boric acid and 10mM EDTA). The agarose was melted and approximately 200µg/ml ethidium bromide was added for visualisation of DNA. The gel was poured into a 7cm by 10cm gel tray containing an 8 well comb and submerged in 1 x TBE buffer in 'Minnie the Gel-Cicle' submarine cell (Hoefer, Newcastle, UK). Plasmid DNA (1µl) was run in a sample solution containing 1µl 'orange juice' (contains 0.25% w/v orange G, 15% w/v ficoll and 0.5M EDTA at pH 7.0) and 8µl water. Samples were separated in parallel with pGem DNA markers (range 36-2645bps, Promega) or Hae III øX174 markers (range 72-1353bp, IBI) by electrophoresis at 100V for 1-2 h in 1x TBE, viewed under UV light and photographed. Pure plasmid DNA appeared as two visible 'bands' of greater than 2kb (depending on plasmid size), one for the circular DNA and the other representing supercoiled DNA which migrates more rapidly through the gel due to its compact form. The approximate concentration of plasmid DNA was determined by comparison with a plasmid DNA sample (pBR322; Promega) of known concentration (200ng) which was also run on the gel.

3.14. RNA extraction and separation

3.14.1. RNA extraction from fresh tissue

Two methods for RNA extraction were employed, the first method was described by Chomczynski & Sacchi (1987) and the second is a modification of this technique using Tri-Reagent™ which allows the simultaneous isolation of RNA, DNA and protein (Chomczynski, 1993).

Method 1

Testes were removed from asphyxiated rats by dissection and were decapsulated. The testicular vein was removed and the tissue was roughly chopped up with scissors before placing each testis in 20ml freshly prepared solution D on ice. Immature rat testis, adrenals and adult rat ovaries were placed in 5ml solution D whilst 10ml was used for RNA extraction from epididymis, kidney, liver, spleen, prostate, brain, muscle, heart, seminal vesicle, uterus and placenta. Solution D contained 4M guanidinium thiocyanate (Fluka Biochemika, Gillingham, Dorset, UK), 0.1M ß-mercaptoethanol (Sigma), 25mM sodium citrate (Sigma) and 0.5% w/v sarcosyl (Sigma).

RNA was extracted according to the method of Chomczynski & Sacchi (1987) with appropriate adjustment of volumes. The tissue in solution D was homogenised for approximately 30 sec until completely dissociated. To this homogenate was added 2ml 2M sodium acetate pH4.0 (Sigma), 20ml water saturated phenol (Gibco) and 4ml chloroform/isoamyl alcohol (49:1) (Sigma). The solution was mixed after each addition and an emulsion formed after the final addition. The mixture was placed on ice for 15 min and then centrifuged at 10,000g for 20 min at 4°C. The top, aqueous phase containing the RNA was removed to a new tube and an equal volume of cold (-20°C) isopropanol (Sigma) was added. The RNA was precipitated at -20°C for at least 1 h, centrifuged at 10,000g for 20 min at 4°C, the supernatant was discarded and the RNA pellet redissolved in approximately 1ml solution D. An equal volume of isopropanol was added and the RNA reprecipitated at -20°C for at least 1 h. RNA was pelleted by centrifugation (10,000g for 20 min), washed in 1ml 75% ethanol, recentrifuged and air dried. The RNA pellet was dissolved in RNase-free water by warming to 65°C for 10 min and stored at -70°C.

RNA was scanned at 260 and 280nm in a spectrophotometer. The 260:280 ratio for each sample was calculated to give an estimation of the purity of the RNA. A ratio of 2.0 was taken to be pure. The concentration of the RNA was calculated from the 260nm value where an optical density of 1.0 is equal to 40µg/ml RNA.

Method 2

Tissue was dissected out as described above and placed in Tri-Reagent™, using the same volumes as used for the extraction with solution D. The tissue was homogenised for approximately 30 sec until completely dissociated and allowed to stand for 5 min at room temperature. To this homogenate was added 4ml chloroform (Sigma) followed by vigorous mixing with the formation of an emulsion. The mixture was allowed to stand for 15 min at room temperature and then centrifuged at 10,000g for 20 min at 4°C. The top, aqueous phase was transferred to a new tube to which an equal volume of cold isopropanol (-20°C) was added. The RNA was precipitated for 10 min at room

temperature and then centrifuged at 10,000g for 20 min at 4°C. The RNA pellet was then washed with 75% ethanol, dissolved in water and purity estimated as described above.

3.14.2. Separation of RNA on denaturing agarose gels

RNA was separated on a 1.5% denaturing agarose gel. The gel was prepared by melting 2.25g Seakem agarose (Flowgen) in 127ml pure water. This was cooled to about 60°C and 15ml of 10x running buffer (containing 200mM MOPS, 10mM EDTA and 50mM sodium acetate at pH7.0) plus 8.1ml 37% formaldehyde was added. The solution was mixed gently and poured into a gel tray (15 x 20cm) containing a 15 well comb in a fume hood. After setting, the comb was removed and the gel submerged in 1x running buffer in a Sub-Cell electrophoresis cell (Bio-Rad).

RNA (15 or 20µg) was prepared by adding 16µl sample buffer and heating at 60°C for 5 min. Sample buffer contained 100µl 10x running buffer, 500µl deionised formamide and 178µl formaldehyde. After heating, 8µl dye solution containing 7.5% w/v ficoll 400 and 0.1% w/v bromophenol blue was added to each sample. RNA was loaded into individual wells of the gel and separated by running overnight at 34V or for 5 h at 120V. The gels were post-stained with ethidium bromide then viewed under UV light and photographed.

3.15. Preparation of radiolabelled probes for Northern blot analysis

3.15.1. Preparation of double stranded DNA

Double stranded DNA for labelling was usually prepared by amplification of the cloned cDNA insert from a plasmid vector using polymerase chain reaction (PCR; Saiki *et al.*, 1988). This was achieved using pairs of T3 and T7 primers (Bluescript SK- vector; Stratagene, Cambridge, UK). The primers were used at a concentration of 0.5µM in a PCR reaction containing 100µM of each dNTP (Pharmacia), Taq polymerase buffer (50mM KCl, 10mM Tris-HCl, 1.5mM $MgCl_2$, 0.01% gelatin, 0.1% Triton X100) and 2.5U Taq polymerase (Promega). Plasmid DNA (100-500ng) containing the desired cDNA insert was used as a template for the reaction. Thirty-five cycles of amplification were performed with an annealing temperatures of 48°C and an extension time of 2 min at 72°C.

Amplification of the correct size DNA insert was checked by running 10µl of the PCR sample on an agarose gel with known DNA markers (see section 3.13.3.). DNA was isolated from the PCR mix using Clontech ChromaSpin +TE-100 columns (Cambridge Bioscience, Cambridge, UK) according to the manufacturers instructions.

3.15.2. Radiolabelling double stranded DNA

Double stranded DNA was radiolabelled using the random primer method (Feinberg & Vogelstein, 1983) using an Amersham 'Megaprime' kit according to the manufacturers instructions. Briefly, DNA (25-50ng) with 5µl primer solution (containing random nonamer primers) was denatured at 98°C for 5 min. DNA was labelled with 50µCi of ^{32}P-[α]-dCTP in a reaction containing dATP, dGTP and dTTP in Tris/HCl (pH 7.5), 2-mercaptoethanol and $MgCl_2$. The reaction was catalysed by addition of 2U Klenow enzyme and incubation was for 30 min at 37°C. Labelled DNA was denatured with 5N NaOH (100µl), neutralised with 1M Tris, pH 7.6 (600µl) and 1M HCl (375µl) and added to the hybridisation mixture (section 3.16.2.).

3.15.3. Synthesis of oligonucleotides

Oligonucleotides (17-24mers) were synthesised using phosphoramadite chemistry on a Model 381 DNA synthesiser (Applied Biosystems, Warrington, UK). Oligonucleotides were recovered into 1ml pure concentrated ammonia, deprotected by incubating overnight at 65°C, recovered by two rounds of ethanol precipitation and resuspended in TE buffer (10mM Tris-HCl, 1mM EDTA, pH8.0). The concentration of the oligonucleotides was estimated by spectroscopy at 260nm in which an optical density of 1.0 is equal to a concentration of 20µg/ml.

3.15.4. Oligonucleotide labelling

Oligonucleotides were labelled at the 5' end using polynucleotide kinase (PNK). DNA was incubated with [γ - ^{32}P]ATP in a reaction mixture containing 50ng DNA, 30µCi [γ-^{32}P]ATP, 1 x kinase buffer (10 x buffer contains 0.5M Tris/HCl, 0.1M $MgCl_2$, 50mM DTT, 1mM spermidine and 1mM EDTA) and 8U T4 PNK (Amersham). The reaction was allowed to continue at 37°C for 30 min. The labelled oligonucleotide was then added directly to the DNA hybridisation solution (section 3.16.2.).

3.16. Northern blot analysis

3.16.1. RNA transfer to membrane

RNA separated on a denaturing gel (section 3.14.2.) was transferred to a nylon membrane (Hybond-N™; Amersham) by capillary blotting as shown in Fig.2. The nylon membrane was pre-wetted with pure water followed by 10x SSC (1x SSC containing 0.15M NaCl and 0.015M sodium citrate, pH 7.0) before placing carefully on the gel. Transfer using 10x SSC was allowed to continue for a minimum of 12 h. After transfer the position of the lanes was marked on the membrane and RNA was bound using a UV crosslinker.

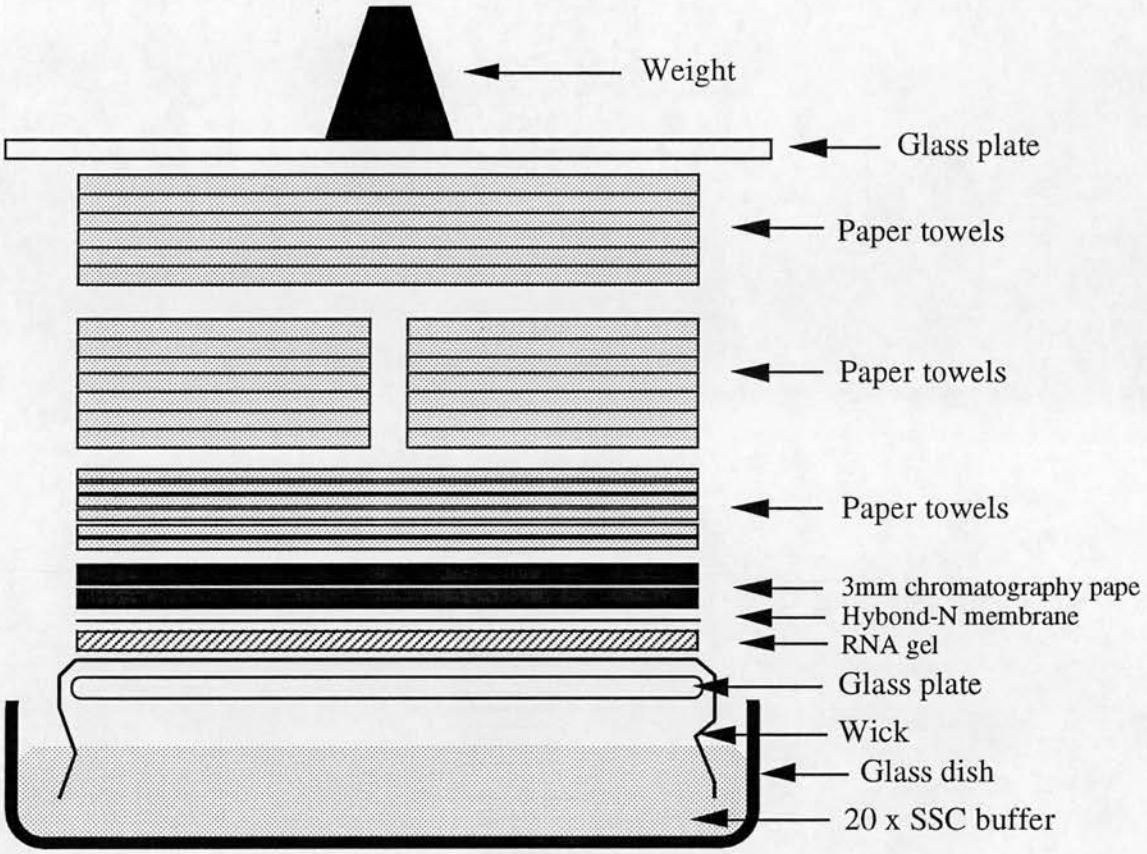


Figure 2. Schematic drawing of the apparatus used to transfer RNA from a denaturing agarose gel to a Hybond-N membrane by capillary action.

3.16.2. Hybridisation of radiolabelled probes to membranes

Double Stranded Probes. Membranes were prehybridised at 65°C for 2-4 hours in buffer containing 0.2M sodium phosphate pH 7.2, 1mM EDTA, 1% w/v BSA, 7% w/v SDS and 15% w/v formamide. Radiolabelled probe was added to the hybridisation solution at a final concentration of 0.5- 1 x 10^6 cpm/ml buffer. Hybridisation was allowed to continue for 24-48 h at 65°C.

Oligonucleotide Probes. Membranes were prehybridised at the melting temperature (Tm) of the probe minus 10°C. Tm is dependent on the nucleotide content of the DNA and was calculated using the formula (4 x G+C) + (2 x A+T). Prehybridisation buffer contained 0.05% w/v BSA, 0.05% w/v polyvinylpyrrolidine, 0.05% w/v ficoll, 0.1% w/v SDS, 0.1% w/v sodium pyrophosphate, 5x SSC and 100µg/ml sonicated salmon sperm DNA

(Sigma). Labelled probe was added to the solution and hybridisation allowed to proceed for 24-48 h.

3.16.3. Post-hybridisation washes

Double Stranded Probes. Membranes were washed twice for 30 min at 65°C with buffer containing 40mM sodium phosphate (pH 7.2), 1mM EDTA and 1% SDS.

Oligonucleotide Probes. Membranes were washed with twice for 30 min with 2x SSC at Tm minus 5°C. For more stringent washes, 1x SSC, 0.5x SSC or 0.1x SSC were used.

3.16.4. Development of signal

After washing, membranes were wrapped in clingfilm. The membrane was then exposed to X-ray film (XAR-5 or X-Omat S; Kodak) in cassettes with Du Pont enhancing screens at -70°C. After a specific exposure time the signal was developed using LX 24 developer and fixed using FX 40 fixative according to the suppliers recommendations (both Kodak).

3.17. Non-radioactive *In situ* hybridisation

Methods are based on those described by Millar *et al.* (1993).

3.17.1. Preparation of probes for *in situ* hybridisation

Plasmid DNA prepared as described previously (see section 3.13.) was linearised in a reaction containing 1µg DNA, reaction buffer (containing 10-50mM Tris/HCl, 5-10 mM $MgCl_2$, 50-100mM NaCl and 1mM dithioerythritol; Boehringer Mannheim), 10U restriction enzyme (Boehringer Mannheim) and pure water to a volume of 40µl. The enzyme used was dependent on the plasmid vector being digested and the direction of synthesis of the riboprobe. The reaction was incubated at 37°C for 1-2 h. Thereafter, 3µl of reaction mix was run on a minigel (0.8% agarose) and compared to an uncut sample of the same plasmid run in a parallel lane to test efficiency of digestion. Digested DNA was then extracted once with Tris-buffered phenol:chloroform:isoamylalcohol 25:24:1 v/v (pH 8.0), precipitated with 1/10th volume 3M sodium acetate (pH 5.5) and 2.5 volumes absolute ethanol overnight at -20°C. Linearised DNA was pelleted, dried and resuspended in 10µl pure water.

3.17.2. Preparation of digoxigenin labelled riboprobes

Synthesis of riboprobes was performed using 1µg linearised template in a reaction mix containing 10mM DTT (Promega), 1mM each rATP, rCTP and rGTP (all from Boehringer Mannheim), transcription buffer (1x transcription buffer containing 40mM

Tris/HCl (pH7.9), 6mM $MgCl_2$, 2mM spermidine and 10mM NaCl; Boehringer Mannheim) and 0.35mM Dig-labelled UTP (Boehringer Mannheim) with 20U RNase inhibitor (Promega). The reaction was catalysed by addition of 40U of the appropriate RNA polymerase (T3 or T7; Boehringer Mannheim) and incubated at 37°C for approximately 2 h. To check that riboprobe synthesis was successful, 2µl of the reaction mix was run on a 0.8% agarose gel to detect the presence of two bands corresponding to 28 and 18S RNA. Enzymes and salts were removed by two rounds of phenol /chloroform extraction, RNA was precipitated with sodium acetate and ethanol overnight at -70°C and recovered by centrifugation as above. The RNA was resuspended in 50µl dd water. The riboprobe was purified further by passage through an RNA spin column (Nu-Clean R50, IBI).

3.17.3. Pretreatment and hybridisation of tissue

Paraffin wax-embedded tissue was cleared in histoclear for 15 min and rehydrated in a series of alcohols of decreasing concentration. Tissue was placed in 0.2N HCl for 20 min followed by two 5 min washes in distilled water. Sections were then incubated in 2µg/ml proteinase K (Sigma) in buffer containing 20mM Tris/HCl (pH 7.4) and 50mM EDTA at 37°C for 40 min. Acid hydrolysis and enzymatic digestion remove excess protein from the tissue section and aids the hybridisation of the riboprobe. This was followed by 0.2% glycine at 4°C for 20 min which inactivates the proteinase K. Sections were washed briefly in 0.1M triethanolamine (TEA) pH 8.0 (Sigma) and acetylated in 0.25% acetic anhydride (Sigma) in 0.1M TEA (pH 8.0) for 10 min. Acetylation reduces ionic charges left by protein digestion. Finally, sections were washed in 6x STE (1x STE contains 150mM NaCl, 2.5mM Tris and 0.25mM EDTA) and prehybridised in buffer containing 6x STE, 1x Denhardt's solution (50x Denhardt's contains 5g BSA, 5g polyvinylpyrrolidine and 5g ficoll in 500ml solution), 125µg/ml salmon sperm DNA (Sigma), 125µg/ml yeast transfer RNA (Sigma) and 50% deionised formamide for 2-4 h in a humidified chamber at 35°C.

Hybridisation was continued overnight using 2µl probe diluted in 40µl buffer /slide. Hybridisation buffer was prehybridisation buffer with 10% v/v dextran sulphate added. Incubation was performed beneath coverslips prepared from gel bond film (Flowgen) in a humidified chamber at 35°C.

Post hybridisation washes were two changes of 4x SSC for 5 min each to remove the coverslip and then RNase A at a concentration of 20µg/ml in buffer for 30 min at 37°C. Slides were then washed in two changes of 2x SSC at room temperature followed by 30 min in 0.1 x SSC/30% v/v formamide at 30°C or 40°C.

3.17.4. Development of *in situ* hybridisation

The slides were further washed with two changes of TBS for 15 min each before incubating in normal sheep serum diluted 1:5 with TBS for 30 min to reduce background staining. The slides were washed briefly in TBS and antibody was added. The antibody (anti-dig conjugated to alkaline phosphatase Fab fragments from sheep; Boehringer Mannheim) was diluted 1:300 in TBS and incubated with the sections for 2 h. Excess antibody was removed with two washes for 15 min in TBS. Development was performed as detailed in section 3.12. The sections were incubated with development solution beneath coverslips at room temperature in the dark until staining was optimal (usually overnight). Sections were washed in water to stop the reaction before dehydrating and mounting.

3.18. DNA sequencing

Most of the sequencing was very kindly performed by Mr. Joe Gaughan.

3.18.1. Preparation of template DNA

Plasmid DNA was prepared as described in section 3.13 Using the Wizard DNA purification system from Promega. Template DNA with a concentration of 100-500ng/µl was used in the sequencing reactions described below.

3.18.2. Automatic sequencing reactions

Automatic sequencing reactions were carried out using the ABI PRISM™ dye terminator cycle sequencing ready reaction kit (Perkin Elmer) and were run on the Applied Biosystems Model 373A DNA sequencing system. The sequencing reaction mixtures were prepared as follows in 0.2ml microcentrifuge tubes. Plasmid DNA template (2µl) was added to separate tubes containing 8µl of terminator ready reaction mix, 3.2 pmol primer and the total volume adjusted to 20µl with water and overlaid with mineral oil. The terminator premix, supplied in the kit, contained A-dye terminator, C-dye terminator, G-dye terminator, T-dye terminator, dITP, dATP, dCTP, dTTP, Tris-HCl (pH 9.0), $MgCl_2$, thermal stable pyrophosphatase and AmpliTaq DNA polymerase, FS. Tubes were placed in a Hybaid Omni-E preheated to 96°C and 25 cycles of thermal cycling carried out as follows; 96°C for 15 sec , 50°C or 52°C for 20 sec and 60°C for 4 min.

3.18.3. Purification of sequencing reactions

On completion of the sequencing reactions, the unincorporated dye terminators were removed from the samples by ethanol precipitation. The entire 20µl contents of each reaction tubes was transferred to a 1.5ml microcentrifuge tube containing 2.0µl 3M sodium acetate (pH 4.6) and 50µl 95% w/v ethanol, vortexed and placed on ice for 10

min. Labelled DNA was pelleted by centrifugation at 13,000rpm for 15-30 min. The supernatant discarded and the DNA washed using 250µl 70% ethanol and allowed to air dry. Just before loading onto the gel, the DNA was resuspended in deionised formamide/EDTA buffer (5µl formamide: 1µl 50mM EDTA, pH 8.0).

3.18.4. Automatic sequencing gel

A sequencing gel mix containing 50g urea, 15ml 40% acrylamide and water made up to a final volume of 80ml was prepared. Amberlite resin (Sigma) was added to remove acrylamide free acid and the mixture was stirred and heated until the urea had dissolved. The solution was then filtered and 10ml 10x TBE (pH 8.4) added. The gel mix was polymerised using 45µl TEMED and 500µl 10% w/v ammonium persulphate and poured carefully between clean glass plates. The gel was prerun for 30 min at 500V. Samples resuspended in buffer were heated to 95°C for 5 min to denature the DNA, then loaded onto the gel. The gel was run overnight using the automatic data collection and analysis programs.

Chapter 4. Evaluation of creatine as a potential marker of testicular damage

4.1. Introduction

The aim of this thesis is to assess the feasibility of monitoring spermatogenesis non-invasively, but the lack of suitable markers of spermatogenic status has so far hindered any significant progress in this area. The studies described in this chapter describe the investigation of creatine as a potential marker of early adverse effects on spermatogenesis. The studies were instigated following several reports that damage to spermatogenesis in rats using testicular toxicants resulted in increased urinary levels of creatine (Rawcliffe *et al.*, 1989; Gray *et al.*, 1990; Moore *et al.*, 1992; Nahas *et al.*, 1993). Within the context of this thesis it seemed appropriate to examine the levels of creatine within the testis, its cell specificity, and to evaluate its potential to be measured in plasma as a non-invasive marker of testicular damage.

Rawcliffe and colleagues (1989), initially examined the effect of a single dose of 500mg/kg of an ethylene glycol momomethyl ether, 2-methoxyethanol (2-ME), given to 6 week old male and female rats. This toxicant specifically destroys early and late stage pachytene spermatocytes (Foster *et al.*, 1984; Creasy *et al.*, 1985). In male rats, this toxicant induced necrosis of pachytene spermatocytes within the same time frame as a decrease in relative testis weight and a significant increase in creatine excretion which was maximal 48 h after dosing. No increase in urinary creatine was observed in female rats suggesting that urinary creatine may be useful non-invasive marker of testicular damage. A similar study using 250 and 750mg/kg doses of 2-ME showed a dose related increase in the creatine/creatinine ratio within 24 h, confirming the results of Rawcliffe *et al.* (Nahas *et al.*, 1993). Both of these studies also measured urinary volume and the increase in levels of urinary creatine was found to be associated with an increase in urinary volume. The increase in urinary creatine might therefore be due to a diuretic effect of 2-ME.

In another study a single dose of 3.23μmol/kg of cadmium chloride administered to 6 week old male rats was also found to cause a significant increase in urinary and plasma creatine concentration at 24-48 h after treatment (Gray *et al.*, 1990). No increase in urinary or plasma creatine was observed in orchidectomised rats or rats orchidectomised and subsequently treated with cadmium. A high dose of cadmium was used in these studies, and this caused widespread and severe damage to both the seminiferous epithelium and the interstitium of the testis. Necrosis of the testis induced by cadmium is due to ischaemia as this chemical is thought to interfere with the vascular supply (for references see Gray *et al.*, 1990). A lower dose of cadmium which was

insufficient to induce testicular damage failed to induce significant increases in either urinary or plasma creatine levels.

To examine the distribution of creatine in the testis, male rats were treated with various cell-specific toxicants (Moore *et al.*, 1992). Rats aged 4 weeks old were treated with different doses of either MAA, a germ cell toxicant, di-n-pentyl phthalate (DPP) or 1,3-dinitrobenzene both of which act on the Sertoli cells. Rats aged 9 weeks were dosed with the Leydig cell toxicant, EDS. MAA is an oxidation product of 2-ME (Miller *et al.*, 1983; Moss *et al.*, 1985), and causes the selective and stage-specific destruction of pachytene spermatocytes (Foster *et al.*, 1983; 1987a; Bartlett *et al.*, 1988). DPP is a phthalate di-ester which has been shown to act on Sertoli cells with germ cell degeneration occurring as a secondary event 24 h after treatment (Creasy *et al.*, 1983; 1987; Gray & Gangolli, 1986). Like DPP, 1,3-DNB also causes initial damage to the Sertoli cell which leads secondarily to germ cell degeneration (Blackburn *et al.*, 1988; Foster *et al.*, 1987b). EDS causes the specific destruction of Leydig cells within 24 h treatment, which results in androgen withdrawal in the testis which adversely affects spermatogenesis (Bartlett *et al.*, 1986; Sharpe *et al.*, 1990). The higher doses of MAA (300mg/kg and 900mg/kg), DPP (2200mg/kg) and 1,3-DNB (60mg/kg) all caused a significant elevation in creatine excretion within 24 h after treatment which coincided with morphological damage to the testis. EDS treatment did not induce significantly increased levels of creatine in urine within 48 h. They concluded from this study that creatine was associated with the cells of the seminiferous epithelium and that its levels in urine were an indicator of damage to the Sertoli cells and/or germ cells.

The increase in urinary creatine within 24-48 h after administration of toxicants known to induce damage to the seminiferous epithelium seems to be a consistent effect, but the data must be interpreted with a certain degree of caution. All of these studies have been carried out in pubertal rather than adult rats, with the exception of EDS treatment. It is possible that the testis is more susceptible to damage at this age in comparison to adult animals. Also some of these results may be due to an increase in urinary volume. The studies using cadmium, MAA, DPP and 1,3-DNB did not measure this parameter and based on the results in studies using 2-ME it is possible that these toxicants may have a similar effect, especially in the case of MAA which is an *in vivo* oxidation product of 2-ME. The toxicants used in the various studies were selected because of their action on the testis but they may also induce effects on other tissues, such as muscle, which would seriously affect measurements of urinary creatine. The increase in urinary creatine is only significant during a period of 24-48 h after testicular damage has occurred. This suggests that creatine is only a marker of active germ cell degeneration within the seminiferous epithelium. An increase in urinary creatine was only observed when the testis was

severely damaged, suggesting that creatine may not be a very sensitive indicator of disruption to spermatogenesis.

Recently, the same group, has tried to address the possibility that the increase in urinary creatine levels could be due to the testicular toxicants also causing muscle damage (Draper *et al.*, 1994). They investigated the effect of increasing doses of a specific muscle toxicant, 2,3,5,6-tetramethyl *p*-phenylenediamine (TMPD), on urinary creatine and other biomarkers when administered to male rats of approximately 5-6 weeks of age. Localised damage to individual muscle fibres was observed 48 h after 50 µmol/kg/day but the higher dose (75µmol/kg/day) was found to result in generalised necrosis at this time point. A significant increase in urinary creatine was observed at 0-24 and 24-48 h after the highest dose of TMPD. However, this dose was also to found to cause testicular damage, specifically abnormal mitoses and spermatid retention at stage XI. Serum levels of LDH-C4 were also significantly raised with this dose which is indicative of active germ cell degeneration. These results do not rule out the possibility that urinary creatine may be due to muscle as well as testicular damage.

The biochemical function of creatine in the testis is poorly understood. Creatine is synthesised mostly in the liver and kidney, then transported in the blood where it is taken up by tissues with high energy demands (Wallimann *et al.*, 1992). Creatine, together with phosphocreatine and the enzyme creatine kinase, are involved in a shuttle mechanism which allows the transport of ATP energy between the subcellular sites of synthesis and utilisation. Isoenzymes of the creatine kinase family catalyse the reversible transfer of a phosphoryl group from ATP to creatine which generates phosphocreatine and ADP via the reaction: Creatine + ATP <=> Phosphocreatine + ADP + H^+. This system is thought to operate in many tissues besides muscle, including the kidney, brain, intestinal epithelium, retina and spermatozoa (Wallimann *et al.*, 1992; Wallimann & Hemmer, 1994). Tombes and Shapiro (1985) were the first to demonstrate the presence of this shuttle system in spermatozoa from sea urchins. They inhibited creatine kinase activity which induced abnormal flagellar motility, particularly in the distal two-thirds of the tail, indicative of limited ATP availability to the tail. They proposed the presence of a shuttle mechanism which allows ATP synthesised in mitochondria in the mid-piece to be transported to the tail to provide energy for motility. This is made possible by the compartmentation of distinct isoenzymes of creatine kinase such that one form is present in mitochondrial membranes and is responsible for the phosphorylation of creatine. Phosphocreatine is not susceptible to ATPases so it acts as a store of energy and is readily diffusable. The tail possesses another form of creatine kinase in the cytosol, plasma membrane and bound to axonemal microtubules which catalyses the release of the phosphate group from phosphocreatine and the regeneration of an ATP molecule. The

shuttle system also plays a complex role in cellular energy homeostasis (Wallimann *et al.*, 1992; Wallimann & Hemmer, 1994).

The aims of the studies described were two-fold. Firstly, to investigate the presence of creatine in the adult rat testis by several approaches. This included determining whether the creatine content of ST was stage dependent or androgen regulated. Stage VI-VIII ST were isolated from MAA treated rats at time points when a specific germ cell type was known to be depleted from the seminiferous epithelium (Bartlett *et al.*, 1988; Allenby *et al.*, 1991a), the ST creatine content was assayed in an attempt to determine which cells of the seminiferous epithelium were able to produce creatine. The levels of creatine in ST and IF during testicular maturation were also investigated to find out if they showed any age dependency and also to assess if creatine was secreted into the interstitium. The second aim was to evaluate whether creatine levels in plasma would be a suitable marker of testicular damage in the adult rat. This involved assessing the relative levels of creatine in RTF, IF, and also testicular and peripheral blood samples, followed by measuring the levels of creatine in samples of IF and PV blood collected from adult rats after treatment with either nitrobenzene (NB), m-dinitrobenzene (mDNB), Heat or EDS. Both NB and mDNB were selected as they are known to be Sertoli cell toxicants (Foster *et al.*, 1986; Blackburn *et al.*, 1988; Hess *et al.*, 1988; Allenby *et al.*, 1990; McLaren *et al.*, 1993a), where as, local heating of the testis (43°C for 30 min) causes degeneration of pachytene spermatocytes and early spermatids (Bartlett & Sharpe, 1987; McLaren *et al.*, 1994) and EDS causes specific degeneration of Leydig cells (Bartlett *et al.*, 1986; Sharpe *et al.*, 1990).

4.2. Experimental procedures

4.2.1. Treatments

EDS. To induce testosterone withdrawal, adult rats (approximately 70-90 days of age) received a single i.p. injection of 75 mg/kg of EDS as described in chapter 3, section 3.2. Some EDS-treated rats were administered 25 mg of testosterone esters on day 0 and on day 3 after treatment to prevent the adverse changes in spermatogenesis induced by testosterone withdrawal. Rats were killed 4 days after the initial treatment. Testes were removed, weighed and ST were isolated from one testis and IF collected from the contralateral testis. PV plasma samples were also collected from two rats on one occasion. EDS treatment was carried out on three separate occasions with the control and treatment groups consisting of three animals.

MAA. In order to assess which cells of the seminiferous epithelium were producing creatine, the testicular toxicant MAA was used to specifically deplete germ cells from the

seminiferous tubules. Adult rats of approximately 75 days of age were administered a single dose of 650 mg/kg MAA by oral gavage as described in chapter 3, section 3.2. Control animals were given an equivalent volume of saline. At specific time points after MAA treatment (24 h, 4, 18 and 30 days) animals were killed and testes removed and weighed. Then ST at stages VI-VIII of the spermatogenic cycle were isolated from the testes of control and MAA-treated rats. Staged tubules were used as this allowed the assessment of effect of depletion of one specific germ cell type from the seminiferous tubules on creatine levels. MAA treatment was carried out on two separate occasions with each treatment group containing three rats; three control rats were matched with each treatment group.

Scrotal heating. Adult rats (aged 70-80 days) were exposed to short-term local testicular heating (43°C for 30 min) to severely disrupt spermatogenesis by inducing germ cell depletion, as described in chapter 3 section 3.2. Animals were killed 24 h after treatment. Control animals were anaesthetized but not heat exposed. Treatment was performed on two separate occasions. Control and treated groups contained at least four animals. Samples of IF and PV were collected from heat-treated and control animals.

NB. At least four adult rats (aged 70-80 days) were given a single oral dose of NB at 300mg/kg as described in chapter 3, section 3.2. NB is a Sertoli cell toxicant but germ cell degeneration is induced as a result of damage to the Sertoli cells. Control animals received vehicle alone, corn oil (1ml/kg). Rats were killed 24 h after treatment and both testicular IF and PV samples were collected.

mDNB. At least three adult rats (aged 70-80 days) were given a single oral dose of mDNB at 50mg/kg as described in chapter 3, section 3.2. mDNB results in similar testicular damage to that observed with NB. Control animals received vehicle alone, PEG 600 (1ml/kg). Animals were killed 24 h after treatment and both testicular IF and PV samples were collected.

4.2.2. Collection of fluid samples

Testicular interstitial fluid. IF was collected from the testes of control adult rats, immature rats at different ages (21, 24, 30, 40 and 45 days), as well as from adult rats treated with either heat, NB, mDNB, EDS or EDS+T. IF was collected overnight (approximately 16 h) at 4°C as described in chapter 3, section 3.3. In one experiment IF was collected after 30 min and then again after 16 h from adult control rats.

Rete testis fluid. This was collected from two control adult rats as described in chapter 3.

Plasma samples. PV plasma samples were collected from control adult rats and from rats which had been subjected to the following treatments: heat, NB, mDNB, EDS and EDS+T. The method of collection was as described in chapter 3, section 3.3. In addition, TV and SV blood samples were also collected from control adult rats as described in section 3.3.

4.2.3. Isolation of seminiferous tubules

ST were isolated from rat testes as described in chapter 3, section 3.4. Both unstaged and staged tubules were dissected depending on the experiment. Unstaged tubules were isolated from immature rats at the following ages: 21, 24, 30, 40 and 45 days; and also from adult rats at 75 days of age. Tubules isolated from control adult rats were also dissected into the stage groupings, I-V, VI-VIII and IX-XIV of the spermatogenic cycle, according to the criteria of Parvinen (1982). This relies on differences in the transilluminated appearance of the ST at each stage as a result of the density and position of the heads of the elongate spermatids. The transillumination pattern for each of the stages of the spermatogenic cycle is shown in Fig. 1. Selection of stage groupings was based on the ease with which particular stages could be distinguished from one another. Tubules at stages VI-VIII were also dissected from EDS, EDS+T and MAA treated animals. ST were cut using a scalpel and only those ST greater than 0.5cm in length were used. ST which showed any signs of stretching or damage were excluded. Samples of 10cm isolated tubules from one testis of each animal were placed in 400µl PBS for homogenisation. Tubule homogenates were either kept on ice to be assayed immediately or were stored at -40°C until assayed.

4.2.4. Preparation of tissue homogenates

Several tissues which included the testis, epididymis, kidney, spleen, liver and muscle were removed from control adult rats. The piece of tissue was weighed and then homogenised in 2.5ml PBS. The homogenates were centrifuged at 9000rpm for 15 min at 4°C to pellet cell debris. Tissue homogenates were either kept on ice to be assayed immediately or were stored at -40°C until assayed.

4.2.5. Creatine assay

Creatine was measured using a Creatinine-PAP assay kit supplied by Boehringer Mannheim based on an enzymatic method described by Siedel *et al.* (1984). The assay is based on the conversion of creatine by several enzymes to a quinone-imine dye which can be measured in a spectrophotometer. The reactions involved are summarised in Figure 2.

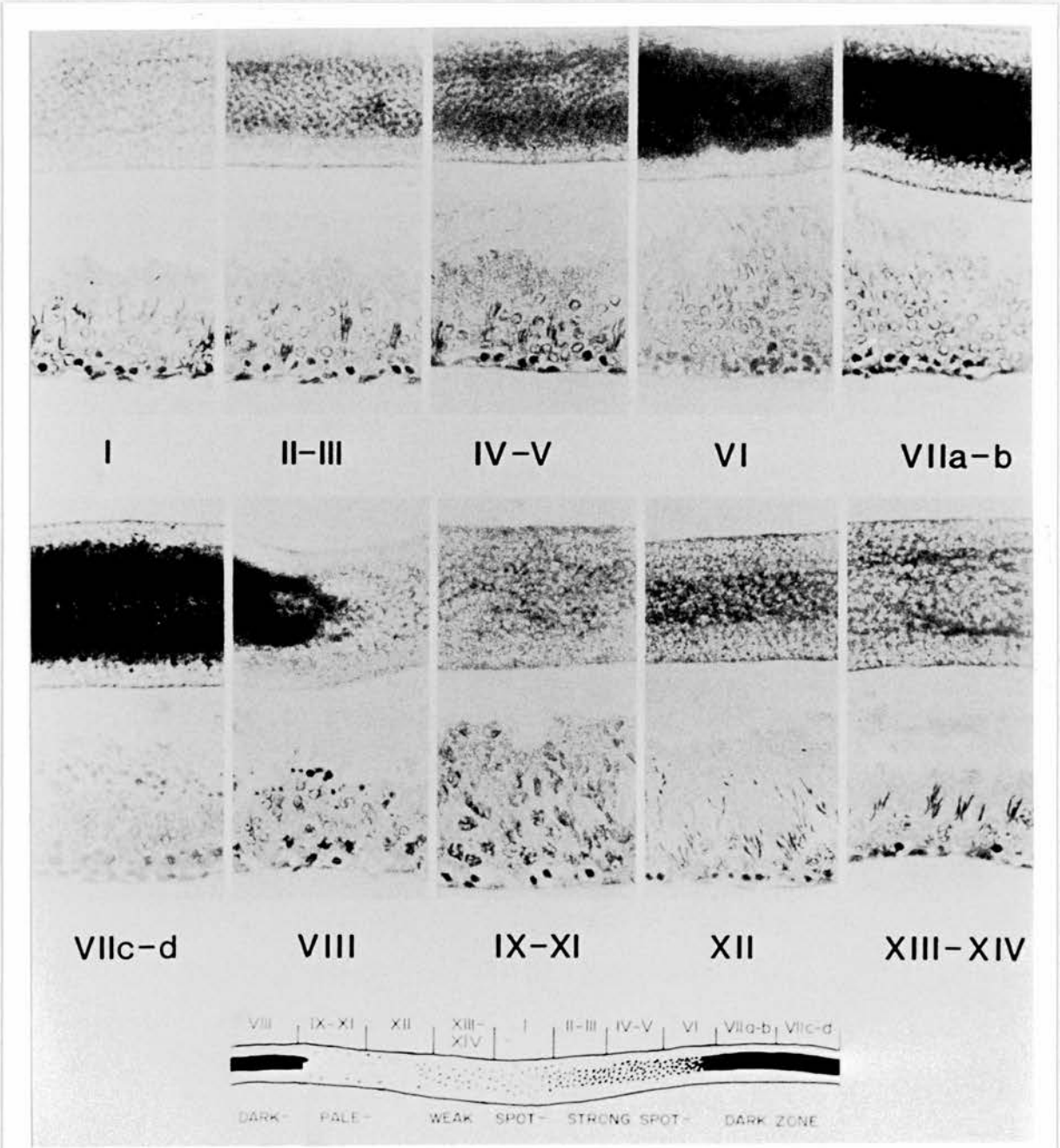


Figure 1. The transillumination pattern of the 14 stages of the rat spermatogenic cycle. When observed under a stereomicroscope, seminiferous tubules show a characteristic light absorption pattern due to differences in the degree of condensation of the nuclei of the elongating spermatids and their positions within the seminiferous epithelium. *Adapted from Parvinen, 1992.*

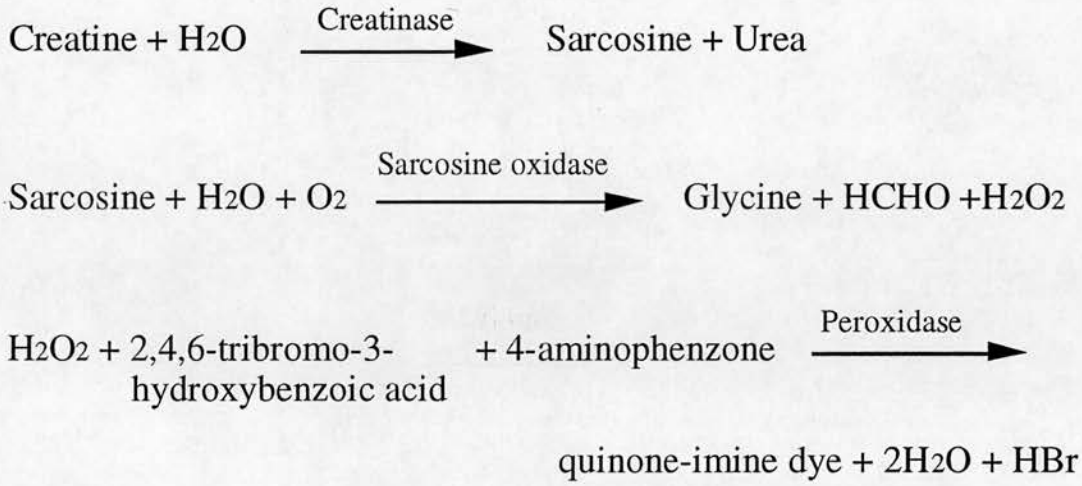


Figure 2. A summary of the reactions involved in producing the dye measured in the creatine assay. *Adapted from Boehringer Mannheim.*

The concentration of creatine standards (Sigma) ranged from 8mg/100ml double diluted to 0.125mg/100ml using PBS (ICN Flow). A standard curve was set up in each assay using standards from the same stock solution frozen in aliquots and stored at -40°C. To the diluted standards, sample or PBS alone (total volume 50µl), was added 1.0ml of the reagent containing enzymes and 4-aminophenazone supplied with the kit. The samples were vortexed gently and incubated for 20 min at room temperature. The absorbance of the dye formed was measured at 510nm using a spectrophotometer. Within each assay a sample of muscle homogenate from the same stock was assayed as an internal control. The levels of creatine in the samples were quantitated using a computer programme, AssayZap (Biosoft, Cambridge), which allowed conversion of the absorbance values into µmoles of creatine. This was based on 2mg creatine in 100ml being equivalent to 153 µmoles creatine in 1 litre (Creatinine PAP assay kit; Boehringer Mannheim).

In order to validate the assay before use, various samples were run as 5 double dilutions in the assay to check that they paralleled the creatine standard curve. The samples used were a muscle homogenate (1:2.5 to 1:40), a ST homogenate (100µl to 6.25µl), testicular IF (1:10 to 1:160) and peripheral plasma (200µl to 12.5µl). This also allowed determination of the optimal concentration/sample volume which was used in the assay. Based on these results the following sample concentrations were used in the assay, muscle homogenate at 1:5, all other tissue homogenates undiluted, ST homogenates undiluted, IF at 1:10, RTF 1:10 and all plasma samples undiluted.

A range of dilutions of creatinine, phosphocreatine and guanidoacetic acid, which are formed during either creatine synthesis or metabolism (Fig. 3), were also tested in the creatine assay to check for any cross-reaction. The following stock concentrations were prepared, creatinine =2mg/100ml (Boehringer Mannheim), phosphocreatine =8mg/100ml (Sigma) and guanidoacetic acid =8mg/100ml (Sigma). A range of 5 doubling dilutions of the stock concentration was assayed in each case.

In addition, the assay was also checked for interference by the testicular toxicant MAA. MAA inhibits the production of sarcosine which is one of the intermediate steps linked to dye production in the colourimetric assay for creatine (Mebus *et al.*, 1989). This is only likely to affect the results of an assay of creatine levels in tubule homogenates 24 h after treatment when residual MAA levels are thought to be in the range of 10^{-4}M. To samples of a stage VI-VIII tubule homogenate from a control adult rat, MAA was added to give a final concentration of either 10^{-3}, 10^{-4} or 10^{-5}M. Both control tubule homogenate and samples to which MAA had been added were assayed for creatine.

4.2.6. Statistical analysis

Data was analysed using Student's t test or analysis of variance. Values of $p<0.05$ were chosen to represent a statistically significant level of difference between groups.

4.3. Results

4.3.1. Validation of creatine assay

The measurement of creatine in doubling dilutions of samples of muscle and tubule homogenates as well as in IF and plasma resulted in curves which paralleled the creatine standard curve, indicating that the creatine in these samples could be measured accurately using this assay (Fig. 4). The assay reagent did not react at all with creatinine, phosphocreatine or guanidoacetic acid showing that the presence of any of these chemicals in samples would not interfere with the specific measurement of creatine (results not shown).

4.3.2. Comparison of creatine levels in various tissue homogenates

Creatine levels were found to be highest in muscle homogenates as expected but the assay also showed that levels of creatine in the testis were approximately half of that found in muscle (Fig. 5). Much higher levels of creatine were measured in the testis in comparison to the other tissues with the exception of muscle. This demonstrated that it would be possible to measure creatine in isolated ST allowing investigation of its presence in the testis.

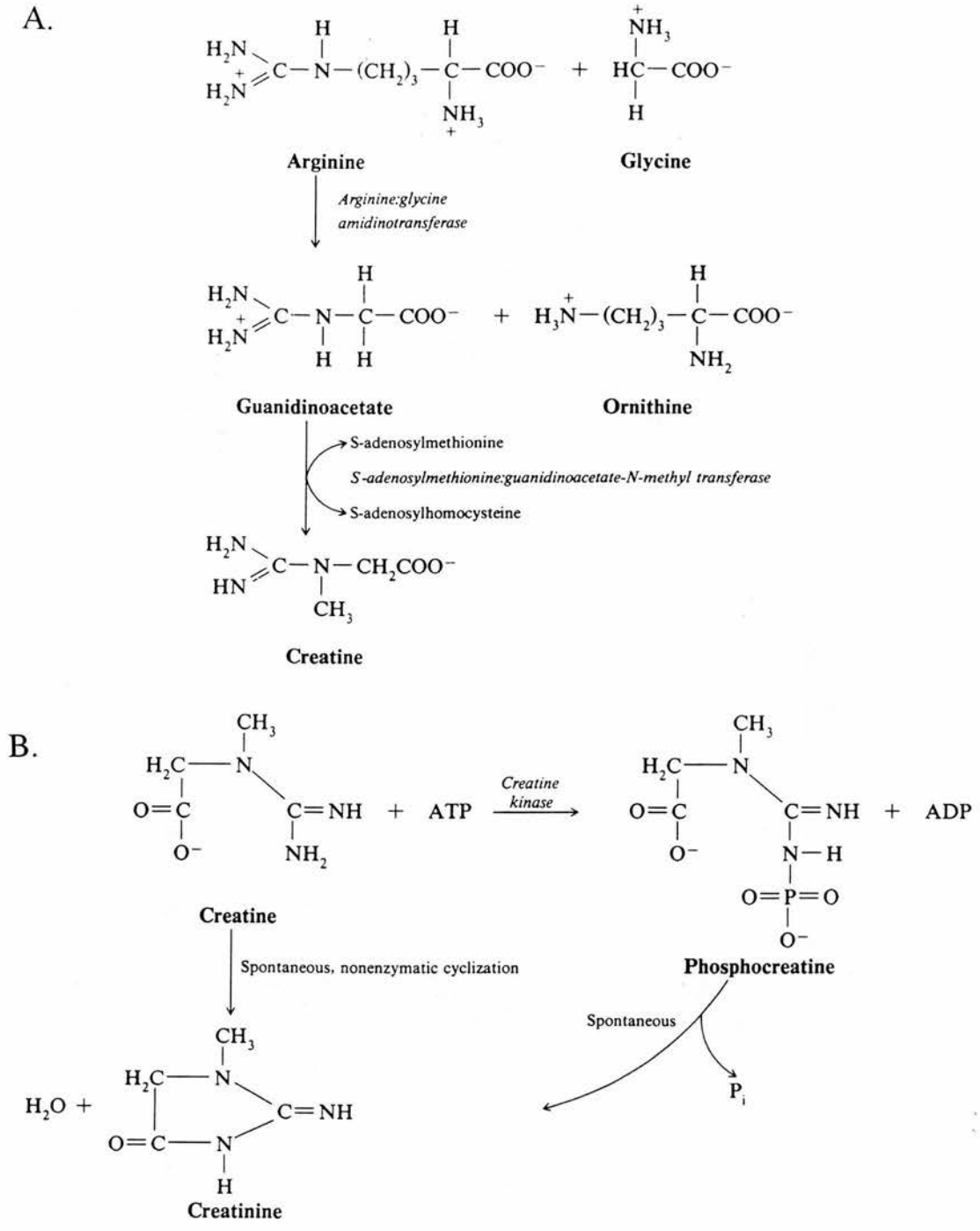


Figure 3. The relationship of creatine to guanidoacetic acid, creatinine, and phosphocreatine. In A, the equations summarise the biosynthesis of creatine showing that guanidoacetic acid is methylated to form creatine. In B, the equation shows the creatine kinase catalysed reaction where phosphocreatine is formed by the transfer of a phosphoryl group to creatine forming ATP in the process and the conversion of creatine to creatinine which occurs in normal metabolism. *Adapted from Tietz, 1986.*

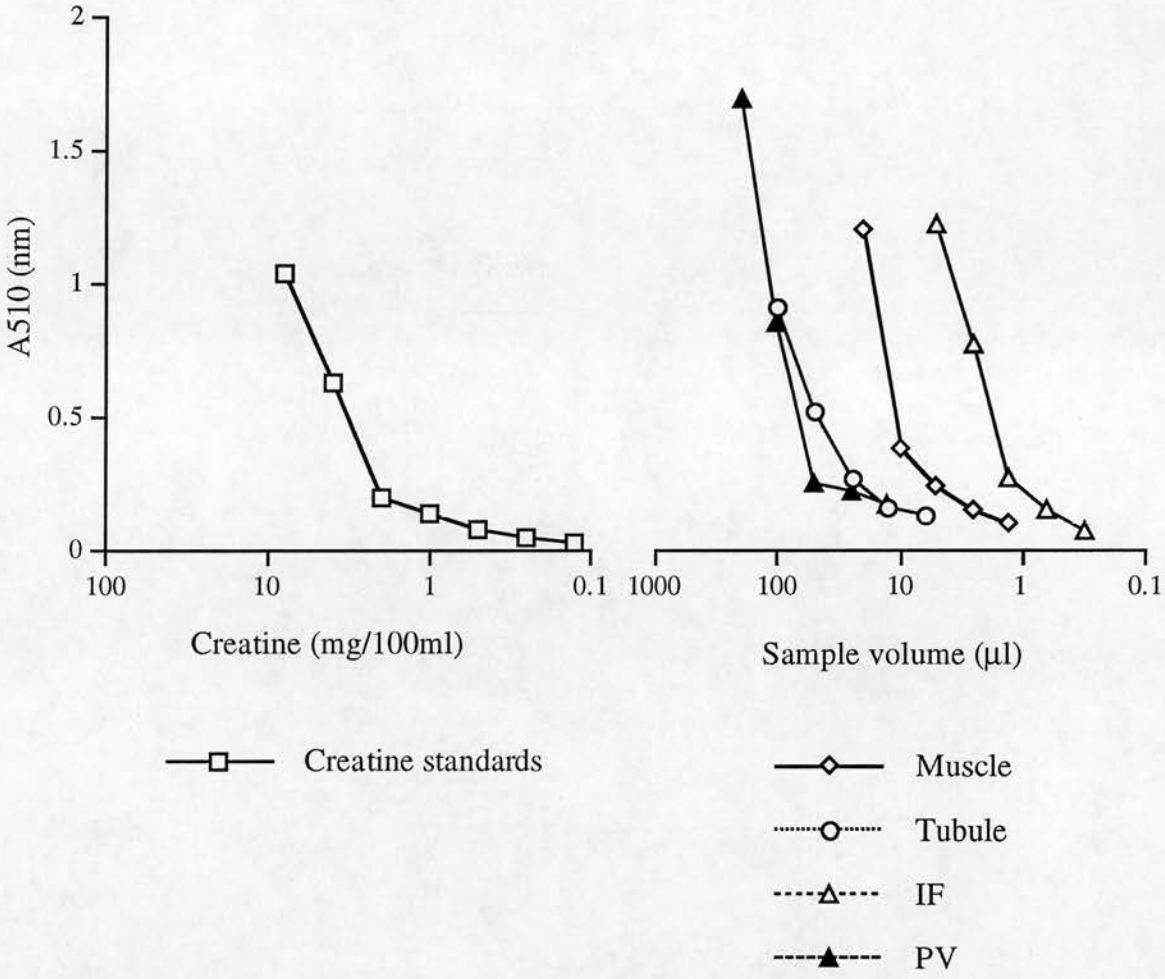


Figure 4. Validation of the creatine assay. Comparison of the creatine levels in 5 doubling dilutions of either muscle homogenate (20-1.25µl), seminiferous tubule (ST) homogenate (100-6.25µl), testicular interstitial fluid (IF; 5-0.31µl) and peripheral venous (PV) plasma (200-12.5µl) with respect to a creatine standard curve (8-0.125mg/100ml). Creatine levels are represented by the absorbance measured at 510nm using a spectrophotometer. This was performed to check that the creatine levels measured in the various samples diluted in parallel to the creatine standard curve.

4.3.3. Comparison of creatine levels in staged ST

ST were dissected from control adult rats and grouped into stages I-V, VI-VIII and IX-XIV. The various stage groupings were homogenised and assayed to assess whether creatine levels within ST showed any regulation with the stage of the spermatogenic cycle. The results obtained from the staged tubules from eleven or twelve rats are shown in Fig. 6. No significant difference in creatine levels, as determined by analysis of variance, was found between the three stage groupings.

4.3.4. Effect of androgen withdrawal on creatine levels in ST

To investigate the effect of androgen withdrawal on the level of creatine in stage VI-VIII ST, staged tubule homogenates were prepared from control rats and from rats treated with either EDS or EDS supplemented with testosterone esters. The results of the assay are shown in Fig. 7. No significant difference between the groups was evident showing that the creatine content of ST is not androgen regulated. The upper graph in Fig. 7 shows the testis weights for each group, demonstrating that EDS has not induced a significant decrease in testis weight at 4 days after treatment, although the decrease in weight in EDS+T treated rats was found to be significant ($p<0.02$).

4.3.5. Effect of specific depletion of germ cells on creatine levels in ST

A preliminary investigation was carried out to check that the presence of any residual MAA in the testis 24 h after treatment would not interfere with the assay. The results demonstrated that the presence of 10^{-3}M MAA in a tubule homogenate had no effect on the level of creatine measured by the assay in comparison to the same sample without the addition of MAA (Fig. 8). The cell types depleted at the selected time points after treatment are summarised in Fig. 9. The effect of MAA on testis weight and on the creatine levels in stage VI-VIII ST homogenates is shown in Fig. 10. At 24 h after a single oral dose of MAA the pachytene spermatocytes at stages VI-VIII of the spermatogenic cycle are degenerating. This induced a small but non-significant decrease in testis weight and a significant decrease in creatine levels ($p<0.01$). With the depletion of pachytene spermatocytes at 4 days after treatment a significant decrease in testis weight was observed ($p<0.01$) but although creatine levels in the isolated tubules were decreased in comparison to control levels this was not found to be statistically significant. In contrast, the depletion of round spermatids at 18 days after MAA treatment from stage VI-VIII tubules resulted in a marked decrease in both testis weight and creatine levels, with a significance of $p<0.001$ for both parameters. At 30 days after treatment, elongate spermatids were depleted, and although this induced a significant decrease in testis weight, the decrease in creatine was not found to be statistically significant. These results are indicative of more creatine being associated with round spermatids and pachytene

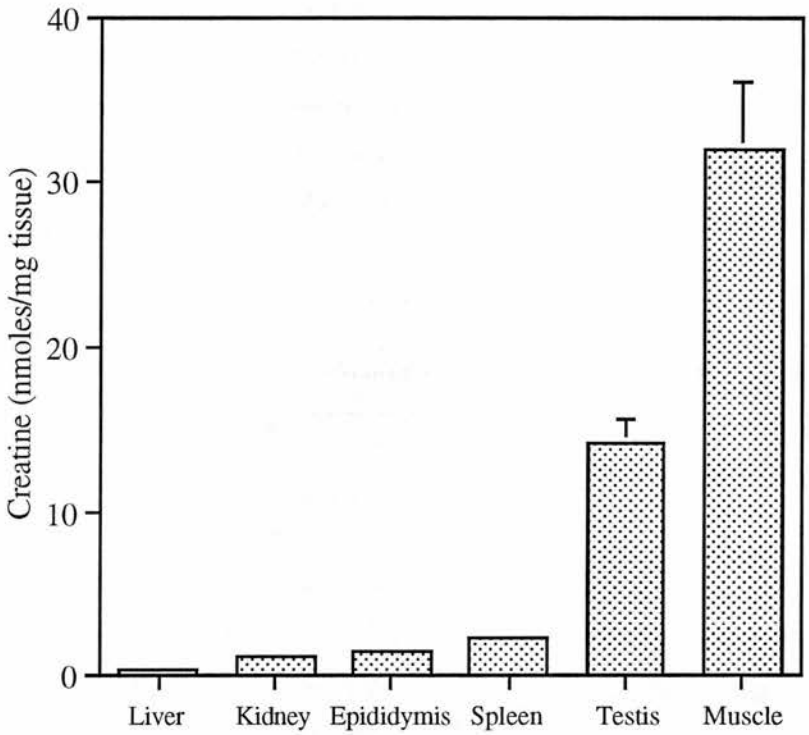


Figure 5. Comparison of the amount of creatine present in liver, kidney, epididymis, spleen, testis and muscle. Results are means of values from four adult control rats ± SD.

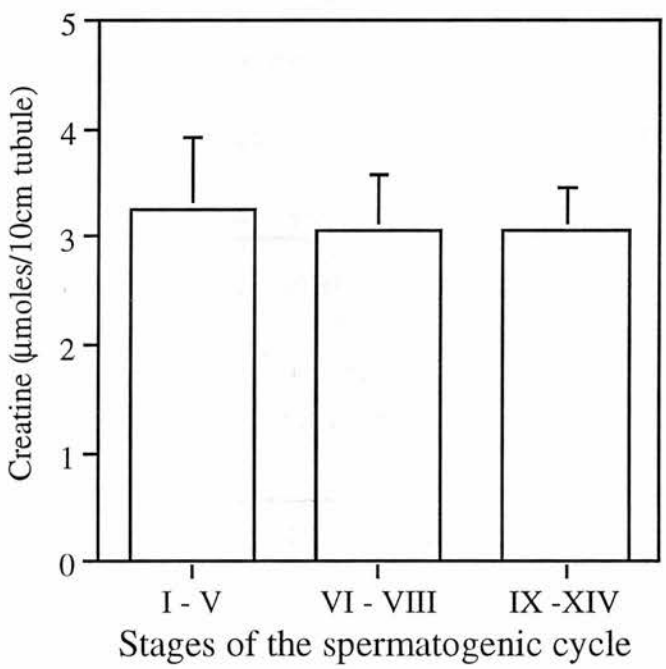


Figure 6. Comparison of the level of creatine in homogenised seminiferous tubules isolated at different stages of the spermatogenic cycle from control adult rats. Values are means ± SD for 11 or 12 rats per stage group.

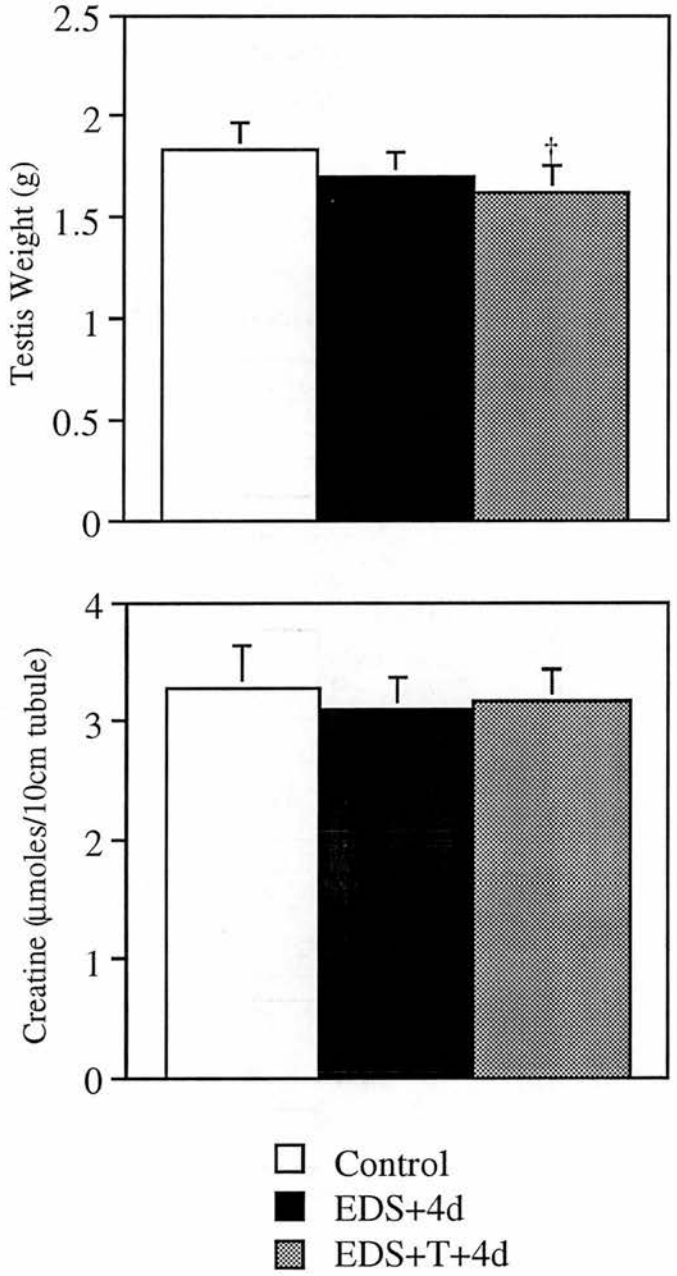


Figure 7. Comparison of the effect of selective withdrawal of testosterone, using a single dose of 75mg/kg EDS, on testicular weight (top) and on the creatine content of homogenised stage VI-VIII seminiferous tubules (bottom). Testicular weights and ST creatine content was measured for control animals (open bars) or rats treated 4 days previously with either EDS alone (solid bars) or EDS + testosterone replacement (shaded bars). Results are shown as the mean ± SD for 6 or 7 rats per group. †$p<0.02$.

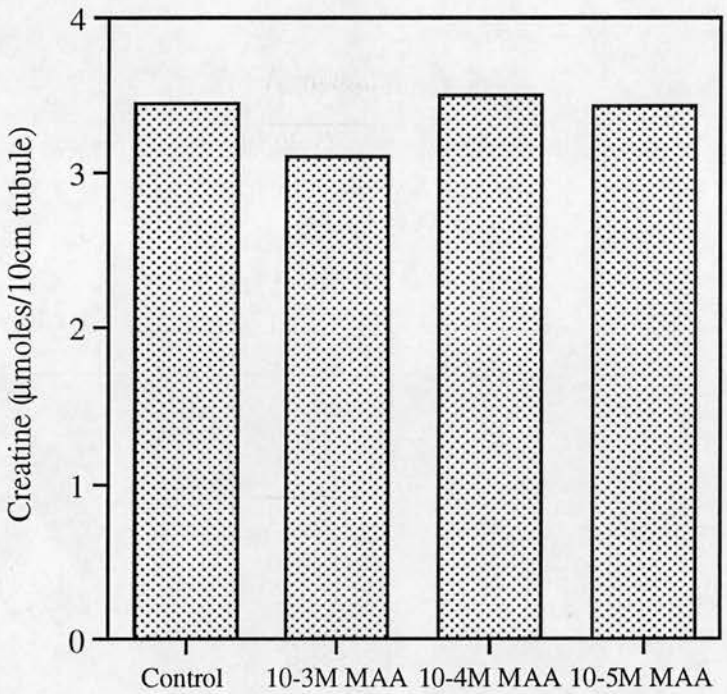


Figure 8. Effect of addition of either 10^{-3}, 10^{-4} or 10^{-5}M MAA to stage VI-VIII ST homogenates, prepared from a control adult rat, on the measurement of creatine.

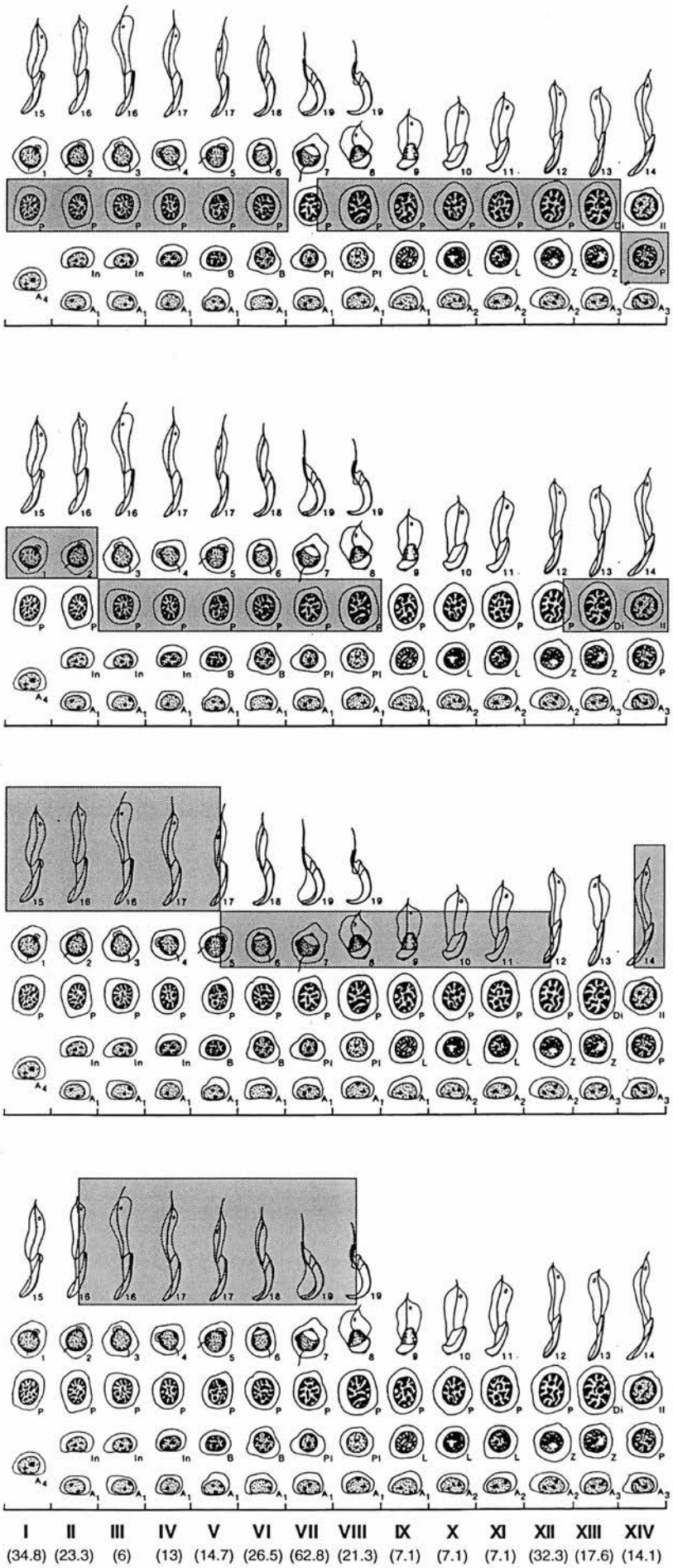
Figure 9. The pattern of depletion of germ cell types observed at 24 hours, 4, 18 and 30 days after administration of a single oral dose of 650mg/kg MAA. The stages (I-XIV) of the spermatogenic cycle and their duration in hours are shown at the bottom. MAA treatment at this dose initially causes a loss of pachytene and later spermatocytes at all stages of the spermatogenic cycle with the exception of early to mid stage VII. As spermatogenesis progresses this initial lesion results in selective loss of round and then elongate spermatids. *Adapted from Leblond & Clermont (1952) and Bartlett et al. (1988).*

24 hours

4 days

18 days

30 days



Stages of the spermatogenic cycle

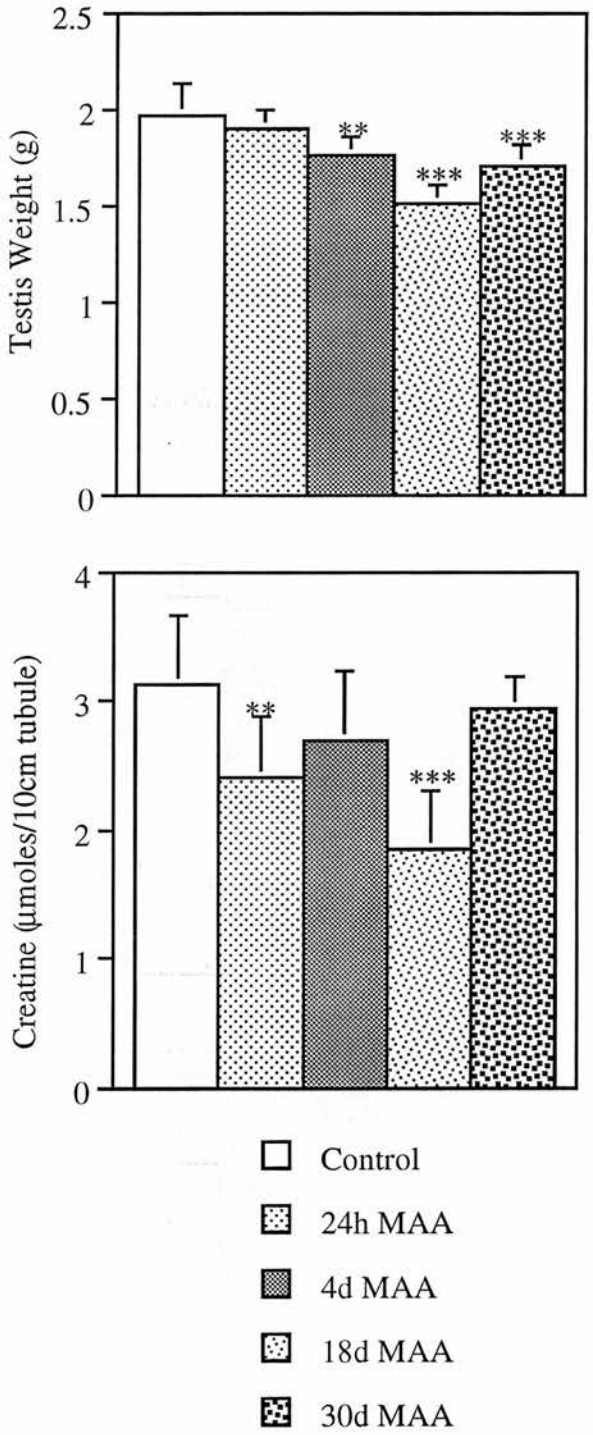


Figure 10. Effect of selective depletion of different germ cell types on testicular weight (top) and on the creatine content of isolated stage VI-VIII seminiferous tubules. Adult rats were pre-treated with a single oral dose of 650mg/kg MAA to induce degeneration of pachytene spermatocytes (24 h) or the selective depletion of either pachytene spermatocytes (4 days), round (18 days) or elongating spermatids (30 days). Results are shown as means ± SD where n=6 or 7 for dosed animals and n=23 for control animals. **p<0.01; ***p<0.001 in comparison with the control group.

spermatocytes than with elongate spermatids. Based on the decrease in creatine induced by the removal of specific germ cells from the seminiferous epithelium, the complete removal of all three types of germ cells would be unlikely to abolish creatine levels completely suggesting that creatine is ubiquitous in all cells within the seminiferous tubules.

4.3.6. Comparison of creatine levels in ST and IF during testicular maturation

Unstaged ST were isolated from one testis and IF collected from the contralateral testis from rats aged 21, 24, 30, 40, 45 and 75 days. The level of creatine in both the tubules and in IF was assessed for at least seven animals for each time point. Testicular weights were also measured for all the animals used in the study. The creatine level in tubule homogenates was found to increase with age and showed a good correlation to increasing testicular weight (Fig. 11). Levels of creatine reached a maximum at around 45 days of age, coinciding with the time when the seminiferous epithelium is known to first possess its full complement of germ cells (Russell *et al.*, 1987). In contrast, the levels of creatine in IF showed an inverse relationship to the levels present in the tubules (Fig. 11). IF creatine concentrations were maximal at around days 20-24, and then declined steadily up to day 45 after which creatine concentration remained at a similar level. The presence of measurable levels of creatine in IF from adult rats suggested that its leakage/secretion from the ST might prove a useful endpoint for detecting testicular damage. The next study attempted to investigate this aspect.

4.3.7. Evaluation of the measurement of creatine in various fluids as a possible marker of testicular damage

A preliminary study attempted to compare the levels of creatine in control adult rats in RTF, testicular IF and also in plasma samples collected from the testicular vein, spermatic vein and the peripheral circulation. Extremely high levels of creatine were found in RTF and IF in comparison to plasma (Fig. 12). The presence of creatine in both RTF and IF suggests that it might be secreted bidirectionally by the seminiferous epithelium. The bottom graph shows a comparison of creatine concentration in various plasma samples. Creatine levels were observed to be slightly higher in SV in comparison to TV and PV but this was not statistically significant as determined by analysis of variance. A sample of peripheral plasma was also assayed from a castrated rat for comparison with levels in an intact animal. Since only one sample was collected from a castrate animal it is not possible to determine the level of significance, but the difference in unlikely to be significant because of the variation seen in plasma creatine levels in intact rats. These results show that the levels of creatine are much higher in RTF and IF in comparison to

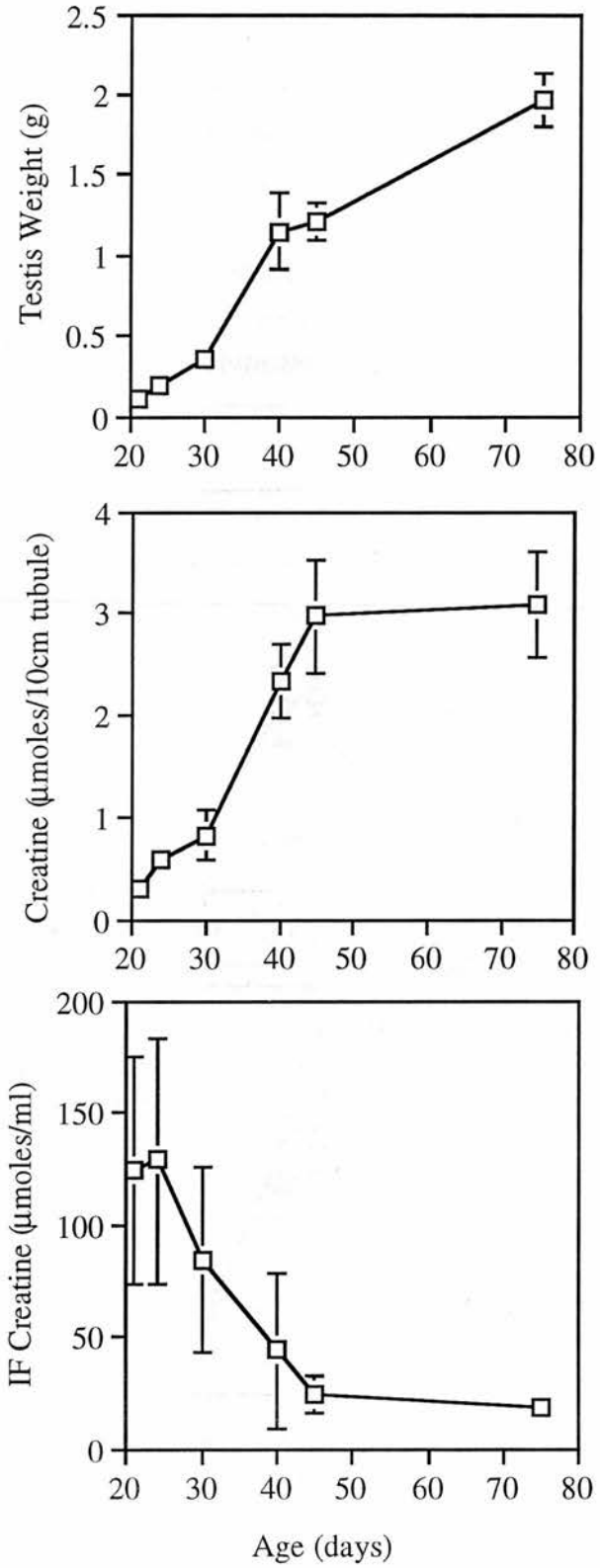


Figure 11. Changes with age in testicular weight (top) and in the levels of creatine in unstaged ST homogenates (middle) and in testicular interstitial fluid (bottom). Results are expressed as mean values ± SD for 7-12 rats.

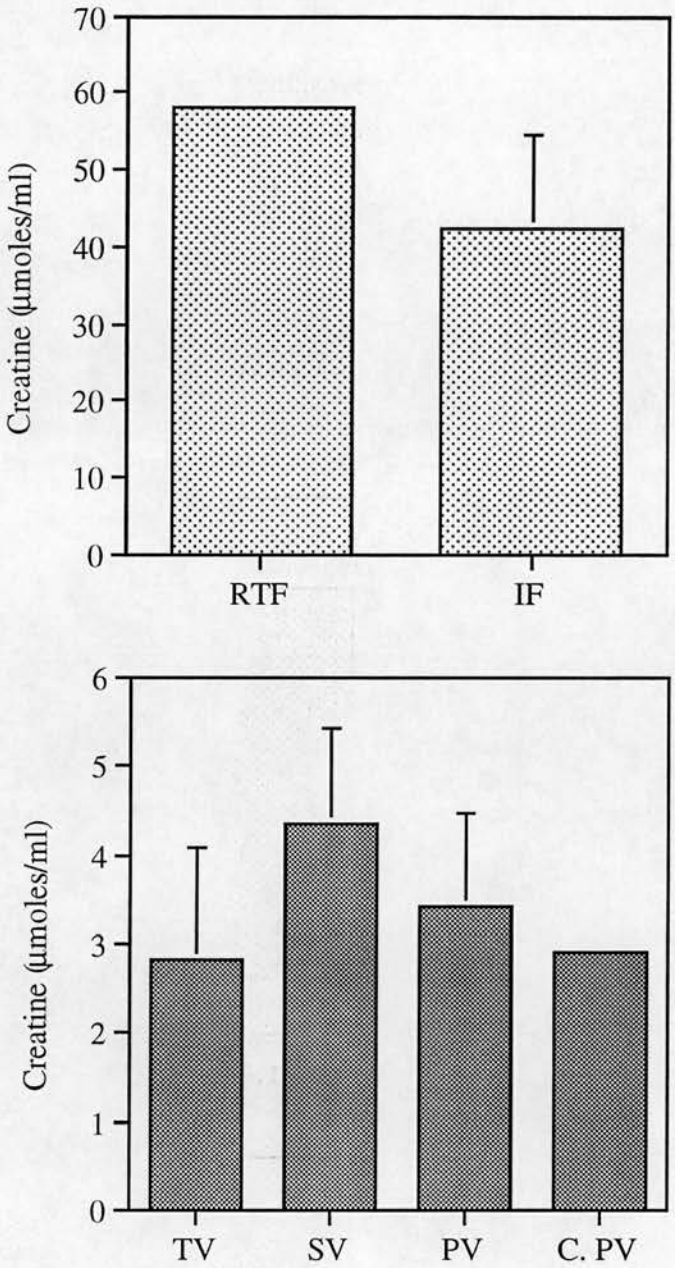


Figure 12. Comparison of the level of creatine in various testicular fluids (top) and plasma samples (bottom) collected from control adult rats. Testicular fluids included rete testis fluid (RTF; n=2) and testicular interstitial fluid (IF; n=17). Plasma samples included testicular venous plasma (TV; n=4), spermatic venous plasma (SV; n=3), peripheral venous plasma (PV; n=10) and peripheral venous plasma from a castrate rat (C. PV; n=1). Results are shown as mean ± SD where possible. Note the different scales used to show the concentration of creatine on the two graphs.

blood.

To assess whether the measurement of creatine is a suitable marker of early adverse effects on spermatogenesis, levels were measured in IF and PV samples collected from rats in which spermatogenesis had been disrupted by the testicular toxicants, EDS, NB and m-DNB, or by scrotal heat-treatment. Animals were killed 4 days after either EDS or EDS plus testosterone treatment, and a sample of IF was collected from each animal but plasma was only collected from two of the animals treated with either EDS or EDS plus testosterone. The levels of creatine in both these fluids was assayed and the results are shown in Figure 13. A marked increase in creatine levels in testicular IF was observed in both EDS and EDS+T treated rats in comparison to controls ($p<0.001$ and $p<0.01$ respectively). Not enough plasma samples were assayed to allow determination of significance. However, the increase in plasma creatine observed in EDS treated animals was very small, and based on the standard deviation of the levels of creatine in control PV samples from another experiment (see Fig. 12) the increase would be unlikely to be significant. For the other methods of inducing testicular damage the level of creatine in testicular IF was found to be increased for all treatments, with a level of significance of $p<0.05$ for NB, $p<0.02$ for mDNB and $p<0.01$ for heat treatment (Fig. 14). Creatine was only significantly increased ($p<0.02$) in PV plasma for animals treated with NB. In contrast, severe local testicular heating induced a significant decrease in plasma creatine ($p<0.02$). A slight increase in plasma creatine induced by mDNB treatment was not found to be significant.

4.3.8. The effect of sample collection procedures on creatine assay results

Further investigation of some of the results gained in these studies led to the realisation that the ST data obtained was severely compromised, since the procedure used to isolate ST was found to result in the measurement of artificially high levels of creatine. Since creatine was measured in both whole testis and in ST homogenates it seemed appropriate to check that the levels measured in 10cm of homogenised ST could be extrapolated to give the same levels as measured in whole testis. This calculation was made based on data published by Wing & Christensen (1982) showing that there is approximately 1240cm seminiferous tubule/g testis in the adult rat. ST homogenates had a mean creatine concentration of 3.091 ± 0.52 µmoles/10cm tubule, which is equivalent to 383.3 nmoles/mg testis. The actual level of creatine measured in whole testis homogenates was found to be only 14.122 ± 1.46 nmoles/mg. This demonstrates that measurements of creatine in tubule homogenates is probably not a true reflection of absolute creatine levels in the testis as a whole. However, since the creatine content of isolated tubules was found to be consistent within groups of animals in all experiments, this might suggest that

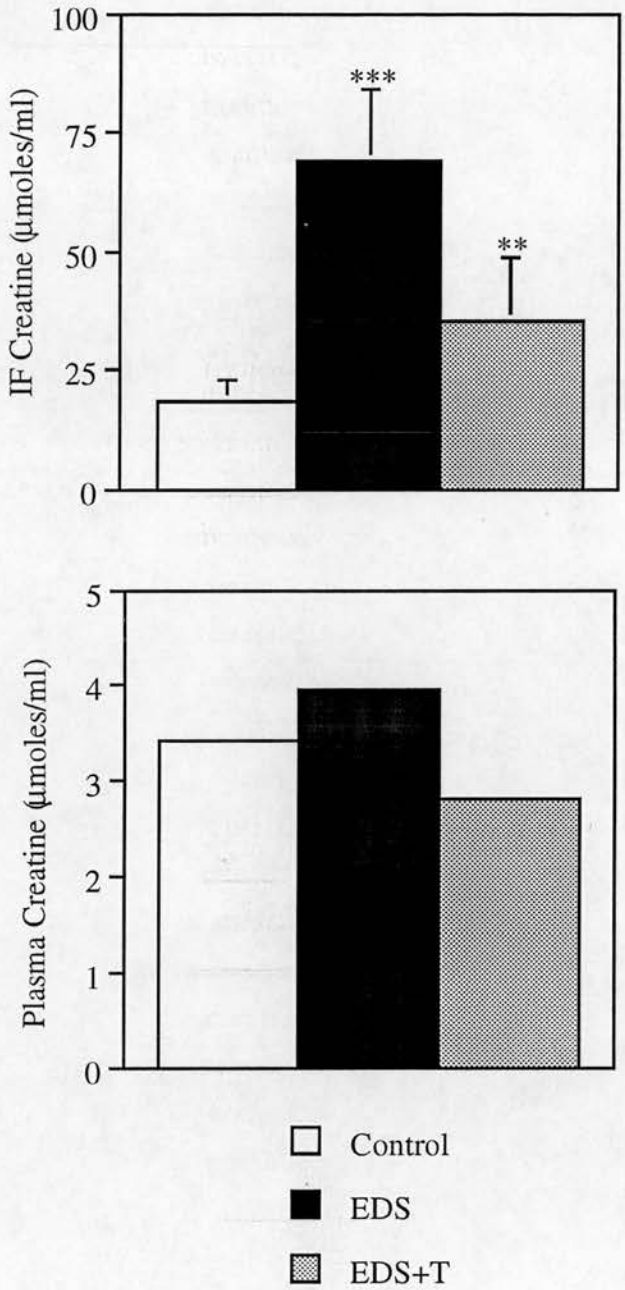


Figure 13. Effect of androgen withdrawal by treatment using EDS on the levels of creatine in testicular interstitial fluid (top) and in peripheral venous plasma (bottom). Samples were collected from control rats (open bars) or from rats treated with EDS 4 days earlier (solid bars) or from rats treated with EDS and supplemented with testosterone (shaded bars). Results are means ± SD for nine rats for the IF results. **$p<0.01$, ***$p<0.001$, in comparison with the respective control value. No SD is shown for the plasma results because blood was only collected from two of the animals in each of the treatment groups. Ideally more samples should have been collected.

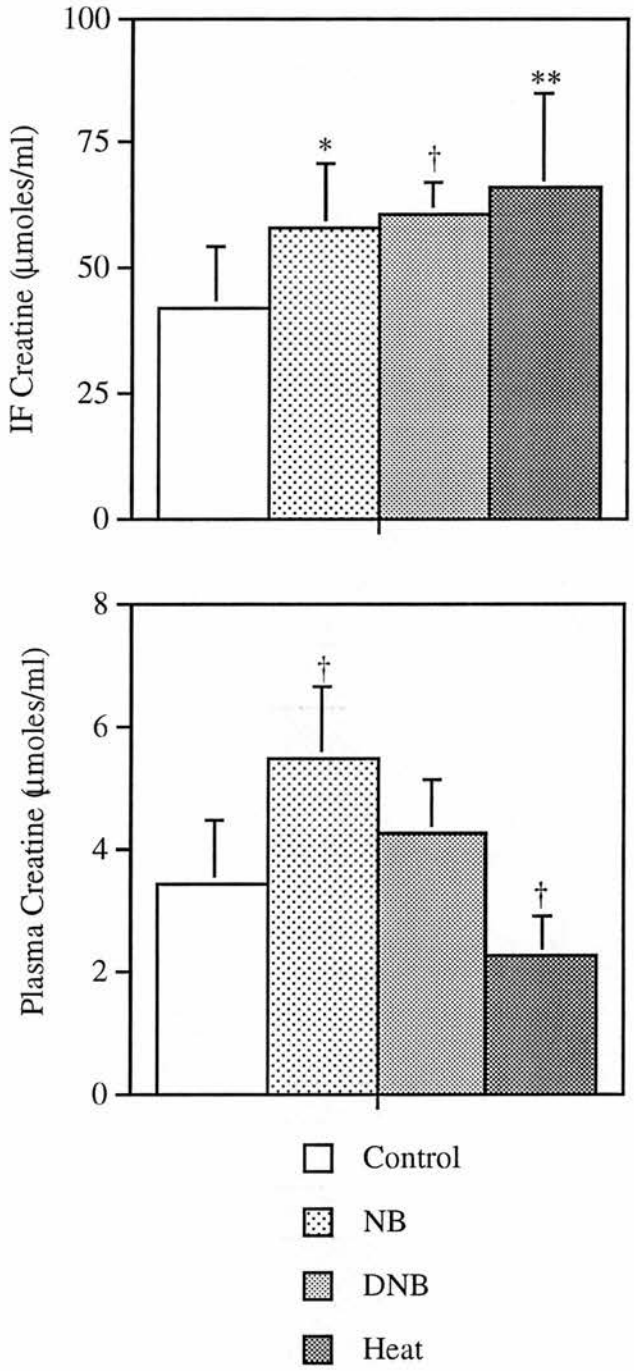


Figure 14. Effect of various testicular toxicants on the levels of creatine in testicular interstitial fluid (top) and in peripheral venous plasma (bottom). Samples were collected 24 h after treatment with either 300mg/kg nitrobenzene (NB; n= 4), 50mg/kg m-dinitrobenzene (mDNB; n=3) or after testicular heating for 30 min at 43°C (Heat; n=7). Results are expressed as means ± SD. For the control animals, IF data is from 17 rats and the plasma results from 10 rats. *$p<0.05$; †$p<0.02$; **$p<0.01$, in comparison with the respective control value.

the levels measured are indicative of relative changes in creatine concentration.

The above results made it necessary to re-examine the IF data to assess if the overnight collection procedure was also resulting in abnormally high levels of creatine in IF. In one experiment a small volume of IF was collected at 4°C from the testes of seven adult rats during the first 30 min after isolation. The creatine levels in these samples were then compared to the levels present in IF collected over 16 h at 4°C. The levels of creatine in the 30 min sample (11.1± 3.6 µmoles/ml) were found to be slightly lower than the levels in overnight samples (14.8± 2.0 µmoles/ml) which was just significant ($p<0.05$). This small difference might be due to slight contamination by intracellular creatine, since care is necessary to prevent damage to the seminiferous tubules beneath the testicular capsule when an incision is made into the capsule to allow IF collection. Intracellular creatine contamination may explain the high degree of variability in the creatine levels measured in immature and pubertal animals (see Fig. 11).

4.4. Discussion

The studies described in this chapter were instigated by several publications suggesting that urinary creatine may be suitable marker of acute testicular damage (Rawcliffe *et al.*, 1989; Gray *et al.*, 1990; Moore *et al.*, 1992; Nahas *et al.*, 1993). The aim of this thesis is to study the feasibility of monitoring the normality or otherwise of testicular function non-invasively. Based on the above reports creatine seemed an ideal candidate for further investigation of its usefulness as a marker of disruption to spermatogenesis. The studies performed had two main aims. Firstly, to investigate the relationship of testicular creatine to spermatogenesis. The experiments were designed to explore the possibility that creatine production might be stage specific or androgen regulated, and also to localise which cells of the seminiferous epithelium were responsible for creatine synthesis. The production of creatine by seminiferous tubules during testicular maturation and its secretion/leakage into interstitial fluid during this period was also investigated. The second aim was to evaluate the potential of measuring changes in the concentration of creatine in blood as a means of detecting testicular damage. Various methods of disrupting spermatogenesis, including scrotal heating and testicular toxicants, were used to establish whether these treatments affected the levels of creatine in testicular interstitial fluid and in peripheral blood.

An initial study investigated the levels of creatine found in various tissues collected from adult rats. The most striking feature of the results is that the testis contains a much higher concentration of creatine than found in either liver, kidney, epididymis or spleen, with levels approximately half of that found in muscle. The concentration of creatine measured in this study (14.12±1.45nmol/mg) is in close agreement with data

reported by Lee *et al.* (1988) for an adult rat testis, 11.2nmol/mg. The *in vivo* synthesis of creatine by the testis has been demonstrated by the injection of radiolabelled arginine or guanidoacetic acid into the testes of rats (Koszalka, 1968). These results demonstrate that the testis is capable of synthesising exceptionally high levels of creatine, suggesting that the creatine-phosphocreatine circuit is operating in the testis to provide an energy transport system necessary for the maintenance of spermatogenesis.

The observation that the testis did contain a high concentration of creatine made it possible to investigate the relationship of creatine to spermatogenesis using isolated seminiferous tubules. Creatine synthesis by isolated rat seminiferous tubules cultured *in vitro* has also been demonstrated (Moore *et al.*, 1989). This approach allowed investigation of whether creatine production was regulated according to the stage of the spermatogenic cycle or by intratesticular levels of testosterone, since stages VI-VIII have been shown to be androgen dependent (Sharpe, 1994b). Stage regulation was assessed by measuring the creatine content of seminiferous tubule grouped into stages I-V, VI-VIII and IX-XIV. No significant difference was found between the creatine concentration in the three stage groupings, suggesting that creatine production is similar at all stages of the spermatogenic cycle. Androgen regulation was investigated by determining the creatine content of stage VI-VIII seminiferous tubules isolated from control rats or rats which had been treated with either EDS or EDS supplemented with testosterone which is able to maintain normal spermatogenesis (Sharpe *et al.*, 1988a,b; 1990). Again, no significant difference in levels of creatine was observed between the different groups of animals. The lack of either stage or androgen dependency of creatine production by seminiferous tubules suggests that creatine is ubiquitous in the seminiferous epithelium and is a basic requirement for cellular metabolism for all of the cell types.

In an attempt to assess which cell types within the seminiferous epithelium were responsible for creatine synthesis the testicular toxicant, MAA, was used to induce the specific depletion of germ cells. Animals were killed at selected time points after treatment when one specific germ cell type was known to be depleted from seminiferous tubules at stages VI-VIII. Tubules were isolated at these stages and their creatine content was assessed. The most marked decrease (40%) in seminiferous tubule creatine concentration was observed when round spermatids were depleted, but a significant decrease (23%) was also induced by the degeneration of pachytene spermatocytes at 24 h after MAA administration. The absence of elongate spermatids from stage VI-VIII tubules resulted in a small decrease (8%) in creatine levels which did not reach statistical significance. These results suggest that the pachytene spermatocytes, round spermatids and elongating spermatids are probably responsible for the synthesis of around 70% of the creatine present in isolated seminiferous tubules. It remains to be determined whether the other 30% is produced by the Sertoli cells and/or the other germ cell types which are

not affected by MAA treatment. It is also possible that the effects on the creatine levels observed in this study are a result of adverse effects on the Sertoli cells due to the removal of germ cells and that the Sertoli cells alone may be responsible for creatine synthesis. However, phosphocreatine has been measured in sea urchin sperm (Tombes & Shapiro, 1985) and also in sperm released from the mouse cauda epididymis (Lee *et al.*, 1988), which would suggest that mature spermatozoa do produce creatine. Therefore, it would seem likely that developing germ cells also have the capacity to synthesise creatine.

Creatine was measured in seminiferous tubules and testicular IF during testicular maturation. The level of creatine in unstaged tubule homogenates increased with age, reaching a maximum at around day 45, and showed a good correlation to testicular weight. Creatine production by STs is maximal when the seminiferous epithelium first attains its full complement of germ cells (Russell *et al.*, 1987). Interestingly, creatine was found to be secreted into the interstitium although, there was a progressive decrease in this secretion during sexual maturation. This follows a similar secretory profile as observed for the Sertoli cell secreted proteins, ABP and inhibin (Sharpe & Bartlett, 1987; Sharpe *et al.*, 1988b; Maddocks & Sharpe, 1990a), which might suggest that the creatine in IF originates from the Sertoli cells. The explanation for this marked reduction with age is unclear. It would correspond with the gradual improvement in efficiency of the inter-Sertoli cell tight junctions which form a major component of the blood-testis barrier which occurs during this time (Setchell *et al.*, 1988). It may also be related to the fall in FSH levels and/or the increase in testosterone levels during testicular maturation (Sharpe & Bartlett, 1987; Sharpe *et al.*, 1988b).

The extracellular distribution of creatine within the testis was investigated by measuring its levels in fluids collected from the rete testis as well as the interstitium. The results show that there is a high concentration of creatine in the extracellular fluids of the testis. The presence of creatine in extracellular fluid of the testis has been demonstrated previously (Lee *et al.*, 1988). They found that greater than 90% of total testicular creatine was found in the soluble fraction of washed testicular slices. No significant creatine kinase was found in the soluble fraction indicating there was no leakage of this enzyme due to damage to the tissue. These authors concluded that most of the creatine in the testis is extracellular. The high levels of creatine in STF and IF would agree with this conclusion.

For comparison, the concentration of creatine was also measured in TV, SV and PV blood. The levels of creatine in blood were approximately 10 fold lower than in rete testis fluid. Although no significant difference was found between the blood samples a slight increase in creatine levels in SV blood was observed. It has been demonstrated in rats that inhibin levels are significantly raised in SV blood in comparison to TV blood showing that inhibin is preferentially resorbed from the rete testis into the venous blood

(Maddocks & Sharpe, 1989a; Maddocks *et al.*, 1993). Due to the variability of creatine levels measured in all the plasma samples and the low number of samples assayed it is not possible to draw similar conclusions about the route of secretion of creatine. A single sample of peripheral blood collected from a castrated rat was found to possess a similar creatine concentration to that of intact rats indicating that testicular creatine does not contribute in a major way to peripheral levels.

To evaluate the usefulness of creatine as a marker of impaired spermatogenesis creatine levels in both testicular IF and PV plasma were measured following various treatments known to disrupt spermatogenesis in rats. EDS was used to induce testosterone withdrawal by the selective depletion of Leydig cells. The Leydig cells are destroyed within 48 hours but at 4 days after treatment the only other abnormal change in testicular morphology is the appearance of a small number of degenerating germ cells at stage VII of the spermatogenic cycle (Sharpe *et al.*, 1992). EDS treatment resulted in a three-fold increase in the IF levels of creatine on day 4. Secretion of both inhibin and ABP into IF is also raised to a similar degree in this situation (Sharpe *et al.*, 1988b; Bartlett & Sharpe, 1987; Sharpe & Bartlett, 1987). Unexpectedly, creatine levels were also significantly increased in IF from rats treated with EDS supplemented with testosterone, which prevents the degeneration of germ cells within the seminiferous tubules. The reason for this is unclear but suggests that the administration of testosterone is insufficient to prevent adverse changes in creatine secretion caused by Leydig cell destruction. This might suggest that paracrine factors secreted by Leydig cells are involved in regulating creatine secretion by the seminiferous tubules. Plasma samples were assayed for creatine from only two individual control rats and from two treated rats, so the sample size was not large enough to determine if there was a statistically significant increase in plasma creatine after EDS-treatment. But based on the results obtained in another experiment where enough control PV blood samples were assayed to show the normal degree of variability, the small increase in plasma creatine concentration induced by EDS treatment is probably not significant. EDS (75mg/kg) was found to cause a slight but significant ($p<0.05$) increase in urinary creatine levels in urine collected 24-48 hours after dosing (Moore *et al.*, 1992). Although EDS treatment is able to alter the pattern of creatine secretion within the testis this effect is not sufficient to affect the levels in plasma. This would suggest that damage to the testis would need to be extremely severe to induce an alteration in plasma, and presumably urinary, creatine.

The adverse testicular effects caused by NB within the 24 hours following dosing consist of Sertoli cell vacuolation with the loss and/or degeneration of pachytene spermatocytes at stages VI-XII (Allenby, 1990). These effects have also been demonstrated *in vitro* on Sertoli cells and on Sertoli-germ cell co-cultures (Allenby *et al.*, 1990). This treatment resulted in a slight increase in levels of creatine in IF which was

only just significant but a more marked increase in plasma creatine concentration. The effects on testicular morphology appear not to be sufficient to induce a large increase in creatine secretion/leakage into the interstitium. These results would suggest that NB is causing damage to other organs besides the testis leading to a greater change in creatine levels in plasma. m-DNB causes similar damage to the testis as observed with NB (Allenby *et al.*, 1990; McLaren *et al.*, 1993a). The first morpological abnormalities observed are the vacuolation and retraction of the Sertoli cells. By 24 hours this is associated with necrosis and loss of pachytene spermatocytes at stages VII-XIII, deformed heads and disorientation of elongating spermatids (Blackburn *et al.*, 1988; Hess *et al.*, 1988). A small but significant increase in creatine levels in IF was induced by this treatment but a slight increase in plasma creatine concentration was not found to be significant. Short-term local testicular heating resulted in the most significant increase in creatine concentration in IF and a marked decrease in plasma creatine levels. At 24 hours after heat treatment the loss or degeneration of pachytene spermatocytes is visible at all stages of the spermatogenic cycle, this is coupled with retention of the heads of elongating spermatids at stages IX-XIV and Sertoli cell vacuolation (McLaren *et al.*, 1994). By relating the changes seen at the morphological level, for the different methods of disrupting spermatogenesis, with the effects on creatine levels in both IF and peripheral blood, it would seem that creatine levels in IF are related to the severity of damage to the seminiferous epithelium. However, changes in plasma creatine induced by these treatments are small even when widespread damage is induced in the seminiferous epithelium, as seen with scrotal heating, indicating that plasma creatine concentration is not a sensitive marker of testicular damage.

Unfortunately, further examination of the data obtained in these studies showed that the results for the creatine content of tubule homogenates were compromised by problems associated with sample collection. These results demonstrated that the initial assumption in the experimental procedure that levels of creatine measured in isolated tubule homogenates were related to the levels measured in whole testis homogenates was incorrect. On extrapolation of the tubule data to give an estimate of total levels in the testis, the estimation was found to be 27 fold higher than what was actually measured in a total testis homogenate. This would indicate that the seminiferous tubule isolation procedure induces an increase in creatine production within the cells of the seminiferous epithelium. One possible explanation is that ATP levels within the tubules have fallen to such an extent that the equilibrium between creatine and phosphocreatine is altered to force the reaction in the direction of creatine production to try and regain cellular energy homeostasis. Testes were kept on ice once removed from the animal and tubules were dissected within 30 min of death on a cooled stage (4°C), in an attempt to prevent any significant changes in tubule viability. ST isolated in this way have been shown in many

studies to be physiologically responsive in culture (Allenby *et al.*, 1991a,b; Sharpe *et al.*, 1992). However, it would seem that these precautions were not sufficient to prevent changes in creatine metabolism. These results perhaps reflect the difficulty in attempting to study a chemical which is an integral part of cellular metabolism using tissue collected post-mortem.

In conclusion, the experiments described in this chapter have shown that the testis produces large amounts of creatine in comparison to other tissues. This suggests that the creatine-phosphocreatine circuit is operating within the seminiferous tubules to supply ATP energy for the maintenance of spermatogenesis. However, creatine levels within the seminiferous tubules do not seem to be related to particular phases in spermatogenesis since creatine production was not regulated by the stage of the spermatogenic cycle or by androgen. Studies involving depletion of germ cells from the seminiferous epithelium suggested that the germ cells contain most of the creatine in the seminiferous tubules but this observation does not exclude the production of creatine by the Sertoli cells. Creatine was observed to have a similar pattern of secretion into IF during testicular maturation as seen for the Sertoli cell secreted proteins, ABP and inhibin, supporting the idea that Sertoli cells synthesise creatine. The fact that creatine was not associated with a particular cell type suggests it would have limited value as an informative marker of spermatogenesis. This was confirmed by results obtained from the studies investigating the effect of disruption of spermatogenesis on creatine levels in IF and blood. These studies showed variable effects on creatine levels, but the changes observed in blood were so small as to allow the conclusion that plasma creatine concentration is not a suitable non-invasive marker of acute testicular damage.

Chapter 5. Detection of seminiferous tubule derived proteins in biological fluids

5.1. Introduction

Disorders of human spermatogenesis are presently only detectable by changes in sperm parameters in the ejaculate, altered hormone levels in peripheral blood or by infertility. When and where in spermatogenesis these defects arise is largely unknown because our understanding of this complex process is poor and there are no means (other than testicular biopsy) of monitoring events within the seminiferous tubules. The ideal would be to identify proteins which play key roles in spermatogenesis and which also find their way into the bloodstream where measurement of their levels might provide important insight into the normality or otherwise of particular steps in spermatogenesis (Sharpe, 1992). The objective of the investigations described in this chapter was to ascertain whether ST secreted proteins can be used to monitor spermatogenesis by determining the feasibility of their detection in biological fluids (interstitial fluid, testicular vein blood, spermatic vein blood and peripheral vein blood) from control adult rats, and also in interstitial fluid and peripheral blood from adult rats in which spermatogenesis has been disrupted by short term local testicular heating. It was hoped that these studies would allow identification of proteins with the potential to act as markers of spermatogenic damage.

The approach used in these studies involved the generation of a polyvalent antiserum raised against seminiferous tubule conditioned medium, prepared by the culture of unstaged seminiferous tubules from control adult rats for 22 hours, which was used in conjunction with Western blot analysis to detect ST derived proteins in various biological fluids. The aim being that the antiserum would contain antibodies against a wide variety of proteins secreted by the seminiferous epithelium. The conditions for culture of STs including culture medium, duration of culture, the total and individual length of tubules have been previously defined in terms of optimising the secretion of immunoactive inhibin in response to FSH and dibutyryl cAMP (Allenby *et al.*, 1991a,b). This culture system has proved useful for investigation of the regulation of protein secretion by seminiferous tubules (Sharpe *et al.*, 1992; McKinnell & Sharpe, 1992; Sharpe *et al.*, 1993; McKinnell & Sharpe, 1995). For the purpose of monitoring spermatogenesis it is more appropriate to investigate protein secretion in the adult rather than immature rat since spermatogenesis is not qualitatively complete until around 45 days of age (Russell *et al.*, 1987), and considerable differences in protein secretion by seminiferous tubules isolated from immature (aged 28 days) to adult (aged 70 days) rats have been demonstrated (McLaren *et al.*, 1993b).

The ability to detect testicular proteins in biological fluids was compared in the normal physiological situation and when testicular damage was induced, to ascertain whether this would affect the secretion or 'leakage' of proteins from the seminiferous epithelium. Rats were exposed to short term scrotal heating (43°C for 30 min) and killed 24 hours later for collection of samples. The effects of testicular heating are well documented and the severity of spermatogenic disruption is related to the temperature used and the duration of heating. Chowdhury and Steinberger (1964,1970) used quantitative analysis to describe germ cell degeneration in the seminiferous epithelium following exposure of the scrotum to 43°C for 15 minutes. They found that primary spermatocytes from the leptotene stage onwards to dividing spermatocytes at stage XIV, except for pachytene spermatocytes in stages V and VI, as well as spermatids in steps 1 and 2 were all selectively damaged by heat and had disappeared within 48 hours after treatment (Chowdhury & Steinberger, 1964). Investigation of changes during the 24 hours after exposure to heat showed that morphological changes could be detected as early as 1 hour later in stage IX to XII pachytene spermatocytes, diakinetic and dividing spermatocytes in stages XIII and XIV and step 1 spermatids, indicating that these cells are the most heat susceptible (Chowdhury & Steinberger, 1970). This pattern of germ cell degeneration has been confirmed in other studies (Jégou *et al.*, 1984; Bartlett & Sharpe, 1987) and Jégou and colleagues demonstrated the complete recovery of spermatogenesis 56 days after the initial exposure to heat. In rats exposed to 43°C for 30 minutes the damage is more widespread but still stage-specific (Bartlett & Sharpe, 1987; McLaren *et al.*, 1994). Morphological examination of testis sections 24 hours later showed widespread degeneration or loss of pachytene spermatocytes, the heads of step 19 spermatids were found to be embedded deeply in the Sertoli cell cytoplasm at stages VI-VIII and extensive vacuolation of the seminiferous epithelium at the level of the inter-Sertoli cell tight junctions was observed (McLaren *et al.*, 1994).

Morphological examination has shown that whilst heat induces degeneration of spermatocytes, round spermatids from step 2 onwards appear to be heat resistant. This may be associated with the susceptibility of meiosis to heat stress. It has been shown in mice exposed to an environmental temperature of 35°C for several days, results in an increased rate of X-Y dissociation in diakinesis-metaphase I spermatocytes in comparison to pachytene spermatocytes (Waldbieser & Chrisman, 1986). The same effect can be induced by heating the testes to 42°C for 20 minutes, suggesting that dissociation of the sex chromosomes is a direct effect of heat (Setchell, 1994). Spermatid resistance to heat may be related to their ability to express members of the heat shock protein (hsp) 70 gene family in an abundant manner even in the absence of heat stress (Zakeri & Wolgemuth, 1987; Zakeri *et al.*, 1990; Krawczyk *et al.*, 1988).

Several studies have investigated the effects of heat induced damage to spermatogenesis on Sertoli and Leydig cell function. Rats exposed to testicular heating at 43°C for 15 minutes resulted in a reduction in the production of STF 26 days later and of ABP production 14 days later; FSH binding declined but serum FSH levels increased during this time (Jégou *et al.*, 1984). These effects are indicative of impaired Sertoli cell function. They also found that Leydig cells showed increased responsiveness to LH-stimulation of testosterone production *in vitro* at 14 days and decreased ability to bind hCG from 7 to 26 days, but serum testosterone and LH were not altered. Since the effects on Sertoli and Leydig cell function do not occur immediately after heat exposure it was concluded that these changes were a result of the altered germ cell complement caused by the degeneration of heat susceptible cells (Jégou *et al.*, 1984). Similar effects on inhibin production have also been shown. Both testicular inhibin content and rate of production were maximally reduced 2 weeks after testicular heating (Au *et al.*, 1987). The effect of heat (43°C for 30 min) on testosterone production *in vivo* and *in vitro* has been compared (Galil & Setchell, 1987b). They also found that stimulation of testosterone production *in vitro* was increased in comparison to control testes. However, hCG stimulation of testosterone production *in vivo* resulted in reduced levels of testosterone in peripheral blood but increased levels in testicular venous blood, suggesting that testosterone secretion is not as effective as in normal testes.

More severe local heating of the scrotum (43°C for 30 min) has also been shown to affect the route of secretion of Sertoli cell proteins (Bartlett & Sharpe, 1987; Sharpe & Bartlett, 1987). ABP secretion into interstitial fluid was significantly increased within 3 days of treatment (Bartlett & Sharpe, 1987). These authors also monitored the bioactivity of a putative regulator of Leydig cell steroidogenesis in interstitial fluid; secretion of this factor was also significantly increased within 3 days after heat, like ABP, suggesting that it might originate from Sertoli cells. Increased secretion of α-inhibin into interstitial fluid is also observed within 3 days of local heating of the scrotum (Sharpe & Maddocks, 1989). These results demonstrate that severe heat treatment results in the increased secretion of specific proteins via the base of the Sertoli cell. It is possible that severe scrotal heating affects the permeability of the blood-testis barrier so that increased leakage of proteins into the interstitium occurs. One study has demonstrated that maintenance of rat testes at 41°C for 1 hour does not significantly alter the integrity of the blood-testis barrier (Main & Waites, 1977). This was assessed by analysis of rete testis composition and measurement of the transfer of various radiolabelled substances (albumin, ions, testosterone and other steroids) from blood into rete testis fluid. There was increased transfer of ions but albumin was unable to penetrate the seminiferous tubules and there was no change in the rapid access to rete testis fluid by testosterone.

Other effects of testicular heating have been reported. A significant decrease in testicular weight is induced by severe heating (Bartlett & Sharpe, 1987; McLaren *et al.*, 1994). It has been demonstrated that in parallel with changes in testis weight there is a decline in blood flow within 2 days of treatment which becomes significant by 7 days, reaches a minimum at 14-21 days and then starts to recover and is normal by 56 days post-heating (Galil & Setchell, 1987a). Temperatures below 41°C had no effect on blood flow. However, vasomotion disappears when rat testes are heated to between 36°C and 42°C (Setchell *et al.*, 1995). The decrease in blood flow would explain why testosterone produced by Leydig cells does not enter the peripheral circulation as readily in heat treated testes (Galil & Setchell, 1987b). Testicular heating has also been implicated in male infertility with effects on sperm production, viability, motility, morphology and fertilising ability (Mieusset & Bujan, 1995).

This chapter describes the experiments performed to assess whether ST derived proteins could be detected in IF and blood, either in the normal physiological situation or in rats in which spermatogenesis had been disrupted by local testicular heating.

5.2. Experimental procedures

5.2.1. Generation of antiserum to seminiferous tubule conditioned medium (STCM)

The methods used have all been described in full in chapter 3, sections 3.4, 3.6 and 3.7. Unstaged seminiferous tubules were isolated from four control adult rats and cultured for 22 h to generate conditioned medium. The STCM was pooled and prepared for immunisation of two rabbits. Serum was collected from the rabbits 3 weeks after a booster injection. The IgG fraction was purified from a portion of the antiserum by Protein A affinity chromatography. The expectation was that the antiserum raised would contain a variety of antibodies against proteins secreted by adult seminiferous tubules.

5.2.2. Immunohistochemistry

Immunostaining was performed on 5μm sections of perfused-fixed control adult rat testis as described in chapter 3, section 3.12. The IgG fraction of the antiserum was used at a dilution of 1:500 and sections were also incubated with an equivalent dilution of normal rabbit serum instead of the immune serum to act as a negative control.

5.2.3. Collection of testicular IF and plasma samples

Samples of testicular IF, TV, SV and PV blood were collected from control adult rats as described in chapter 3, section 3.3. PV blood was also collected from a castrated rat administered with testosterone esters to maintain normal testosterone levels. In one

experiment, IF was collected over 30 min from the testes of four control rats and pooled; the same testes were then maintained overnight (approximately 16 hours) at 4°C and a further sample of IF then collected and pooled. This was to ensure that results gained by the analysis of IF collected overnight at 4°C were not an artefact of the collection procedure.

5.2.4. Specificity of antiserum

STCM, prepared as described in chapter 3, was resolved on 7-15% w/v gradient acrylamide gels using 1-D and 2-D SDS PAGE, electroblotted onto a PVDF membrane and then probed with STCM antiserum, (see sections 3.4, 3.8, 3.9 and 3.10 of chapter 3 for methods). This was to allow assessment of how many proteins in STCM were recognised by the polyvalent antiserum in comparison to equivalent blots stained for total protein using 0.1% w/v Coomassie blue (Bio-Rad) in a solution of 50% w/v methanol and 5% w/v acetic acid. To analyse the specificity of the antiserum, membranes were incubated with preabsorbed antiserum or normal rabbit serum instead of the immune serum during Western blot analysis. The antiserum was preabsorbed as follows. A total of 16mls of STCM (= 7.5mg protein) was prepared from two adult rats as described in section 3.4 and was used to coat one side of several nitrocellulose membranes (Hybond™-C, Amersham) which were allowed to dry. The antiserum was diluted 1:500 in 5% serum and TBS-Tween and then incubated with an STCM coated membrane at 4°C with shaking. After a couple of hours the membrane was removed and replaced with a fresh piece of membrane. This was performed over a 24 hour period after which the antiserum was used in Western blot analysis. Normal rabbit serum was diluted to an equivalent protein concentration as the immune serum. Gels were loaded with 15µg protein on 1-D gels and with 30µg on 2-D gels. Protein concentration was estimated using a Bradford assay as described in chapter 3, section 3.5.

5.2.5. Optimisation of conditions for the detection of testicular proteins

It was obvious at the start of these studies that the extremely high albumin content of plasma and IF was going to hinder the detection of testicular proteins. Only a limited amount of protein can be resolved on acrylamide gels by 1-D or 2-D electrophoresis; if ST-derived proteins are able to gain access to the bloodstream they will be present in extremely small amounts. Two methods were attempted to remove albumin from samples of testicular IF, TV, SV and PV blood. The first approach involved the use of microconcentrators with a molecular weight cut off of 30 kDa. The second method used a blue dye affinity column specific for albumin.

Microconcentrators (Centricon™-30; Amicon® Ltd., Stonehouse, Gloucestershire, UK) are designed for use in conjunction with centrifugation to allow

concentration of samples. The centrifuge tubes have an upper and lower chamber divided by a membrane filter which has a specific molecular weight cut off so that, in theory, proteins below this molecular weight should end up in the lower chamber during centrifugation. A cut-off of 30 kDa was selected as this was below the molecular weight of albumin, 67 kDa, and it was expected that low molecular weight proteins would be more likely to leak from the seminiferous tubules. The Centricon-30™ tubes were prewashed by filling the upper chamber with dd H_2O and centrifuging for 30 min at 4500 rpm at 4°C. The upper chamber of the centricon-30 tube was then loaded with either 200µl of IF, TV or PV plasma diluted with dd H_2O to a total volume 500µl or with 500µl of STCM. Samples were centrifuged for 1 h at 4500 rpm at 4°C. The fluid present in each chamber was collected and stored at -20°C. The proteins present in the <30 kDa and the >30kDa fractions prepared from STCM, IF, TV and PV were analysed by 1-D SDS PAGE followed by silver staining as described in chapter 3.

An affinity column (Econo-pac Blue cartridge; Bio-Rad) was also used to remove albumin from samples of TV and PV according to the manufacturers instructions. A lml sample of either SV or PV plasma was dialysed overnight at 4°C against 1 litre 0.02M Na_2HPO_4 (pH 7.2). The column was connected up to a pump, UV monitor, fraction collector and chart recorder as described for IgG purification in section 3.7. The column was equilibrated with the buffer used for dialysis and then loaded with 400µl of dialysed plasma. Once the plasma sample had run into the column the phosphate buffer was pumped through the column until all of the protein had washed through. The UV monitor and chart recorder were used to monitor the elution of protein from the column. Fractions containing protein were pooled and assayed for protein content (section 3.5). The protein profile of the 'albumin stripped' plasma samples was analysed by 2-D SDS PAGE followed by silver staining (section 3.9).

5.2.6. Detection of testicular proteins

Samples of IF and plasma from testicular, spermatic and peripheral veins were resolved on 7-15% w/v gradient acrylamide gels using 1-D and 2-D SDS PAGE and then electroblotted onto PVDF membranes for Western blot analysis using the STCM antiserum. Gels were loaded with equivalent protein amounts of the IF and plasma samples, 100µg protein on 1-D gels and 500µg on 2-D gels. Blotted membranes were incubated with normal rabbit serum for control purposes. IF and PV samples from eight individual control rats were analysed by 1-D Western blotting and three individual samples of both IF and PV were also subjected to 2-D Western blot analysis. SV from three control rats and TV from 2 control rats were analysed by both 1-D and 2-D Western blotting.

5.2.7. Heat treatment

Testicular IF and PV plasma samples were collected from adult rats whose testes had been exposed to heat (43°C for 30 min) 24 hours earlier. Treatment was performed on two separate occasions and each treatment group contained four rats. IF and PV samples were also collected from four control adult rats of the same age or of the same litter for each treatment group. Treatment and sample collection is described in chapter 3, sections 3.2 and 3.3. Severe heat treatment disrupts spermatogenesis and it was of interest to investigate whether ST derived proteins could be more easily detected in biological samples taken from these animals in comparison to animals with normal spermatogenesis. IF and PV samples from six individual control rats (three from each treatment session) were compared with six individual samples from heat treated rats (three from each treatment session) by 1-D western blot analysis. Each gel was loaded with three individual control samples and three individual samples from heat treated rats, Western blot analysis was performed on at least four occasions to confirm reproducibility of results. IF and PV samples from three control and three heat-treated animals were also subjected to 2-D Western blot analysis.

5.2.8. Investigation of proteins detected in biological fluids

A peptide antiserum was raised to PEBP as described in chapter 3, section 3.6. PEBP is a protein secreted by round spermatids which was isolated and microsequenced from STCM (Saunders *et al.*, 1995). The PEBP antiserum was used in conjunction with Western blot analysis to confirm the identity of one of the proteins detected in IF. Samples of STCM and IF from both control and heat treated rats were resolved by 1-D SDS PAGE. STCM was also separated by 2-D SDS PAGE using a 15% acrylamide gel. IF samples from three control and from three treated rats were analysed by 1-D Western blotting. The PEBP antiserum was diluted 1:500 and Western blot analysis was performed using the same protocol as described for the STCM antiserum, see section 3.10.

Protein extracts were prepared from various tissues from adult control rats to investigate the tissue specificity of proteins detected in IF. Pieces of testis, caput epididymis, corpus epididymis, cauda epididymis, prostate, kidney, liver and brain were homogenised in a solution containing 20mM Tris-HCl, 150mM NaCl (pH 7.4) and protease inhibitors. A protease inhibitor cocktail tablet (Complete™, Boehringer Mannheim) was added to 50ml of the extraction solution. After homogenisation the extracts were centrifuged at 10,000g for 20 min at 4°C to pellet the cell debris. The supernatants were removed, assayed for protein concentration and then stored at -20°C. Protein extracts were separated on 7-15% w/v acrylamide gels using 1-D SDS PAGE,

electroblotted and then Western blotting was performed using the STCM antiserum. Lanes were loaded with 10μg protein.

5.3. Results

5.3.1. Immunolocalisation of antiserum to STCM

The antiserum raised to STCM localised proteins in Sertoli cells in a stage dependent manner (Fig. 1a). Intense staining was also seen associated with elongate spermatids, more weakly with round spermatids but was not detected in any other cell types within the seminiferous epithelium. Specificity was confirmed as no staining was apparent when normal rabbit serum was used as the primary antibody (Fig. 1b).

5.3.2. Analysis of specificity of antiserum to STCM

1-D Western blot analysis of the proteins present in STCM (the immunogen) prepared from control adult rats revealed that as expected the antiserum recognised many of the proteins present in STCM in a specific manner (Fig. 2, lanes 1 and 2); these were not detected when the antiserum was preabsorbed (Fig. 2, lane 3) and only one protein of approximately 80 kDa cross-reacted with normal rabbit serum when this was used as the primary antibody (Fig. 2, lane 4).

The number of proteins in STCM recognised by the antiserum was also assessed using 2-D SDS PAGE. A blot stained for total protein using coomassie blue was compared with a Western blot using the antiserum (Fig. 3). This confirmed that the antiserum was able to recognise around one hundred proteins in the conditioned medium. Comparison of the Western blot profile with 2-D autoradiographs of the secreted proteins in STCM radiolabelled with methionine, revealed that the antiserum was unable to recognise SGP-1 and SGP-2, the two most abundant secretory products of Sertoli cells (McKinnell & Sharpe, 1995).

5.3.3. Removal of albumin from IF and plasma

Analysis of the proteins present in the two fractions separated using microconcentrators demonstrated that the centricon-30 tubes were not able to separate the <30 kDa proteins from the samples of STCM, IF and plasma. 1-D SDS PAGE demonstrated that very little protein was fractionated into the lower chamber; that protein samples from the upper chamber showed the same protein profile by electrophoresis as observed for samples which had not been put through microconcentrators (not shown). This indicated that the microconcentrator approach was not suitable for the removal of albumin.

Figure 1. Immunohistochemical localisation of the proteins recognised by an antiserum raised against seminiferous tubule conditioned medium in control adult rat testis. In panel A the tissue was incubated with the antiserum whilst in panel B normal rabbit serum was used instead of the primary antibody. A x99 magnification and B x99 magnification.

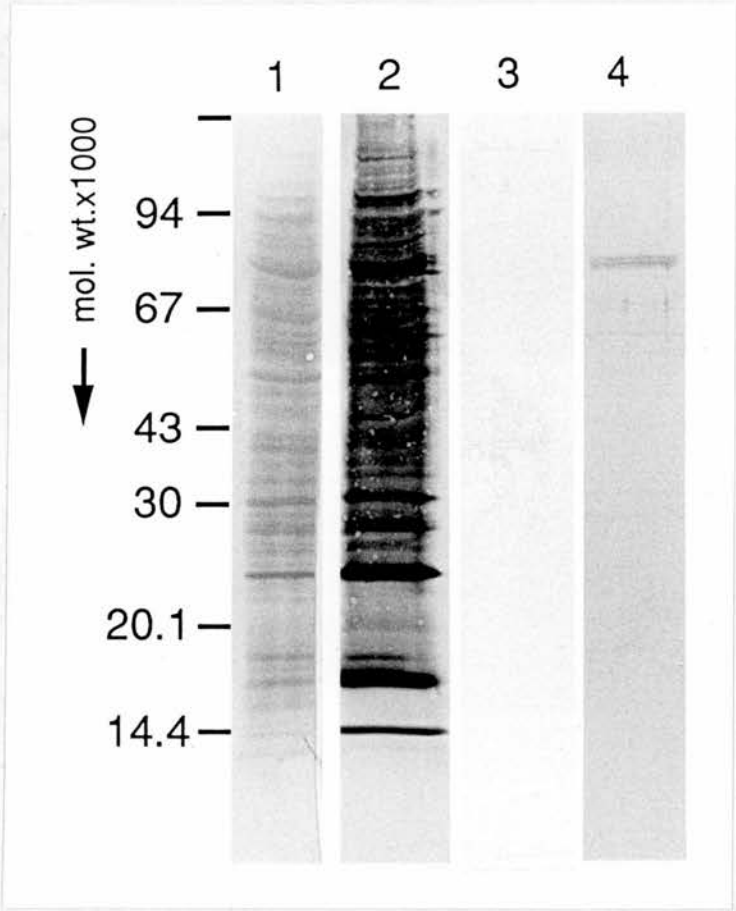
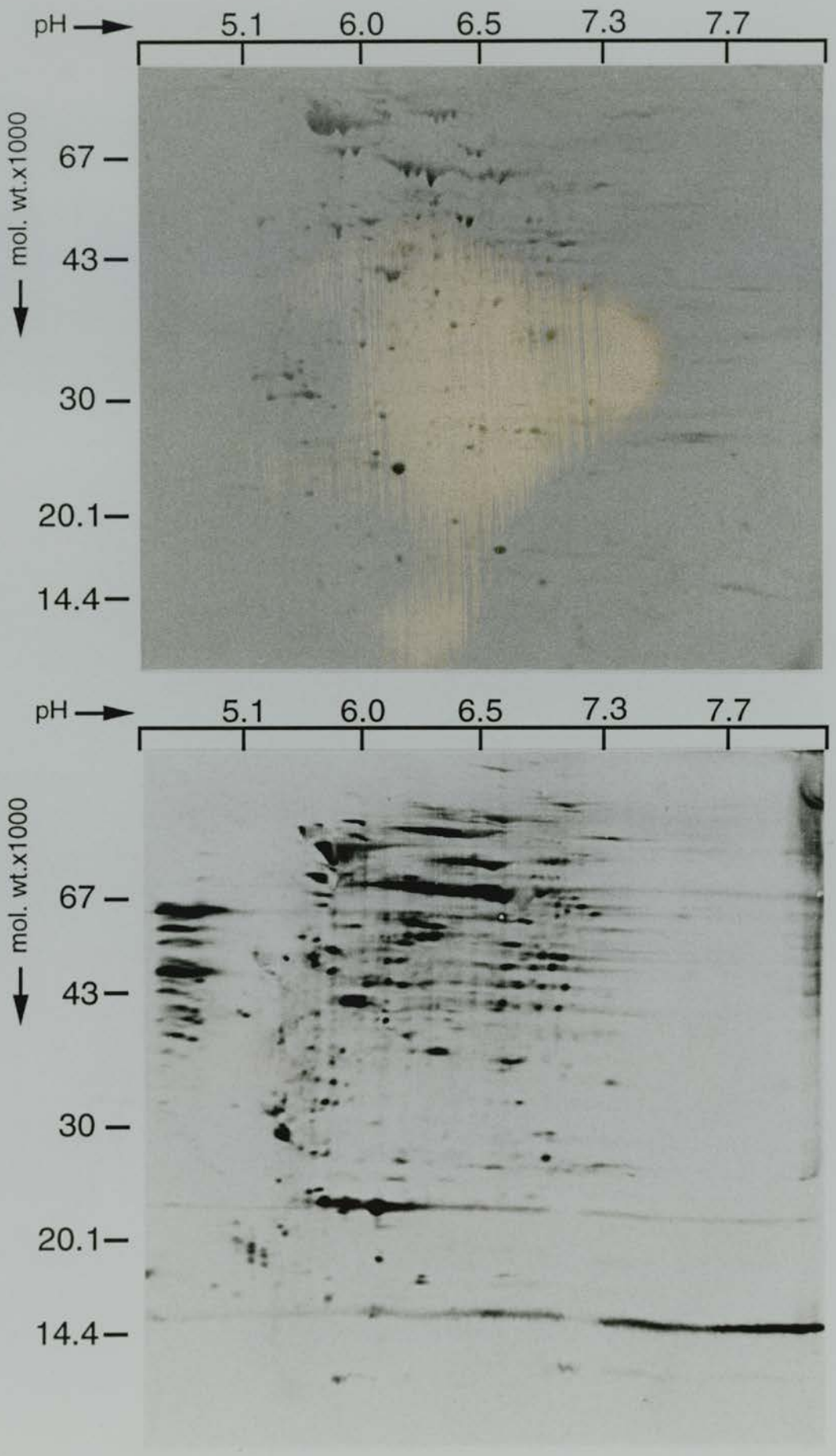


Figure 2. Specificity of antiserum raised against STCM as analysed using 1-D SDS PAGE on 7-15% w/v acrylamide gels. Each lane was loaded with 15µg protein and the gel was blotted after electrophoresis. In lane 1 the membrane was stained with coomassie blue to represent the total protein profile and lanes 2, 3 and 4 was subjected to Western blot analysis (lane 2 = STCM antiserum; lane 3 = preabsorbed STCM antiserum; and lane 4 = normal rabbit serum instead of the antiserum).

Figure 3. Representative blots from gels loaded with STCM (30μg protein) and separated on 7-15% acrylamide gels by 2-D SDS PAGE. In the top panel, the blot was stained with coomassie blue to illustrate the total protein profile of STCM. In the bottom panel, Western blot analysis was performed using the antiserum raised against STCM.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7.3
7.7
mol. wt.x1000
67
43
30
20.1
14.4
pH
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6.5
7.3
7.7
mol. wt.x1000
67
43
30
20.1
14.4

The removal of albumin using an affinity column also proved unsuccessful. The protein profile of the pooled fractions eluted from the column, which was loaded with either SV or PV plasma, was analysed by 2-D gel electrophoresis. This demonstrated that the column had removed the majority of proteins from the samples and not just albumin (not shown). This suggests that the proteins present in plasma are interacting with the albumin, which might have been expected since albumin is a binding protein.

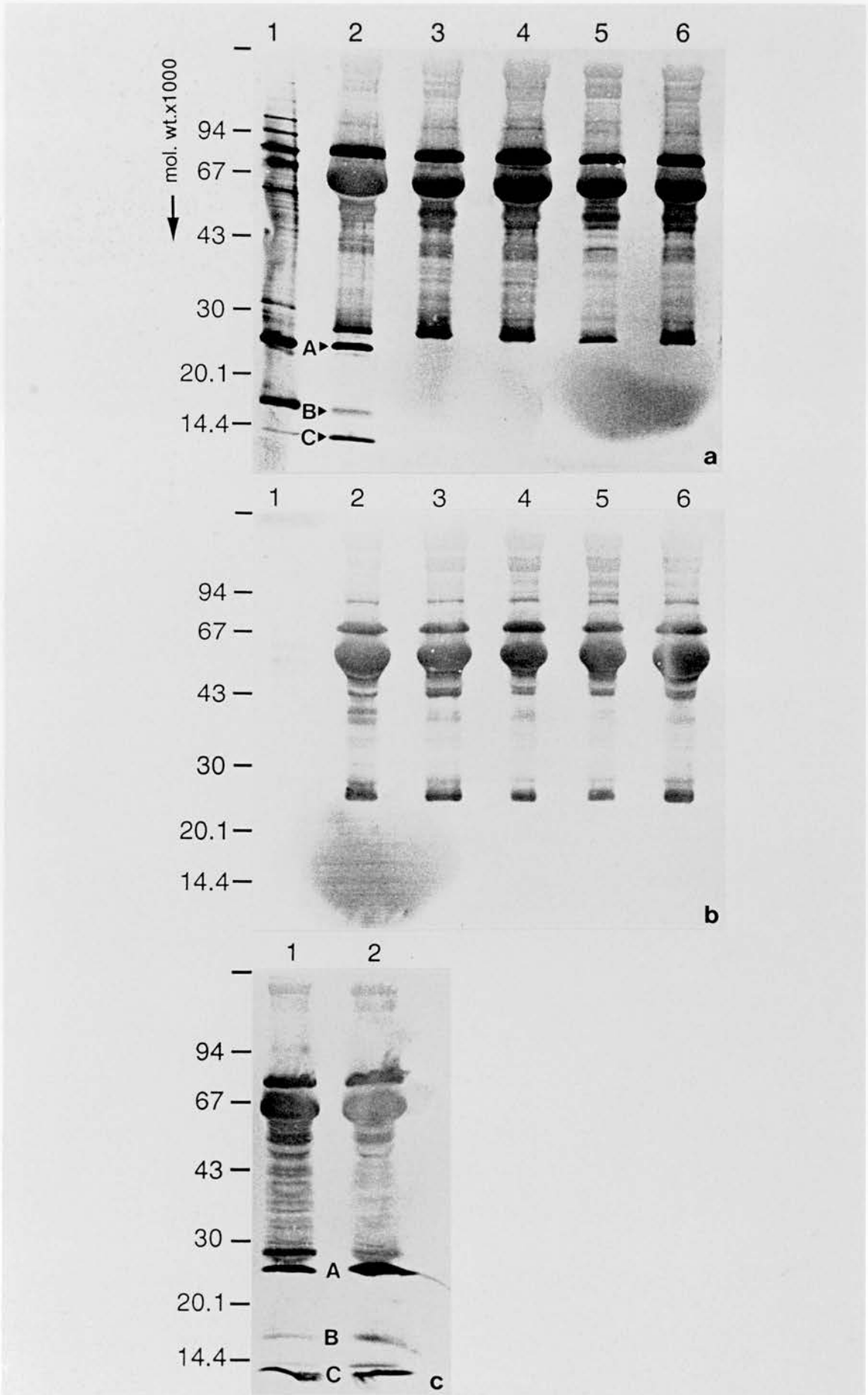
The inability of these methods to remove albumin from samples of IF and plasma meant that the conditions for Western blotting had to be optimised to allow detection of testicular proteins in whole IF and plasma. Some time was spent improving the separation of proteins on gradient acrylamide gels and determining the maximum amount of protein which could be loaded onto 1-D and 2-D gels without any loss of resolution. Different blocking buffers and antibody dilutions were used to try and reduce non-specific staining by the Western blot procedure.

5.3.4. Detection of testicular proteins in biological fluids from control animals

Three proteins which were present in STCM were also detected by Western blotting in every sample of IF that was analysed; their molecular masses were estimated to be 24 (A), 16 (B) and 14 kDa (C) respectively (Fig. 4a, lane 2). These proteins were equally evident in Western blots of IF which had been collected over 30 min (Fig. 4c, lane 1) or over 16 h at 4°C (Fig. 4c, lane 2), indicating that their presence in the latter was not because of their artifactual release during the overnight collection period. Western blot analysis of plasma, whether of TV, SV or PV, failed to detect any proteins in the plasma samples which were not also evident with the control rabbit serum (Fig. 4a and 4b, lanes 3-5). Similar results were obtained using PV collected from a rat two weeks after castration in which peripheral testosterone levels had been restored by injection (Fig. 4a and 4b, lane 6). Although several proteins greater than 27 kDa were detected in IF and the plasma samples it was not possible to analyse these effectively because normal rabbit serum also bound non-specifically to proteins in this molecular weight range.

Western blot analysis of TV, SV and PV plasma using 2-D SDS PAGE was also undertaken to ascertain whether the increased sample load of 500µg protein instead of 100µg as used on 1-D gels would improve the sensitivity of detection of ST derived proteins. The antiserum was still unable to detect any proteins in these plasma samples which were not also apparent with control rabbit serum (Fig. 5).

Figure 4. Representative Western blots showing the detection of seminiferous tubule (ST) secreted proteins in ST-conditioned medium (STCM), testicular interstitial fluid (IF) and samples of testicular (TV), spermatic (SV) and peripheral (PV) venous plasma from control adult rats by Western blot analysis. The samples were resolved using 1-D SDS PAGE on a 7-15% w/v acrylamide gel. After electrophoresis, the proteins were electroblotted onto a membrane and detected using the IgG fraction of an antiserum raised against STCM (a) or control rabbit serum (b) as the primary antibody. Lane 1= STCM (2μg protein); lane 2= IF (100μg); lane 3= SV (100μg); lane 4= TV (100μg); lane 5= PV (100μg); and lane 6= PV (100μg) from a castrate rat supplemented with testosterone. In (c) representative Western blots against IF (100μg) collected over 30 min (lane 1) and over 16 h (lane 2) are shown. Three proteins detected by the antiserum (A, B and C : arrowed) present in both STCM and IF are indicated.


1
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6
mol. wt.x1000
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67
43
30
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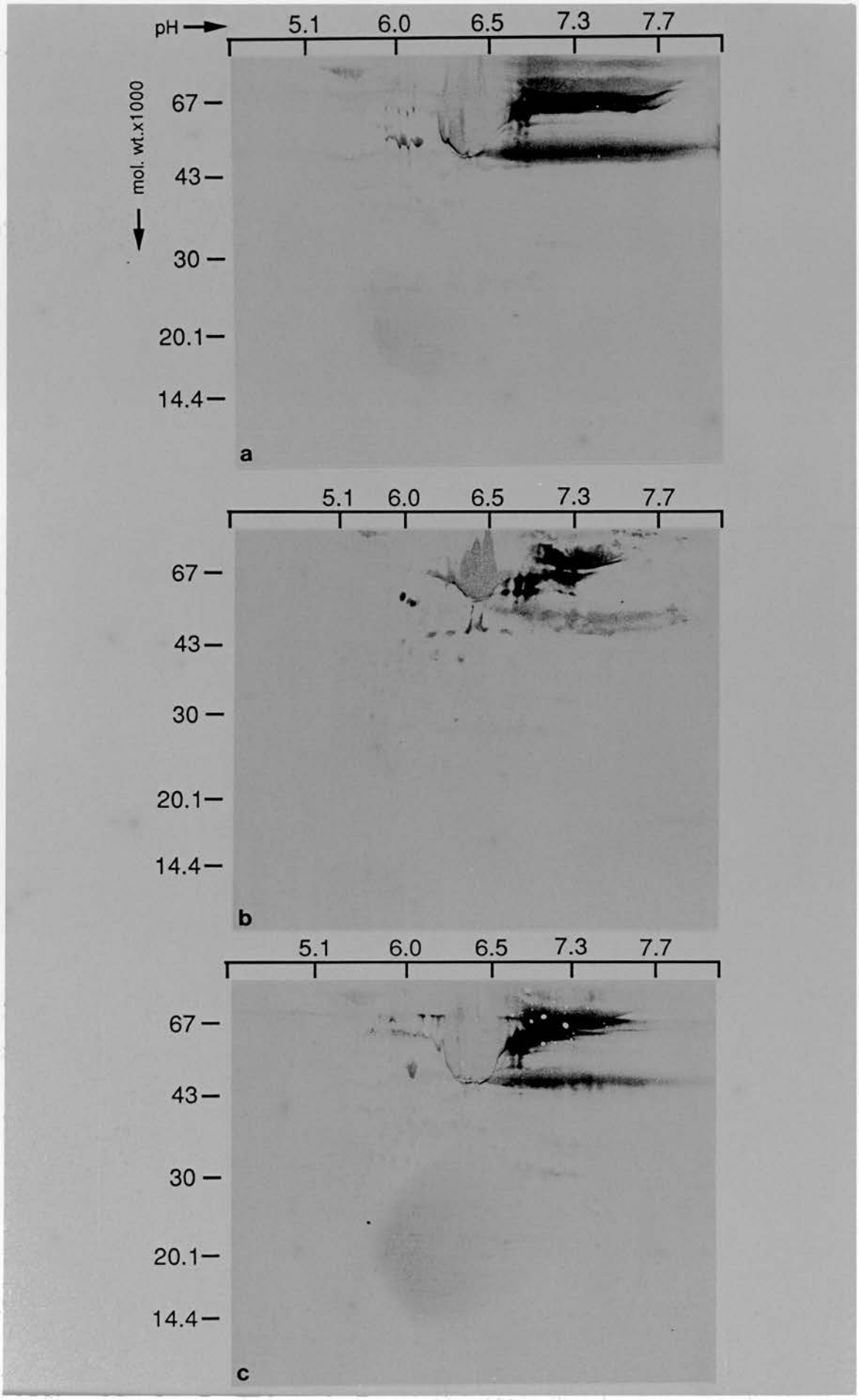
A
B
C
a
b
c

Figure 5. Representative Western blots of TV, SV and PV from control adult rats (500μg protein) resolved by 2-D SDS PAGE, electroblotted onto a membrane and probed with an antiserum raised against STCM. In panel (a) the gel was loaded with TV, panel (b) with SV and panel (c) with PV.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5.3.5. Effect of heat treatment on the detection of testicular proteins in interstitial fluid

Western blot analysis was used to assess whether the abundance of the proteins A, B and C detected in control IF changed or if any new proteins could be detected after damage to spermatogenesis was induced by scrotal heating 24 h earlier (Fig. 6, lanes 2, 4, 6). All three proteins increased in abundance in each of three heat treated animals compared with three control rat samples (Fig. 6, lanes 1, 3, 5), as judged by the greater NBT staining intensity; this increase was less marked for protein A than for proteins B and C. This was observed in IF samples from a total of eight control and six heat treated rats on several occasions. An additional faint band, with a molecular mass of approximately 18 kDa, was detected in IF samples from heat-treated animals, though this band is difficult to visualise on photographs of the blots.

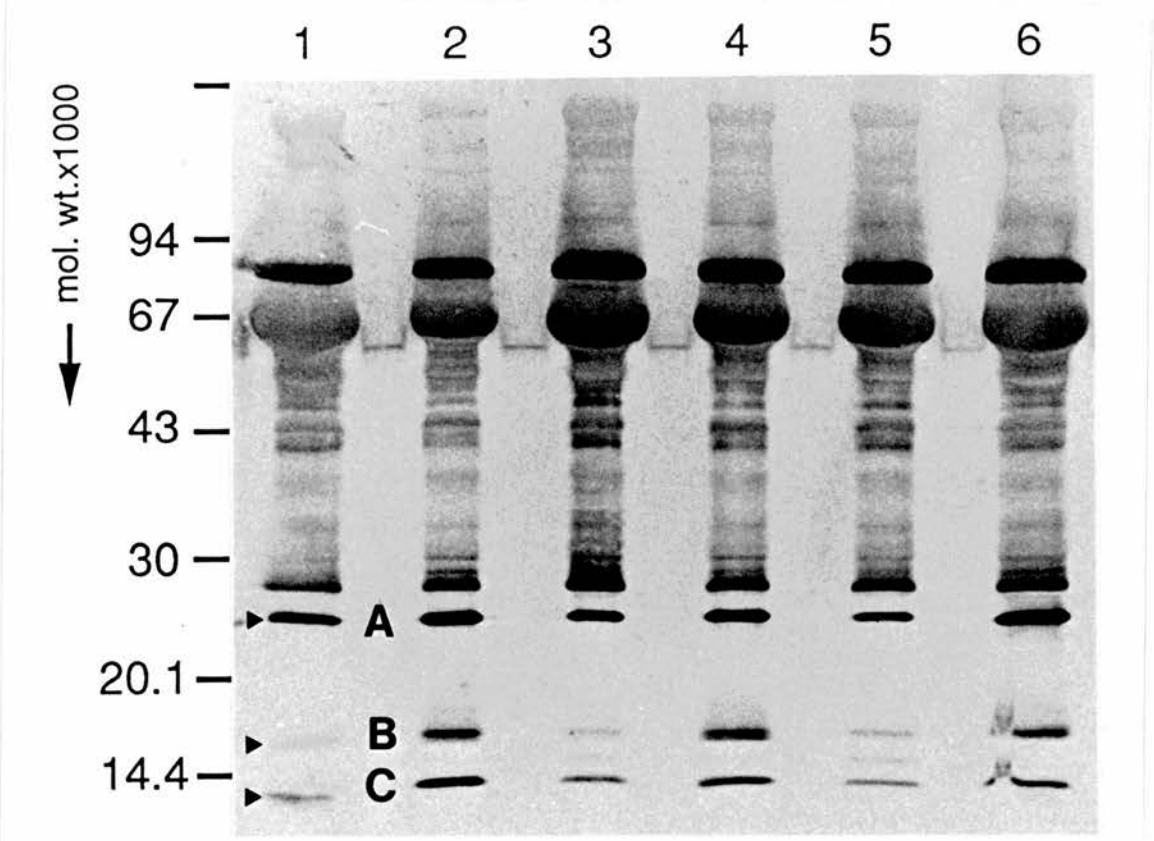


Figure 6. A representative Western blot showing the detection of ST secreted proteins in IF from control adult rats and from animals in which spermatogenesis had been impaired by heating the scrotum (43°C for 30 min) 24 h earlier. Lanes 1, 3 and 5 were loaded with IF (100μg protein) from 3 control rats whilst lanes 2, 4 and 6 were loaded with IF (100μg) from 3 heat-treated rats. Three proteins (A, B, C) which showed changes in IF abundance between control and heat-treated animals are indicated by arrows.

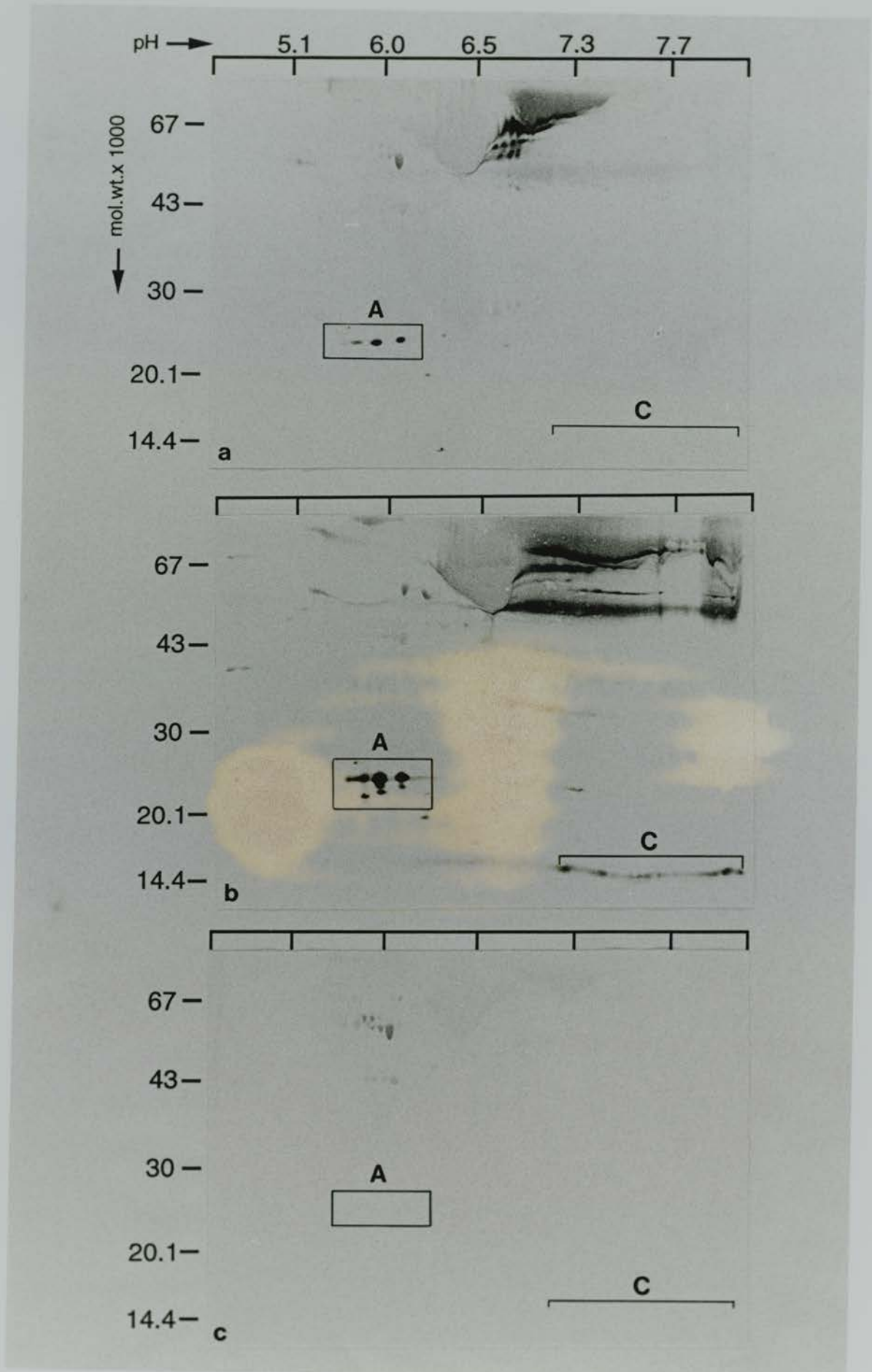
Western blot analysis using 2-D SDS PAGE was undertaken to provide additional information on possible identities of the ST-derived proteins detected in IF by comparison with the 2-D profile of STCM, for which a number of the proteins have been characterized and their cellular origin established (see Sharpe *et al.*, 1994; McKinnell & Sharpe, 1995). This analysis confirmed that heat-induced testicular damage led to an increased leakage of proteins from the seminiferous tubules (Fig. 7, compare panel b with a). Protein A (24 kDa) appeared as four distinct charge isomers in both STCM and IF and these proteins appeared to be increased in IF samples from heat-treated rats (Fig. 7). In the latter, a further three proteins were detected which resolved with the same isoelectric point (pI) as protein A but with a slightly lower molecular weight, raising the possibility that they are related to protein A. Protein B (16 kDa) could not be detected in IF from control or treated animals on the 2-D Westerns, in contrast to the results on the 1-D blots. This could mean that the pI of this protein is not within the range (4.8-8.0) which was resolved on the 2-D gel. On the 1-D blots, protein C (14 kDa) showed an increase in abundance in IF from heat-treated rats compared with controls; on the 2-D blots it appeared as a broad unresolved smear and stained very intensely in both STCM from normal rats (Fig. 3b) and in IF from heat treated animals (Fig. 7b). Several other much less abundant proteins (approximately twenty) were detected in IF samples after heat-treatment which were not present in the control samples, but these stain too weakly to be visualised in photographs of the blots.

The position of proteins A and C is indicated on each of the Western blots (Fig. 7). Based on the Mr and pI, protein A was putatively identified as PEBP which has been isolated and microsequenced from STCM (Saunders *et al.*, 1995). Protein C was more tentatively identified as an androgen regulated protein, ARP-2, whose secretion by isolated seminiferous tubules is significantly decreased by EDS treatment *in vivo* (Sharpe *et al.*, 1992; McKinnell and Sharpe, 1995). Both of these proteins derive specifically from round and elongating spermatids. The sequence and nature of ARP-2 is unknown.

5.3.6. Effect of heat treatment on the detection of testicular proteins in peripheral venous plasma

PV samples from control and heat-treated rats were also resolved by 1-D SDS PAGE and subjected to Western analysis to ascertain whether induction of damage to spermatogenesis might allow the detection of ST secreted proteins in the bloodstream. However, this analysis failed to detect any proteins which were not also evident with control rabbit serum (Fig. 8). PV samples from control and heat-treated rats were also subjected to 2-D Western blot analysis but again failed to detect the ST-secreted proteins found in IF (Fig. 9).

Figure 7. Representative Western blots of testicular IF (where each gel was loaded with 500μg protein) resolved by 2-D SDS PAGE, electroblotted onto a membrane and probed with an antiserum raised against STCM (panels a and b) or with normal rabbit serum (panel c). In panels (a) and (c) the gel was loaded with IF from a control rat whereas IF from a heat-treated rat was used in panel (b). Those proteins identified by 1-D Western blot analysis are labelled where possible. Considerable variation was seen in the visualization of antigens by the normal rabbit serum in comparison to the STCM antiserum. Effort was made to try and dilute the normal rabbit serum to be comparable with the antiserum but on occasions the non-specific staining observed with the normal rabbit serum was not as strong as seen with the antiserum. Ideally, the best control would have been a pre-immune serum from the same rabbit that was immunised with STCM.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mol.wt.x 1000
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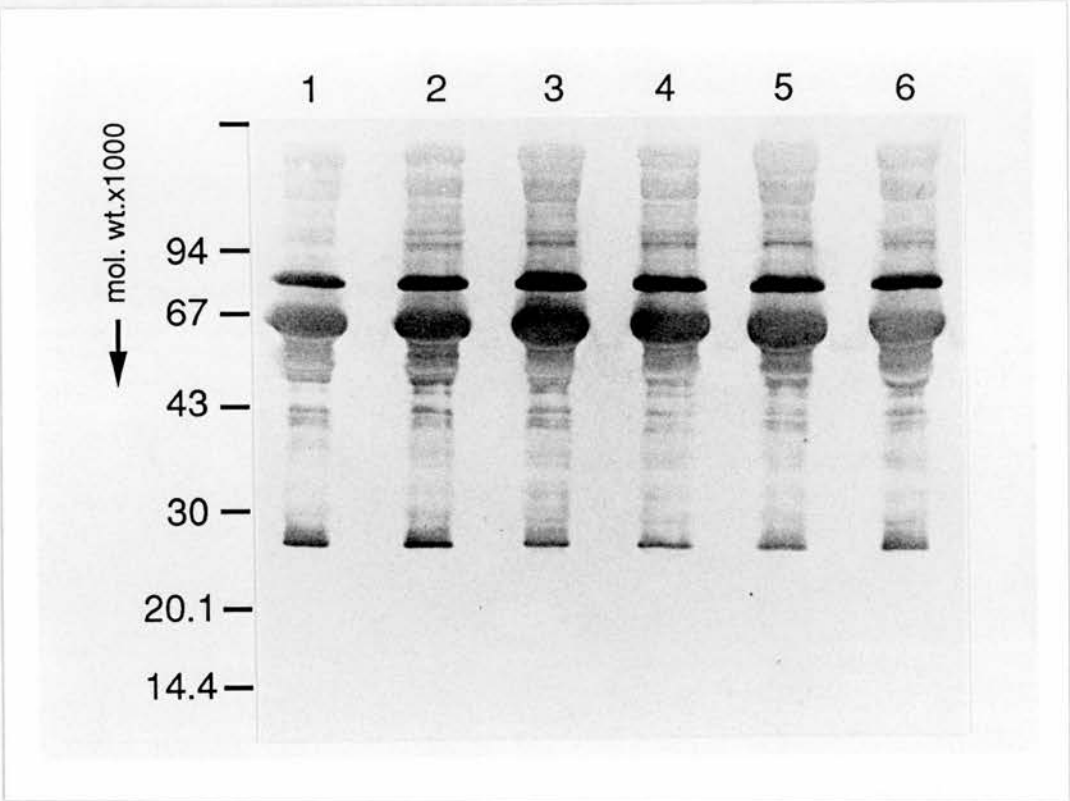
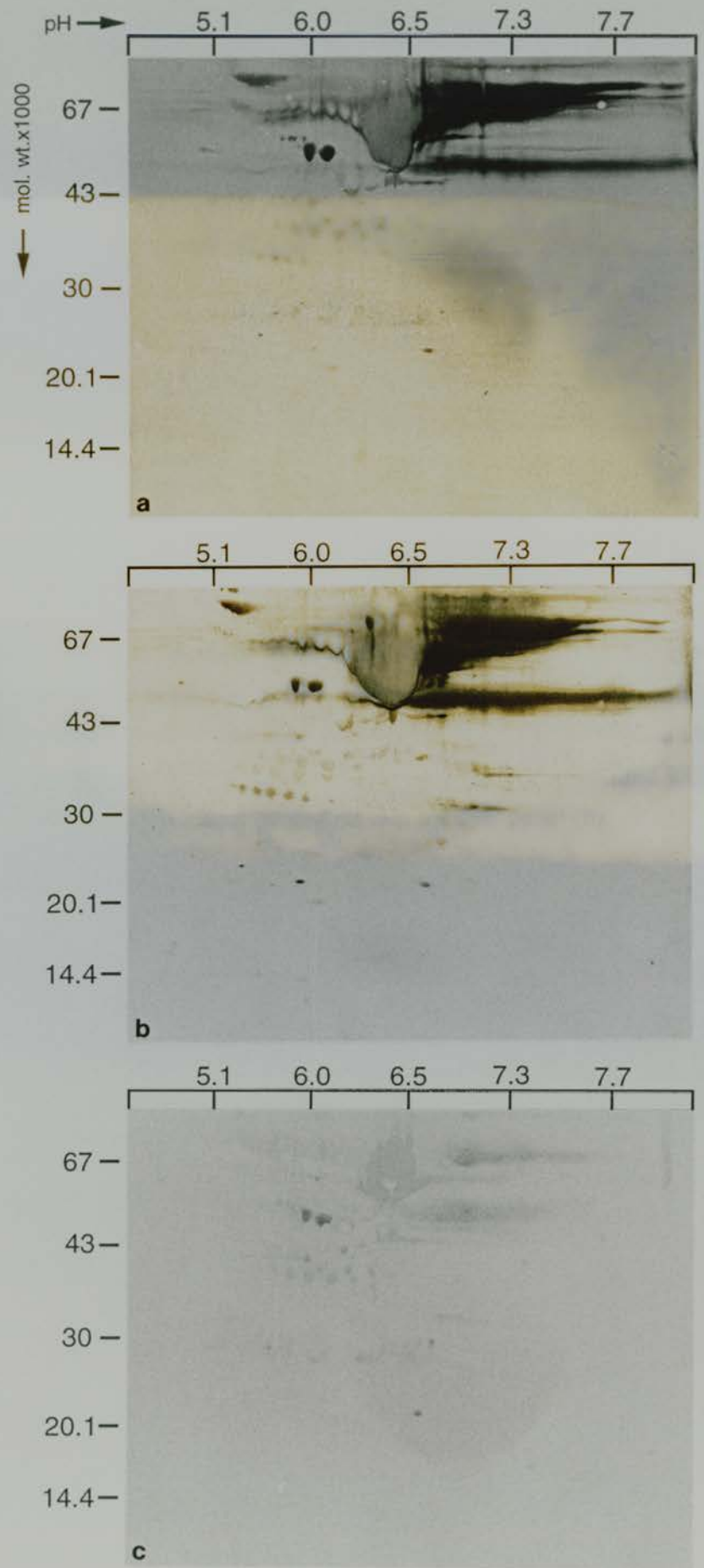


Figure 8. A representative Western blot of a gel loaded with samples of peripheral venous plasma (PV) from control adult rats and from animals in which spermatogenesis had been impaired by heating the scrotum (43°C for 30 min) 24 h earlier. Samples were resolved by 1-D SDS PAGE then electroblotted onto a membrane and probed with an antiserum raised against ST-secreted proteins. Lanes 1, 3 and 5 were loaded with PV (100μg) from 3 control rats whilst lanes 2, 4 and 6 were loaded with PV (100μg) from 3 heat-treated rats.

Figure 9. Representative Western blots of PV (gels were loaded with 500µg protein) resolved by 2-D SDS PAGE on 7-15% w/v acrylamide gels, electroblotted onto a membrane and probed with an antiserum raised against STCM (panels a and b) or with normal rabbit serum (panel c). In panels (a) and (c) the gel was loaded with PV from a control rat whereas PV from a heat-treated rat was used in panel (b).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5.3.7. Identification of a protein in interstitial fluid as PEBP

Confirmation that protein A was PEBP was gained by the use of an antiserum generated against a PEBP peptide. Western blot results are shown in Fig. 10. A protein band of 24 kDa, resolving on 2-D blots as several charge isomers, was detected in STCM using the antiserum to PEBP peptide (Fig. 10b). This corresponds to the molecular weight and pI of PEBP (Saunders *et al.*, 1995). A few fainter staining bands of a higher molecular weight were also detected which may be non-specific, as comparable staining was evident in an equivalent blot incubated with normal rabbit serum (not shown). The 24 kDa band was detected with the PEBP antiserum in all IF samples and was found to be increased after heat treatment (Fig. 10a). 1-D Western blot analysis failed to detect PEBP in samples of SV, TV and PV (results not shown).

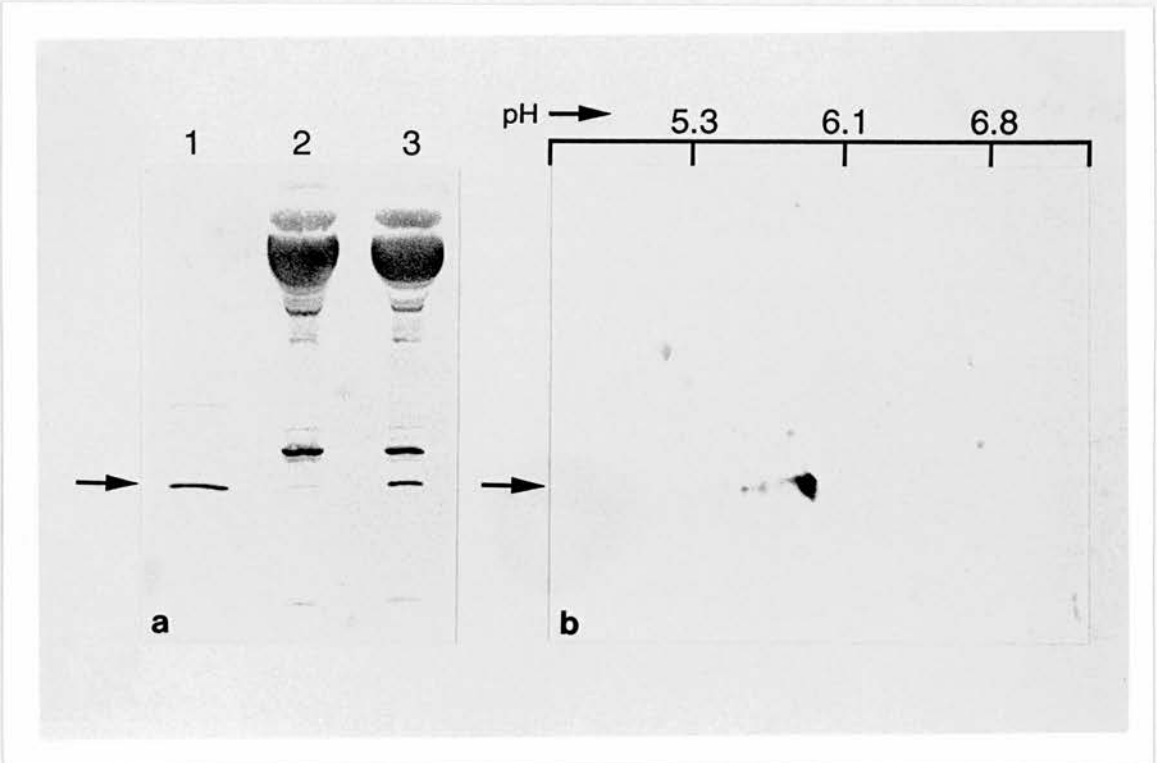


Figure 10. Identification of the 24 kDa protein present in both STCM and IF as PEBP, by Western blot analysis using an antiserum generated against a PEBP peptide. In panel (a), STCM and IF from control and heat-treated animals was resolved by 1-D SDS PAGE and then blotted. Lane 1= STCM (10µg protein); lane 2= IF (100µg) from control and lane 3= IF (100µg) from a heat-treated rat. In panel (b), STCM (30µg) was resolved by 2-D SDS PAGE on a 15% acrylamide gel. Arrows indicate the position of PEBP on both Western blots.

5.3.8. Investigation of the tissue specificity of the antiserum

Protein extracts prepared from a range of tissue homogenates were also subjected to 1-D Western blot analysis using the STCM antiserum to ascertain whether the proteins detected in testicular IF were specific to the testis. The antiserum was able to recognise several proteins in a specific manner in all the tissue extracts, which included the caput, corpus and caudal regions of the epididymis, the prostate, kidney, liver and brain (Fig. 11). Protein A, identified as PEBP, was present in all tissues and the intensity of staining seen on the Western blot would suggest that this protein is highly expressed in all tissues except for the prostate. Protein B was found to be an abundant product of the testis and the amount of protein detected in the epididymis declined from the caput to caudal regions. This protein appeared as a very faint band in the prostate, kidney, liver and brain. Protein C, tentatively identified as ARP-2, was expressed in a similar manner to protein A, in that it is detected in all tissues and stained quite strongly in all tissues with the exception of the prostate.

Figure 11. Analysis of the proteins in various rat tissues recognised by the STCM antiserum by Western blotting using 1-D SDS PAGE. The gels were loaded with 10μg of tissue extract as follows, lane 1= testis; lane 2= caput epididymis; lane 3= corpus epididymis, lane 4= cauda epididymis, lane 5= prostate, lane 6= kidney, lane 7= liver; and lane 8= brain. In panel (a) the blotted membrane was incubated with the STCM antiserum and in (b) an equivalent blot was incubated with normal rabbit serum instead of the primary antiserum. Three proteins detected by the STCM antiserum in IF (A, B, and C) are indicated.

5.4. Discussion

The primary purpose of this investigation was to assess whether proteins deriving from the seminiferous epithelium can gain access to the bloodstream and thus provide a potential means of monitoring the process of spermatogenesis. An antiserum was raised against STCM and in conjunction with Western blot analysis was used to assess whether the seminiferous tubule-derived proteins recognised by this antiserum could be detected in blood or in testicular interstitial fluid from normal rats, and to assess whether exposure of adult rats to scrotal heating (43°C) for 30 minutes affected levels of such proteins by inducing germ cell degeneration (Chowdhury & Steinberger, 1964, 1970; Bartlett & Sharpe, 1987: McLaren *et al.*, 1994) and/or by increasing the 'leakiness' of inter-Sertoli cell tight junctions. Investigation of the effects of testicular heat treatment on Sertoli cell function has demonstrated that it causes increased secretion of both ABP and α-inhibin from the base of the Sertoli cells into interstitial fluid (Bartlett & Sharpe, 1987; Sharpe & Bartlett, 1987; Sharpe & Maddocks, 1989). There are several possible routes by which ST derived proteins may enter the bloodstream. They could be resorbed from STF in the rete testis or epididymis (Maddocks & Sharpe, 1989a), secreted by Sertoli cells or 'leak' via Sertoli cell tight junctions into the interstitium and then enter the bloodstream (Sharpe, 1992). Samples of testicular IF and TV, SV and PV blood were all screened using the antiserum in an attempt to detect ST-derived proteins.

Before attempting to screen IF and blood samples the specificity of the antiserum for testicular proteins was investigated using immunohistochemistry and Western blotting. Immunocytochemical localisation of the proteins recognised by the antiserum on rat testis sections demonstrated that the antiserum contained antibodies predominantly against elongate spermatid and Sertoli cell proteins. Much weaker staining could be seen in round spermatid cytoplasm but in no other cells of the seminiferous epithelium. Western blot analysis demonstrated that the antiserum could specifically recognise around one hundred proteins present in STCM, and none of these proteins were detected when the antibody was preabsorbed or when normal rabbit serum was substituted for the antiserum. Analysis of a 2-D Western blot against STCM further revealed that the antiserum did not contain antibodies against the major Sertoli cell secretory proteins, SGP-1 and SGP-2. This would imply that these two proteins are very similar in the rat and rabbit and are therefore not very immunogenic. Demonstration that the antiserum contained a wide range of antibodies against both Sertoli cell and germ cell proteins suggests that it would be a useful tool for attempting to detect ST derived proteins in various biological fluids.

The antiserum detected three proteins in testicular IF from control rats. Heat treatment increased the abundance of these proteins and induced the appearance of several

other proteins which were recognised by the STCM antiserum. Two proteins present in IF from control rats and which were increased in abundance by testicular damage were identified as PEBP and ARP-2, both of which are known to be major secretory products of round spermatids (Sharpe *et al.*, 1992, 1994; Saunders *et al.*, 1995; McKinnell and Sharpe, 1995). PEBP is secreted by round spermatids in culture and expression of the mRNA has been localised by *in situ* hybridisation to pachytene spermatocytes at stage VII and thereafter in round and elongating spermatids until step 14 in the adult rat testis (Saunders *et al.*, 1995). Immunocytochemistry in the mouse testis has localised the protein to the cytoplasm of elongate spermatids, residual bodies and cytoplasmic droplets of spermatozoa (Vierula *et al.*, 1992). PEBP is present in high abundance in both testicular and epididymal secretions as well as being associated with membrane preparations of spermatozoa in the rat and mouse (Jones & Brown, 1987; Jones & Hall, 1991; Rankin *et al.*, 1992; Araki *et al.*, 1992; Vierula *et al.*, 1992; Perry *et al.*, 1994; Saunders *et al.*, 1995). The function of PEBP is unresolved but it is thought to be involved in membrane remodelling during sperm maturation and may have a similar role in organising membrane components in spermatogenesis (Saunders *et al.*, 1995).

Evidence that ARP-2 is secreted by germ cells comes from two sources. Firstly, analysis of changes in radiolabelled proteins secreted by stage VI-VIII tubules from MAA treated adult rats showed that ARP-2 disappeared when round spermatids, but not when pachytene spermatocytes or elongate spermatids, were selectively depleted (McKinnell & Sharpe, 1992). Secondly, analysis of radiolabelled proteins secreted by round spermatids in culture confirmed that ARP-2 is produced by round spermatids (Sharpe *et al.*, 1994). At present we have no further information regarding identity or possible function of this protein. These results suggest that germ cell secretory products can gain access to the interstitium under both normal physiological conditions and more easily after testicular damage. This may mean that if suitable methodology can be devised, germ cell proteins could also be detectable in blood, thus providing a non-invasive way of monitoring spermatogenesis. This possibility was investigated in a preliminary way in the present studies using Western blotting, but no proteins recognised specifically by the STCM antiserum were detectable in blood samples (see below).

Detection of germ cell derived proteins in IF with the STCM antiserum could be due to contamination of the IF by RTF or the consequence of mechanical damage to the seminiferous tubules during the IF collection procedure. The former possibility was excluded by ligating the efferent ducts prior to testicular isolation. The possibility of mechanical damage during the IF collection procedure cannot be completely excluded but three pieces of evidence suggest this is unlikely to explain these findings. First, IF collected over periods of either 30 min or 16 h at 4°C exhibited similar protein profiles by Western analysis, indicating that lysis of cells or artifactual disruption of inter-Sertoli cell

junctions during the longer of these collection periods did not explain the presence of germ-cell derived proteins in IF. A similar conclusion has been made before for the measurement of ABP in interstitial fluid, where the level of this protein in IF collected over 10 min was comparable to that measured in IF collected over 16 h for several rats (Sharpe & Bartlett, 1987). Second, mechanical damage would be expected to have resulted in the release of a wider (and perhaps more variable) range of ST-derived proteins, detectable by the STCM antiserum, rather than three specific proteins which were detected in every sample of IF analysed. Of particular significance is the fact that all of the ST-derived proteins detected in IF were below 25 kDa in molecular weight whereas the STCM antiserum also recognised many proteins of much higher molecular weight in STCM. In contrast, 'leakage' of inter-Sertoli cell junctions would be expected to preferentially affect low molecular weight proteins, and this is what was observed. Third, mechanical trauma would fail to explain why these specific proteins were increased in abundance in IF collected from heat-treated animals, whereas this treatment is known to increase leakage / secretion of Sertoli cell-derived proteins into IF (Sharpe, 1992).

Another explanation for these findings is that homologues of ST-derived proteins are secreted by Leydig cells. Again several pieces of evidence indicate that this is unlikely. First, analysis of the 2-D profile of radiolabelled proteins secreted by adult Leydig cells in culture shows that no proteins corresponding in Mr and pI to PEBP and ARP-2 are secreted by isolated Leydig cells (Qureshi, 1992). Second, immunostaining of the mouse testis with an antiserum to PEBP (Vierula *et al.*, 1992) and analysis of PEBP mRNA expression by *in situ* hybridisation on adult rat testis (Saunders *et al.*, 1995) showed no reaction with peritubular cells or cells of the interstitium. Therefore, all of the available data indicates that this protein is unlikely to originate from a source other than germ cells. This thinking is reinforced by the fact that PEBP seems to be a non-secreted cytosolic protein outside of the male reproductive tract (Jones and Brown, 1987; Jones and Hall, 1991; Saunders *et al.*, 1995).

The presence of germ cell proteins in the interstitial fluid compartment of the testis in control adult rats is a surprising observation. The inter-Sertoli cell tight junctions act as a selective permeability barrier between IF and STF, the function of which is to provide a unique environment for germ cell development (Setchell *et al.*, 1994). Studies to date have concentrated on investigating transport of molecules from the interstitial fluid compartment into the tubules (Setchell *et al.*, 1994) and have shown little effect of heat exposure on this permeability (Main and Waites, 1977). Little is known about how effective the barrier is to leakage in the opposite direction. The present results suggest that germ cell secretions can gain access to the IF presumably by leakage, although it is conceivable that they might be transported actively across the Sertoli cells. This observation has interesting implications concerning the possible function of these germ

cell proteins in the interstitium. It is well established that depletion of germ cells from ST can result in marked changes in Leydig cell structure and function (Sharpe, 1993), effects which are thought to be mediated by effects on the Sertoli cells. However, the present observations suggest that it is perhaps possible for germ cell secretions to directly influence Leydig cells.

The tissue specificity of the proteins detected in IF was assessed in a preliminary manner by Western blotting of tissue extracts prepared from a control adult rat. All three proteins were expressed in other tissues besides the testis. Protein A (PEBP) was an abundant protein in testis, epididymis, kidney, liver and brain but was only weakly expressed in the prostate. This is in agreement with published data demonstrating that PEBP is a ubiquitous protein present in the cytosols from a wide range of tissues; besides the above tissues it is also present in spleen, muscle and mammary gland (Jones & Brown, 1987). PEBP does not possess a classic signal peptide (Perry *et al.*, 1994), however published data supports a secretory role for PEBP within the testis and epididymis. This conflicting information suggests that this protein has an unusual secretory mechanism. Protein B, which could only be identified in IF on 1-D Western blots, was found to be an abundant product of the testis. Its expression decreased from the caput to the cauda region of the epididymis and only a faint band was present in the other tissues. This suggests that it would be of interest to identify this protein as its abundance in the testis in comparison to other tissue indicates that it may be a potential candidate for monitoring spermatogenesis. Protein C showed a similar tissue distribution to that of protein A, since Western blotting detected strongly staining bands in all tissues with the exception of the prostate. However, none of these three proteins were detectable by Western blotting in any of the blood samples which would suggest that they are not secreted into the peripheral circulation in substantial amounts by tissues outside of the male reproductive tract.

The possibility of detecting ST-derived proteins in blood as means of providing non-invasive monitoring of spermatogenesis was investigated in the present studies using Western blotting. The STCM antiserum failed to recognise the three proteins detected in IF in either testicular venous, spermatic venous or peripheral venous blood samples or in peripheral blood after testicular damage induced by heat treatment. It seems likely that this negative result is the consequence of the insensitivity of the present techniques which were limited technically by the protein resolving capacity of the acrylamide gels (100µg and 500µg total protein for 1- and 2-D gels respectively). Since both IF and plasma have a similar protein concentration (approximately 50µg/µl), only 2-10µl of sample could be analysed by this method. It is possible that improved detection methods might allow the assay of germ cell proteins in the peripheral circulation. In support of this is the fact that LDH-C4, a cytosolic enzyme present in meiotic and post-meiotic germ cells, can be

measured enzymatically in plasma from control rats and its levels are increased following testicular damage (Reader *et al.*, 1991). Development and application of specific immunoassays for individual ST-derived proteins (e.g. PEBP) should overcome this drawback and enable unequivocal assessment of whether such proteins are present in PV blood under normal or experimental conditions (e.g. after testicular damage). In view of these findings, PEBP and ARP-2 are obvious candidates for such investigations.

This work has provided preliminary evidence that when the testis is damaged by acute heat exposure, increased numbers and amounts of proteins deriving from the seminiferous tubules can be found in IF. The conclusion from these results is that the increased numbers of proteins detected in IF caused by acute damage is due to ongoing damage to the germ cells. It remains to be determined whether these proteins would be useful markers of chronic spermatogenic defects but they have the potential to be diagnostic markers of germ cell degeneration. However, the present results also demonstrate clearly that more sensitive methods will have to be developed if non-invasive monitoring of spermatogenesis using such an approach is to become a realistic probability. This will involve targeting of specific proteins for isolation and sequencing (McKinnell and Sharpe, 1995) and then the generation of specific antibodies which can be used to establish assays for the sensitive detection of such proteins in blood. The present results have identified two possible candidates for such an approach. Moreover, as a homologue of one of these proteins may be secreted by human ST (ARP-2, McKinnell *et al.*, 1995), this approach may have clinical applications. The observation that the large number of proteins secreted by isolated ST *in vitro*, and which are recognised by the polyvalent antiserum to STCM, do not have homologues of major abundance in peripheral blood is an encouraging finding in terms of the potential specificity of any assays for ST-derived proteins which might be developed.

Chapter 6. The use of immunoscreening to identify cDNAs for novel secreted proteins

6.1. Introduction

The preceding chapter described the results of using an antiserum raised against STCM to detect ST derived proteins in testicular IF and blood. This antiserum was able to recognise many of the proteins present in STCM and several proteins in testis homogenates by Western blot analysis. This chapter describes the use of this antiserum to screen the proteins expressed by a directionally cloned testis cDNA library. The aim of this study was to identify novel proteins secreted from testicular cells which might have the potential to act as markers of testicular function.

The concept of screening proteins within bacterial colonies or phage plaques using antibodies was first proposed by Broome and Gilbert (1978), but it was only when the construction of cDNA libraries in expression vectors became established that the use of antibody probes for isolating genes became a widely used technique (Young & Davis, 1983). In order to screen a cDNA library using antibodies the library must be cloned into either a plasmid or phage expression vector. The cDNA fragment must be inserted adjacent to a promoter site, which is functional in *E.coli*, in the correct orientation and reading frame for the correct protein product to be produced. The product of translation is a fusion protein in which amino acids from a prokaryotic protein are incorporated at one end of the eukaryotic protein derived from the inserted cDNA. These recombinant proteins may be recognised using a primary antibody directed against the protein of interest in conjunction with a detection system.

In this study an antiserum, which was raised to the medium conditioned by the culture of unstaged seminiferous tubules isolated from control adult rats, was used to screen a library constructed from mRNAs isolated from a testis cell fraction containing Sertoli cells and late spermatids from control adult rats. In the previous chapter, immunohistochemistry had demonstrated the ability of the antiserum to recognise predominantly Sertoli cell and elongate spermatid proteins, and the fact that the antiserum could recognise denatured proteins on Western blots suggested that this screening approach would be able to isolate novel cDNAs encoding proteins involved in spermatogenesis.

6.2. Experimental procedures

Library screening using an antiserum was performed according to manufacturer's instructions supplied with the picoBlue™ immunoscreening kit and the ZAP-cDNA® synthesis kit (both from Stratagene, La Jolla, California). The strategy followed during immunoscreening is outlined in Fig. 1.

6.2.1. Details of the cDNA library

A directionally cloned cDNA library was prepared from mRNA isolated from a mixture of Sertoli cells and late spermatids isolated from untreated adult rats by Dr. Sharon Maguire (Maguire, 1994). Synthesis of cDNA and preparation of the library was carried out using the Stratagene Zap-cDNA synthesis kit, following the manufacturer's instructions, based on the method of Okayama & Berg (1982). The library used for the studies described in this chapter was cloned into the Lambda Zap® expression vector in which the cloning site is downstream of the *E.coli lac*Z promoter responsible for β-galactosidase production. The library had a titre of 7.3 x 10^5 plaque forming units (pfu)/ml.

6.2.2. Preliminary testing of the antiserum

Before attempting to screen the library, the antiserum raised against STCM was subjected to preliminary testing using a dot blot assay, the aim of which was to confirm the ability of the antiserum to detect at least nanogram quantities of antigen. This assay also allowed determination of the appropriate dilution of the antiserum to be used during screening and whether there was any cross-reaction with the *E.coli*/phage lysate. PVDF membrane (Millipore) was cut into 6 strips (2 x 3cm) and marked with a grid pattern as shown below in Fig. 2. Each square of the first row was spotted with 1µl of a serial dilution of the antigen, in this case STCM, such that the first square contained 10µg whilst the last square contained 10pg protein. The squares in the second row were spotted with 1µl of a serial dilution of the antiserum in a blocking buffer of TBS containing 1% BSA w/v (Sigma), ranging from a 1:100 to 1:1500 dilution. In the final row the squares were spotted with 1µl of a serial dilution of the *E.coli*/phage lysate in blocking buffer, ranging from undiluted to a 1:1000 dilution. The *E.coli*/phage lysate used was supplied with the picoBlue™ immunoscreening kit (Stratagene).

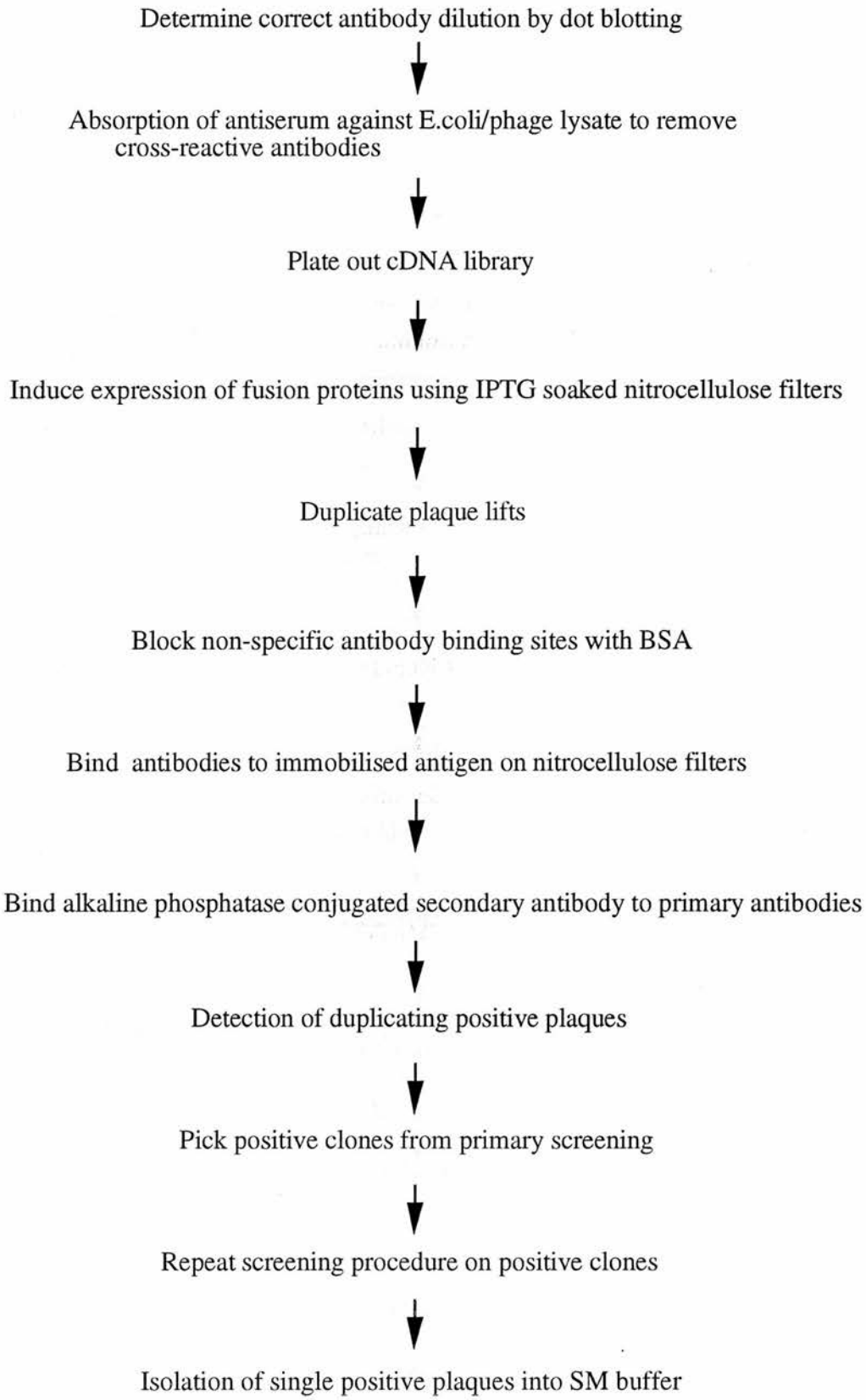


Figure 1. A flow chart outlining the main steps performed in the technique of immunoscreening.

Antigen (STCM)	1µg	100ng	10ng	1ng	100pg	10pg
Antiserum	1:100	1:250	1:500	1:750	1:1000	1:1500
E.coli/phage lysate	Neat	1:10	1:100	1:1000		

Figure 2. A representative dot blot test strip for analysis of antiserum specificity.

The strips were air dried for 5 min and then incubated in blocking buffer for 1 h at room temperature with shaking. Five strips were incubated at room temperature for 2 h each with a different dilution of primary antiserum (1:250, 1:500, 1:750, 1:1000 and 1:1500) and the final strip was incubated in blocking buffer to act as a control. Following incubation with the antiserum, the strips were washed three times for 10 min in TBS-Tween. All strips were incubated for 1 h at room temperature with an alkaline phosphatase conjugated secondary antibody (immunoaffinity purified goat anti-rabbit conjugate) provided in the kit, which was diluted 1:4000 in blocking buffer. This was followed by four washes for 5 min each in TBS-Tween and then development as described in the Western blot protocol, section 3.10.

6.2.3. Absorption of the antiserum with *E.coli*/Phage lysate

Since polyclonal antisera often contain antibodies that cross-react with *E.coli* and phage proteins, the STCM antiserum was preabsorbed against *E.coli*/phage lysate to eliminate any contaminating antibodies which could cause false positives and a high background. The lysate was diluted 1:10 in TBS-Tween and four nitrocellulose filters (Hybond-C™, Amersham) were incubated in this solution for 30 min at room temperature with shaking. The filters were removed and allowed to air dry after which they were washed three times in TBS-Tween for 5 min each. They were then incubated in blocking buffer for 30 min at room temperature with shaking. Following this, a 1:5 dilution of the STCM antiserum was incubated with a lysate coated filter for 10 min at 37°C, after which the filter was replaced. This was repeated for all the filters and then the antibody solution was collected and stored in aliquots at -20°C.

6.2.4. Immunoscreening of testis cDNA library

Plating of library. XL1-blue *E.coli* (Stratagene) for plating were grown to log phase (O.D.$_{260}$=0.5) overnight at 30°C with shaking in LB-broth (Appendix) containing 10mM $MgSO_4$ and 0.2% maltose. The cells were centrifuged at 1600g for 10 min and resuspended in 0.5 volumes cold, filtered 10mM $MgSO_4$. Cells were stored at 4°C and used within 24 h. NZY agar (Appendix) plates (150mm diameter) were poured and allowed to dry for at least 24 h. The library was diluted to 1:1000 so that when plated out 10µl of diluted library would result in approximately 5 x 10^4 plaques on a 150mm diameter Petri dish. Aliquots of XL1-blue cells (600µl) were combined with 5 x 10^4 pfu from the Sertoli cell enriched testis library, incubated at 37°C for 20 min to allow phage to adsorb to cells, mixed with 10ml NZY top agarose (Appendix) at 50°C and each poured onto an NZY bottom agar plate which had been prewarmed (37°C). Plates were allowed to solidify for 10 min and then incubated inverted at 42°C until small plaques became visible after approximately 3.5 h.

Expression of fusion proteins. Thirty minutes before use, nylon backed nitrocellulose filters (Hybond™-C extra; Amersham) were wetted by submersion in 10mM isopropyl-β-D-thiogalactopyranoside (IPTG) (Gibco; Paisley, UK), then placed on blotting paper to air dry. IPTG-soaked filters were numbered and placed on individual agar plates following their 3.5 h incubation period. Care was taken to make sure no air bubbles became trapped between the filter and the surface of the agar. A wide bore needle was used to mark the orientation of the filter in relation to the plate. After incubation at room temperature overnight the nitrocellulose filter was removed from the plate and placed into TBS-Tween. Additional filters were IPTG-treated, numbered, placed on the surface of their agarose and their orientation marked. This second filter was left on the plate for 4 h at 37°C. Once filters had been removed, the plates containing the library were stored at 4°C.

Detection of positive plaques. Nitrocellulose filters were washed three times for 10 min in TBS-Tween. Plaques recognised by the STCM antiserum were detected using a similar protocol to that used for Western blot analysis as described in section 3.10 but with minor modifications. Filters were blocked in TBS containing 1% w/v BSA (Sigma) for at least 1 h at room temperature or overnight at 4°C with shaking. This was followed by incubation with a 1:500 dilution of the antiserum in blocking buffer for either 2 h at room temperature or overnight at 4°C, with shaking. During incubation with primary and secondary antibodies the nitrocellulose filters were placed in individual Petri plates. Any residual unbound antibody was removed by three washes for 10 min in TBS-Tween. The filters were then incubated with the goat anti-rabbit alkaline phosphatase conjugate diluted 1:5000 in blocking buffer for 2 h at room temperature with shaking. The filters were washed twice for 10 min in TBS-Tween followed by a further two washes in TBS

only. After this, alkaline phosphatase staining was detected as described in section 3.10. After colour development the filters were air-dried, and the patterns of stained plaques detected on the duplicate filters taken from each plate were compared to identify which plaques immunolocalised on both filters.

Isolation of positive clones. Single plaques or an area of agar containing several plaques in close association which were identified from the nitrocellulose filters as expressing proteins recognised by the antiserum, were removed from the agar plates by coring the top agarose using a pipette tip. This was placed into 1ml SM buffer (Appendix) in a glass tube and the phage allowed to disperse at 4°C overnight.

NZY agar plates (80mm diameter) and fresh XL1-blue plating cells (see above) were prepared. To determine the phage titre of these samples, a 1:1000 dilution of the SM solutions containing the primary positives were prepared using SM buffer and either 5µl or 15µl of this solution added to 100µl cells, incubated at 37°C for 15 min in top agarose and plated on the 80mm plates. After incubation at 37°C overnight, plaques were counted and the pfu of the primary positive stock determined. Primary positives were then replated at a dilution which would give well spaced plaques (approximately 100-250 plaques) on 150mm diameter plates. These plates were then incubated with duplicate IPTG-impregnated membranes and positive plaques were detected as described above. Individual plaques containing cDNAs capable of synthesising protein recognised by the antiserum were cored out of the agar and placed in 0.5ml SM buffer with 20µl chloroform. These were incubated overnight at 4°C before *in vivo* excision of recombinant plasmid DNA.

In vivo excision of individual recombinant phagemids. LB-agar (Appendix) plates containing 100µg/ml ampicillin were poured. XL1-blues were grown up as described previously. SOLR® cells (Stratagene) were grown up overnight in LB broth (containing no supplements) to an $O.D._{600}$ of 1.0. XL1-blue cells (200µl) were combined with 150µl of selected phage stock (containing >1 x 10^5 phage particles) and 2µl of ExAssist™ helper phage (>1 x 10^6 pfu/µl) (Stratagene) and incubated at 37°C for 15 min prior to addition of 3ml LB broth and incubation at 37°C for 2.5 h (with shaking at 200 rpm). Helper phage were inactivated by heating at 70°C for 15 min and the cells sedimented by centrifugation at 3000 rpm for 15 min. The supernatant containing phagemid as filamentous phage particles was transferred to a fresh tube and stored at 4°C.

Aliquots of supernatant, 50 or 100µl, were combined with 100µl freshly prepared SOLR cells, incubated at 37°C for 15 min and 25µl of the cell suspension was spread onto LB-amp plates (see above) and incubated at 37°C overnight. Individual colonies of ampicillin-resistant bacteria were picked from these plates, restreaked onto numbered segments of fresh LB-amp plates and incubated at 37°C overnight.

6.2.4. Analysis of positive clones

The strategy used for the analysis of clones identified by immunoscreening is outlined below in Fig. 3.

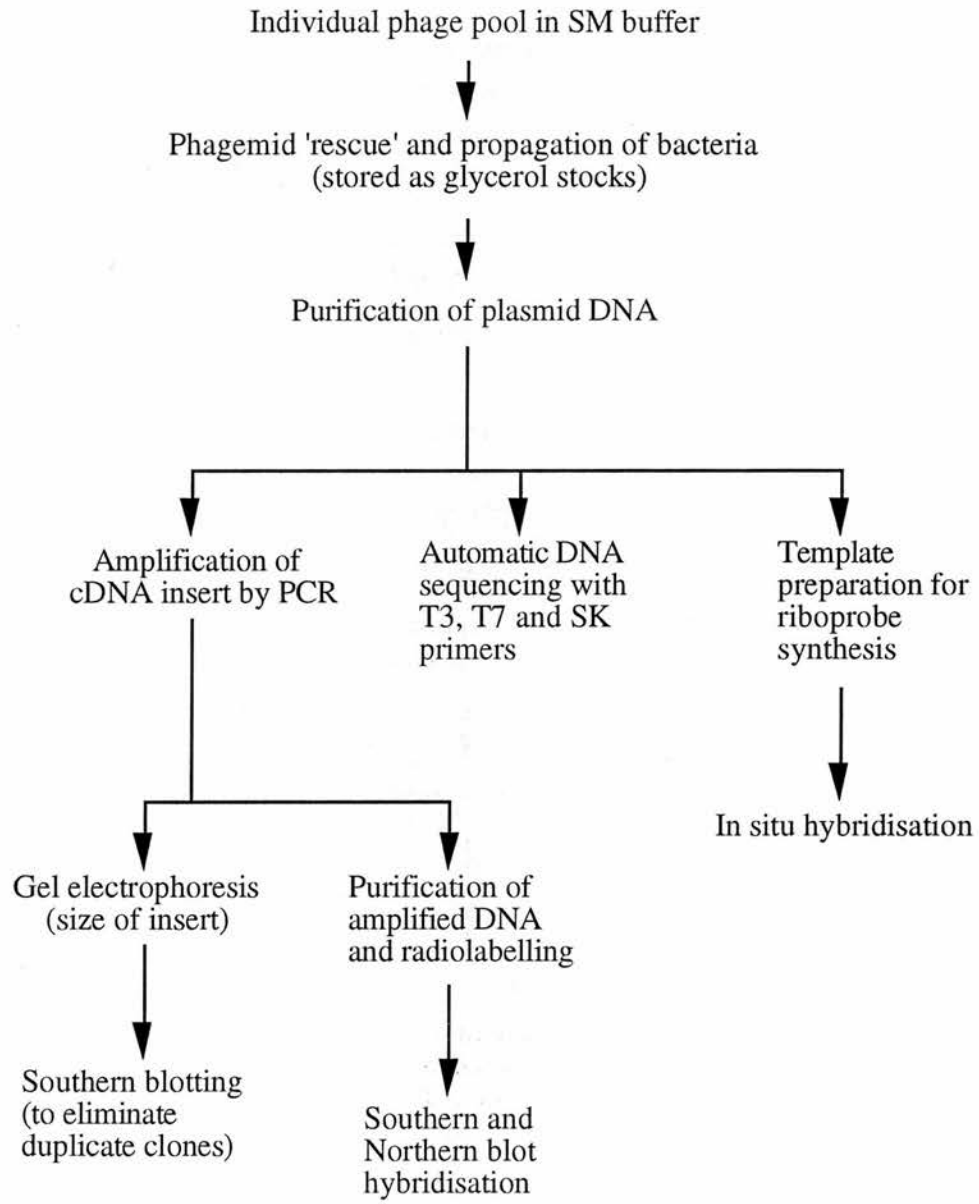


Figure 3. A flow chart outlining the techniques used to analyse clones isolated by screening a Sertoli cell and spermatid cDNA expression library using an antiserum raised against STCM.

Plasmid preparation. Single colonies from the restreaked ampicillin resistant bacteria were used to inoculate LB-broth containing 50µg/ml ampicillin, and cultured overnight at 37°C. Glycerol stocks were taken and plasmid DNA was prepared using the Promega Wizard™ purification system as described in section 3.13.

PCR of cDNA inserts. The cDNAs in this library were all originally cloned between the Eco R1 and Xho I sites of Lambda Zap. After excision rescue they were propagated in the Bluescript SK plasmid (Fig. 4). Inserts were amplified by PCR using primers corresponding to the T3 and T7 RNA polymerase sites present in this vector. The PCR method is described in section 3.15. A 10µl aliquot of each PCR reaction was separated on a 1.5% agarose gel and run in parallel with DNA size markers to allow determination of the size of the amplified cDNA inserts (see section 3.13). Amplified DNA was purified from the PCR reaction mixes using Chroma Spin+TE-100 columns according to the manufacturer's instructions (Clontech, Cambridge Bioscience, Cambridge, UK).

Southern blot analysis. DNAs amplified by PCR were separated by electrophoresis and then transferred to nylon membranes (Hybond™-N; Amersham). The agarose gels were incubated in denaturing solution (0.5M NaOH, 1.5M NaCl) for 20 min at room temperature on a rocker platform. The gel was then placed on a plastic tray and a piece of nylon membrane was laid directly onto the gel and covered with several layers of 3mm blotting paper (Whatman), paper towels and a glass plate. A weight was placed on top and left for 4 h at room temperature to allow transfer of the DNA by capillary action. DNA was fixed to the membrane by UV cross linking and the blot wrapped in clingfilm and stored at 4°C until hybridisation. Membranes were probed with ^{32}P-dCTP labelled positive cDNA inserts to identify duplicate clones using methods described in section 3.15.

Northern blot analysis. To allow an initial evaluation of the tissue specificity of mRNA expression for the isolated cDNAs, RNAs were separated on a denaturing agarose gel and blotted onto a nylon membrane as described in section 3.14. The RNAs used were isolated from a range of adult rat tissues including testis, epididymis, prostate, kidney, liver and brain, from testis of immature rats aged 20 and 23 days old, and from the ovaries of adult female rats. RNA was also isolated from enriched Sertoli cell and germ cell fractions isolated according to methods described by Saunders *et al.* (1993). Membranes were hybridised to ^{32}P-dCTP labelled cDNA inserts as described in section 3.15 and visualised on a phosphoimager and using X-ray film. Northern blots were stripped with boiling 0.1% SDS w/v and reprobed with an oligonucleotide to 18S ribosomal RNA (section 3.15).

Sequencing. Recombinant plasmids were sequenced using primers corresponding to the T7, T3 and SK regions of the DNA using the PCR based sequencing reaction described

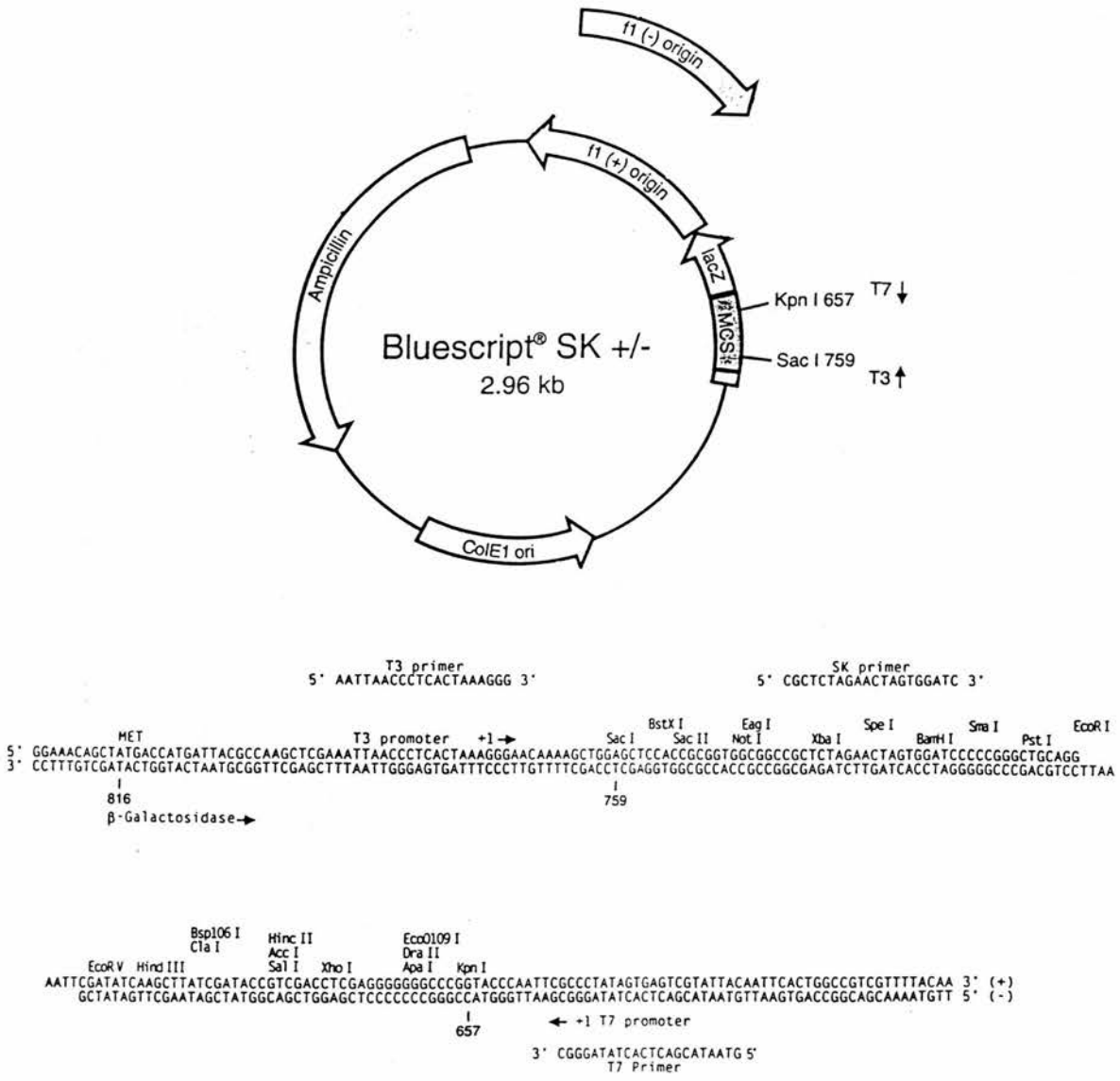


Figure 4. A map of the Bluescript SK- cloning vector showing the polylinker region, sites for RNA polymerase binding (T3 and T7) and the genes for ampicillin resistance and β-galactosidase which are the basis for the selection of recombinants (A). Below is shown the sequence of the polylinker region including the restriction enzyme sites (B).

in section 3.18. The majority of the sequencing was kindly performed by Mr. Joe Gaughan. Sequence data was analysed and error rate determined using the Genejockey computer programme (Biosoft, Cambridge, UK) and portions corresponding to vector sequences discarded prior to determination of sequence homology. Searches for homology to sequences lodged in Genbank were achieved by accessing the MRC human genome mapping project (HGMP) sequence analysis facility and made use of a specific package, basic local alignment search tool (Blast) (Altschul *et al.*, 1990).

In situ hybridisation. For some of the clones, non-radioactive *in situ* hybridisation was performed using digoxigenin labelled riboprobes (the full methods are described in section 3.17). Plasmid DNA was digested with restriction enzymes to give linearised templates for preparation of sense and antisense riboprobes. A map of the Bluescript SK- vector is shown in Fig. 4 detailing the restriction enzyme sites which can be used for linearisation. 828B8 was cut with Kpn 1 to provide template for sense riboprobe synthesis and Not 1 for the antisense template. Riboprobes were prepared from the templates as detailed in section 3.17, using T3 polymerase to generate the sense riboprobe and T7 polymerase for the antisense. *In situ* hybridisation was carried out on tissue sections from control rats.

6.3. Results

6.3.1. Dot blot analysis

From the results of the dot blot test a 1:500 dilution of the antiserum was selected as the optimal dilution to use for immunoscreening since it was sensitive enough to give a strong positive reaction with 1ng of antigen (STCM) but without crossreacting with a 1:1000 dilution (equivalent to 1ng protein) of the *E.coli*/phage lysate. There was some non-specific reaction by the antiserum when incubated with the higher concentrations of bacterial proteins (e.g. neat and the 1:10 dilution) and the antiserum was therefore preabsorbed before further use in immunoscreening.

6.3.2. Identification of positive clones

Immunoscreening with the STCM antiserum did result in some non-specific background staining of plaques which were not duplicated on both membranes. This suggests that the antiserum was not fully preabsorbed and still contained antibodies against bacterial proteins.

A total of sixteen plaques were identified after two rounds of screening which synthesised proteins which were immunolocalised on duplicating membranes. These plaques were isolated and following, Bluescript phagemid rescue, plasmid DNA was purified. The insert DNA was amplified by PCR; amplified DNAs with sizes ranging

from 400-2600 base pairs were detected. Several of the inserts were found to be identical in size and Southern blotting was carried out to eliminate duplicate clones from the analysis. Two clones were selected for use as probes on Southern blots; these were numbered 813B4 and 828A5 and had lengths of 750 and 2600bp, respectively. A total of four clones were identified as being 813B4 and two clones as 828A5; representative Southern blots are shown in Fig. 5. Duplicate clones were excluded from further analysis and therefore the results described in the next section are based on the analysis of eight unique clones.

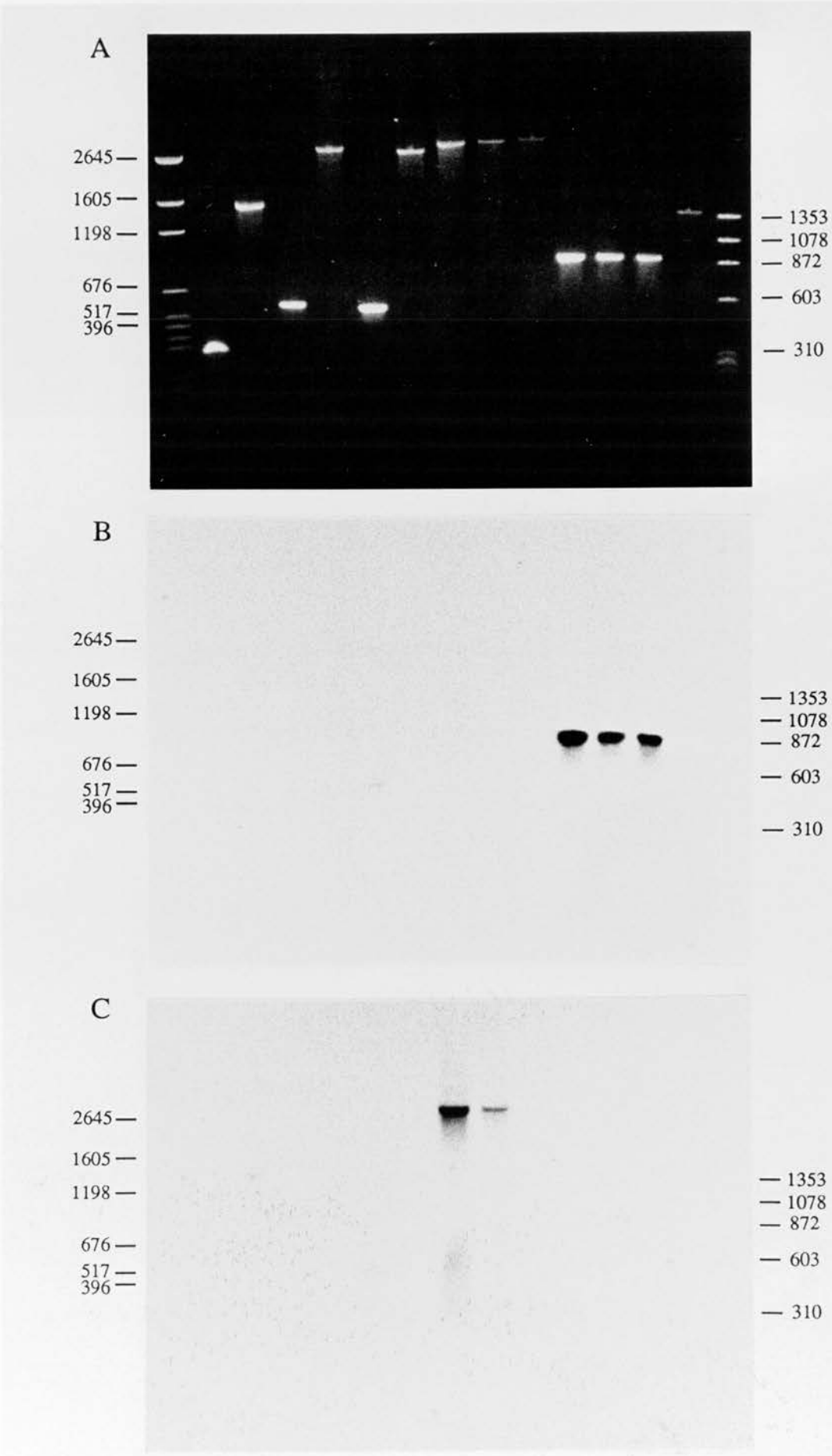
6.3.3. Analysis of selected positive clones

A summary of the positive clones identified by immunoscreening which were selected for further analysis, indicating the cDNA insert size and any homology to sequences lodged in Genbank is shown in Table 1. Northern blot hybridisation was performed for all clones and *in situ* hybridisation was attempted for some of the clones. Full details of the results obtained for each clone are described below.

Table 1. Characterisation of positive clones

Clone	Insert size (bp)	Sequence data
712A5	1500	significant homology to cyclic protein-2 (CP-2)
712B5	400	no significant homologies
712B8	2700	some homology to P-cadherin and MHC class 1 H-2-D at 5' end only
712C8	600	identity to rat ribosomal protein S17
813B4	750	no significant homologies
828A5	2600	significant homology to c-H-ras proto-oncogene mechanism sequence and endogenous retroviral sequence
828B8	2700	weak homology to a rabbit endometrial progesterone induced protein and to a human TNF induced mRNA sequence
828C8	2700	significant homology to a chloride channel (ClC-2)

Figure 5. This shows representative Southern blots used to identify duplicate clones. Panel A shows PCR of selected Bluescript clones rescued from positive plaques. Markers shown are pGem digested with Hinf I, Rsa I and Sin I (left hand side; IBI) and phi X digested with Hae III (right hand side; Promega). Panel B shows an autoradiograph of a Southern blot of the agarose gel in (A) probed with ^{32}P-labelled clone 813B4. Panel (C) shows a duplicate Southern blot probed with ^{32}P-labelled clone 828A5. In both autoradiographs several clones are shown to have the same identity.


A
2645
1605
1198
676
517
396
1353
1078
872
603
310
B
2645
1605
1198
676
517
396
1353
1078
872
603
310
C
2645
1605
1198
676
517
396
1353
1078
872
603
310

Clone 712A5

A Northern blot loaded with total RNA (20μg) from a range of tissues, including adult testis, day 20 testis, epididymis, kidney, prostate, brain and ovary was probed with 712A5 and a 1.7kb transcript was detected in all tissues after exposure to X-Omat AR film for 4 days (Fig. 6, panels A and B). The level of expression was highest in the ovary. Hybridisation of this cDNA to both adult testis and day 20 testis total RNA resulted in a similar intensity of signal, suggesting that this mRNA was expressed by Sertoli cells rather than germ cells. This was confirmed by another Northern blot (not shown) which showed that this transcript was present in total RNA from Sertoli cells but was not present in total RNA isolated from either pachytene spermatocytes or round spermatids. Sequencing of this clone generated around 500 bases of sequence from both the 3' and 5' ends of the cDNA insert. Comparison of this data with sequences lodged in Genbank showed very good homology to cyclic protein-2 (CP-2) (accession no.: S85184; Erickson-Lawrence *et al.*, 1991). A stretch of 375 bases of sequence from the 5' end of 712A5 showed 96% homology to CP-2. This protein is the proenzyme form of cathepsin L, a cysteine protease, the secretion of which by Sertoli cells has already been well characterised, so no further work was carried out on this clone.

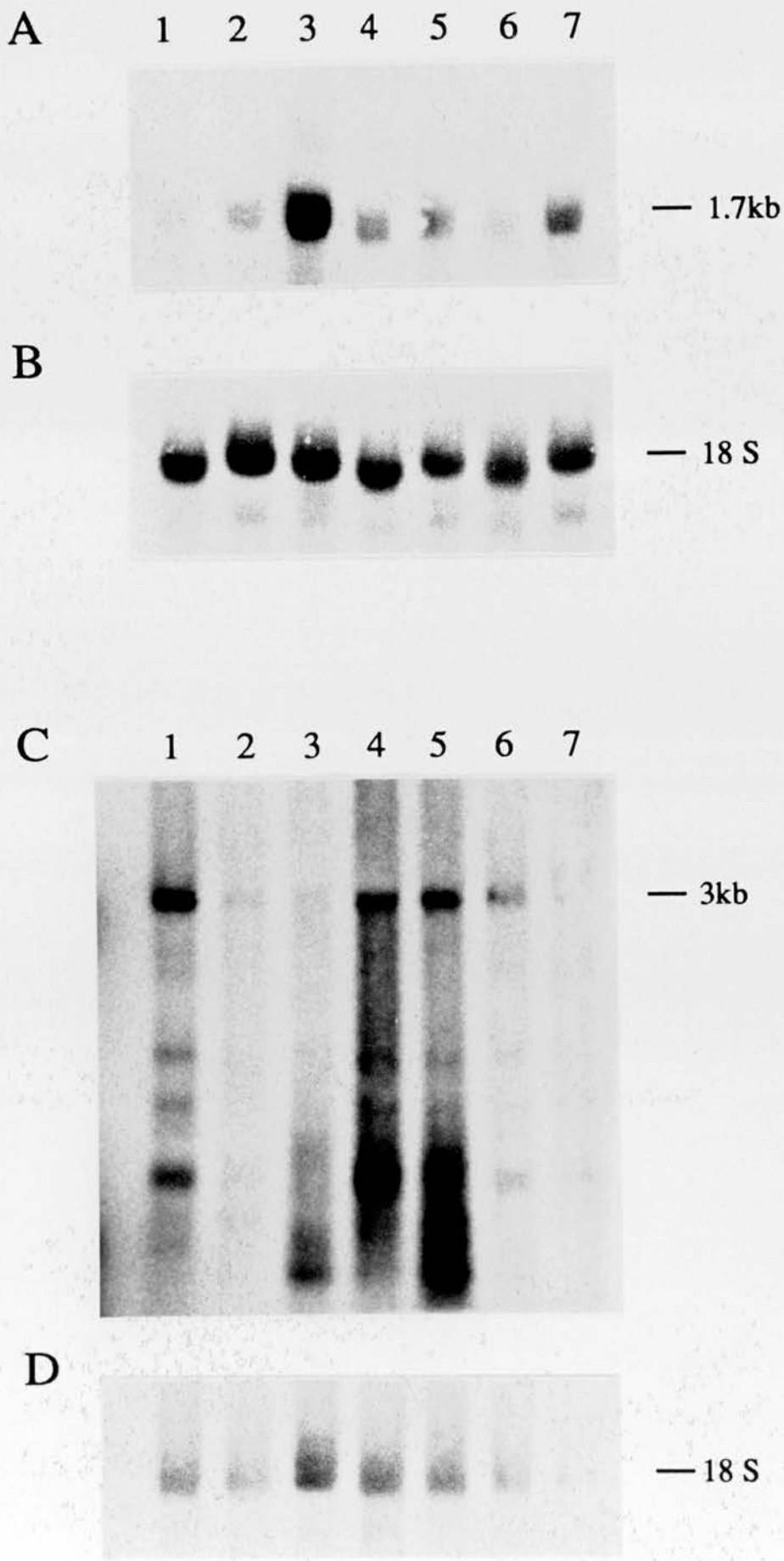
Clone 712B5

Hybridisation of a Northern blot with 712B5 was only able to detect several very faint transcripts in poly A^+ RNA from adult testis and in total RNA from kidney, liver and prostate after 5 days on a phosphoimager screen (not shown). No further analysis by Northern blot hybridisation was attempted. Sequencing generated approximately 300 bases from both the 3' and 5' ends of the insert which covered the full cDNA length, 419bp. A comparison of this sequence with those present in Genbank failed to find any significant homologies. The lack of testis specificity of this transcript combined with the difficulty of its detection by Northern blot hybridisation resulted in no further analysis being attempted.

Clone 712B8

Northern blot hybridisation with 712B8 detected multiple transcripts in total RNA from the following tissues: adult testis, day 23 testis, epididymis, prostate, kidney, liver and ovary after exposing the autoradiograph for 2 days to X-Omat AR film (Fig. 6, panels C and D). The most abundant transcript had an approximate size of 3kb and the highest level of expression of this transcript was seen in the kidney and testis. Sequencing generated 350 bases at the 3' end of the insert and 200 bases at the 5' end. A search of the sequences in Genbank revealed some homology of the 5' sequence to a mouse P-cadherin gene (accession no.: D12688; Nose *et al.*, 1987) and also to the mouse major

Figure 6. Northern blot analysis of 712A5 and 712B8 mRNA expression. In panel A, the gel was loaded with 20μg total RNA as follows: lane 1= kidney; lane 2= prostate; lane 3= ovary; lane 4= adult testis; lane 5= day 20 testis; lane 6= epididymis and lane 7= brain. The blot was hybridised with ^{32}P-labelled 712A5 cDNA and exposed to X-Omat AR film for 4 days. In panel C, the gel was loaded with 15μg total RNA as follows: lane 1= kidney; lane 2= prostate; lane 3= ovary; lane 4= adult testis; lane 5= day 23 testis; lane 6= epididymis and lane 7= liver. The blot was hybridised with ^{32}P-labelled 712B8 cDNA and exposed to X-Omat AR film for 2 days. The blots were stripped and reprobed with an oligonucleotide probe for the 18S ribosomal RNA to check that all lanes contained equivalent amounts of RNA (panels B and D).


A
1 2 3 4 5 6 7
— 1.7kb
B
— 18 S
C
1 2 3 4 5 6 7
— 3kb
D
— 18 S

histocompatibility complex (MHC) class I H-2D-k (accession no.: M18524; Watts *et al.*, 1987) and H-2D-b (accession no.: M18523; Watts *et al.*, 1987) genes but no homology was found to the 3' sequence. A stretch of 217 bases of sequence including 4 gaps showed 79% homology to the P-cadherin sequence; a stretch of 226 bases of sequence including 1 gap showed 75% homology to MHC class I H-2D. No further analysis was attempted since expression of the mRNAs did not appear testis specific.

Clone 712C8

Northern blot hybridisation with clone 712C8 detected an abundant, low molecular weight transcript of less than 1kb in total RNA in all the tissues on the blot which included adult testis, day 23 testis, epididymis, prostate, kidney, liver and ovary (Fig. 7, panels A and B). Another Northern blot showed that this transcript was present in total RNA from Sertoli cells and pachytene spermatocytes but not round spermatids (not shown). Sequencing generated 300 bases at the 3' end which showed a very good homology to the S17 rat ribosomal protein (accession no.: K02933; Nakanishi *et al.*, 1985). Comparison of 279 bases of sequence with the ribosomal protein sequence gave an homology of 95%. No further analysis of this clone was undertaken.

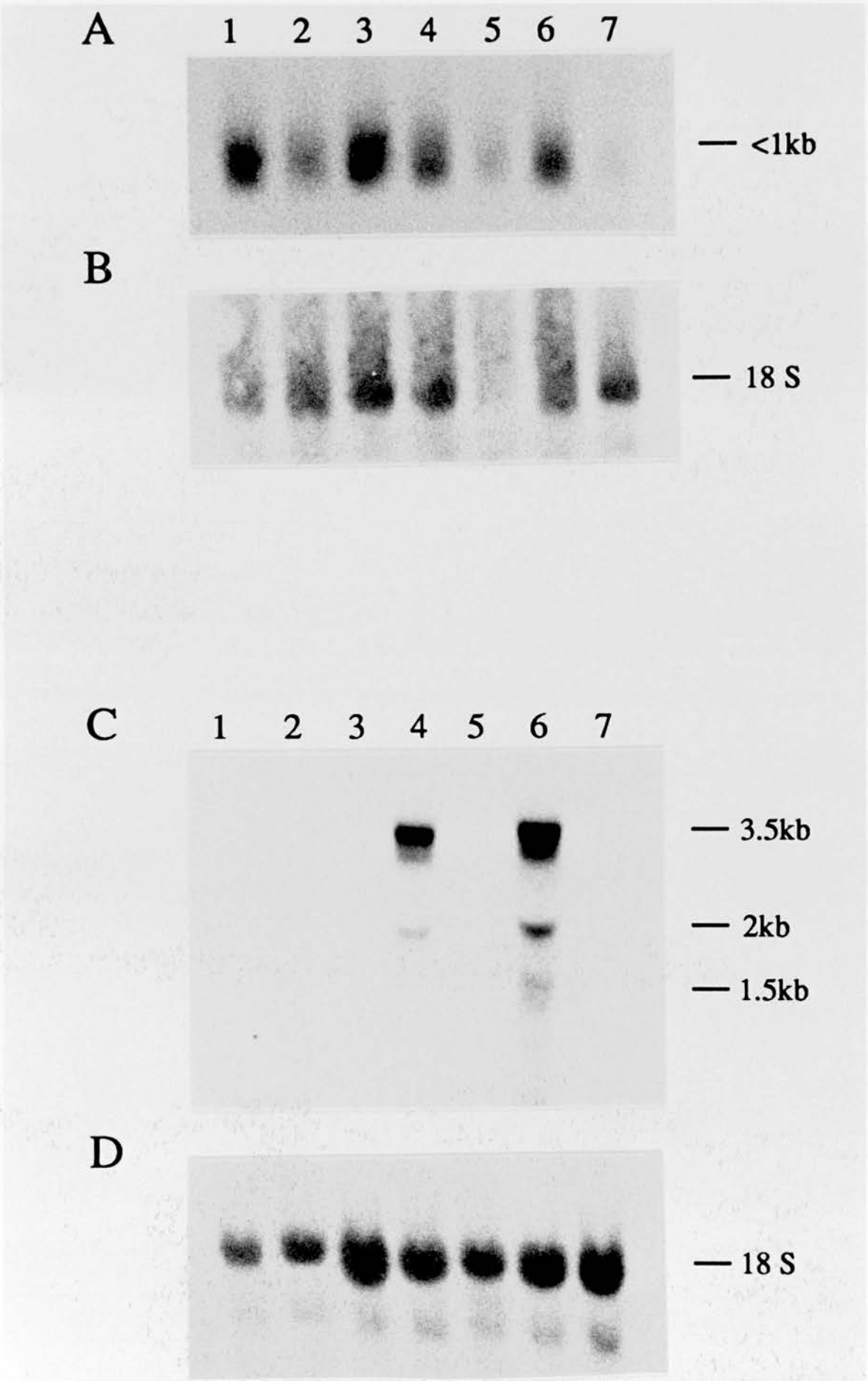
Clone 813B4

Hybridisation of this probe on a Northern blot detected two abundant transcripts of 3-3.5kb and 2kb in both testis and epididymis, plus a third transcript of 1.5kb was also expressed in the epididymis of adult rats. These transcripts were detected within 12 h of exposure to X-Omat LS film (Fig. 7, panels C and D). Two much fainter transcripts were also detected in total RNA from day 23 rat testis after a longer exposure time. The stronger signal in adult testis in comparison to immature testis is probably indicative of these transcripts being produced by the germ cells. No expression of this transcript could be detected in total RNA from prostate, kidney, brain or ovary, even after the autoradiograph had been exposed for a week. The entire cDNA insert (750bp) was sequenced using T3, SK and T7 primers but no significant homology to any of the sequences lodged in Genbank was found. Since these transcripts are specifically expressed in the testis and epididymis and the lack of sequence homology indicated this clone encoded a novel gene product, 813B4 was therefore subjected to further analysis which is described in the following chapter.

Clone 828A5

Automatic sequencing was able to generate 360 bases at the 3' end of the cDNA insert and a further 433 bases at the 5' end. Comparison of this sequence data with that present in Genbank resulted in good homology to a rat c-H-ras proto-oncogene mechanism

Figure 7. Northern blot analysis of 712C8 and 813B4 mRNA expression. In panel A, the gel was loaded with 15μg total RNA as follows: lane 1= kidney; lane 2= prostate; lane 3= ovary; lane 4= adult testis; lane 5= day 23 testis; lane 6= epididymis; and lane 7=liver. The blot was hybridised with ^{32}P-labelled 7.1.2 C8 cDNA and exposed to X-Omat AR film for 2 days. In panel C, the gel was loaded with 20μg of total RNA as follows: lane 1= kidney; lane 2= prostate; lane 3= ovary; lane 4= adult testis; lane 5= day 20 testis; lane 6= epididymis, lane 7= brain. The blot was hybridised with ^{32}P-labelled 813B4 cDNA and exposed to X-Omat LS film for 12 hours. The membranes were stripped and reprobed with an oligonucleotide probe for the 18S ribosomal RNA to check that all lanes contained equivalent amounts of RNA (panels B and D).


A
1 2 3 4 5 6 7
<1kb
B
18 S
C
1 2 3 4 5 6 7
3.5kb
2kb
1.5kb
D
18 S

sequence. Comparison of 216 bases of sequence from the 3' end of 828A5 showed 93% homology, whilst comparison of 241 bases of sequence from the 5' end resulted in 83% homology to a rat c-H-ras mechanism sequence (accession no.: L06433; Makris *et al.*, 1993) and to endogenous retroviral sequence (accession no.: D90005; Nakamuta *et al.*, 1989).

Clone 828B8

Several attempts to examine mRNA expression of 828B8 by Northern blot hybridisation failed to detect any clear transcripts. Due to these difficulties two attempts were made to try and localise the cellular pattern of mRNA expression by non-radioactive *in situ* hybridisation on sections of control rat testes and on uterus and ovary from a rat at day 17.5 of gestation but without any success. Sequencing was able to generate 300 bases at the 3' end and 270 bases at the 5' end of the cDNA insert. A comparison of these sequences with those present in Genbank resulted in some homology of the 3' sequence to the mRNA of a rabbit endometrial progesterone-induced protein (EPIP) (accession no.: M17099; Misrahi *et al.*, 1987) and a human TNF (Tumour necrosis factor)-induced mRNA (accession no.: U30158; unpublished) (Fig. 8). Comparison of 194 bases of sequence resulted in 78% homology to EPIP and 71% homology to the human TNF-induced mRNA. Translation of the putative open reading frame for 828B8 resulted in a peptide of 72 amino acids showing 71% homology to the C-terminus of EPIP and 95% homology to the TNF-induced mRNA (Fig. 8).

Clone 828C8

Northern blot hybridisation detected several very weakly expressed transcripts in testis and other tissues after 4 days on a phosphoimager screen (not shown). Automatic sequencing generated 250 bases of sequence at the 3' end of the insert which showed good homology to the mRNA for a rat chloride channel, CIC-2 (accession no.: X64139; Thiemann *et al.*, 1992). A stretch of 239 bases showed 98% homology to CIC-2. No further analysis of this clone was attempted.

6.4. Discussion

Antibody screening of cDNA expression libraries is a useful technique for detecting the cDNA sequences of proteins for which an antiserum has already been generated. For a cDNA library to be translated in an *in vitro* system the cDNAs must be cloned into expression vectors in the correct orientation and reading frame next to a promoter site functional in *E.coli*. The quality of the mRNA used to prepare the library will also affect screening results and may reduce the likelihood of isolating cDNAs encoding high

```
  29 GGTAACAGGAGGGGGGATGAAGCTCTGGAGAAGCGATTTCTTGACAAGGCCGTAGAACTC 88
     || |||   | |||||| |||||| | || || || || || || || ||| | || |||
1010 GGCAACGCCAAGGGGGACGAAGCTTTAGAAAAACGGTTCCTCGATAAAGCCCTGGAGCTC 1069

  89 AACATGATCTCCTTGAAGGGGCACAGGTCGGTGGGCGGCATCCGAGCGTCTCTGTATAAT 148
      | |||||||||||||| || |||||||| ||||| ||| |||| |  ||||| ||||||
1070 CATATGATCTCCTTGAAAGGACACAGGTCCGTGGGAGGCGTCCGGGTCTCTCTCTATAAT 1129

 149 GCTGTCACCACTGAAGATGTGGAGAAGTTGGCTGCCTTCATGAAGAACTTCCTGGAGATN 208
     ||||||||||  ||||| ||  ||||| | ||  |||||||||| || ||  |||||||
1130 GCTGTCACCATCGAAGACGTTCAGAAGCTAGCATCCTTCATGAAAAATTTTTTGGAGATG 1189

 209 NATCAGCTGTGAAT 222
      ||||||| |||||
1190 CATCAGCTATGAAT 1203
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                 10        20        30        40        50
                 |         |         |         |         |
     GNAKGDEALEKRFLDKALELHMISLKGHRSVGGVRVSLYNAVTIEDVQKLSFMKNFLEM
      ..  ............ .. ........... . ....... ... ..
XXGFEDSPRGNRRGDEALEKRFLDKAVELNMISLKGHRSVGGIRASLYNAVTTEDVEKLAAFMKNFLEXXQL
        |         |         |         |         |         |         |
        10        20        30        40        50        60        70
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107 GGGCACAGGTCGGTGGGCGGCATCCGAGCGTCTCTGTATAATGCTGTCACCACTGAAGAT 166
    |||||||||| ||||| ||||| || || |||||||||| |||||||| || |||||
  1 GGGCACAGGTCAGTGGGAGGCATTCGTGCCTCTCTGTATAACGCTGTCACAACCGAAGAC 60

167 GTGGAGAAGTTGGCTGCCTTCATGAAGAACTTCCTGGAGATNNATCAGCTGTGAATGTGA 226
    || |||||| |||| |||||||||||||| ||| ||||||| |||||||||||  |||
 61 GTTGAGAAGCTGGCGGCCTTCATGAAGAATTTCTTGGAGATGCATCAGCTGTGAACATGA 120

227 CCAGCCTCACCC 238
    |||||| | | |
121 CCAGCCACGCTC 132
```

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                                          10        20        30
                                          |         |         |
                                GHRSVGGIRASLYNAVTTEDVEKLAAFMKNFLEMHQL
                                ..................................  ..
XXGFEDSPRGNRRGDEALEKRFLDKAVELNMISLKGHRSVGGIRASLYNAVTTEDVEKLAAFMKNFLEXXQL
        |         |         |         |         |         |         |
        10        20        30        40        50        60        70
```

Figure 8. Sequence alignment of 828B8 with a rabbit endometrial progesterone-induced protein (EPIP) mRNA (A and B) and with human TNF-induced mRNA (C and D). A shows the alignment of the clone 828B8 using sequence obtained using SK and T3 primers (top line) to bases 1010-1203 of the rabbit EPIP mRNA (bottom line). B shows the alignment of the peptides translated from the putative open reading frames for EPIP (top line) and 828B8 (bottom line). In C, the 828B8 DNA sequence (top line) is aligned with bases 1-132 of the human TNF-induced mRNA (bottom line). D shows the alignment of the peptide sequences for 828B8 (bottom) and the TNF induced mRNA (top).

molecular weight proteins or those encoded by short-lived or rare messenger RNAs. The main limitation of using antibodies to screen expression libraries is that this method of detection relies on the proteins translated by bacteria being able to fold into a conformation which preserves the epitope recognised by the antibody. Since the mechanisms responsible for post translational modifications are different in bacteria in comparison to mammalian cells and, further more, many of the cDNAs will not encode the complete protein sequence, the proteins formed by this method of expression may be unable to fold correctly. It is therefore an advantage if the antiserum used for the screening can recognise epitopes present on denatured proteins. The previous chapter of this thesis presented the results of an investigation into the feasibility of detecting ST derived proteins in testicular interstitial fluid and blood, work which was based on using a polyvalent antiserum raised to STCM. Western blot analysis demonstrated that this antiserum could recognise many of the proteins present in STCM when they were resolved on gels under denaturing conditions. Since very few of the proteins secreted by seminiferous tubules have been characterised, this antiserum represented a tool which might allow the identification of novel proteins secreted by the seminiferous epithelium.

The present study used an anti-STCM antiserum to screen a cDNA expression library prepared from cells isolated from adult rat testes in an attempt to identify the cDNAs of novel proteins involved in spermatogenesis, which might be useful as potential markers of spermatogenesis. The only directionally cloned testis cDNA library available in the laboratory at the time this study was initiated had been prepared for the purpose of identifying Sertoli cell gene products, the synthesis of which was modulated by elongate spermatids (Maguire, 1994). The RNA used to prepare the library was not isolated from total testis tissue but from a cell fraction which was enriched in Sertoli cells (approximately 60%) (Meistrich *et al.*, 1981), but which also contained significant numbers of spermatids which were predominantly of the elongate type (Maguire, 1994). It would also have been useful to screen a cDNA library prepared from mRNA isolated from total testis from adult rats since this would have contained cDNAs representing the total cell population of the seminiferous epithelium. However, since immunohistochemistry had demonstrated that the anti-STCM antiserum contained antibodies which recognised proteins localised to Sertoli cells and elongate spermatids, in preference to other cell types, as judged by the intensity of the immunostaining (see chapter 5, Fig. 1). The cDNA library was deemed suitable for the purposes of this preliminary study.

Eight individual clones recovered following two rounds of screening were subjected to analysis by sequencing and Northern blot hybridisation; in some cases *in situ* hybridisation was also performed. Northern analysis of RNA from various rat organs demonstrated that clone 712A5 was widely expressed as an mRNA was detected in all the

tissues tested. The most abundant transcript had an apparent size of 1.7 kb and the mRNA was present in RNA from immature testis (day 20) as well as in that from adult rats, suggesting that this was likely to be expressed in Sertoli cells, a hypothesis subsequently confirmed by Northern analysis of RNA from enriched preparations of Sertoli cells. Partial sequencing of clone 712A5 showed that this cDNA insert had a sequence identical to that of the Sertoli cell secreted protein cyclic-protein 2 (CP-2), also known as the pro-form of cathepsin L (Wright *et al.*, 1989; Erickson-Lawrence *et al.*, 1991). Both the transcript size detected on Northern blots and its expression by Sertoli cells and other tissues from male rats are in agreement with published data on CP-2 mRNA expression (Erickson-Lawrence *et al.*, 1991, Maguire *et al.*, 1993). CP-2 is secreted in a stage dependent manner such that secretion is maximal at stages VI-VII of the spermatogenic cycle (Wright *et al.*, 1983). More recently, mRNA expression has been shown to be regulated by both elongate spermatids (Maguire *et al.*, 1993) and FSH (Penttila *et al.*, 1995a). The function of CP-2 within the testis is unknown but since it is the pro-form of a cysteine protease it has been proposed that it may act on the extracellular matrix at stages VI/VII of the spermatogenic cycle to allow movement of the elongate spermatids from the base to the lumen of the seminiferous tubule in preparation for spermiation (Erickson-Lawrence *et al.*, 1991).

The measurement of the abundance of CP-2 secretion suggests it is unlikely to be a suitable marker of the efficiency of spermatogenesis. Western blot analysis has detected similar concentrations of CP-2 in the rete testis and caput epididymal fluid but the level in cauda epididymal fluid is reduced (Zabludoff *et al.*, 1990a). This appears to be due to degradation. Western blot analysis has shown that as CP-2 moves down the length of the epididymis there are subtle changes in its molecular weight and in cauda epididymal fluid a much lower molecular weight peptide can be detected. No attempt has been made to measure the levels of CP-2 in blood, but since this protein is produced not only by the Sertoli cells but also by other tissues of the body such as the kidney, liver and brain it seems highly likely that a high degree of homology will exist between these proteins. Even if testicular CP-2 could gain access to the blood, assay results would probably be confounded by CP-2 secreted from other tissues.

Clone 712B5 proved difficult to assess since Northern analysis failed to reliably detect mRNA transcripts in either poly A^+ or total RNA from adult rat testes, and faint mRNA transcripts were also observed in several other tissues. Sequencing failed to detect any significant homologies resulting in a complete lack of information on the identity of this cDNA. This meant that this clone was not a suitable candidate for further investigation.

Clone 712B8 was also found to be unsuitable for further analysis. This cDNA was expressed in all the tissues examined by Northern blotting. Sequence analysis of

712B8 revealed some homology of the 5' sequence to the mouse P-cadherin and major histocompatibility complex (MHC) class I H-2-D genes. This suggests that the cDNA cloned possesses a domain present in all of these mouse sequences. P-cadherin mRNA has been shown to be expressed in the testes of immature rats by Northern analysis, with a 3.2-3.5 kb transcript detected in both testis and Sertoli cell RNA (Cyr *et al.*, 1992; Wu *et al.*, 1993), and also the epididymis (Cyr & Robaire, 1991). The most abundant transcript detected on Northern blots using 712B8 cDNA had a similar size to that reported for P-cadherin. Cadherins are a family of transmembrane proteins which are involved in calcium dependent intercellular adhesion and are important in the organisation of tissue structure (Takeichi, 1988). P-cadherin is thought to play a role in the cellular organisation of the seminiferous epithelium during testicular development in immature rats (Cyr *et al.*, 1992; Wu *et al.*, 1993). The MHC class I genes encode transmembrane proteins which are expressed on virtually all nucleated cells. These proteins are important in the immune response as they are involved in presenting peptides to T cells whose role is to discriminate between foreign and self antigens. The ubiquitous expression of H-2D in adult mouse tissues, including testis, has been demonstrated using RNase mapping (Drezen *et al.*, 1993). Because both MHC class I proteins and P-cadherin have a transmembrane structure it is possible that the protein encoded by 7.1.2 B8 also has a similar conformation. To obtain more information on the possible structure and identity of this clone would entail sequencing the complete cDNA insert. This was not attempted as the protein was not testis specific and was unlikely to be secreted, therefore would not be a useful marker of testis function.

The isolation of clone 712C8 is thought to represent a false positive since this cDNA was found to encode a rat ribosomal protein, S17. Ribosomal proteins are involved in the structure and function of ribosomes but their role is not well characterised. The prokaryotic homologue of S17 has been shown to be involved in ribosome assembly and translational fidelity (Golden *et al.*, 1993). It is highly improbable that the antiserum raised against STCM would contain antibodies against a ribosomal protein. A more likely explanation is that this clone was probably rescued due to its close association to a positive plaque recognised by the antiserum. Ribosomal proteins are expressed in an abundant manner and therefore are likely to be well represented in the cDNA library Although care was taken to isolate pure plaque populations, contamination and false positives are not unusual during library screening.

The preliminary analysis of 813B4 was encouraging since it fitted some of the criteria for a marker of spermatogenesis. Northern analysis indicated that the mRNA to which it hybridised was abundantly expressed in the testis and also the epididymis but in no other tissues screened in the initial investigation of tissue specificity of mRNA expression. Sequencing of the cDNA insert, of 750bp, failed to find any homology to

sequences lodged in Genbank indicating that this cDNA sequence has not been reported before. These results were sufficiently encouraging that additional analysis of 813B4 was performed as reported in chapter 7.

Sequencing of both ends of the 828A5 cDNA insert resulted in sequence data which showed significant homology to a proto-oncogene c-H-ras mechanism sequence and to the long terminal repeat regions of a rat endogenous retroviral sequence. These correspond to retroviral sequences which have become integrated into the rat genome, they are usually not expressed but are thought to be fairly common elements within the genome. The identification of this cDNA probably represents the isolation of a mixed plaque population.

Sequence analysis of 828B8 suggested that the cDNA encoded by this clone might be of interest, since a search in Genbank showed weak homology to both the nucleotide and amino acid sequence of a rabbit endometrial protein, the expression of which was induced by progesterone (Misrahi *et al.*, 1987). It is therefore possible that the cloned cDNA might encode a steroid regulated secreted protein and which has a role in spermatogenesis. In addition, this cDNA was also found to show a similar level of homology to a human TNF-induced mRNA. This expressed sequence tag was isolated from a subtraction library derived from TNF-treated BG9 human fibroblasts but has not yet been published. Unfortunately, several attempts to analyse the expression of this clone by Northern blot analysis failed to detect expression of an mRNA transcript in any tissue tested and non-radioactive *in situ* hybridisation was also unsuccessful. Further analysis of this clone was not undertaken because of lack of time.

The final clone that was analysed, 828C8, was found to have significant sequence homology to a chloride channel (CIC-2). This voltage gated chloride channel is ubiquitously expressed in rat tissues (Thiemann *et al.*, 1992), and is thought to be important in the regulation of cell volume (Grunder *et al.*, 1992). The isolation of this cDNA suggest s two possibilities. That this cDNA might be an aberrant pick from the library as it would seem unlikely that an antiserum raised to STCM would contain antibodies against a transmembrane ion channel. The other alternative is that chloride channels could be involved in reducing the cytoplasmic volume of spermatids prior to spermiation. It is possible that chloride channels are present on the residual cytoplasm formed during cytoplasmic elimination which might be released into the medium during ST culture.

The preliminary studies described in this chapter were undertaken with the aim of isolating genes encoding proteins secreted by the seminiferous epithelium. Of the eight cDNAs identified by immunoscreening only three encode potentially secreted proteins, 712A5 (CP-2), 813B4 and 828B8 (EPIP). The incidence of false positives suggests that plaques were not plated at a sufficiently low density to allow the isolation of pure plaque

populations resulting in contamination by other cDNAs in the library, as exemplified by the cloning of 712B8, 712C8, 828A5 and 828C8. Additional rounds of screening at low density would have helped to optimise the detection of duplicating true positive plaques against the background staining. Detection of clones by immunoscreening is dependent on the epitopes recognised by the antiserum being preserved following *in vitro* translation in the *E.coli* and that the cDNAs in the library are of sufficient size to generate a protein containing the epitope in the first place. These limitations mean that this method of library screening has more drawbacks when compared to screening using DNA probes. Future planned studies, which were abandoned due to lack of time, would have involved screening of cDNA libraries containing a greater proportion of germ cell derived cDNAs, size selected to encode complete protein sequences. The problem of non-specific background staining could be reduced by further preabsorption of the antiserum against phage/*E.coli* lysate. However, in spite of the preliminary nature of these investigations one clone was isolated which fitted the criteria set by this study. The cDNA, 813B4, was found to be abundantly expressed in the adult testis and epididymis but in no other tissues. In addition, comparison of the sequence of this 750bp insert failed to find any significant sequence homologies. The further study of this clone is described in the next chapter.

Chapter 7. Analysis of a testis specific cDNA

7.1. Introduction

The previous chapter described the use of an antiserum, raised against rat STCM, to screen a rat testis cDNA library. The aim of this approach was to identify novel proteins secreted by the testis which could then be evaluated as to their usefulness for monitoring spermatogenesis by non-invasive means. Several cDNAs were cloned by immunoscreening and initial analysis suggested that one cDNA was suitable for further investigation. On Northern blots, clone 813B4 recognised mRNA transcripts expressed in the rat testis and epididymis but not the prostate, kidney, brain or ovary. In total testis RNA, two transcripts with approximate sizes of 3kb and 2kb were identified and it was concluded that the cloned 813B4 cDNA, which was 750bp in length, did not therefore encode the full length cDNA. In addition, preliminary sequence analysis and comparison to sequences lodged in Genbank failed to identify homologues that had already been cloned. This preliminary data suggested that clone 813B4 was a partial cDNA of a potentially novel sequence, the expression of which was confined to the testis and epididymis.

The present chapter describes the results of a more detailed study of expresssion of the clone 813B4 mRNA and the complete sequence of the cDNA of which it forms a part, hereafter termed germ cell specific cDNA 1 (GCS1). Clone 813B4 was used to initially screen a rat testis library in order to identify a cDNA encoding the additional sequence of GCS1. Thereafter, the cDNAs isolated were sequenced and analysed for the presence of motifs and domains which might give some indication of the function of the protein it encodes. Northern blot analysis and *in situ* hybridisation were used to further investigate the tissue specificity and cellular pattern of mRNAs recognised by the cDNA encoded by clone 813B4. Southern blot analysis was used to determine whether the gene encoding GCS1 was conserved amongst several species.

7.2. Experimental procedures

7.2.1. Isolation and sequencing of 813B4 and homologous cDNAs of GCS1

The strategy involved in gaining the complete sequence is outlined in Figure 1 and described in full below. Due to the shortage of time, some library screening was undertaken by Dr. Philippa Saunders and the sequencing by Joe Gaughan with the help of Julie Wilson.

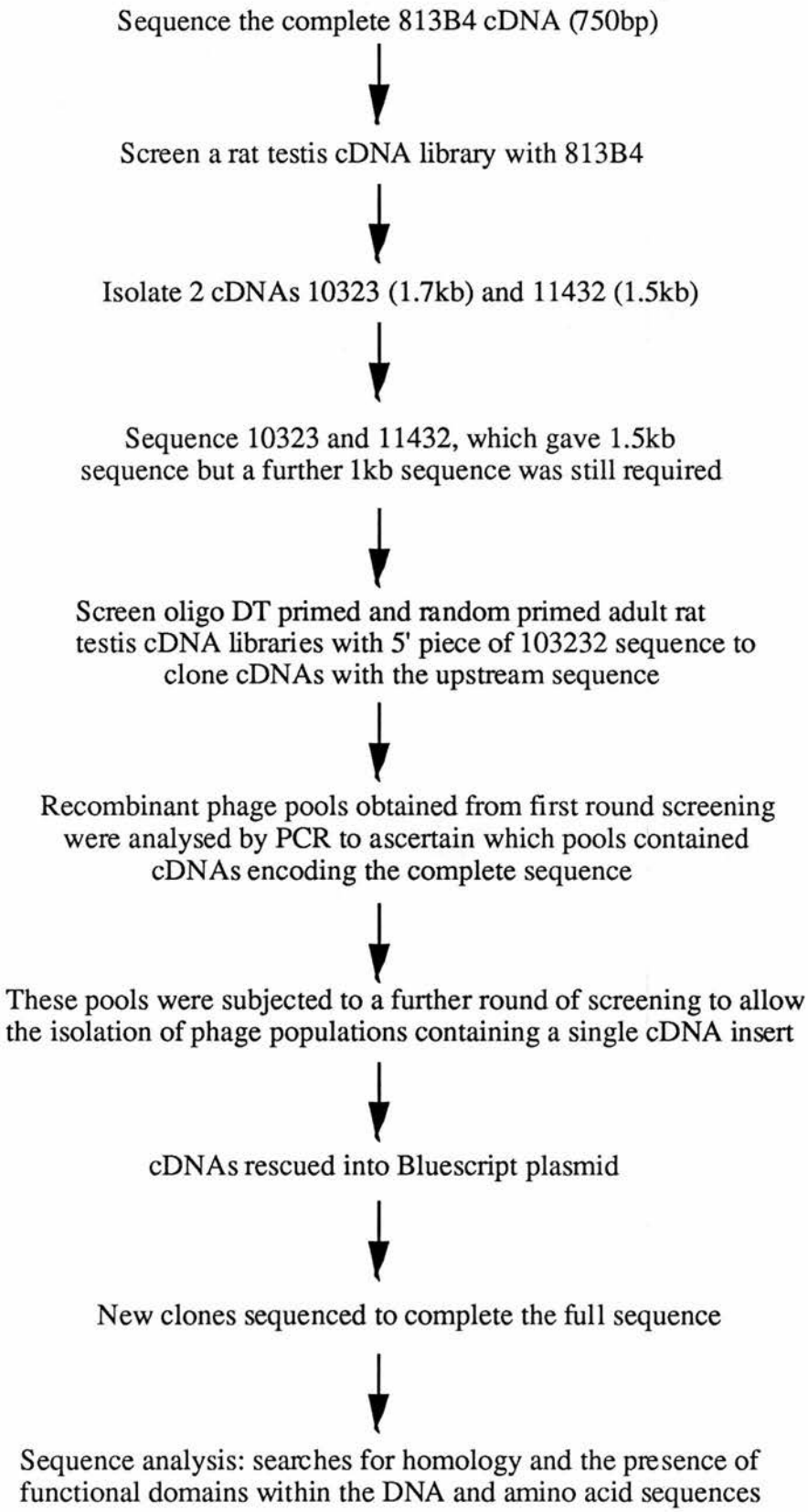

Figure 1. Strategy used to obtain the complete sequence for cDNA GCS1.

Sequencing of GCS1 clone 813B4

813B4 recombinant plasmid DNA was sequenced using primers corresponding to the T7, T3 and SK regions on the Bluescript SK vector as described in chapter 6 and section 3.18. This experimental approach resulted in 200bp of sequence data from either end of the 813B4 cDNA, the full length of which was calculated to be 750bp. In order to obtain the complete sequence of the clone, two internal oligonucleotide primers (1a and 2a) were synthesised on an Applied Biosystems PCR mate (Warrington, Cheshire, UK). They were recovered using ammonia, precipitated and quantified. The primers were based on the 3' and 5' sequences already gained for clone 813B4 (see Table 1 for details of sequence and direction). The sites of these two primers in relation to the full length of the cDNA are shown in Figure 2. These primers were used in PCR based sequencing reactions as described in section 3.18.

Table 1. Summary of the sequences and directions of the internal oligonucleotide primers used to sequence GCS1 Alignment of primers is designated relative to the complete sequence of the cDNA 1721.

Primer	Sequence (5'-3')	Orientation	Sequence alignment
1a	CTCACTTGCAGGTCCAGCAAC	Sense	1921-1941
1b	GCGCGCCTGCTGTTACTACTG	Sense	1260-1281
2a	ATGGCTGTGAGGTCGATGTCC	Antisense	2235-2214
2b	AGCCACTTCGTCAGCCTCCAATC	Antisense	2029-2006
3	GAACATCGACCTCACAGCCATC	Sense	2216-2238
4	TGCTGCCTCGCACATTCAGGAAC	Antisense	1155-1132
5	AGTGGCAACCAGCACAAGGCGG	Sense	1464-1486
7	CAGTAATGCACCTTGTTGCTC	Antisense	1229-1207
10	GTCCTTCTCCTCCTTGATGCG	Antisense	850-829
12	CGCCGTACATTCACGGTGTC	Antisense	504-484

Library screening with 813B4

A directionally cloned oligo DT primed adult rat testis cDNA library had been prepared previously in Lambda Zap by Dr. Philippa Saunders according to manufacturer's instructions (Stratagene). The library was screened with a ^{32}P-dCTP labelled 813B4 cDNA insert according to the following methods.

The library was plated out according to the methods described in chapter 6 and incubated overnight at 37°C. The plates were chilled for 2 h at 4°C to prevent the NZY top agar from sticking to the nitrocellulose membrane. The DNA produced by the

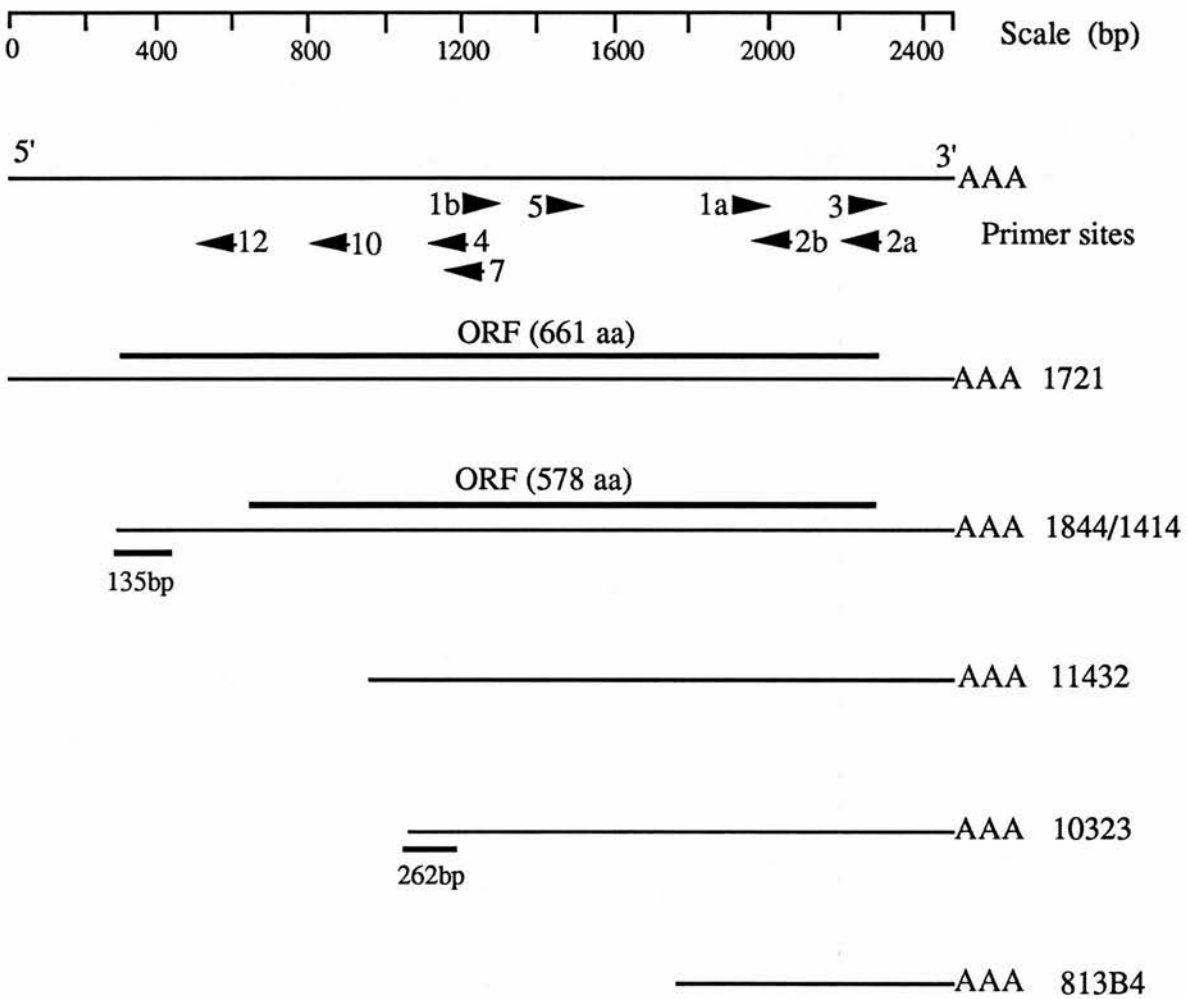

Figure 2. Schematic diagram showing the alignment of the individual cDNAs to each other and to the complete sequence. In addition the regions of the sequence to which internal primers were synthesised and their orientation in relation to the cDNAs is illustrated. The region of 10323 which was amplified by PCR to use for library screening is underlined, as is the region of clones 1414 and 1844 which do not show homology to 1721. The scale at the top of the diagram represents the lengths of the cDNAs in base pairs.

plaques was transferred to nylon membranes (Hybond™-C extra; Amersham) by the following procedure. The membranes were numbered and placed on individual agar plates for 2 min. A wide bore needle was used to mark the orientation of the filter in relation to the plate. A second membrane was placed on the agar plates and allowed to transfer for 4 min. The transferred DNA was denatured by placing face up on 3MM paper soaked in a solution 1.5M NaCl and 0.5M NaOH for 5 min. The membrane was neutralised by placing on 3MM paper soaked in a solution of 1.5M NaCl and 0.5M Tris-HCl (pH8.0) for 5 min and then rinsed for 30 sec in 2x SSC. Membranes were blotted briefly and baked for 2 h at 80°C. The agar plates were stored at 4°C. Positive plaques were detected by the hybridisation of radiolabelled 813B4 cDNA insert to the membranes and visualised by exposure to film using methods described in section 3.15. The film exposures from the duplicate membranes were lined up in the correct orientation to determine which plaques were duplicated. The positive plaques were then isolated from the agar plates by coring the top agarose using the large end of a pipette tip. Each piece of agar was placed into 1ml of SM buffer. These primary positives were then subjected to a second round of screening using the same methods as described above and individual recombinant phage were placed into 0.5ml SM buffer. Recombinant cDNAs were rescued into Bluescript plasmid by *in vivo* excision (described in chapter 6). This library screening identified several clones, the insert size of these cDNAs was determined by PCR as described in chapter 6 and section 3.15. The two cDNAs with the largest inserts were selected for propagation and sequencing; clone 10323 was estimated to be approximately 1.4kb and clone 11432 to be 1.5kb.

Sequencing of GCS1 clones 10323 and 11432

These two cDNAs were initially sequenced using primers made to the T3, SK and T7 polymerase regions of the Bluescript plasmid. Following analysis of sequence data, additional internal oligonucleotide primers were synthesised (primers 1b, 2b, 3, 4 and 5). The sequences and the direction of these primers is shown in Table 1, and their position in relation to the complete sequence of 1721 and the cDNAs, 10323 and 11432, is shown in Figure 2. The sequence generated at the 3' ends of clones 10323 and 11432 using a T7 primer confirmed that these cDNAs possessed poly A tails. Comparison of the sequence gained for 10323 and 11432 with 813B4 showed that they aligned to the complete length of 813B4 with 100% homology. Combining the overlapping sequence data generated from the cDNAs 813B4, 10323 and 11432 generated 1500 bases of sequence data, which was still too short to code for the larger transcript observed on Northern blots (approximately 3-3.5kb).

Library screening with 5' portion of DNA amplified from 10323

In order to clone further cDNAs which would encode the remaining 5' sequence, a piece of the 5' sequence of 10323, approximately 260bp, was amplified by PCR using SK primer and internal oligonucleotide primer 7, see Table 1 and Figure 2. This PCR product was labelled with ^{32}P-dCTP by random priming (section 3.15) and used to screen both oligo DT primed and random primed directionally cloned adult rat testis cDNA libraries, which had been previously prepared by Dr. Philippa Saunders. The aim of this strategy was to identify full length cDNAs. Screening was performed as described above. The oligo DT library had a titre of 1.7 x10^6 pfu/ml and the random primed library a titre of 1.9 x10^6 pfu/ml. After the first round of screening, the areas of agar containing a positive plaque(s) were isolated into SM buffer. These recombinant phage pools were then screened using PCR to identify which pools contained cDNA inserts with homology to 813B4 and a significant stretch of cDNA 5' to the portion already cloned. Two PCR reactions were performed in parallel on 10µl each phage pool (for details of PCR method see section 3.15). The first PCR reaction used primers 1a and 2a to amplify 314 bases from the 3' end of the sequence. This acted as an internal control to ensure that the pool was a true positive and demonstrated the presence of the 3' end of the sequence. Forty cycles of amplification were performed with an annealing temperature of 55°C and a 1 min extension time. The second PCR utilised an SK primer (recognising the Lambda vector) in conjunction with an internal antisense oligonucleotide, primer 7 present close to the 5' end of clones 11432 and 10323 (see Table 1 and Figure 2). This amplified the 5' region of the cDNAs and allowed estimation of the size of the upstream sequence. The PCR was performed as above but the extension time was lengthened to 2 min. The PCR reactions were run on 1.5% agarose gels with DNA size markers (section 3.13). Those phage pools found to contain over 1.5kb of upstream sequence were then subjected to a second round of screening to ensure the isolation of a single recombinant phage population from each pool. The resulting recombinant plasmids were rescued using *in vivo* excision (for methods see chapter 6).

Sequencing of full length cDNAs

The new longer length cDNAs (1414, 1721 and 1844) were sequenced as described in previous sections using T7, SK and T3 primers together with the internal oligonucleotide primers 4, 7, 10 and 12 (see Table 1 and Fig. 2). PCR indicated that cDNAs 1414 and 1844 were found to be a similar size, 2.2kb, and 1721 slightly bigger, 2.4kb.

7.2.2. Sequence analysis

Sequence analysis and comparisons were carried out using Genejockey software (Biosoft). Searches for homology to sequences lodged in Genbank were achieved by

accessing the MRC human genome mapping project (HGMP) sequence analysis facility and making use of several search packages. Sequence homology of both nucleotide and amino acid sequences were assessed using Blast (Altschul *et al.*, 1990), which allows comparison of the sequence of interest with sequences present in several databases including Genbank, EMBL and SwissProt. The presence of functional sites within the translated open reading frame were identified using Prosite (Bairoch & Bucher, 1994), which was accessed via the Genejockey software. The amino acid sequence was also analysed to determine if it contained a signal peptide sequence which is indicative of secretory proteins. The Genejockey software was used to estimate the molecular weight and pI of the peptide to assess whether a protein with these characteristics was present on 2-D Western blots against STCM.

7.2.3. RNA isolation

RNA was extracted from tissues from both control adult male and female rats and also from the testes of immature and pubertal rats according to the methods described in section 3.14. In addition, RNA was also isolated from the testes of adult mice and from an adult marmoset. RNA was extracted from the testes of adult rats which had been previously treated with either EDS or EDS supplemented with testosterone esters and also from rats treated with MAA (see section 3.2 for details of the different treatments).

7.2.4. Northern blot hybridisation

Either 15 or 20μg of total RNA was separated on 1.5% denaturing agarose gels and blotted as described in sections 3.14 and 3.16. The 813B4 cDNA insert was labelled with ^{32}P-dCTP in a random primed reaction (see section 3.15). Northern hybridisation to RNA was performed overnight at 65°C in 15% formamide buffer followed by two post-hybridisation washes at 65°C (detailed in section 3.16). Northern blots were reprobed with an oligonucleotide to 18S ribosomal RNA (sections 3.15 and 3.16).

7.2.5. Non-radioactive *In situ* hybridisation

813B4 plasmid DNA was linearised with Xba I and labelled with Dig-UTP to give a riboprobe antisense to 813B4 mRNA; transcription was catalysed using T7 RNA polymerase (see section 3.17). For synthesis of the sense riboprobe the plasmid DNA was linearised with Kpn I and transcription was catalysed using T3 polymerase. *In situ* hybridisation was performed as described in section 3.17. Testis sections from control and MAA-treated adult rats were used to investigate the cellular pattern of 813B4 mRNA expression and its stage specificity during spermatogenesis. Testis sections from immature and pubertal rats were used to assess the developmental expression of 813B4 mRNA. Localisation of message expression was also attempted on rat epididymal

sections as well as on the mouse and marmoset testis.

7.2.6. Southern blot hybridisation

To assess whether the gene for 813B4 was conserved amongst several species a genomic DNA zoo blot was purchased from Clontech. This membrane contained Eco R1 digested DNAs from the following species: human, rhesus monkey, rat, mouse dog, cow, rabbit, chicken and yeast. Hybridisation and post-hybridisation washes were performed according to the manufacturer's instruction as follows. The Zoo blot was prehybridised for 4 h at 65°C in hybridisation solution which consisted of 5x SSPE (see appendix, 10x Denhardt's solution (50x stock; Sigma), 100µg/ml freshly denatured, sheared salmon sperm DNA (Sigma) and 2% w/v SDS. 813B4 cDNA insert was labelled with ^{32}P-dCTP as detailed in section 3.15. Hybridisation was performed at 65°C for 24 h. Following hybridisation the membrane was rinsed twice with a solution of 2x SSC and 0.05% w/v SDS. Then the membrane was washed twice in 0.1x SSC and 0.1% w/v SDS for 40 min at 60°C. After washing, the membrane was wrapped in clingfilm and exposed to XAR-5 film (Kodak).

7.3. Results

7.3.1. Isolation and sequencing of 813B4 and related cDNAs encoding GCS1

The cDNA clones 813B4, 10323 and 11432, which encoded the partial cDNA of GCS1, were sequenced using T3, T7 and SK primers and a selection of internal oligonucleotide primers complementary to their sequence. The sizes of the three cDNAs are shown in Table 2 together with their relationship to the complete sequence of GCS1 (clone 1721). The sequences obtained for each cDNA were found to possess a poly A tail, indicating that these cDNAs encoded the 3' end of the mature mRNA. The three sequences showed 100% homology to each other, and when assembled this encoded a total of 1529 nucleotides. Consideration of results obtained from Northern blots lead to the conclusion that this was insufficient to encode the larger of the two transcripts (see Fig. 8). Further screening was therefore undertaken to isolate longer cDNAs including nucleotides 5' to those already obtained.

Following isolation of these three partial cDNAs, two testis cDNA libraries, one random primed and the other oligo DT primed, were screened with a small portion of cDNA amplified from the 5' end of clone 10323. The positive phage pools isolated after primary screening of the two libraries, 20 in total, were then analysed using PCR to identify which pools contained cDNAs with homology to GCS1 but with additional sequence at the 5' end to that already identified. Two PCR reactions were performed on

each phage pool. Pools 1-7 were isolated from the random primed testis cDNA library and pools 8-20 from the oligo DT primed library. The first PCR reaction used SK (sense) and oligonucleotide 7 (antisense) primers to amplify stretches of cDNAs in the region 5' to those of clone 10323; the results of these reactions are shown in lanes 1a - 20a in Figure 3. The second PCR reaction used oligonucleotide primers 1a (sense) and 2a (antisense) homologous to sequences present in cDNAs 813B4, 10323 and 11432; these results are shown in lanes 1b - 20b in Figure 3. These reactions resulted in amplification of a DNA fragment of approximately 1000bp from pools 17 and 18 using SK plus primer 7 (Fig. 3, lanes 17a and 18a). The second PCR confirmed that cDNA present in pool 17 contained DNA homologous to that in clone 813 B4 (see Fig. 3, lane 17b).

Phage pools 14, 17 and 18, all isolated from the oligo DT primed library, were subjected to a second round of screening to allow isolation of pure populations of recombinant phage, hereafter referred to as 1414, 1721 and 1844. Plasmid DNA was isolated and propagated from these phage and sequenced. All three cDNAs analysed were found to possess poly A tails and at their 3' ends. The cDNA sequences obtained for clones 1414 and 1844 matched exactly and were also homologous to that of clone 1721 with the exception of 135bp at the extreme 5' ends of cDNAs 1414 and 1844 (Fig. 4). Clone 1721 was found to possess an extra 234 nucleotides in comparison to clones 1414 and 1844, and this was followed by the 135bp of sequence where clone 1721 was not homologous to clones 1414 and 1844 (Fig. 4). Thereafter, the sequences showed 100% homology to each other and to the other partial cDNAs. The complete sequence was compiled for both 1721 and 1414/1844 using sequence data generated from all the cDNAs. A summary of all the clones comprising GCS1 and their regions of alignment is shown in Table 2 and Figure 2.

Table 2. Summary of the sequence lengths of the various cDNAs comprising the full length of GCS1. Sequence alignment is given relative to the sequence of clone 1721.

cDNA	**Sequence length (bp)**	**Sequence alignment**
813B4	754	1698-2452
10323	1433	1019-2452
11432	1529	923-2452
1414	2202	368-2452
1844	2202	368-2452
1721	2452	0-2452

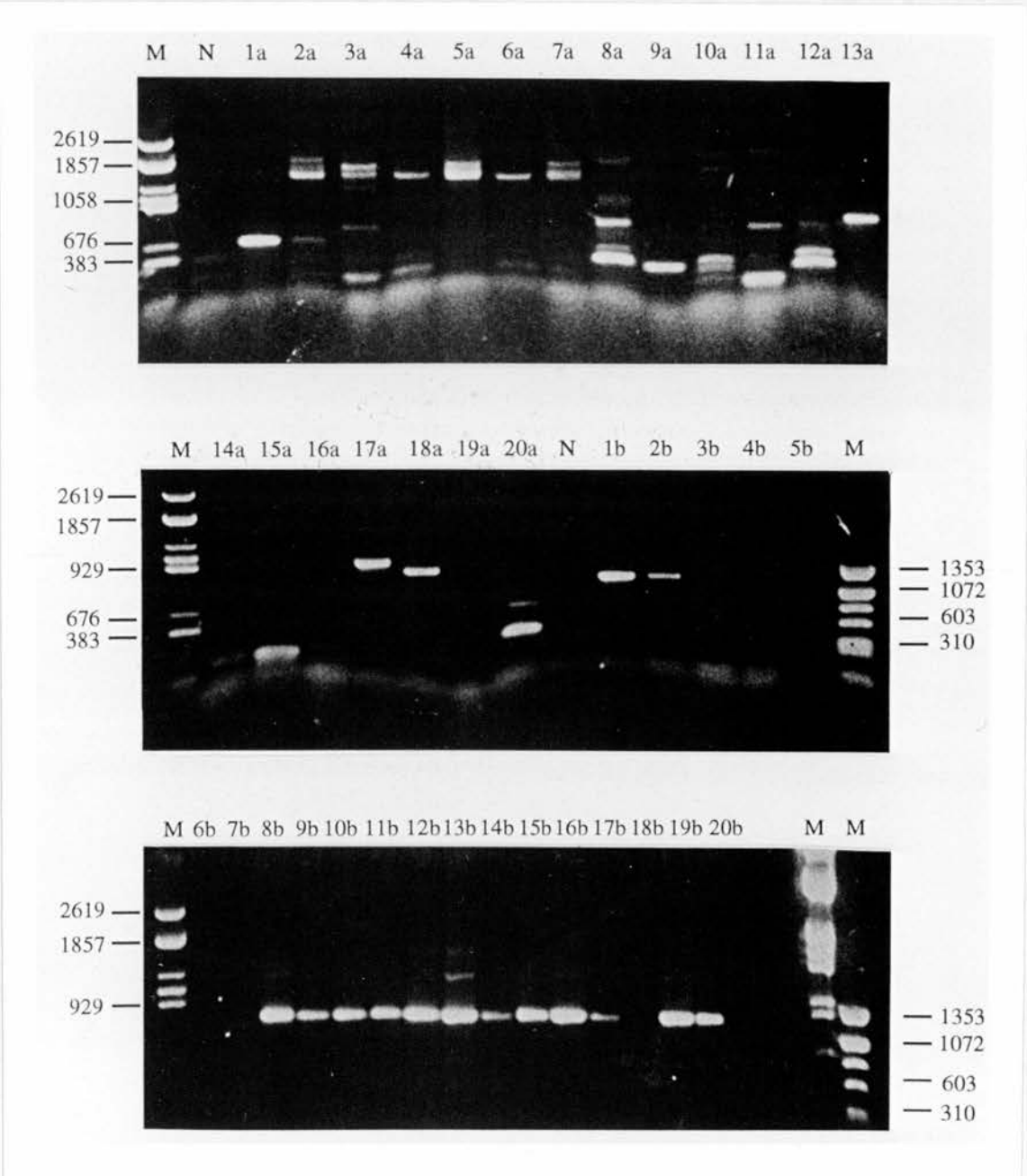

Figure 3. Identification of cDNAs containing long stretches of 5' sequence by PCR. Lanes labelled M are loaded with DNA size markers. Markers shown are pBR322 digested with Alw 44I and Mra I on the left (MBI) and phi X digested with Hae III on the right (Promega). Lanes labelled N were loaded with 'no DNA' controls from the PR reactions. Reactions 1-7 used cDNA pools isolated from the random primed library and 8-20 cDNA pools from the oligo DT primed library. Lanes 1a-20a were loaded with DNA from the PCR reactions using SK and 7 primers. The aim of this was to amplify the 5' sequence of the cDNA and allow estimation of its size. Lanes 1b-20b were loaded with DNA from the PCR reactions using primers 1 and 2. The aim of this was to amplify part of the known sequence at the 3' end to check that this was present. The numbers correspond to the cDNA pool used in the reaction.

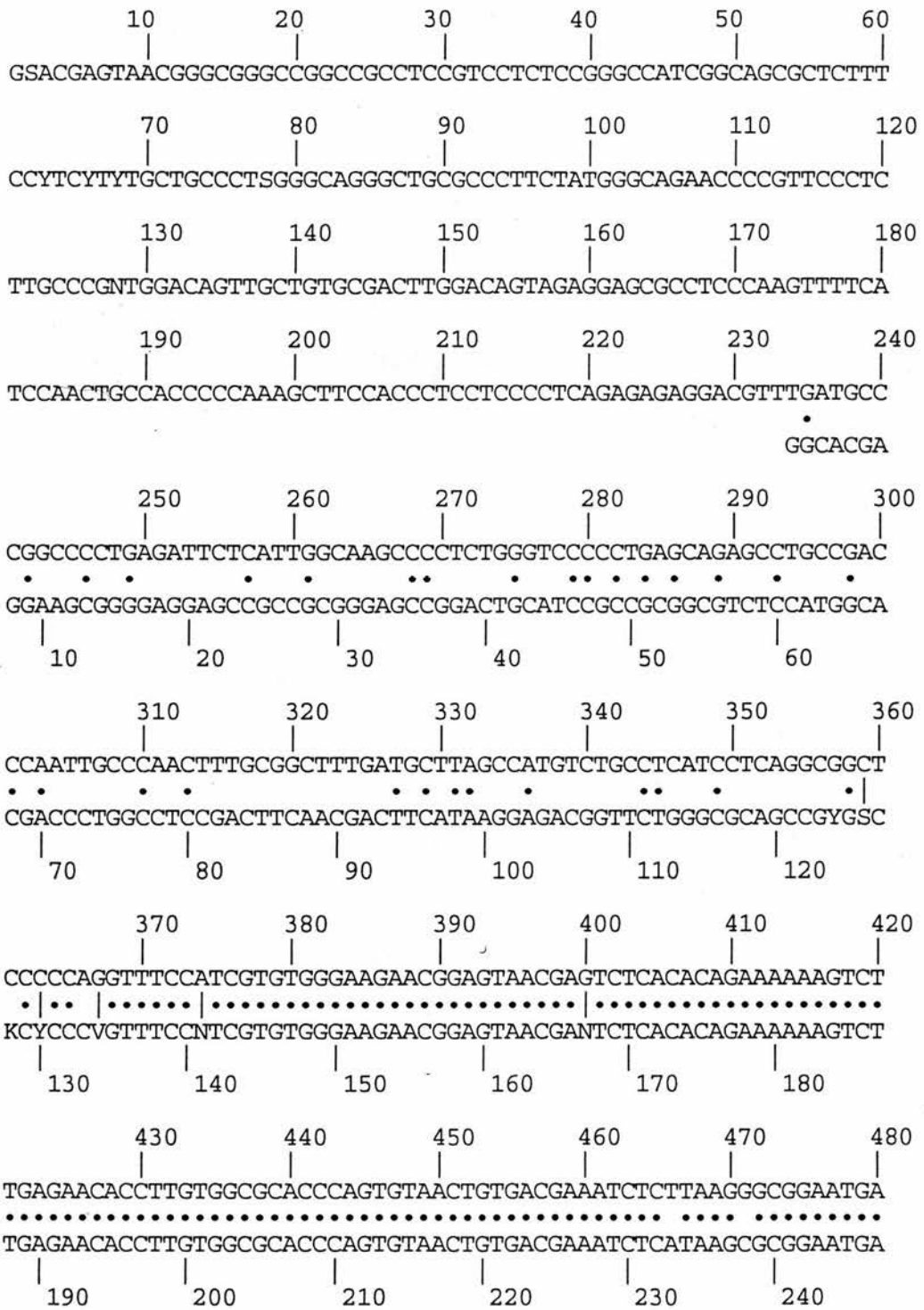

Figure 4. Alignment of the 5' sequence obtained from the cDNA 1721 (top) in comparison to that from the cDNAs 1414 and 1844 (bottom). 1721 possesses an extra 234 nucleotides of sequence and the first 135 bases of the 1414/1844 sequence do not show good homology to 1721. However, the sequences show 100% homology after this stretch of sequence.

7.3.2. Sequence analysis

Putative open reading frames (ORF) for GCS1 were generated using the Genejockey programme. A single long ORF aligned with the deduced cDNA sequence for clones 1414 and 1844 in the sense orientation starting at an ATG at nucleotide 353, a TGA stop codon at nucleotide 2087 and a putative polyadenylation signal at nucleotide 2172 (Fig. 6). The cDNA encodes a predicted protein of 578 amino acids (Fig. 6). The cDNA sequence for 1721 also possessed a single long ORF with an ATG start codon at 336 and a TGA stop codon at 2323, encoding a protein of 661 amino acids. Both the nucleotide and deduced peptide sequences for 1721 and 1414/1844 were compared to Genbank, EMBL and SwissProt databases. These sequences were found to have homology to four expressed sequence tags (EST). Three of the ESTs were reported as being isolated from an adult mouse testis library, MMTEST 29 (accession no.: Z31099), MMTEST 128 (accession no.: Z31029) and MMTEST 384 (accession no.: Z31122) (Yuan *et al.*, 1995). The fourth, EST 111685 (accession no.: H34612), was isolated from rat adrenal chromaffin PC-12 cells (Lee *et al.*, 1995). The sequence of GCS1 showed 97% homology to MMTEST 29 over 129bp, 95% homology to MMTEST 128 over 168bp, 96% homology to MMTEST 384 over 208bp and 99% homology to EST 111685 over 383bp (Fig. 5).

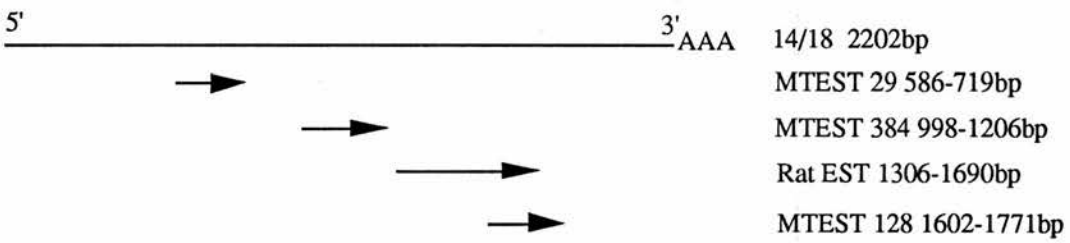

Figure 5. Schematic diagram showing the regions where the 4 ESTs align to the sequence of clones 1414 and 1844 .

The deduced protein sequence of GCS1 was analysed using the Prosite programme which searches for potential functional motifs within the sequence (Bairoch & Bucher, 1994). This analysis revealed that the protein contained two potential leucine zipper domains. The consensus pattern for this motif being 4 leucine residues each separated by 6 amino acids, L-x(6)-L-x(6)-L-x(6)-L (Landschulz *et al.*, 1988). The leucine zipper regions of the peptide sequence are shown in Figure 6. Prosite also showed the presence of one potential N-glycosylation motif (amino acid 165) and nine protein kinase C phosphorylation sites (amino acids 135, 164, 167, 204, 223, 265, 322, 364 and 456) (Fig. 6), but further investigation would be needed to confirm whether this protein undergoes these forms of modification. Examination of the deduced amino acid sequence

Figure 6. The complete DNA and amino acid sequence of cDNA GCS1 as compiled from clones 1414, 1844, 11432, 10323 and 813B4. The two leucine zipper domains are highlighted in colour, the positions of the leucines in red and the intervening amino acid repeats in blue. A putative glycosylation site and a putative poly (A) addition signal are underlined.

```
   1                                                                GGCACGAGGAAGCGGGG
  18 AGGAGCCGCCGCGGGAGCCGGACTGCATCCGCCGCGGCGTCTCCATGGCACGACCCTGGCCTCCGAC
  85 TTCAACGACTTCATAAGGAGACGGTTCTGGGCGCAGCCGTGCCGCTCTTGGTTTCCATCGTGTGGGA
 152 AGAACGGAGTAACGAGTCTCACACAGAAAAAAGTCTTGAGAACACCTTGTGGCGCACCCAGTGTAAC
 219 TGTGACGAAATCTCATAAGCGCGGAATGAAAGGGGACACCGTGAATGTACGGCGGAGTGTCCGGGTG
 286 AAAACCAAGAATCCACCTCATTGCCTGGAGATCACACCACCATCTTCAGAAAAAGCTGGTCTCGGTG
     Met Arg Leu Ser Asp Leu Ser Thr Glu Glu Glu Asp Ser Gly His Cys Lys     17
 353 ATG CGA TTG AGT GAC CTT TCT ACA GAA GAA GAA GAT TCT GGT CAC TGT AAG
     Met Asn Arg Tyr Asp Lys Lys Ile Asp Ser Leu Met Asn Ala Val Gly Cys     34
 404 ATG AAC CGT TAC GAT AAG AAG ATT GAC AGT CTA ATG AAC GCG GTC GGT TGT
     Leu Lys Ser Glu Val Lys Met Gln Lys Gly Glu Arg Gln Met Ala Lys Arg     51
 455 CTC AAA TCT GAG GTC AAG ATG CAG AAA GGT GAA CGC CAA ATG GCC AAA AGG
     Phe Leu Glu Glu Arg Lys Glu Glu Leu Glu Glu Val Ala His Glu Leu Ala     68
 506 TTC CTG GAG GAG AGG AAG GAA GAA CTG GAG GAG GTA GCA CAT GAG CTT GCA
     Glu Thr Glu His Glu Asn Thr Val Leu Arg His Asn Ile Glu Arg Ile Lys     85
 557 GAG ACG GAG CAT GAG AAC ACA GTG CTC AGA CAC AAC ATC GAG CGC ATC AAG
     Glu Glu Lys Asp Phe Thr Met Leu Gln Lys Lys His Leu Gln Gln Glu Lys    102
 608 GAG GAG AAG GAC TTC ACC ATG CTT CAA AAG AAA CAC CTC CAG CAG GAG AAG
     Glu Cys Leu Met Ser Lys Leu Val Glu Ala Glu Met Asp Gly Ala Ala Ala    119
 659 GAA TGC CTC ATG TCC AAA CTG GTG GAG GCT GAA ATG GAT GGG GCG GCT GCT
     Ala Lys Gln Val Met Ala Leu Lys Asp Thr Ile Gly Lys Leu Lys Thr Glu    136
 710 GCC AAA CAA GTC ATG GCC TTG AAG GAT ACC ATC GGG AAG CTG AAA ACC GAG
     Lys Gln Met Thr Cys Thr Asp Ile Asn Thr Leu Thr Arg Gln Lys Glu Leu    153
 761 AAA CAG ATG ACT TGC ACC GAC ATC AAC ACC TTA ACG AGG CAG AAG GAA CTT
     Leu Leu Gln Lys Leu Ser Thr Phe Glu Glu Thr Asn Arg Thr Leu Arg Asp    170
 812 CTC CTG CAG AAG CTG AGC ACC TTT GAA GAG ACC AAC CGC ACC CTC CGA GAT
     Leu Leu Arg Glu Gln His Cys Lys Glu Leu Cys Leu Lys Val Pro Glu Cys    187
 863 CTG TTG AGG GAG CAG CAC TGC AAA GAG CTG TGC CTG AAA GTT CCT GAA TGT
     Ala Arg Gln His Arg Pro Gly Arg Glu Arg Gln Glu Asp Cys Gln Asp Ser    204
 914 GCG AGG CAG CAC AGG CCT GGG AGG GAG AGG CAG GAG GAT TGT CAG GAT TCC
     Glu Arg Leu Met Glu Gln Gln Gly Ala Leu Leu Lys Arg Leu Ala Glu Ala    221
 965 GAG AGA CTA ATG GAG CAA CAA GGT GCA TTA CTG AAA CGT CTG GCA GAG GCC
     Asp Ser Glu Lys Ala Arg Leu Leu Leu Leu Leu Gln Asp Lys Asp Lys Glu    238
1016 GAC TCA GAG AAA GCG CGC CTG CTG TTA CTA CTG CAA GAC AAG GAC AAG GAA
     Val Glu Glu Leu Leu Gln Glu Ile Gln Cys Glu Lys Ala Gln Ala Lys Thr    255
1067 GTG GAA GAA CTC CTC CAG GAG ATA CAA TGT GAG AAG GCT CAA GCA AAG ACA
     Ala Ser Glu Leu Ser Lys Ser Met Glu Ser Met Arg Gly His Leu Gln Ala    272
1118 GCG TCT GAG CTC TCC AAG TCC ATG GAG TCC ATG CGG GGG CAT TTG CAG GCA
     Gln Leu Arg Cys Lys Glu Ala Glu Asn Ser Arg Leu Cys Met Gln Ile Lys    289
1169 CAG CTT CGC TGC AAA GAG GCT GAG AAC AGC CGC CTG TGC ATG CAG ATC AAG
     Asn Leu Glu Arg Ser Gly Asn Gln His Lys Ala Glu Val Glu Ala Ile Met    306
1220 AAT TTA GAG CGC AGT GGG AAC CAG CAC AAG GCG GAA GTA GAG GCC ATC ATG
     Glu Gln Leu Lys Glu Leu Lys Gln Lys Gly Asp Arg Asp Lys Glu Thr Leu    323
1271 GAG CAG CTA AAG GAA CTG AAG CAA AAG GGA GAC CGA GAC AAA GAG ACC CTG
     Lys Lys Ala Ile Arg Ala Gln Lys Glu Arg Ala Glu Lys Ser Glu Glu Tyr    340
1322 AAG AAG GCC ATC CGA GCC CAG AAG GAG CGA GCT GAG AAG AGT GAG GAG TAT
     Ala Glu Gln Leu His Val Gln Leu Ala Asp Lys Asp Leu Tyr Val Ala Glu    357
1373 GCC GAG CAG CTA CAT GTG CAG CTG GCC GAC AAG GAC CTT TAT GTT GCT GAA
     Ala Leu Ser Thr Leu Glu Ser Trp Arg Ser Arg Tyr Asn Gln Val Val Lys    374
1424 GCT TTA TCT ACT CTG GAG TCA TGG AGG AGC CGT TAC AAC CAA GTT GTG AAA
     Asp Lys Gly Asp Leu Glu Leu Glu Ile Ile Val Leu Asn Asp Arg Val Thr    391
1475 GAC AAA GGA GAC CTT GAG TTG GAG ATC ATT GTC CTA AAT GAC CGG GTG ACA
     Asp Leu Val Asn Gln Gln Gln Ser Leu Glu Glu Lys Met Arg Glu Asp Arg    408
1526 GAT CTT GTA AAC CAA CAA CAG AGC TTG GAG GAG AAG ATG CGG GAA GAC CGG
     Asp Ser Leu Val Glu Arg Leu His Arg Gln Thr Ala Glu Tyr Ser Ala Phe    425
1577 GAC AGC TTG GTA GAG AGA CTG CAC CGG CAA ACT GCT GAG TAT TCT GCC TTC
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     Lys Leu Glu Asn Glu Arg Leu Lys Ala Ser Phe Ala Pro Met Glu Asp Lys    442
1628 AAG CTA GAG AAC GAG AGG CTC AAG GCT AGC TTT GCC CCT ATG GAG GAC AAG
     Leu Asn Gln Ala His Leu Glu Val Gln Gln Leu Lys Ala Ser Val Lys Asn    425
1679 CTC AAT CAG GCT CAC TTG GAG GTC CAG CAA CTG AAG GCA TCA GTG AAG AAC
     Tyr Glu Gly Met Ile Asp Asn Tyr Lys Thr Gln Val Met Lys Thr Arg Leu    476
1730 TAC GAG GGG ATG ATC GAC AAC TAC AAG ACC CAG GTG ATG AAA ACA AGA TTG
     Glu Ala Asp Glu Val Ala Ala Gln Leu Glu Arg Cys Asp Lys Glu Asn Lys    493
1781 GAG GCT GAC GAA GTG GCT GCG CAG CTA GAG CGC TGC GAC AAA GAG AAC AAG
     Met Leu Lys Asp Glu Met Asn Lys Glu Ile Glu Ala Ala Arg Arg Gln Phe    510
1832 ATG CTT AAA GAC GAG ATG AAC AAA GAA ATC GAG GCG GCC CGT CGG CAG TTC
     Gln Ser Gln Leu Ala Asp Leu Gln Gln Leu Pro Asp Ile Leu Lys Ile Thr    527
1883 CAG TCA CAG CTG GCT GAC CTA CAA CAG CTG CCT GAC ATC CTC AAG ATC ACG
     Glu Ala Lys Leu Ala Glu Cys Gln Asp Gln Leu Gln Gly Tyr Glu Arg Lys    544
1934 GAG GCC AAG CTG GCT GAG TGT CAA GAC CAG CTG CAG GGC TAC GAG AGG AAG
     Asn Ile Asp Leu Thr Ala Ile Ile Ser Asp Leu His Ser Arg Val Arg Asp    561
1985 AAC ATC GAC CTC ACA GCC ATC ATT TCA GAC CTG CAC AGC CGG GTA AGG GAC
     Trp Gln Lys Gly Ser His Glu Leu Ala Arg Ala Gly Ala Arg Leu Pro Arg    578
2036 TGG CAG AAG GGA TCC CAC GAA CTG GCC CGA GCA GGG GCC CGC TTA CCG AGA
     Stop                                                                  578
2087 TGA GCTGCACGCCCCCCAAGGGAGGACCACTTCCTTTTTCTTGGCTGCCGATTTCTGAAAGGAGTG
2153 AGCTATCATCAGTGCTGTGAAATAAAAGTCTGGTGTGCCATAAWCTAAAAA
```

of GCS1 cDNA revealed that it did not appear to possess a classic signal peptide at its N-terminal.

Other information on the peptide sequence was gained using the Genejockey software. The peptide of 578 amino acids was deduced to have a molecular weight of 67.4 kDa and a potential isoelectric point of 6.06. There are several proteins with these characteristics resolved from STCM by 2-D SDS PAGE which are also recognised by the STCM antiserum (Fig. 7). It is not currently possible to determine which, if any, of these proteins is GCS1 since the values determined for the molecular weight and pI are only theoretical based on the properties of the amino acids which compose the sequence. These values do not however take into account the effect of glycosylation at the potential site present in the predicted GCS1 protein on these parameters.

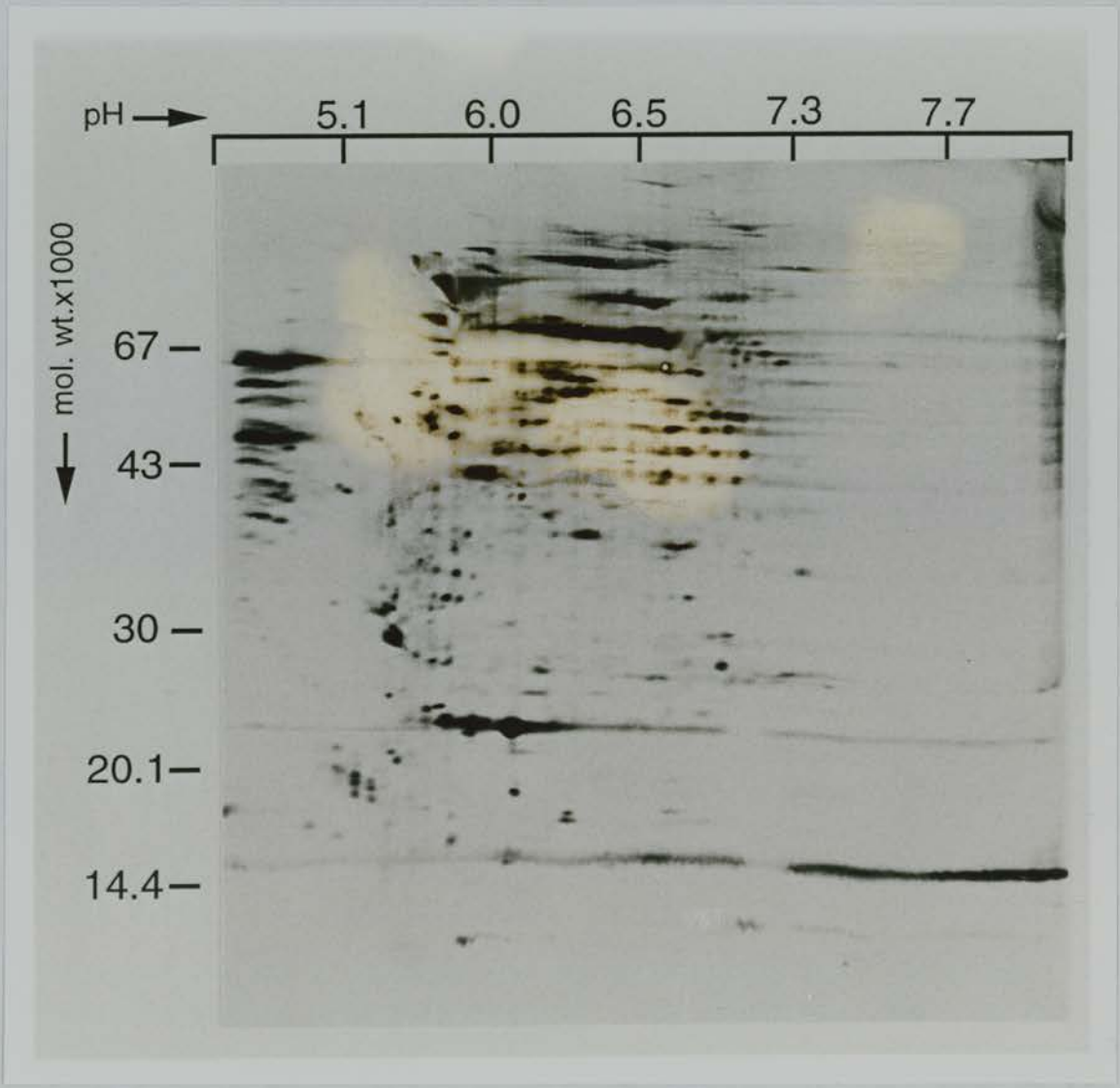


Figure 7. Representative Western blot of STCM (30µg) resolved by 2-D SDS PAGE, blotted onto PVDF membrane and then incubated with antiserum raised against STCM. As this membrane shows there are several proteins recognised by the antiserum which have an approximate MW of 67 kDa and pI of 6.0.

7.3.3. Analysis of the tissue specificity of GCS1 mRNA expression

The tissue specificity of GCS1 mRNA expression was determined using Northern blots which were hybridised to clone 813B4. Expression of GCS1 mRNA was identified to the testis and epididymis but in no other tissues tested from adult male and female rats even after extended exposure to films (Fig. 8). Northern hybridisation was repeated several times using RNA from different animals. These experiments revealed that testicular expression of GCS1 transcripts was highly consistent, however levels of mRNA in the epididymis were more variable. In some adult rat epididymal samples the three mRNA transcripts which hybridised to clone 813B4 could not be detected or were found to be much less abundant in comparison to the RNA sample shown in Fig. 8; the reason for this is not clear.

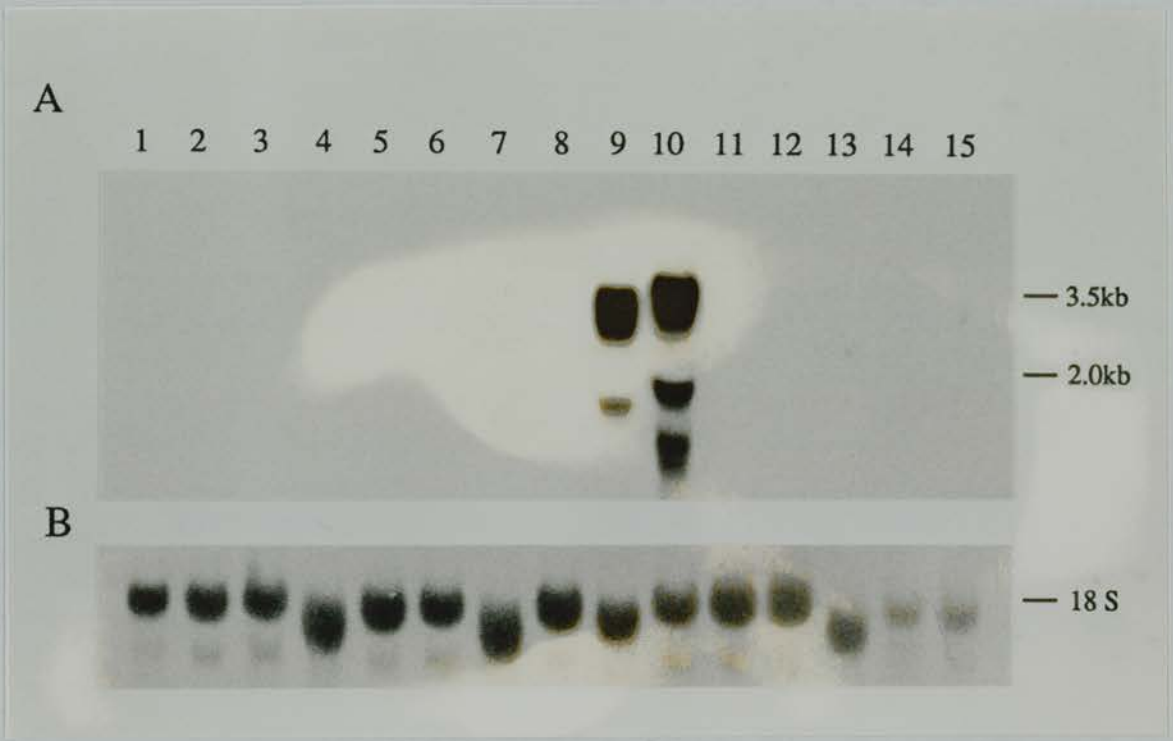


Figure 8. Tissue specificity of GCS1 mRNA expression. The gel was loaded with 20μg RNA as follows: lane 1= liver; lane 2= muscle; lane 3= heart; lane 4= spleen; lane 5= lung; lane 6= brain; lane 7= kidney; lane 8= adrenal; lane 9= testis; lane 10= epididymis; lane 11= prostate; lane 12= seminal vesicle; lane 13= ovary; lane 14= placenta (day 17.5 gestation) and lane 15= uterus (non-pregnant). All tissues were from adult male rats except for the female reproductive tissues. The blot was hybridised with ^{32}P-labelled 814B4 cDNA and exposed to X-Omat LS film for 12 h (panel A). The membrane was stripped and reprobed with an oligonucleotide probe for 18S ribosomal RNA to check that all the lanes contained equivalent amounts of RNA (panel B).

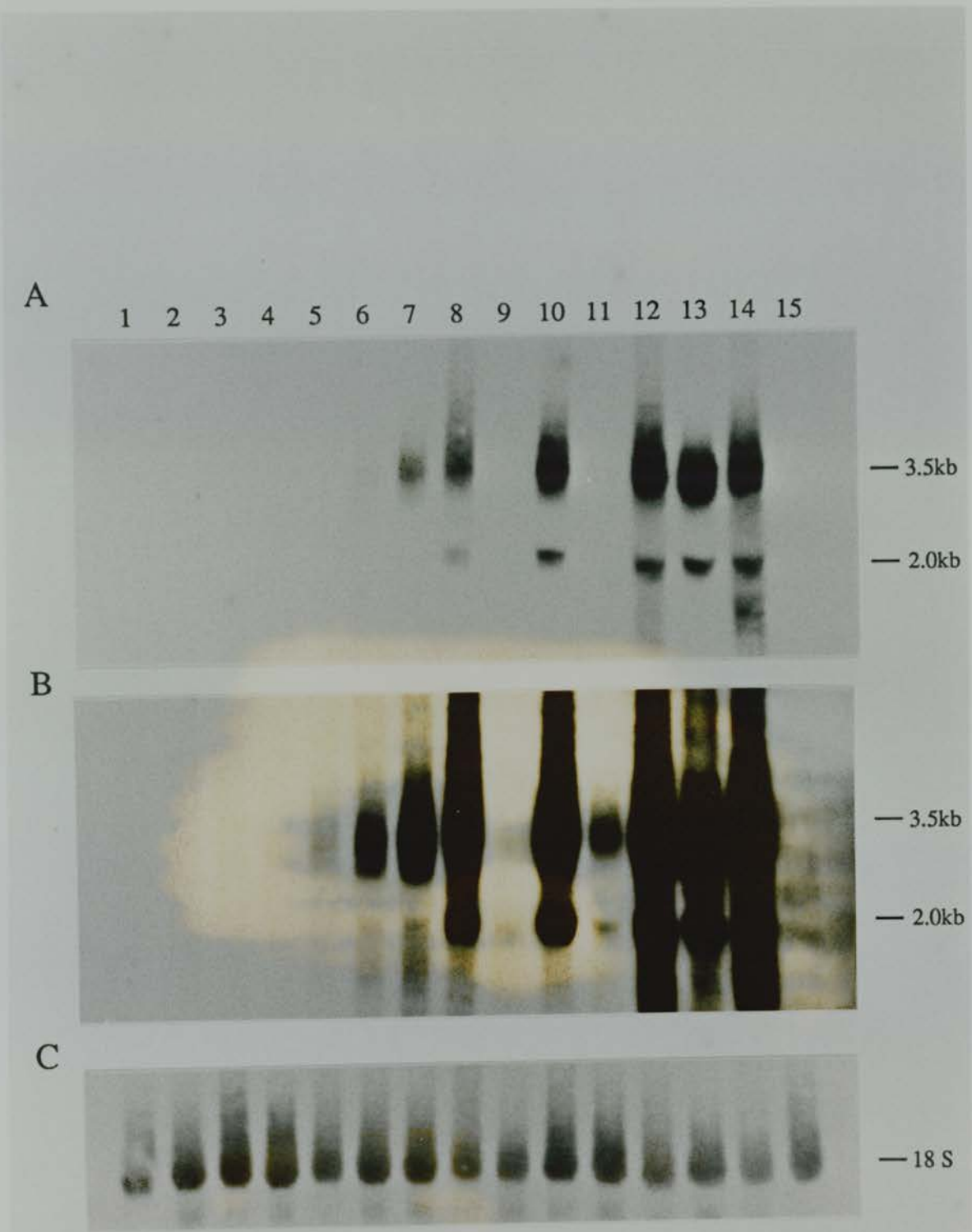
7.3.4. Testicular expression of GCS1 mRNA

The expression of GCS1 mRNA in testicular RNA samples was further investigated using Northern blot hybridisation with clone 813B4 (Fig. 9). A single mRNA transcript of approximately 3-3.5kb was first detected at low abundance in testicular RNA isolated from 20 day old rats (Fig. 9, lane 5). This mRNA transcript increased in abundance in samples from testes of rats aged 23 and 27 days (Fig. 9, lanes 6 and 7). These results would be consistent with expression of GCS1 mRNA by germ cells. The smaller transcript (2kb) was only present in total RNA from adult testis (Fig. 9, lanes 8 and 12). There was no obvious change in the abundance of the two transcripts in testicular RNA isolated from EDS-treated rats in comparison to that from control untreated rats or animals treated with EDS and supplemented with testosterone (Fig. 9, lanes 12 to 14). 813B4 cDNA also hybridised to two mRNA transcripts in RNA isolated from mouse and marmoset testes, which were of identical size to the mRNA transcripts present in adult rat testis (3.5 and 2kb) (Fig. 9, lanes 10 and 9 respectively). The amount of mRNA transcripts detected in total RNA from marmoset testis was low indicating that either the rat cDNA was not completely homologous to the mRNA present in marmoset testis or that GCS1 is less abundant in this species.

7.3.5. Cellular localisation of GCS1 and stage specificity

GCS1 mRNA was localised by *in situ* hybridisation on sections of fixed adult rat testis using a digoxigenin labelled antisense riboprobe generated from clone 813B4. In adult rat testis, expression of GCS1 mRNA was confined to the germ cells as anticipated from the Northern results (Fig. 10); no hybridisation signal was detectable using a sense cRNA (Fig. 10c). The mRNA was first detectable in the cytoplasm of pachytene spermatocytes at stage VII of the spermatogenic cycle and was present in all subsequent germ cells until step 15 spermatids. The strongest signal was observed in the cytoplasm of the round and elongating spermatids. This pattern of localisation was demonstrated repeatedly using fixed tissue from different control animals. The pattern of germ cell expression was confirmed by the cellular localisation of 813B4 cRNA to sections of testis from rats treated with 650mg/kg MAA. This dose results in the depletion of specific germ cells. Results from a rat 14 days after MAA treatment are shown when round spermatids are depleted from the seminiferous tubules (Fig. 11). The amount of positive staining for GCS1 mRNA was reduced markedly compared with control sections. No hybridisation to Sertoli cells or to any of the cells of the interstitium of adult rat testes was detected in the presence or absence of germ cells. The pattern of expression is summarised in Fig. 12.

Figure 9. Northern analysis of mRNA expression of GCS1. Each lane of the gel was loaded with 15μg total RNA as follows: lane 1= liver; lane 2= kidney; lane 3= testis day 7; lane 4= testis day 14; lane 5= testis day 20; lane 6= testis day 23; lane 7= testis day 27; lane 8= adult testis; lane 9= marmoset testis; lane 10= mouse testis; lane 11= epididymis; lane 12= adult testis; lane 13= EDS+6 days testis; lane 14= EDS+T+6 days testis and lane 15= spleen. All tissues were taken from rats unless otherwise stated. The membrane was hybridised with ^{32}P-labelled 813B4 cDNA and exposed to X-Omat LS film for 7 hours (shown in panel A) or for 48 hours (shown in panel B). The longer exposure shows the presence of a transcript in day 20 testis (lane 5) and two transcripts in marmoset testis (lane 9) and mouse testis (lane 10). The membrane was stripped and reprobed with an oligonucleotide probe for 18S ribosomal RNA to check that all the lanes were evenly loaded (panel C).


A
1 2 3 4 5 6 7 8 9 10 11 12 13 14 15
— 3.5kb
— 2.0kb
B
— 3.5kb
— 2.0kb
C
— 18 S

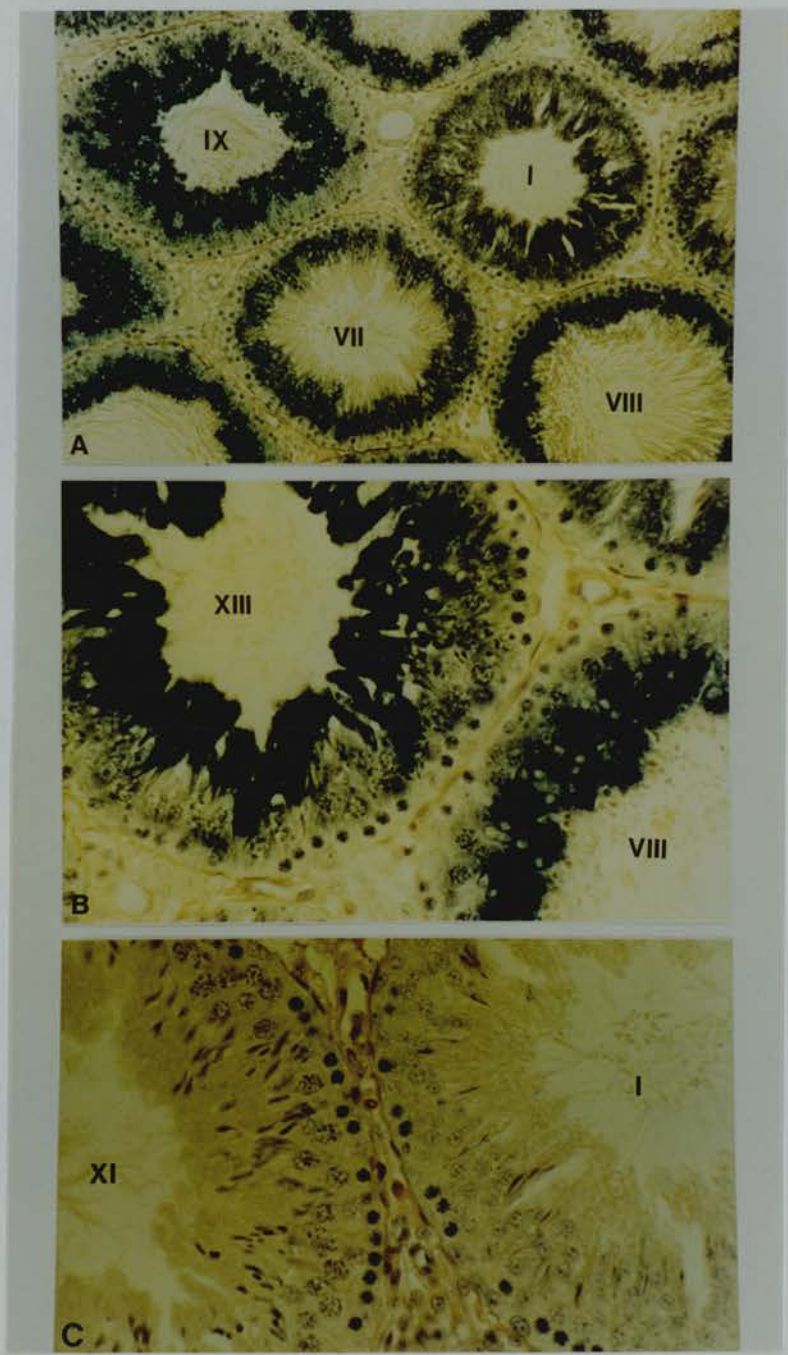


Figure 10. Cellular localisation of GCS1 mRNA expression in control adult rat testis. Fixed rat testis sections were probed with antisense (panels A and B, magnification x99 and x248 respectively) or sense (panel C; magnification x248) Dig-labelled riboprobes generated from cDNA 813B4. The photomicrographs A and B clearly show the expression of mRNA recognised by the probe is confined to the germ cells, with the strongest signal in the cytoplasm of round and elongating spermatids and fainter staining in pachytene spermatocyte cytoplasm. No staining was evident with a sense riboprobe (C). The tissue sections were counterstained with haematoxylin. Roman numerals indicate the stage of the spermatogenic cycle.

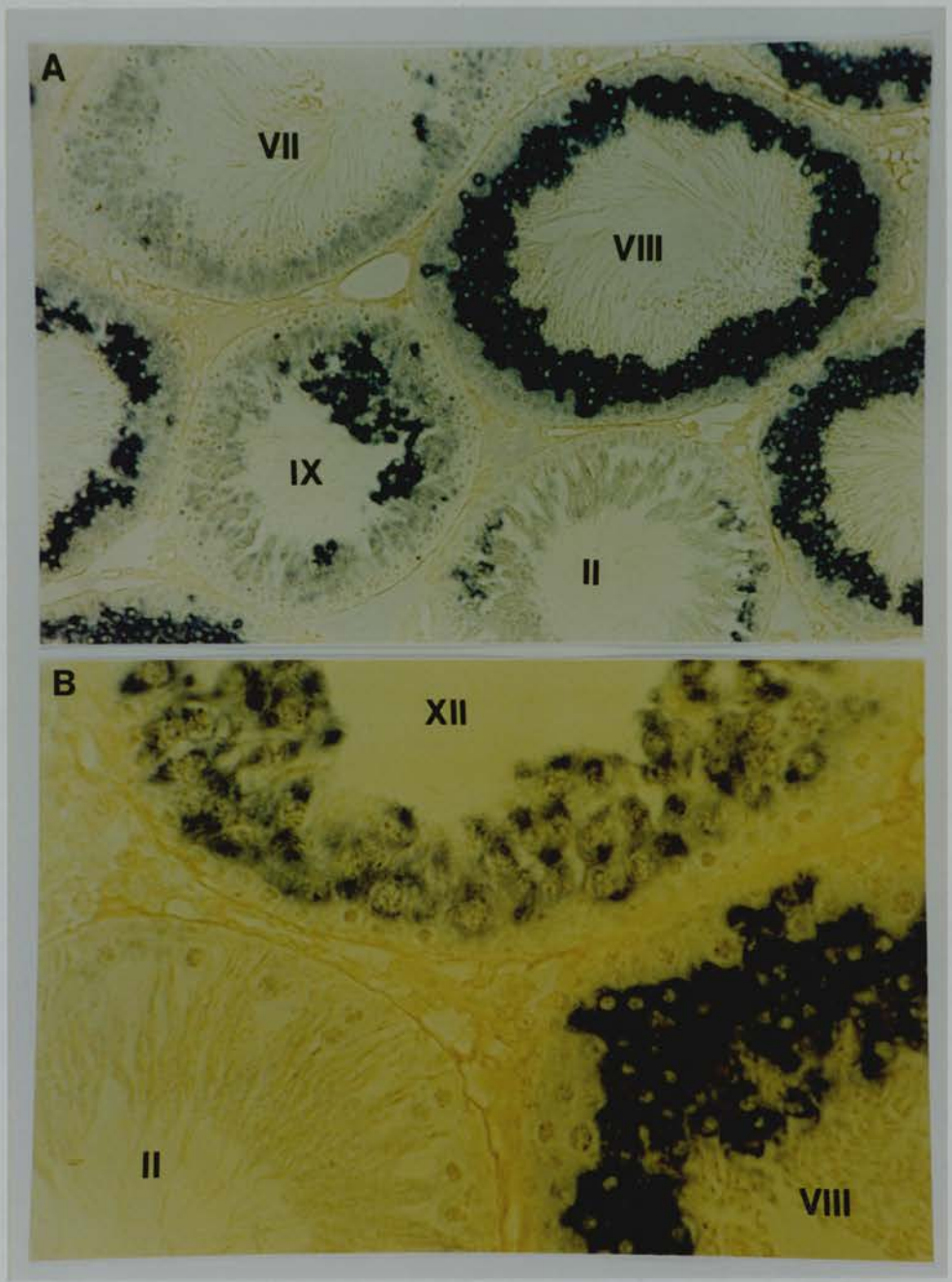


Figure 11. Cellular localisation of GCS1 mRNA expression in the rat testis 14 days after MAA treatment when mainly round spermatids are depleted. The tissue was probed with Dig-labelled 813B4 cRNA. The photomicrographs show the loss of GCS1 mRNA expression from those seminiferous tubules where germ cells are missing (A= x99 magnification and B= x248 magnification). Roman numerals indicate the stage of the cycle.

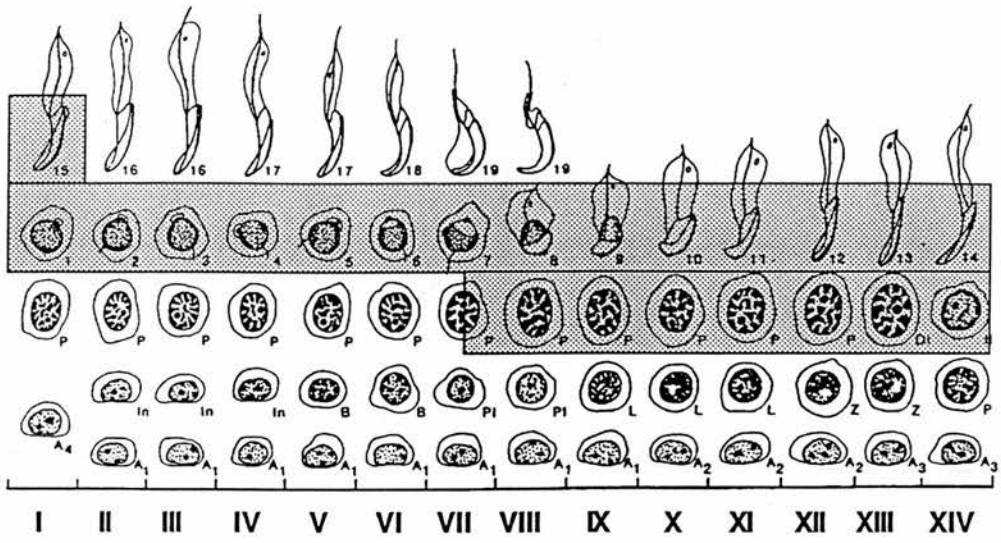


Figure 12. A summary of the stage dependent pattern of GCS1 mRNA expression in adult rat testis detected by *in situ* hybridisation with clone 813B4. The shaded regions indicate which germ cells types express this transcript in their cytoplasm.

The hybridisation of 813B4 cRNA to fixed sections of mouse and marmoset testis was also undertaken. In both species the cRNA hybridised specifically to germ cells within the seminiferous epithelium (not shown). Consistent with results from Northern hybridisations, the positive hybridisation signal on sections of marmoset testis was very low.

A distinct pattern of GCS1 mRNA expression was also observed in some fixed sections of epididymis from adult rats. A strong positive reaction product was apparent in the basal cytoplasm of the epithelial cells lining the caput region of the epididymis, whilst mRNA expression within the epithelia of the corpus and cauda was lower than that in the caput (Fig. 13). The localisation of mRNA expression in the epididymis detectable by *in situ* hybridisation was also found to be extremely variable between samples taken from different adult rats, consistent with results observed with Northern blotting, and expression was not detectable in all epididymal samples.

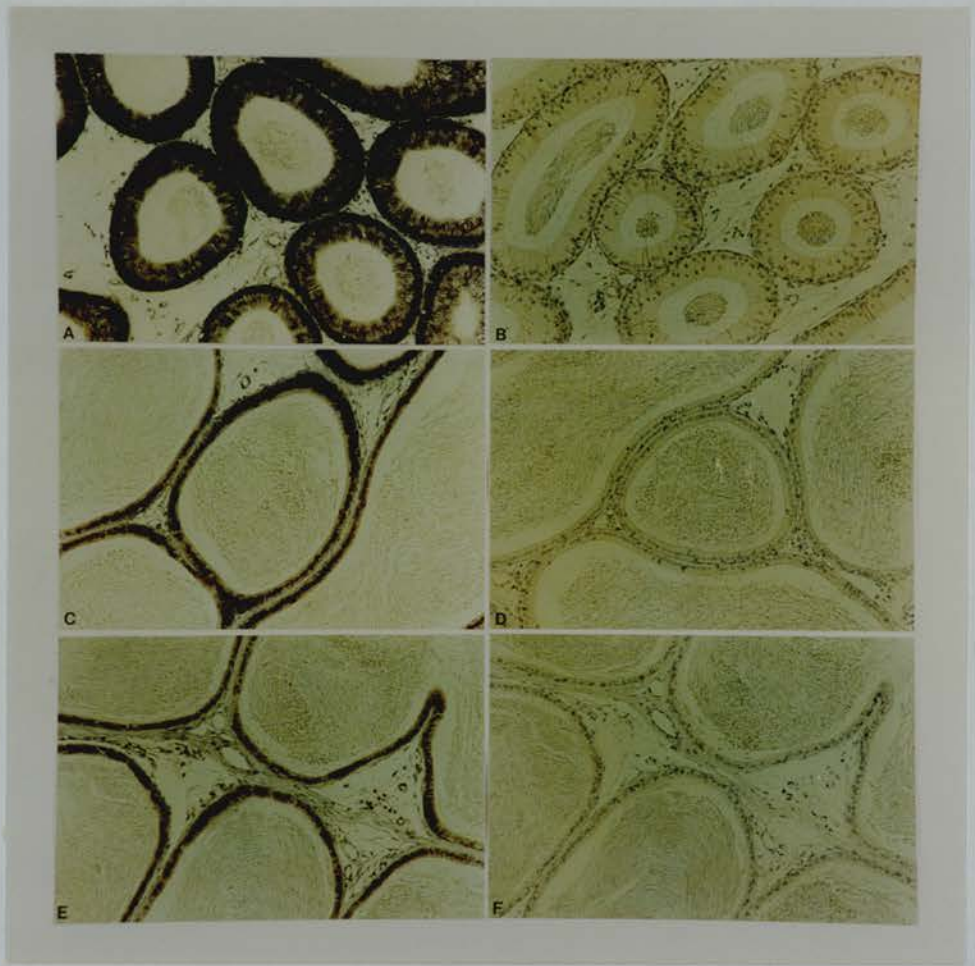


Figure 13. The cellular localisation of GCS1 mRNA expression in the adult rat epididymis by *in situ* hybridisation. Photomicrographs A and B show tissue sections from the caput region of the epididymis, C and D from the corpus and E and F from the cauda. A, C and E show hybridisation of a Dig-labelled riboprobe antisense to 813B4 whereas a sense riboprobe was used on sections B, D and F. All photomicrographs are shown at x99 magnification. Tissue sections were counterstained with haematoxylin.

7.3.6. Onset of expression of GCS1 during development

Expression of GCS1 mRNA in immature and pubertal rat testes was also studied using 813B4 cRNA. No hybridisation was observed on sections of testes taken from 7 day old (not shown) or from 15 day old rats (Fig. 14, panel A). However, by day 21 of postnatal life a strong positive signal was observed in the cytoplasm of the few pachytene spermatocytes now present around the edge of the lumen of the seminiferous tubules (Fig. 14, panel B). Stage specificity of the signal became more apparent by day 28, with different intensities of cellular staining being observed between different seminiferous tubule cross-sections, and not all tubules contained cells expressing GCS1 mRNA (Fig. 14, panel C). In testes from rats aged 35 days, GCS1 mRNA in the round spermatids was more abundant than in the pachytene spermatocytes, and was similar to that seen in the adult rat testis (Fig. 14, panel D).

7.3.7. Conservation of the gene encoding GCS1 amongst several species

Southern hybridisation of 813B4 cDNA to a Zoo blot containing genomic DNA from a range of species resulted in a positive signal on DNA from human, rhesus monkey, rat, mouse, dog and cow but not that from rabbit, chicken or yeast (Fig. 15). Based on the number of bands detected on the Southern blot, there would appear to be only one copy of this gene in the genome of monkeys and humans.

7.4. Discussion

The objective of the experimental work described in this and the previous chapter was to assess whether immunoscreening would prove a useful technique for identifying novel testicular cDNAs encoding secreted proteins, which may have future application for monitoring spermatogenesis. This chapter has described the isolation and sequencing of a full length transcript of the mRNA now called GCS1 for convenience, of which the cDNA 813B4 identified in chapter 6 is a partial clone. Initial studies (see chapter 6) had suggested that 813B4 encoded a novel sequence and Northern blot analysis showed that mRNA expression was limited to the testis and epididymis. The expression of GCS1 mRNA within the testis was analysed by both Northern blotting and *in situ* hybridisation to try and define when and where it was expressed during spermatogenesis. Finally, Southern blotting was used to determine if the gene was expressed in species other than the rat.

The complete sequence of GCS1 was compiled from several overlapping cDNAs. The approximate size of the transcripts was estimated from Northerns to be 3-3.5kb and 2kb. Two sequences were identified which were 100% homologous except for a short stretch at the 5' ends. This was due to differences in the sequence obtained from the 5' region of the clones 1414 and 1844 in comparison to clone 1721. Clone

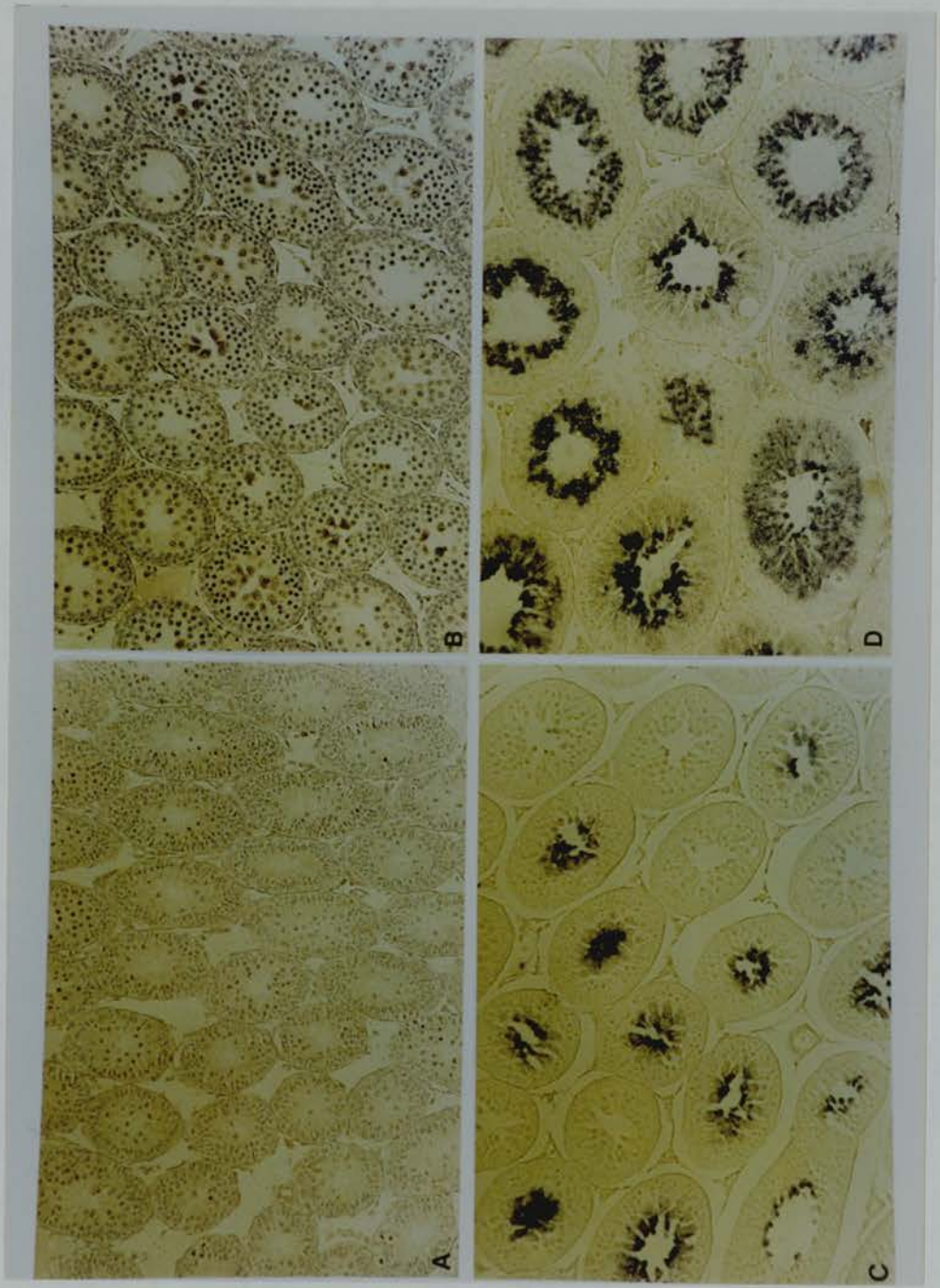


Figure 14. Localisation of GCS1 mRNA to fixed sections of rat testis during testicular maturation. The tissues were probed with Dig-labelled antisense riboprobes. No signal was detected at day 15 (A) but by day 21 (B) strong staining was evident in the cytoplasm of the germ cells surrounding the lumen of the seminiferous tubules. The level of mRNA expression became more abundant with increasing maturation of the testis. Stage specificity of expression of GCS1 by pachytene and round spermatocytes became apparent by day 28 of age (C). Further maturation of the seminiferous epithelium meant that by day 35 the pattern of expression most closely resembled that observed in the adult testis (D). All photomicrographs are shown at x99 magnification.

Figure 15. A Zoo blot showing conservation of the DNA sequence between several species. The membrane supplied by Clontech contained genomic DNA cut with Eco R1 from several species as follows: lane 1= human; lane 2= monkey (rhesus); lane 3= rat (Sprague-Dawley); lane 4= mouse; lane 5= dog; lane 6= cow; lane 7= rabbit; lane 8= chicken and lane 9= yeast. The membrane was hybridised with ^{32}P-labelled 813B4 cDNA and exposed to X-Omat AR film for 5 days.

1721 was found to have a length of 2452 bases with an ATG start site 336 bases into the sequence which generated an ORF of 661 amino acids. In contrast, clones 1414 and 1844 (which showed 100% homology to each other) were 2202 bases in length with an ATG at position 353, and gave an ORF of 578 amino acids. Alignment of all the full length cDNA sequences showed that clone 1712 possessed an extra 234 nucleotides at its 5' end in comparison to clones 1414 and 1844, and that the first 135 nucleotides of 1414 and 1844 sequences were not homologous to the corresponding region of clone 1721. It is possible that these sequence differences have been generated by alternative splicing, such that two different exons of the gene are responsible for coding for the 5' regions of these cDNAs. Another explanation is that these sequences arise from different genes. These questions can only be resolved by cloning the gene(s) and looking at its structure, which was not possible during the time available for the current study. If more time had been available it would have been of interest to establish whether the non-homologous region at the 5' end of clone 1721 hybridises exclusively to the larger mRNA transcript of GCS1 on Northern blots, since the incomplete cDNA clones 813B4, 10323 and 11432 encoding the 3' end of the mRNA (results not shown) were all able to hybridise to both testis transcripts. The availability of cDNA/cRNA probes which could differentiate between the two sizes of mRNA transcript would allow investigation of whether both the transcripts are expressed in all germ cell types. There may be a difference in the expression of the two mRNA transcripts since only the larger transcript is present in total RNA from immature testes (day 20) suggesting that this species of mRNA is present in less mature germ cells e.g. pachytene spermatocytes.

A search of the Genbank database with the complete sequence of GCS1 revealed that it had homology to four expressed sequence tags but no homology to any other complete sequences with known functions. Three of the ESTs had been obtained by partial sequencing of cDNA clones isolated at random from an adult mouse testis cDNA library, MTESTs 29, 128 and 384 (Yuan *et al.*, 1995). These three ESTs were reported to be germ cell specific by Northern blot analysis, consistent with my own findings. MTEST 29 hybridised to poly $(A)^+$ RNA from adult mouse testis and also to RNA from isolated round spermatids but not to any other tissues or testicular cell fractions (Yuan *et al.*, 1995). MTESTs 128 and 384 hybridised to poly $(A)^+$ RNA from isolated pachytene spermatocytes and round spermatids in addition to adult testis but not to any other tissues (Yuan *et al.*, 1995). The fourth EST was obtained from the partial sequencing of cDNAs randomly isolated from cDNA libraries of untreated and 9 day nerve growth factor (NGF)-treated PC-12 cells, where NGF induces the differentiation of adrenal chromaffin-like PC-12 cells to a neuronal phenotype (Lee *et al.*, 1995). There is no specific information on EST 111685 within this publication, the objective of which was to investigate the expression profiles of ESTs in PC-12 cells before and after NGF

treatment. This lack of information suggests that the mRNA expression of this EST was not regulated by NGF. The significant sequence homology of this EST to clones 1414, 1844 and 1712 is surprising since Northern analysis failed to detect any evidence of GCS1 mRNA in total RNA from rat adrenal or brain, although poly $(A)^+$ RNA from these tissues was not tested.

Further analysis of the protein sequence obtained from the ORFs of the cDNAs was undertaken for functional domains using Prosite. This revealed the presence of two potential leucine zipper domains. This motif is a common characteristic of DNA binding proteins, in which the leucine zipper structure is situated adjacent to a DNA binding domain composed of basic amino acids (Busch & Sassone-Corsi, 1990). This domain is organised so that two regions of basic residues, such as the amino acids arginine and lysine, are separated by a region of non-basic amino acids. However, in the protein sequence predicted for GCS1, a region of basic amino acids is not present suggesting that this protein is unlikely to be a transcription factor. The leucine zipper domain is composed of four or five leucine residues precisely six amino acids apart and has an α helical structure which places each leucine residue along the same face of the helix (Landschultz *et al.*, 1988). This provides a hydrophobic surface for the interaction of two protein subunits known as dimerisation. This generally occurs between two identical proteins, homodimers, but related proteins may dimerise to form heterodimers. The presence of a leucine zipper but without a DNA binding domain in the protein sequence of GCS1 would suggest that the leucine zipper has only a structural role allowing the protein to dimerise with itself to generate a coiled coil structure which increases stability of the tertiary structure of the protein (O'Shea *et al.*, 1989). Many fibrous proteins such as intermediate filaments, tropomyosin, keratin and laminin possess coiled coil structures (Cohen & Parry, 1986). This is achieved by a heptad repeat pattern of amino acids interspersed with hydrophobic residues (not necessarily leucine) to allow hydrophobic interactions between the two α helices. Computer searching of databases can identify hundreds of proteins which contain the leucine zipper domain, showing that this is a common motif in many types of proteins.

RT7 is a testis specific protein which localises to the sperm tail and is thought to be a structural component of outer dense fibres (Higgy *et al.*, 1994). This gene is expressed in round spermatids and encodes a protein of 26-27 kDa. Sequence analysis has predicted that the RT7 protein may have an N-terminal α-helix which resembles a leucine zipper domain (van der Hoorn *et al.*, 1990). Similarly, this protein does not possess a basic DNA binding domain. Recent studies have shown that the RT7 protein can form stable complexes with itself, which are associated with the N-terminal region of RT7 which contains the putative leucine zipper domain (Higgy *et al.*, 1994). The function of this protein in spermiogenesis remains to be defined.

The final part of analysis of the predicted protein sequence of GCS1 involved estimation of its molecular weight and isoelectric charge. Since the original cDNA clone 813B4 was isolated based on the ability of this cloned cDNA to express a fusion protein recognised by the antiserum raised to STCM, it is assumed that a protein with similar properties to the predicted protein encoded by full length GCS1 should be present in STCM, and detectable by Western blot analysis using the original antiserum. A computer programme calculated that the putative GCS1 protein would have a molecular weight of 67.4kDa and an isoelectric point of 6.0. However, this estimation is unlikely to be completely accurate because it does not take into account the effects of post-translational modifications such as glycosylation, which effects the ability of a given protein to migrate through a gel under 2-D SDS PAGE conditions. In the present study, several proteins were resolved on 2-D gels with similar properties to those predicted from the amino acid sequence, but it was not possible to identify which, if any, of these proteins is the correct one. Future studies to resolve this could involve generating a specific antiserum to the recombinant protein or to a peptide and then using this to identify which protein in STCM is recognised on a 2-D gel.

The original aim of the immunoscreening approach was that the antiserum raised to STCM would allow the cloning of cDNAs specific for secreted proteins, as these proteins might have potential use as markers of testicular function. The classic secretory pathway in eukaryotic cells involves the translocation of proteins across the endoplasmic reticulum membrane and their transport in vesicles through the Golgi apparatus to the plasma membrane. Most secretory proteins are synthesised as precursor polypeptides containing a cleavable N-terminal signal. This signal peptide does not have a conserved sequence but is characterised by a three domain structure (von Heijne, 1985). It has a variable length of around 16-26 amino acid residues consisting of a positively charged N-terminal region, a central region of at least 6 or 7 hydrophobic amino acids and a more polar C-terminal region which defines the cleavage site. The sequence GCS1 cDNA described above does not appear to contain amino acids encoding a classical signal peptide at its N-terminal, suggesting that this protein may be cytosolic or not secreted by the classical route. Interestingly, several proteins have been identified which lack a signal sequence which are secreted and these findings have led to the proposal of alternative route(s) of secretion (Muesch *et al.*, 1990). It has been proposed that these may involve the use of ATP driven transporters to translocate proteins across membranes (Muesch *et al.*, 1990; Kuchler & Thorner, 1990), interaction of proteins with phospholipids or calcium present in the membrane (Manin *et al.*, 1995) or apocrine secretion.

Recent evidence suggests that apocrine secretion may exist in the male reproductive tract (Manin *et al.*, 1995). During this process the epithelium forms

abundant apical protrusions which become detached so that fluid together with degenerating apical cytoplasm is 'secreted'. A protein without a signal peptide, produced by the epithelium of the mouse vas deferens has been reported to be secreted in an apocrine manner based on ultrastructural studies in conjunction with immunocytochemistry (Manin *et al.*, 1995). These authors cite other publications which have reported the presence of apical protrusions in the epithelia of male accessory sex organs of several species including the rat and human. This method of secretion may account for the fact that PEBP appears to be secreted into the extracellular fluids of the male reproductive tract of the rat and mouse even though this protein does not possess a signal peptide and is cytosolic in other tissues (Jones & Brown, 1987; Perry *et al.*, 1994; Saunders *et al.*, 1995). It has also been demonstrated that annexin 1, also known as lipocortin, is secreted by the human prostate gland (Christmas *et al.*, 1991). Annexins are intracellular proteins which bind to certain phospholipids and are abundant in many cell types, and this is reflected by their lack of a signal peptide. However, Christmas *et al.* (1991) demonstrated that human seminal plasma contained high concentrations of annexin 1 which appeared to be secreted by prostatic cells via a selective mechanism. More recently, two proteins have been identified in STCM by microsequencing as being lactate dehydrogenase-B (LDH) and β-actin (McKinnell & Sharpe, 1995). β-Actin was also shown to be present in rete testis fluid. These proteins would normally be considered to be intracellular. However, other publications have reported the presence of LDH in Sertoli cell conditioned medium, seminal plasma and prostatic fluid (for references see McKinnell & Sharpe, 1995). Taken together these reports suggest that apocrine secretion might be a common mechanism of protein secretion in the male reproductive tract.

Northern blot analysis showed that GCS1 mRNA was expressed abundantly in the adult rat testis, more variably in the epididymis but not in any other tissues. The precise cellular localisation and stage-dependent mRNA expression pattern of GCS1 was analysed using *in situ* hybridisation, and found to be confined to the germ cells, with expression of mRNA increasing in abundance from stage VII pachytene spermatocytes to step 15 spermatids. Many testis specific transcripts have been identified which are expressed only in the germ cells (for example Erickson, 1990; Wolgemuth & Watrin, 1991; Yuan *et al.*, 1995; Penttila *et al.*, 1995b). An example of an mRNA expressed in pachytene spermatocytes at high levels at stages VII-XI of the cycle is mitochondrial cytochrome C oxidase II, which is the terminal enzyme in the electron transport chain located in mitochondria (Saunders *et al.*, 1993). In contrast, most germ cell specific mRNAs which have been identified are expressed in haploid cells. The best characterised are those mRNAs representing proteins involved in nuclear condensation, the transition proteins and protamines (Hecht, 1990). Transition protein 2 mRNA is first expressed in

step 7 spermatids, is maximal during steps 10 and 11 before decreasing until it is undetectable in step 14 spermatids at stage XIV (Saunders *et al.*, 1992). GCS1 mRNA shows a different pattern of expression to either of these two mRNAs as it is expressed in both pachytene spermatocytes and in round and elongating spermatids. Germ cell transcripts tend to be long lived in comparison to those of other cell types, and this is probably a reflection of the fact that transcription ceases in step 8 spermatids. Therefore, proteins needed in the later stages of spermiogenesis have be transcribed some time before they are translated. An example of this is transition protein 1, which shows a similar pattern of mRNA expression to transition protein 2, though immunocytochemistry has demonstrated that the protein is not translated until step 12-13 spermatids (Heidaran *et al.*, 1988). Some messages may even be transcribed in mid to late pachytene spermatocytes when RNA synthesis is maximal (Monesi *et al.*, 1978). It is therefore possible that the protein encoded by GCS1 mRNA is not translated until the spermatid steps of spermatogenesis.

The gene coding for GCS1 appears to be highly conserved amongst several species. Southern blot hybridisation detected the presence of homologous genes in rat, mouse, dog, cow, monkey and human DNA. Northern blot hybridisation was also able to detect the presence of two mRNA transcripts in mouse and marmoset testis. It would be of interest to investigate the localisation of this protein in the testis as this might help to establish its functional role in spermatogenesis. However, this would necessitate the generation of a peptide antiserum to the protein. This antiserum could then be used to resolve the issue of whether this protein is secreted into seminiferous tubule fluid and STCM or whether it is cytosolic, and would be an essential first step towards an assay for this protein which could be used to determine whether it has potential use as a marker of germ cell development.

The method of immunoscreening was chosen to clone cDNAs from a testis library since it was hoped this would allow the selection of cDNAs specifically encoding secreted proteins. The results described in this chapter and also in chapter 6 would suggest that this approach has not been as staightforward as was hoped. One explanation might be that the STCM used for immunisation is not a true reflection of the types of proteins secreted by the seminiferous epithelium *in vivo*. The technique of immunoscreening still has potential for future studies to identify proteins important in spermatogenesis, although it would probably be more successful with a better defined antiserum. One alternative would be to raise an antiserum to proteins present in seminiferous tubule fluid, since these proteins are secreted *in vivo*. Cloning strategies involve a lot of time and effort to determine whether a cDNA is of interest and to investigate its role in spermatogenesis, but it is likely that only through this type of approach will proteins important in the regulation of germ cell development be identified.

Chapter 8. General Discussion

There are increasing numbers of reports which suggest that a decline in male reproductive health is occurring highlighted by decreases in semen quality and an increasing incidence of abnormalities in the male genitourinary tract (Giwercman, 1995). Sperm concentration data for normal men from over 60 publications during the period 1938-1990 were subjected to statistical analysis (Carlsen *et al.*, 1992). This paper indicated that mean sperm concentrations had declined from $113x10^6$/ml to $66x10^6$/ml. This observation is supported by other studies based on semen quality analysis within single laboratories during the past 20 years (Auger *et al.*, 1994; Irvine, 1994; van Waeleghem *et al.*, 1994). During the same time period there has been an increase in incidence of testicular cancer and of two congenital abnormalities, hypospadias and cryptorchidism (Giwercman, 1995). These reports give cause for concern and highlight the lack of understanding of the mechanisms responsible for determining male fertility. The deterioration in semen quality indicates that spermatogenesis is impaired but, unfortunately, there are no means of monitoring this process at present other than by testicular biopsy, hormone levels or semen analysis. There is a real need to identify markers of spermatogenesis which can be measured non-invasively, for example in peripheral blood, and which are able to provide information on the normality or otherwise of germ cell development. Such markers would be invaluable for assessing male fertility in the clinical situation, and may aid in the management of human male infertility. In addition, non-invasive markers would also be useful in the screening of drugs and chemicals for deleterious effects on laboratory animal or human testicular function during toxicological studies or clinical trials. The studies described in this thesis had the objective of establishing whether it would be feasible or not to monitor spermatogenesis by non-invasive means by measuring products of the seminiferous epithelium.

The first objective was to assess whether creatine could be used as a non-invasive marker of acute testicular damage (chapter 4). This study was instigated following reports that the administration of testicular toxicants to rats resulted in increased levels of urinary creatine in male but not female rats (Rawcliffe *et al.*, 1989; Gray *et al.*, 1990; Moore *et al.*, 1992; Nahas *et al.*, 1993). These results led these authors to suggest that urinary creatine might be a marker of impaired testis function. The first part of the studies described in chapter 4 was to investigate the relationship of creatine to spermatogenesis, to try and determine whether creatine was associated with a specific cell type within the seminiferous epithelium or with specific stages of the spermatogenic cycle. Creatine levels within ST were found to be related to the germ cell complement of the seminiferous epithelium, since levels increased with testicular maturation and were decreased by the depletion of specific germ cells using MAA. However, from the results of these studies it was not possible to exclude the fact that creatine might be produced by both germ cells

and Sertoli cells, and it was concluded that creatine could not be used as a marker of damage to a specific cell type within the seminiferous epithelium. The levels of creatine in testicular IF, following the deleterious effects of various toxicants or local testicular heating, seemed to be related to the severity of damage induced to the seminiferous epithelium. However, plasma creatine levels following testicular damage showed little change, demonstrating that creatine is not a suitable marker for the non-invasive detection of testicular damage. The increase in urinary creatine observed following the administration of testicular toxicants might be due to leakage from the testis, but it is also possible that it originates from other damaged tissues since creatine is ubiquitous in many tissues.

The conclusions gained from evaluation of creatine as a marker suggests some points which need to considered for the identification of protein markers of spermatogenesis. Firstly, it would be preferable for marker proteins to be produced only by the testis and be secreted by a specific cell type within the seminiferous epithelium. Secondly, for non-invasive monitoring purposes the protein should gain access to the peripheral circulation. Thirdly, if the protein is produced by other tissues, assays should be able to discriminate the testicular protein from that originating from other sources. Fourthly, it would be useful if a marker protein had a known function in relation to spermatogenesis so that it is able to provide information on the nature of the defect to germ cell development. Finally, measurement of the levels of a suitable marker protein should be sensitive enough to detect early adverse changes to spermatogenesis before more severe testicular damage arises.

The aim of the studies described in chapter 5 was to ascertain whether proteins derived from ST in the adult rat testis are able to gain access to testicular IF and/or blood in the normal physiological situation, and whether this was altered by the impairment of spermatogenesis. In order to investigate whether ST secreted proteins are detectable in biological fluids, an antiserum was generated to STCM for use in conjunction with Western blotting. This provided a means of detecting many proteins, the majority of which have not been identified. STCM was selected as the source of secreted proteins since ST can be isolated from adult rat testes and cultured with ease, allowing generation of enough STCM for immunisation purposes. This method has the advantage over using isolated cells in that cell-cell interactions within an isolated length of ST are more comparable with the *in vivo* situation. Therefore proteins secreted in this culture system are probably more likely to reflect the situation *in vivo*. In addition, it was important to investigate proteins secreted by the seminiferous epithelium in the adult testis since it has been demonstrated that the pattern and level of protein secretion by adult ST is very different to that observed in immature animals (McLaren *et al.*, 1993b). Western blot analysis showed that this antiserum could recognise over a hundred of the proteins

secreted by the ST during culture, suggesting that it could be a useful tool to evaluate whether any of these proteins might be markers of spermatogenesis *in vivo* and also for the identification of testicular cDNAs encoding novel secreted proteins by immunoscreening. Western blot analysis was able to detect three proteins (A=24 kDa; B=16 kDa and C= 14 kDa) in IF, but not in SV, TV or PV plasma from control rats. Induction of damage to spermatogenesis 24 hours earlier by local testicular heating (43°C for 30 min) increased the abundance of these three proteins in IF, and it also induced the appearance of several other less abundant proteins which had molecular masses below 25 kDa. Proteins A and C were identified as germ cell secretory products. Protein A was demonstrated to be phosphatidylethanolamine binding protein (PEBP) by Western blotting using an antiserum raised against a PEBP peptide. Protein B was identified as androgen regulated protein-2 (ARP-2) based on its molecular weight, pI and localisation on 2-D Western blots. The identification of germ cell secreted proteins in IF was a totally unexpected result, as it has generally been assumed that only Sertoli cell proteins are secreted into the interstitium because of the presence of the inter-Sertoli cell tight junctions. The detection of PEBP and ARP-2 in IF suggests that low molecular weight germ cell secreted proteins can also gain access to IF, although the mechanism by which this is achieved is unknown. One explanation might be that the tight junctions are selectively permeable or 'leaky' to small molecules. This is an encouraging finding as it suggests that non-invasive monitoring of testicular function might be feasible, provided that the germ cell proteins which 'leak' into IF find their way from here to the bloodstream.

These studies failed to identify ST derived proteins, by Western blot analysis, in TV, SV and PV blood samples from both control adult rats and also in peripheral blood from rats after induction of testicular damage. However, this result is most likely a reflection of the insensitivity of the technique used and cannot be viewed as definitive evidence for the absence of these proteins from blood. The enzyme LDH-C4, which is a cytosolic enzyme unique to meiotic and post-meiotic germ cells, is detectable by enzymatic assay in peripheral blood of control rats and is increased after toxicant-induced damage to the germ cells (Reader *et al.*, 1991). It is concluded that a definitive assessment of whether ST derived proteins can be detected in blood will involve generating sensitive immunoassays to specific proteins. Currently, the main problem remains the identification of proteins suitable for this approach. PEBP would be an obvious candidate since the sequence of this protein in the rat and monkey is known (Perry *et al.*, 1994; Saunders *et al.*, 1995), therefore antisera against defined peptides can be generated. Recently, the human homologue of PEBP has been sequenced from the brain (Moore *et al.*, 1995), placenta (Tohdoh *et al.*, 1995) and from a liver cancer cell line, HepG2 (Hori *et al.*, 1994). The predicted human amino acid sequence was found to

have >90% homology to the monkey and rat sequences (Moore *et al.*, 1995). It would be of interest to determine whether PEBP is produced by the human testis and epididymis. PEBP is a ubiquitous protein present in the cytosols of a wide range of tissues, but it only appears to be secreted by the testis and epididymis (Jones & Brown, 1987; Jones & Hall, 1991; Rankin *et al.*, 1992; Araki *et al.*, 1992; Vierula *et al.*, 1992; Saunders *et al.*, 1995). This suggests that if PEBP can be measured in peripheral blood it is likely to have originated from the testis and/or the epididymis rather than from other tissues. In order to establish whether PEBP is a potential marker of spermatogenesis or sperm maturation (since it is produced also in the epididymis) a specific immunoassay needs to be generated. Preliminary studies using rats would then involve determining whether PEBP can be measured in TV, SV and/or PV blood samples from control and castrate rats and assessing whether levels are altered by the induction of testicular damage. If this approach provides encouraging results then studies could be performed to evaluate the usefulness of PEBP as a non-invasive marker of spermatogenesis in humans, by comparing the levels in blood between fertile, infertile and castrate men. Preliminary evidence suggests that ARP-2 is also secreted by human seminiferous tubules (McKinnell *et al.*, 1995), but this protein has not yet been isolated and sequenced so its identity and function remains unknown. Until this information becomes available, PEBP remains the best available candidate for assessing whether non-invasive monitoring of spermatogenesis is feasible.

To be fully comprehensive, non-invasive monitoring will require measurement of a spectrum of proteins, preferably from different testicular cells, and therefore further candidate proteins need to be identified and their usefulness evaluated. Screening an testis cDNA expression library was selected as a suitable strategy to identify cDNAs for novel secretory proteins of the testis (chapters 6 and 7). Preliminary analysis of eight individual cDNAs cloned by this approach revealed only one clone (712A5) which enoded a partial cDNA for a protein which was already known to be secreted, this protein being cyclic protein-2 (CP-2), previously identified as a product of Sertoli cells (Zabludoff *et al.*, 1990a; Erickson-Lawrence *et al.*, 1991). Another clone (813B4) was selected for further study since it was found to localise to mRNA exclusively in the testis and epididymis. This cDNA was used for further screening and a full length cDNA (GCS1) was assembled from overlapping clones. Sequence analysis of the GCS1 cDNA showed significant homology to four ESTs but failed to reveal any domains indicative of a specific function. The lack of a peptide sequence with obvious homology to that of the signal peptide means that further work is necessary to determine whether the predicted protein encoded by this cDNA is secreted by germ cells by alternative routes, although leakage of cytosolic proteins from damaged or dying cells might also be informative. The technique of immunoscreening has not proved to be completely straightforward but it still

has potential to be extremely useful in future studies. The main reason for our inability to monitor spermatogenesis stems from the lack of understanding of this process and the fact that many of the key proteins important for the regulation of germ cell development have yet to be identified. The identification of gene products which are important for the maintenance of spermatogenesis, is complicated by the fact that the testis expresses a great number of mRNAs which are not translated into functional proteins (Ivell, 1992). This problem suggests that the random cloning of cDNAs from a testis library is not the most efficient method of identifying novel genes which are functional within the testis. Immunoscreening avoids this problem since cDNAs are selected based on their ability to translate a fusion protein recognised by an antiserum. The identification of novel testicular secretory proteins still remains the key area of study if non-invasive monitoring of testicular function is ever to become a reality.

In conclusion, the studies described in this thesis have made some progress towards evaluating whether monitoring of spermatogenesis by non-invasive means is a likely prospect for the future. It has been demonstrated that some germ proteins can gain access to the interstitium but it remains to be determined whether such proteins can also be measured in blood. However, PEBP has been identified as a suitable candidate to allow a definitive assessment of this possibility. The overall conclusion from this work is that more effort is required to identify secreted proteins which are important in the regulation of spermatogenesis and which have well characterised functions. Hopefully, this will identify potential markers of spermatogenesis and increase our understanding of this complex process. This will prove invaluable for elucidating the causes of male infertility.

Appendix: Some Commonly Used Buffers and Agars

Buffers

1x SSC
0.15M sodium chloride
0.015M sodium citrate
at pH7.0

1x TBE
0.089M Tris base
0.089M boric acid
10mM EDTA

1x SSPE
0.15M sodium chloride
0.01M NaH_2PO_4, anhydrous
1mM EDTA
at pH7.4

TE buffer
10mM Tris-HCl
1mM EDTA
at pH8.0

SM buffer
10mM Tris, pH7.4
10mM $MgSO_4$
0.01% gelatin

Agars

Luria Bertani (LB) broth
10g bacto-tryptone
5g bacto-yeast extract
10g NaCl
in 1 litre water

LB-agar
LB-broth
with 1.5% bacto-agar

NZY agar
15g agar
5g NaCl
2g $MgSO_4.H_2O$
5g yeast extract
10g NZ amine
in 1 litre water at pH7.0

NZY top agar
7g agarose
5g NaCl
2g $MgSO_4.H_2O$
5g yeast extract
10g NZ amine
in 1 litre water at pH7.0

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