GENETIC DETERMINANTS OF HUMAN SEX HORMONE-BINDING GLOBULIN EXPRESSION

by

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in
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ABSTRACT

Sex hormone-binding globulin (SHBG) is the major sex steroid-binding protein in human plasma. Hepatocytes are the primary source of plasma SHBG production, and changes in blood levels are influenced by genetic, hormonal, metabolic, and nutritional factors. The rodent *SHBG* gene is expressed in the fetal liver, but is inactive postnatally. By contrast, the human *SHBG* gene is expressed throughout life, and this is recapitulated in transgenic mice expressing 4 kb (*SHBG*4) or 11 kb (*SHBG*11) human *SHBG* transgenes. Serum levels of human SHBG are sexually dimorphic in *SHBG*11 transgenic mice, but are similar in male and female *SHBG*4 mice. However, the hepatic expression of the human *SHBG* gene is highly regulated by dietary carbohydrate content and metabolic status in both *SHBG*4 and *SHBG*11 transgenic mice. The human *SHBG* promoter that regulates the production of plasma SHBG contains a $(TAAAA)_n$ polymorphism, and the activity of the *SHBG* promoter in human HepG2 hepatoblastoma cells is influenced by the number of TAAAA repeats. In addition, two novel variations in the human SHBG coding sequence contribute to low serum SHBG levels in humans, and are associated with hyperandrogenism and ovarian dysfunction. The *SHBG* gene is also expressed in the testis. While *SHBG* expression in the Sertoli cells of the rodent testis is regulated by sequences flanking the exon 1 that encodes the secretion signal polypeptide, this promoter is inactive in transgenic mice containing a 4 kb human *SHBG* transgene. However, human *SHBG* transcripts containing a non-coding alternative exon 1 sequence accumulate in a spermatogenic stage-dependent manner in the germ cells of transgenic mice containing an 11 kb human *SHBG* transgene. In these mice, human SHBG isoforms that bind steroid accumulate in the acrosome of developing germ cells, and are present in the acrosome of epididymal sperm, as well as ejaculated human sperm. Female *SHBG*11 transgenic mice that contain these alternative *SHBG* transcripts in the brain display a maternal phenotype of maternal neglect. These studies have greatly improved our understanding of how human *SHBG* gene expression is regulated by genetic, hormonal, metabolic and nutritional factors, and has provided valuable insight into the function of SHBG isoforms within the brain and testis.

KEYWORDS: Sex hormone-binding globulin, sex steroids, spermatogenesis, polymorphisms, transcription.

CO-AUTHORSHIP

The research presented in this thesis is a result of a substantial collaborative effort of research conducted both within the laboratory of Dr. Geoff Hammond, and with international collaborators. Work described in this thesis has either been published, or is intended for publication.

In studies described in Chapter 2, the author was responsible for characterizing serum levels of human SHBG, and SHBG mRNA levels in tissues of transgenic mice expressing human *SHBG* transgenes throughout development. The author also contributed to the writing of the manuscript.

Work outlined in Chapter 3 was performed in collaboration with Dr. David M. Selva, a post-doctoral fellow in the laboratory of Dr. Geoff Hammond. Dr. Francis Tekpetey provided human sperm samples.

Chapter 4 describes experiments conducted by the author and Dr. Marja Talikka. The author identified the $(TAAAA)_n$ polymorphism in the human *SHBG* promoter, and characterized its function in the transcriptional regulation of human *SHBG* gene expression. The author also made substantial contributions in writing the manuscript.

The results presented in Chapter 5 are intended for publication. Dr. Marja Talikka identified the USF site in the human *SHBG* promoter. The author performed all of the other experiments presented in this chapter.

Studies outlined in Chapter 6 were performed in collaboration with Dr. Michel Pugeat and Dr. Patrice Cousin (Lyon, France) and Dr. Didier DeWailly and Benoit Soudan (Lille, France). The group in Lille brought to our attention a patient with extremely low serum SHBG levels and severe hyperandrogenism. Work in Dr. Hammond's laboratory identified the two SHBG coding sequence variations in her *SHBG* alleles, and the author developed a screening assay for identification of these alleles in the population. The author also characterized the expression of these alleles *in vitro*, and contributed to the writing of the manuscript. Dr. Patrice Cousin screened a large group of patients for the two novel *SHBG* variants.

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ABBREVIATIONS

ABP	androgen binding protein
BMI	body mass index
bp	base pairs
cAMP	cyclic adenosine monophosphate
cDNA	complementary deoxyribonucleic acid
CBG	corticosteroid binding globulin
C/EBP	CCAAT/Enhancer binding protein
COUP-TF	chicken ovalbumin upstream promoter – transcription factor
DCC	dextran-coated charcoal
DHT	5α-dihydrotestosterone
DNA	deoxyribonucleic acid
dpc	days post-coitus
DTT	dithiothreitol
EDS	ethane dimethane sulfonate
EDTA	ethylenediaminetetraacetic acid
EMSA	electrophoretic mobility shift assay
FP	DNaseI footprint
FSH	follicle stimulating hormone
HNF-4	hepatocyte nuclear factor-4
IGF-I	insulin-like growth factor I
kb	kilobase pairs
kDa	kilo Dalton
LG	laminin G-like
Lp(a)	lipoprotein (a)
M	Molar
MCR	metabolic clearance rate
mRNA	messenger ribonucleic acid
NHR	nuclear hormone receptor
nt	nucleotides

PAGE	polyacrylamide gel electrophoresis
PBS	phosphate-buffered saline
PCOS	polycystic ovarian syndrome
PCR	polymerase chain reaction
RFLP	restriction fragment length polymorphism
RT	reverse-transcription
SHBG	sex hormone-binding globulin
SNP	single nucleotide polymorphism
SP1	sephacryl and phosphocellulose protein-1
STZ	streptozotocin
TBG	thyroxine-binding globulin
TBP	TATA binding protein
TBS	tris-buffered saline
USF	upstream stimulatory factor

CHAPTER 1

INTRODUCTION

1.1 Sex Steroid Hormone Action

1.1.1 Introduction

The sex steroid hormones exert fundamental roles in a vast number of physiological events, including the development of reproductive tissues during embryonic life and maturation of these structures during puberty. Androgens and estrogens exert profound effects on bone and the cardiovascular system, and modulate male and female-type behaviour patterns. The mechanism by which sex steroids affect these physiological processes is complex, and involves regulation of sex steroid hormone activity at a number of levels. In essence, sex steroid hormone action encompasses steroid synthesis, transport and delivery, signal transduction and metabolism, which all serve to modulate the physiological response. The activities of sex steroid hormones are regulated at multiple levels. The amounts of circulating sex steroids in the blood is tightly regulated through the hypothalamus-pituitary-gonadal axis, and although their synthesis occurs predominantly in the testis and ovary , the adrenal is also a major source of precursor steroids which can be converted into biologically active sex steroids in peripheral tissues including the breast, fat, and brain. In the blood circulation, specific hormone-binding proteins transport sex steroids, and modulate their entry and accumulation within target tissues. At the cellular level, steroid hormone activity is regulated by metabolic conversions (Simpson, 2000), and/or the binding of steroids or their metabolites to the classical nuclear receptors (Tsai and O'Malley, 1994).

1.1.2 Genomic Effects of Steroid Hormones

The traditional view of how sex steroids mediate their biological effects is that steroids entering target cells by passive diffusion interact with cognate nuclear hormone receptors (NHR), which act as ligand-dependent transcription factors. Accordingly, this interaction allows the hormone-receptor complex to bind with high affinity to specific DNA response elements in the promoters of responsive genes (Tsai and O'Malley, 1994). Nuclear hormone receptors also undergo a steroid-dependent (Beekman *et al.*, 1993), and DNA response element-dependent (Hall *et al.*, 2002) conformational change which facilitates an exchange of co-regulatory proteins. The NHR-dependent assembly of a multi-protein coactivator complex directly stabilizes the basal trancriptional machinery,

and results in a modification of the chromatin context to a form more permissive to transcription (Spencer and Davie, 1999). This complex series of interactions directs changes in the expression of specific genes to effect a physiological response. In addition, in response to extracellular signals, NHR's can be activated in a steroid ligand-independent manner through phosphorylation of an amino-terminal activation function-1 domain (Weigel and Zhang, 1998).

1.1.3 Non-Genomic Effects of Steroid Hormones

This classical view of steroid hormone action is lacking in that it does not explain the rapid, non-genomic effects of sex steroids which occur on a time-scale inconsistent with changes in transcription (Simoncini and Genazzani, 2003). Binding sites for sex steroids have been localized at the plasma membrane of responsive cells (Pietras and Szego, 1977), yet the identity of these receptors remains to be characterized. Several studies have provided evidence that the membrane receptor that mediates the rapid non-genomic effects of sex steroids is an isoform of the traditional nuclear receptor which targets to the cell membrane (Razandi *et al.*, 1999; Watson *et al.*, 2002). In the case of the estrogen receptor, the membrane-associated pool of nuclear hormone receptors can interact with G-proteins (Wyckoff *et al.*, 2001), and activate signal transduction through phospholipase C (PLC) (Le Malley *et al.*, 1997), protein kinase A (PKA) and protein kinase C (Kelly *et al.*, 1999) and endothelial nitric oxide synthase (Chen *et al.*, 1999). The relevance of rapid signaling events of sex steroids originating at the plasma membrane is supported by effects on the cardiovascular system (Simoncini and Genazzani, 2000) and neural activity (McEwan, 1991), which are independent of transcription.

1.2 Sex Hormone Binding Globulin (SHBG)

1.2.1 Introduction

Although sex steroids exert effects in the endocrine glands in which they are synthesized, their actions extend to target tissues remote from their sites of biosynthesis, and the transport and delivery of steroid hormones to these sites is an important component in the regulation of steroid hormone action. Sex steroids circulate in the

blood distributed in a state of dynamic equilibrium between a protein-bound fraction, and a free fraction which accounts for approximately 1-3 % of the total circulating steroid (Dunn *et al.*, 1981). In the blood, androgens and estrogens are bound reversibly and with low affinity (10^{-5}-10^{-6} M) to albumin, which has a considerable impact on the plasma distribution of these steroids primarily due its large capacity. Although present in the serum at concentrations much lower than albumin, a specific plasma steroid binding protein that binds biologically active androgens and estrogens with high affinity, which is known as sex hormone-binding globulin (SHBG), plays a major role in regulating sex steroid distribution (Westphal, 1986). Regulation of the distribution of sex steroids in the blood is fundamental to the classical view of their action, as only the free sex steroid is considered to be biologically active and able to enter target cells to initiate a response (Siiteri *et al.*, 1982).

1.2.2 SHBG/Androgen-Binding Protein (ABP)

The major determinant of sex steroid distribution in human serum is the concentration of sex hormone binding globulin (SHBG), a specific androgen and estrogen-binding protein first identified in the β-globulin fraction of human serum (Rosner *et al.*, 1966; Vermeulen and Verdonck, 1968). Later, a high affinity androgen binding protein (ABP) was found in rat testis and epididymis (Ritzén *et al.*, 1973) with binding characteristics distinct from that of the androgen receptor (French and Ritzén, 1973; Danzo *et al.*, 1974). Although plasma SHBG and testicular ABP have been shown to be physicochemically related (Cheng *et al.*, 1984a) and are derived from the same gene (Gershagen *et al.*, 1987; Hammond *et al.*, 1989; Sullivan *et al.*, 1991), the testicular protein is still generally referred to as ABP, whereas the serum protein is termed SHBG. Throughout this thesis, these terms will therefore be used interchangeably.

1.3 Structural Composition of SHBG

1.3.1 Precursor Polypeptide

The primary structure of human SHBG was first determined by direct sequencing of the protein isolated from serum (Walsh *et al.*, 1986), followed by confirmation of this sequence by deduction from complementary DNA (cDNA) and gene sequences

(Hammond *et al.*, 1987; Gershagen *et al.*, 1987). These latter studies revealed that SHBG is synthesized as a 402 amino acid precursor protein with a hydrophobic 29 residue polypeptide leader sequence required for secretion, which when cleaved gives rise to a 373 amino acid mature polypeptide with a molecular weight of 40.5 kDa. In biological fluids, SHBG exists as a homodimer composed of two identical subunits, and there is evidence that divalent cations (Rosner *et al.*, 1974; Bocchinfuso and Hammond, 1994) and steroid ligand (Bocchinfuso and Hammond, 1994) both serve to stabilize the dimer, probably through independent effects.

Each SHBG monomer is composed of two laminin G-like (LG) domains, and disulphide bridges within each LG domain (Cys^{164}-Cys^{188} and Cys^{333}-Cys^{361}) likely provide structural contributions (Walsh *et al.*, 1986). The amino-terminal LG domain contains all the structural elements necessary for subunit dimerization and high affinity sex steroid binding (Hildebrand *et al.*, 1995), as well as a site for O-linked glycosylation at Thr^{7} (Walsh *et al.*, 1986), and a domain which is suggested to be important for interaction with cell membranes (Khan *et al.*, 1990; Joseph and Baker, 1992). The carboxy-terminal LG domain is less well characterized, but two sites for N-linked glycosylation have been identified at residues Asn^{351} and Asn^{367} (Walsh *et al.*, 1986). Although little is known about the role of the C-terminal LG domain in SHBG function, it may contribute to the structural stability of the protein, especially during synthesis. In support of this, even small carboxy-terminal truncations prevent secretion, and the truncated protein appears to be retained within the insoluble fraction of cell extracts (Joseph and Lawrence, 1993).

1.3.2 Steroid Binding Domain

The N-terminal LG domain of SHBG when expressed in *E. coli* is capable of binding steroids with high affinity, and this is accompanied by spontaneous subunit dimerization (Hildebrand *et al.*, 1995). The steroid binding site of human SHBG has been well characterized, and the amino acids required for high affinity steroid binding have been identified by several independent methods. Photoaffinity labeling with Δ6-[^{3}H] derivatives of testosterone and estradiol revealed that Met^{139} interacted with these steroids (Grenot *et al.*, 1992), and the importance of this residue in steroid binding was

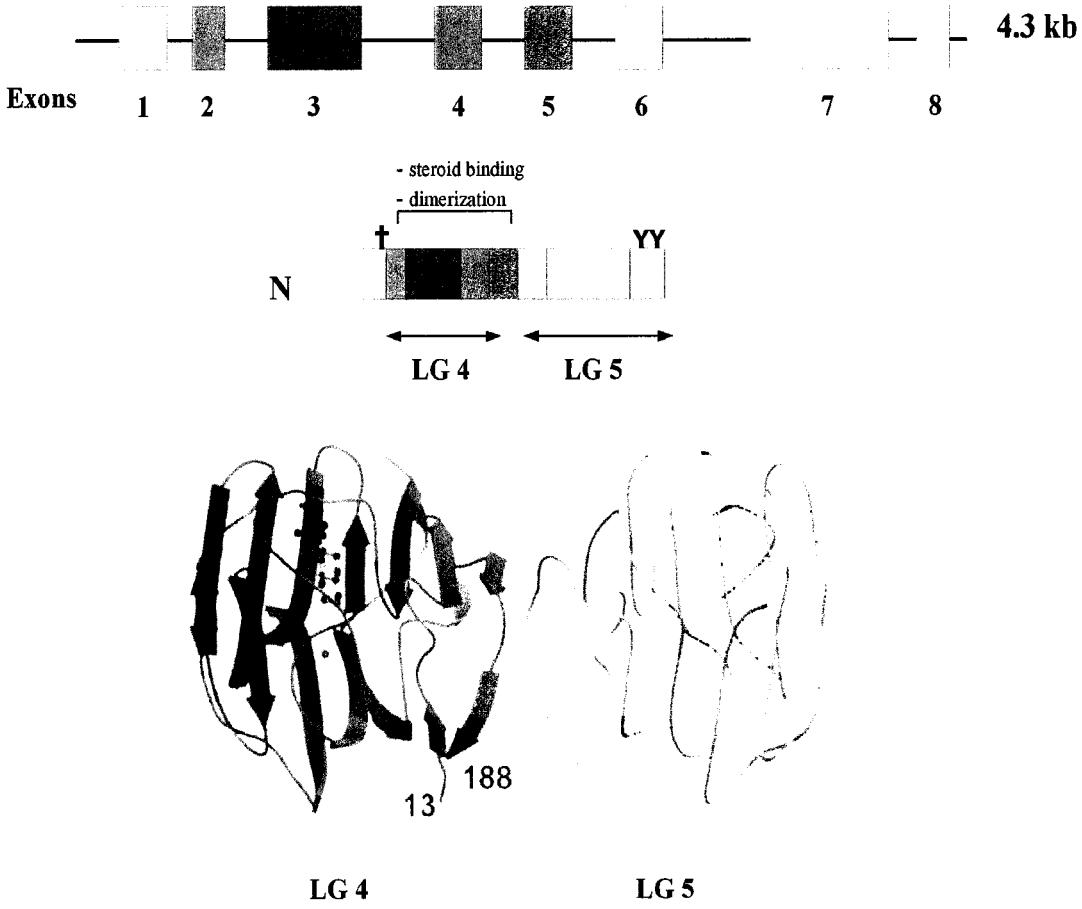

Figure 1.1 Structure of human SHBG. Structure of the human *SHBG* gene (A) and linear representation of SHBG protein structure (B). Exons in the *SHBG* gene are represented as boxes, colour-coded with respect to their position in the human SHBG crystal structure (Grishkovskaya *et al.*, 2000). (C). SHBG coding regions not resolved in the crystal structure are shaded grey. An O-linked glycosylation site (†) is present within the N-terminal LG domain, and the C-terminal LG domain contains two sites for N-linked glycosylation (Y). All amino acids necessary for dimerization and high affinity ligand binding are present within the N-terminal LG domain.

confirmed by site-directed mutagenesis (Bocchinfuso *et al.*, 1992a; Sui *et al.*, 1992). Using 17β-bromoacetoxy-DHT for affinity labeling, one group identified Lys^{134} (Namkung *et al.*, 1990) as a labeled residue, whereas Khan and Rosner (1990) using the same label, identified His^{235}. Using site-directed mutagenesis, substitution of Lys^{134} with a histidine residue resulted in a reduction of binding affinity for estradiol, but not for other SHBG ligands (Bocchinfuso and Hammond, 1994). Moreover, substitution of residues 134-139 all resulted in significant changes in SHBG affinity for estradiol, with little effect on binding of other steroid ligands (Bocchinfuso and Hammond, 1994). This was an interesting observation given that these residues are poorly conserved between species, and was interpreted as an explanation for the fact that SHBG from most sub-primate species binds estrogens with lower affinities.

1.3.3 Glycosylation

In the blood, SHBG exists as a homodimeric glycoprotein with a relative molecular size of 90 kDa (Westphal, 1986). Under conditions of denaturing polyacrylamide gel electrophoresis (PAGE), SHBG from human serum migrates as a mixture of heavy (52 kDa) and light (48 kDa) protomers in a 10:1 ratio (Cheng *et al.*, 1983; Danzo *et al.*, 1989; Hammond *et al.*, 1986). This molecular size heterogeneity can be explained by differential utilization of N-linked glycosylation sites on each monomer, and is eliminated by enzymatic deglycosylation of the protein (Danzo *et al.*, 1989) or removal of sites of carbohydrate attachment by site-directed mutagenesis (Bocchinfuso *et al.*, 1992b). The SHBG in testis extracts has a different ratio of heavy and light protomers (Cheng *et al.*, 1984b; Cheng *et al.*, 1985). At the present time, it is not known if this difference in SHBG isoforms in testicular extracts is due to differences in glycosylation during synthesis within the testis, or selective sequestration of a particular glycoform from the blood. The latter is a distinct possibility because transgenic mice which do not express human SHBG in the testes accumulate significant amounts of immunoreactive SHBG in the interstitial compartment surrounding the Leydig cells (Jänne *et al.*, 1998).

The function of two N-linked glycosylation sites in the C-terminal LG domain is unknown, but an SHBG mutant lacking these sites displays normal steroid binding

characteristics, although secretion of this protein from Chinese Hamster Ovary cells is impaired (Bocchinfuso *et al.*, 1992b). It is also of particular interest that the N-linked glycosylation site at Asn^{367} is strictly conserved between mammalian species despite genetic drift in nucleotide sequence within this region (Hammond, 1993). Whereas SHBG from the serum has a bi-antennary oligosaccharide at this position (Avvakumov *et al.*, 1983), based on its inability to interact with concanavalin A, the testicular form probably has a more highly branched oligosaccharide at this site (Hsu and Troen, 1978; Hammond, 1995). Moreover, Avvakumov *et al.* (1988) demonstrated that carbohydrate moieties in the SHBG molecule provide structural features required for interaction with cell membranes (see section 1.5.3). This is of interest because it has been suggested that variability in carbohydrate composition of glycoprotein hormones alters their ability to interact with receptors at the cell membrane (Combarnous, 1992) and activate signal transduction cascades (Sairam and Bhargavi, 1985). It is therefore tempting to speculate that this site may have some function in terms of either the distribution of SHBG between the serum and the extravascular compartment, or its ability to interact with components of cell membranes.

In addition to two sites for N-linked glycosylation in the C-terminal LG domain of SHBG, an O-linked glycosylation site at Thr^{7} is present in the N-terminal LG domain (Walsh *et al.*, 1986). Very little is known about the significance of this glycosylation, but a single study has demonstrated it to be important for interaction with cell membranes and initiation of SHBG-mediated signal transduction in MCF-7 cells (Raineri *et al.*, 2002). However, this O-linked glycosylation site is not conserved between species (Griffin *et al.*, 1989; Hammond, 1993), and is probably not critical for its biological activity.

1.3.4 Insight into Human SHBG Gained by Crystal Structure

The crystal structure of the N-terminal LG domain of human SHBG has recently been solved (Grishkovskaya *et al.*, 2000). The steroid binding domain of the SHBG protein is arranged such that the steroid ligand is intercalated between two seven-stranded β-sheets in a jellyroll motif, and shows no similarity to the androgen or estrogen receptors in which the ligand-binding domain consists of mostly α-helices (Shiau *et al.*,

1998). Crystal structures of this region of human SHBG solved in the presence of steroid revealed how amino acids demonstrated by affinity labeling and site-directed mutagenesis contribute to steroid binding, and identified key residues that are critical for high affinity binding interactions. In particular, residues Ser^{42} Phe^{67}, Met^{107} and Met^{139} provide important contacts with DHT, and additional hydrophobic interactions are provided by Asp^{65} and Asn^{82} (Grishkovskaya *et al.*, 2000). Crystal structure analysis has also revealed that SHBG is a zinc binding protein, and occupancy of the zinc binding site reduces the affinity of SHBG for estradiol with no change in its affinity for androgens (Avvakumov *et al.*, 2000). This may have a functional impact on steroid hormone action in tissues where concentrations of zinc are high, such as the male reproductive tract (Hammond, 1995; Avvakumov *et al.*, 2000).

The crystal structures of SHBG complexed to steroid has also provided data demonstrating that each SHBG monomer is capable of binding a steroid ligand (Avvakumov *et al.*, 2001), and disproves previous assumptions that a single steroid binding site is located at the homodimer interface (Petra, 1991). Furthermore, recent data have shown that androgens and estrogens reside within the steroid binding pocket of SHBG in opposite orientations, and the side groups of specific amino acid residues interact with different regions of the steroid molecule (Grishkovskaya *et al.*, 2002). As a result, there are distinct conformational changes in the SHBG molecule when complexed with androgens or estrogens (Grishkovskaya *et al.*, 2002), and these may explain the preferential association of estrogen-bound SHBG with cell membranes (Avvakumov *et al.*, 1986, see section 1.5.3).

1.3.5 Similarity of SHBG to Other Proteins

The SHBG structure comprises a repeated laminin G-like domain, and this SHBG-like element is found within a number of other proteins with diverse biological functions such as the vitamin K dependent protein S (Gerhagen *et al.*, 1987), Gas6, and several extracellular matrix associated proteins including laminin, agrin and merosin (Joseph and Baker, 1992). Furthermore, G domains are found in the neuronal cell surface protein neurexin (Villoutreix *et al.*, 2001), the *Drosophila* developmental proteins Slit and Crumb, and the tumour suppressor Fat (Mahoney *et al.*, 1991; Joseph, 1997). The

structural similarities between these proteins is of interest because some of them are ligands for plasma membrane receptors, or interact with other proteins through their SHBG-like domain. For example, protein S interacts with complement regulatory protein C4b-binding protein through its SHBG-like domain (Nelson and Long, 1992), and is a ligand for the Tyro 3 receptor-like tyrosine kinase (Stitt *et al.*, 1995). Moreover, proteins containing LG domains influence diverse biological functions including cell signaling, adhesion, migration and differentiation (Grishkovskaya *et al.*, 2000). The SHBG-like domain of these proteins, and more specifically the N-terminal LG domain, may therefore provide a platform to mediate cellular effects through protein-protein interactions (Rudenko *et al.*, 2001), including the proposed interaction between SHBG and a plasma membrane receptor (Rosner, 1990).

1.4 Spatial and Temporal Expression of SHBG

1.4.1 Species Distribution

A protein with steroid-binding properties consistent with SHBG has been found in the plasma of most mammalian species, and several species of amphibians, reptiles and fish (Westphal, 1986; Joseph, 1994). In most species, SHBG preferentially binds androgens (Westphal, 1986), but in primates SHBG has a high affinity binding site for both androgens and estrogens (Renoir *et al.*, 1980). This difference in steroid binding characteristics likely reflects differing roles for SHBG in regulating the androgen/estrogen balance in different species (Hammond, 1990). Interestingly, although mice and rats express SHBG transiently in the liver during fetal life at a time coincident with the growth and development of reproductive tissues (Wilson, 1978), levels of SHBG in the blood are undetectable at birth (Corvol and Bardin, 1973; Sullivan *et al.*, 1991). Low plasma levels of SHBG are typically found in mammals at birth, but unlike most other species, there is no resumption of SHBG expression in the liver of rodents postnatally, and the very low levels of the protein present in immature male rats probably originates from the testis prior to the formation of the blood-testis barrier (Gunsalus *et al.*, 1978). Despite phylogenetic differences in hepatic expression of SHBG postnatally, SHBG expression appears to be conserved in the fetal liver and adult testes of all mammalian species examined to date. Detailed studies of temporal and tissue-specific

expression of SHBG in different species during development are lacking, and could provide insight into the fundamental role of SHBG in the regulation of steroid hormone action.

1.4.2 SHBG During Human Development

Serum SHBG concentrations in newborn humans are relatively low, and rise rapidly within the first 30 days to levels that exceed those in adult females (August *et al.*, 1969) and are maintained until puberty (Forest *et al.*, 1986; Wenn *et al.*, 1977). At this time, the blood levels of SHBG in males declines to a level maintained throughout adulthood. In girls, the decrease in serum SHBG levels is less pronounced (Apter *et al.*, 1984), and they are two fold higher in women than in men. In old age, serum SHBG levels in men increase, and is thought to be a result of changes in the androgen/estrogen balance caused by decreasing Leydig cell function.

1.4.3 SHBG Expression in the Liver

Studies performed with a transformed human hepatoblastoma cell line (HepG2) provided the first evidence that hepatocytes of the liver are the major source of circulating SHBG (Khan *et al.*, 1981). This was later confirmed by the isolation of SHBG cDNAs from a human liver library (Hammond *et al.*, 1987; Gershagen *et al.*, 1987; Que and Petra, 1987) and direct demonstration of SHBG mRNA in the liver by northern blotting (Hammond *et al.*, 1989). The liver is the source of circulating SHBG in rats during days 15-17 of fetal life (Sullivan *et al.*, 1991), and SHBG immunoreactivity can be detected in hepatocytes at this time (Becchis *et al.*, 1996). Transgenic mice expressing either 4.3 kb or 11 kb human *SHBG* transgenes express very high levels of SHBG mRNA and protein in the hepatocytes, and the 0.8 kb of promoter sequence present in the 4 kb human SHBG transgene appears to be sufficient for its liver-specific expression (Jänne *et al.*, 1998). The hepatic expression of SHBG *in vivo* is regulated by a complex array of steroidal, hormonal, and metabolic factors (Table 1.1, section 1.7), but the mechanisms responsible for this are largely unknown.

1.4.4 SHBG Expression in Testes

In addition to the liver, the testes are a major site of SHBG expression in rats (Reventos *et al.*, 1988), mice (Wang *et al.*, 1989), and humans (Hammond *et al.*, 1989). In rodents, ABP produced by Sertoli cells is secreted into the lumen of the seminiferous tubule, where it is thought to regulate the sex steroid-dependent processes of spermatogenesis and sperm maturation (Hansson *et al.*, 1975; Ritzén *et al.*, 1982). Approximately 20% of ABP produced by rat Sertoli cells is secreted basolaterally into the blood (Gunsalus *et al.*, 1980), and elevated levels of SHBG can be detected in the blood of immature male rats (Gunsalus *et al.*, 1981). It is generally assumed that SHBG expression in human testes resembles that in the rat testis, but evidence supporting this is lacking. However, there is evidence that expression of SHBG in the human testes arises from utilization of an alternative transcription unit, and SHBG cDNAs isolated from a human testicular library contain sequences that are derived from an alternative exon 1. These cDNAs represent alternative transcripts that lack the coding sequence for a secretion polypeptide that is in frame with the coding sequence for the mature polypeptide (Hammond *et al.*, 1989).

1.4.5 SHBG Expression in the Brain

There is evidence that SHBG is expressed in the male and female rat brain (Wang *et al.*, 1990). In the rat, SHBG mRNA has been detected in all regions of the brain by Northern blotting, and immunoreactive rat ABP was located in neuronal cell bodies of the hypothalamic median eminence, and the supraoptic and paraventricular nuclei (Wang *et al.*, 1990). Although the rat brain contains an SHBG mRNA similar to that coding for testicular ABP, it also contains mRNA with an alternative exon 1 which would encode a protein with an alternate N-terminal sequence that is not secreted (Sullivan *et al.*, 1993). The role of SHBG expression in the brain has not been studied, but sex steroids are critical for the masculinization of the male brain, as well as the initiation and maintenance of maternal behaviour (McEwan, 1983), and reproductive behaviour is abolished in mice lacking estrogen receptors (Ogawa *et al.*, 1998; Ogawa *et al.*, 2000). It is therefore likely that expression of SHBG in the rat and human brain is important for regulating sex hormone activity within specific cell types.

1.4.6 SHBG Expression in Other Tissues

In transgenic mice expressing rat ABP transgenes, ABP mRNA and immunoreactive rat ABP are present in tissues of the female reproductive tract including the uterus and ovary (Joseph *et al.*, 1997). In humans, SHBG mRNA has been found in the ovary (Misao *et al.*, 1998), uterine endometrium (Misao *et al.*, 1994), and placenta (Larrea *et al.*, 1993). Both SHBG mRNA and immunoreactivity have been detected in the human fallopian tube (Noe, 1999). The kidney is also a site for SHBG expresssion in the hamster (Cates and Damassa, 1997), and in transgenic mice expressing human *SHBG* transgenes (Jänne *et al.*, 1998). The latter study demonstrated that human SHBG is produced in the epithelial cells of the proximal convulated tubule, and a fully functional SHBG can be detected in the urine of these mice. The function of SHBG expression in the kidney is not known, but could be important in the rodent kidney which is well known to be a classic androgen target tissue (Catterall *et al.*, 1986).

Variant human SHBG transcripts lacking the exon 7 sequence have been found in many human cancer tissues including breast carcinoma (Murayama *et al.*, 1999; Moore *et al.*, 1996), cervical cancer (Misao *et al.*, 1997), and well as prostate (Mercier-Bodard *et al.*, 1991; Plymate *et al.*, 1991). The function of the proteins produced from these variant SHBG mRNAs is unknown, but Misao and co-workers (1998a, 1998b) demonstrated that the abundance of these transcripts increases in malignant tissues as the state of histological dedifferentiation advances.

1.5.1 SHBG Function

1.5.1 Regulation of Sex Steroid Hormone Bioavailability

Serum SHBG binds androgens and estrogens with high affinity, and a primary function of SHBG is to modulate the bioavailability and transport of these sex steroids in the blood (Siiteri *et al.*, 1982). Human SHBG interacts with DHT with highest affinity ($K_d = 5 \times 10^{-10}$), and has a 5-fold lower affinity for testosterone and a 20-fold lower affinity for estradiol when compared to DHT (Dunn *et al.*, 1981; Westphal, 1986). The metabolic clearance rate (MCR) of these steroids is inversely proportional to their affinity for SHBG (Siiteri *et al.*, 1982), and there is a significant negative correlation between serum SHBG levels and the relative amounts of free testosterone and estradiol

(Anderson, 1974) that are considered to be accessible to target cells (Siiteri *et al.*, 1982). Accordingly, fluctuations in serum SHBG levels under normal conditions, or in response to pathological states alter the distribution of sex steroids in the blood, and control the exposure of target tissues to these steroids.

1.5.2 Sequestration Within Target Tissues

Immunoreactive SHBG accumulates within the stromal compartment of various sex steroid dependent tissues such as the breast, prostate and uterine endometrium (Bordin and Petra, 1980; Sinnecker *et al.*, 1988; Sinnecker *et al.*, 1990). It is of interest that the concentrations of sex steroids within these tissues are elevated considerably when compared to circulating levels. For example, in normal breast tissue, estradiol levels are twenty fold greater than in peripheral blood (Santen, 1986). Although this may represent the local production of estradiol by aromatase within the breast, the accumulation of SHBG within the stromal compartment may play an active role in the elevation of estradiol levels in this tissue. In support of this, in female rats which do not contain circulating SHBG, pretreatment with human SHBG increased the uptake of [^{3}H]-estradiol in the uterus and oviducts coincident with an accumulation of SHBG in these structures (Noe *et al.*, 1992). This sequestration of SHBG by the interstitial compartment of various sex steroid responsive tissues may therefore represent a mechanism by which SHBG facilitates the transport of sex steroids to target tissues.

1.5.3 Interactions with Cell Membranes

According to the free hormone hypothesis, only the non-bound, or "free" steroid fraction gains access to target tissues by passive diffusion and mediates steroid dependent responses (Mendel, 1989). However, many studies suggest that the role of SHBG in the regulation of sex hormone action extends beyond that of a carrier and transport protein, and may contribute directly to the hormonal effect. Early evidence demonstrated that SHBG interacts with binding sites on cell membranes prepared from human decidual endometrium (Strel'chyonok *et al.*, 1984). This binding site exhibited a strong interaction with SHBG in a steroid ligand dependent manner, such that only SHBG complexed with estradiol bound the cell membranes with high affinity (K_d=10^{-12}M)

(Avvakumov *et al.*, 1986). Binding sites for SHBG have also been detected in normal breast tissue and on the membranes of MCF-7 breast cancer cells (Frairia *et al.*, 1991; Porto *et al.*, 1992a), epididymis (Porto *et al.*, 1992b), prostate (Hryb *et al.*, 1985), testis (Frairia *et al.*, 1994; Porto *et al.*, 1992b) and liver but not on lymphocytes, colonic mucosa, or muscle (Frairia *et al.*, 1992). Interestingly, when SHBG is injected in brains of female mice, sexual receptivity is increased only when SHBG is unliganded or coupled to estradiol (Caldwell *et al.*, 2000), but not when complexed with dihydrotestosterone (Caldwell *et al.*, 2002). Furthermore, this same group has demonstrated the presence of membrane binding sites for SHBG in the hypothalamus and medial preoptic area (Caldwell, 2001) which may mediate this response.

Kahn *et al.* (1990) have identified a peptide region spanning residues 48-57 within the N-terminal laminin G-like domain which is part of a putative SHBG receptor-binding domain. This region of the protein is highly conserved between species, and has the highest sequence similarity to corresponding regions of proteins containing SHBG-like domains. The assumption that this sequence can act as a receptor interacting region has recently been called into question by crystal structural data obtained from the N-terminal LG domain of human SHBG, since only 5 of the 9 amino acids in this region are exposed on the surface of the protein (Grishkovskaya *et al.*, 2000). However, the latter study suggests an alternate domain located in close proximity to the steroid binding domain that could function as a receptor interacting region and could explain the steroid dependence of SHBG interactions with cell membranes (Rudenko *et al.*, 2001). It must be noted however, that despite several affinity and signal transduction studies, the putative SHBG receptor has not been cloned, and the identity of this receptor has not been defined.

1.5.4 Signal Transduction and Cellular Effects

The current model explaining the interaction of SHBG with cell membranes details a specific series of events that are required for the initiation of signal transduction through this receptor system. Central to this theory is the concept that SHBG contains a docking site for the membrane receptor, as well as a steroid binding site. Several studies have indicated that SHBG interacts with membranes only in the unliganded state (Hryb *et*

al., 1989; Hryb *et al.*, 1990), and binding of biologically active steroid ligand inhibits this interaction presumably through ligand-dependent conformational changes in SHBG structure. Although a crystal structure of human SHBG in the absence of ligand is lacking, the N-terminal LG domain of SHBG undergoes discrete steroid-dependent structural conformations (Grishkovskaya *et al.*, 2002), and unliganded SHBG may exhibit a unique structure that could present a membrane receptor interacting domain. Upon the interaction of membrane-bound unliganded SHBG with steroid, structural changes in the SHBG molecule could therefore initiate the activation of a signal transduction cascade and the dissociation of the SHBG from the receptor (Hryb *et al.*, 1990; Khan *et al.*, 2002), or internalization of the receptor-SHBG-steroid (Porto *et al.*, 1991).

Several studies have demonstrated that an interaction between SHBG and cell membranes from MCF-7 cells (Fortunati *et al.*, 1993; Fortunati *et al.*, 1996), LNCaP prostate cancer cells (Nakhla *et al.*, 1990), as well as human and dog prostate (Nakhla *et al.*, 1994; Nakhla *et al.*, 1995), activates a signal transduction cascade involving an increase in intracellular levels of cAMP through a G-protein coupled mechanism (Nakhla *et al.*, 1999). This signal transduction cascade mediated through SHBG has been shown to have downstream biological effects. For instance, in prostate explants treated with an SHBG-estradiol complex, activation of this receptor system increased the secretion of prostate-specific antigen (PSA) in a PKA-dependent manner (Nakhla *et al.*, 1997). In addition, SHBG signaling through its receptor in the presence of steroid ligand has also been shown to influence the proliferation of cancer cell lines *in vitro* (Khan *et al.*, 2002). In MCF-7 cells, interaction of an SHBG-estradiol complex with cell membranes resulted in the accumulation of cAMP and inhibition of estradiol-induced cell proliferation in a PKA dependent pathway (Fortunati *et al.*, 1993; Fortunati *et al.*, 1996). In contrast, the growth of the human prostate cancer cell line ALVA-41 is stimulated by DHT and estradiol in the presence of SHBG (Nakhla and Rosner, 1996). This cell-type specific effect of SHBG on proliferation is likely a result of a different subset of signaling components in these cells, and selective modification of the interaction of SHBG with cell membranes in cancer tissues could be used to slow the growth of cancer.

There is evidence that SHBG is internalized by specific cell types, for example in MCF-7 breast cancer cells (Porto *et al.*, 1991), the principal cells of the epididymus (Gerard *et al.*, 1990) and spermatogenic germ cells in the monkey testis (Gerard *et al.*, 1991) through a process resembling receptor-mediated endocytosis (Gerard *et al.*, 1988; Gerard *et al.*, 1990). Although the biological function of this internalization is not known, it is believed to target the delivery of androgens or estrogens to specific responsive cell-types, and consequently to regulate the expression of sex-steroid responsive genes.

1.5.5 SHBG in the Male Reproductive Tract

1.5.5.1 Introduction

Spermatogenesis is the process through which diploid spermatogonial stem cells undergo mitotic and meiotic divisions and a dramatic morphological restructuring to form mature haploid spermatozoa. The process of spermatogenesis and sperm maturation is highly dependent on a tightly controlled balance of androgens and estrogens. In experimental animal models where this balance has been disrupted by knockout or overexpression of components of sex steroid action including estrogen receptor α (Eddy *et al.*, 1996), androgen receptors (Fritz *et al.*, 1983; Yeh *et al.*, 2002), P450 aromatase (Robertson *et al.*, 1999; Li *et al.*, 2001) and androgen-binding protein (Selva *et al.*, 2000), severe impairment of testicular function is observed. Moreover, testosterone depletion in rats by ethane dimethane sulfonate (EDS), a Leydig cell toxin, results in apoptosis of spermatocytes in stage VII of spermatogenesis (Henriksen *et al.*, 1995). Despite the importance of sex steroids in maintenance of testicular function and male fertility, very little is known about the regulation of sex hormone action within the seminiferous tubule and the mechanism by which sex steroids act to regulate spermatogenesis.

1.5.5.2 Rat ABP in the testis

Expression of SHBG is strictly conserved in the testis of all mammalian species examined to date, and the expression has been the best characterized in rat Sertoli cells (Joseph, 1994). The Sertoli cells of the seminiferous tubule provide support to developing germ cells through extensive contacts with germ cells at varying stages of

development. Sertoli cells have a cyclical function that depends on the stage of the seminiferous epithelium (Parvinen, 1993), and the levels of a number of testicular proteins vary throughout the spermatogenic cycle. Using the technique of transillumination assisted microdissection of rat seminiferous tubules, Ritzén *et al.* (1982) demonstrated that rat ABP mRNA accumulated in a spermatogenic stage dependent manner with the highest mRNA levels found in stages VII and VIII, and the ABP protein was detected in Sertoli cells at stages IX and X. The spermatogenic stage dependent regulation of ABP expression in rat Sertoli cells appears to be dependent directly on FSH (Joseph *et al.*, 1988; Reventos *et al.*, 1988), and indirectly on testosterone, presumably mediated through peritubular myoid cells (Skinner and Fritz, 1985).

In rodents, ABP/SHBG is secreted from Sertoli cells (Ritzén *et al.*, 1982) into the lumen of the seminiferous tubules and travels through the reproductive tract to the epididymus where it is internalized by luminal epithelial cells (Feldman *et al.*, 1981; Gerard *et al.*, 1988). It is thought that the function of ABP/SHBG secreted into the lumen of the seminiferous tubule is to maintain an appropriate steroid environment necessary for spermatogenesis and sperm maturation, and the regulation of androgen responsive genes in the epididymis (Turner *et al.*, 1984; Joseph, 1994). In support of this model, spermatogenic arrest and increased levels of apoptosis are observed in pachytene spermatocytes in the testes of transgenic mice over-expressing rat ABP (Selva *et al.*, 2000; Jeyaraj *et al.*, 2002). In older animals, this results in a progressive loss of fertility (Reventos *et al.*, 1993). This hypothesis needs to be more rigorously tested, and identification of the biological role for SHBG in the rodent testis awaits the generation of mice in which testicular expression of ABP has been disrupted.

1.5.5.3 SHBG in Human Testes

Very little is known about SHBG expression in the human testis, and it is generally assumed that its function and regulation are conserved phylogenetically. However, transcripts containing the exon 1 sequence that encodes the leader sequence for secretion of ABP/SHBG have not been identified in the human testis, and the presence of alternatively spliced SHBG transcripts lacking a signal for secretion in the human testis and the testes of mice that express a human *SHBG* transgene (Jänne *et al.*, 1998) argues

for a distinct role for SHBG in human testes versus testes of rodents. In this context, it is possible that the expression of secreted SHBG by Sertoli cells may be limited to species in which circulating SHBG is lacking.

Transgenic model systems have provided insight into human SHBG expression in different tissues, and are a convenient means of studying the expression of human SHBG in the testes. The testes of transgenic mice expressing a 4 kb human *SHBG* transgene, which contains all eight exons required for production of secreted SHBG, and a region of 800 bp of promoter sequence necessary for SHBG expression in the liver, contain undetectable levels SHBG mRNA by northern blotting (Jänne *et al.*, 1998). By contrast, transgenic mice expressing a rat ABP transgene containing a similar amount of promoter sequence have very high levels of rat ABP mRNA in their Sertoli cells (Reventos *et al.*, 1993) and this suggests that human *SHBG* expression in the testis may be controlled by unique gene sequences. In support of this, human SHBG transcripts are present in the testes of transgenic mice containing an 11 kb transgene that includes an alternative exon 1 sequence together with its regulatory elements (Jänne *et al.*, 1998), and the vast majority of the SHBG transcripts in the testes of these mice contain the alternative exon 1 sequence. Taken together, these data indicate that expression of the rat and human *SHBG* genes in the testes are regulated from different transcription units (see section 1.6.2), and the protein products derived from these transcripts function in distinct ways to regulate sex hormone action in the testes of different species.

The human SHBG transcripts in the 11 kb human *SHBG* transgenic mouse testis accumulate in a spermatogenic stage dependent manner, with levels reaching a peak between stages VI and IX (Jänne *et al.*, 1998). The specific cell type containing the human *SHBG* mRNA was not determined in that study, but transcripts changed localization throughout the spermatogenic cycle, from a basal location at the seminiferous epithelium to a more adluminal position, consistent with expression in germ cells. A direct role for SHBG in germ cells has been suggested by evidence that human SHBG is internalized by monkey germ cells (Gerard *et al.*, 1991) and ejaculated rabbit spermatozoa contain immunoreactive SHBG (David *et al.*, 1985). It is therefore likely that SHBG expression in the testis of rat and mouse exerts a different function than that

in other mammalian species, and any SHBG within germ cells may regulate intracellular steroid concentrations.

1.6 The Gene Encoding SHBG

1.6.1 *SHBG* Gene Structure

The human *SHBG* gene is located on the short arm of chromosome 17 (p12-p13) (Bérubé *et al.*, 1990), in a region known to be rearranged or deleted in many cancers (Baker *et al.*, 1989; Cousin *et al.*, 2000), and a distance of only 30 kb from the p53 tumour suppressor gene (Bérubé *et al.*, 1990; Kahn *et al.*, 2002). The rat and mouse *SHBG* genes are located on chromosome 10 and 11 respectively, in a region with homology to the human *SHBG* locus (Joseph *et al.*, 1991). The *SHBG* genes of all species examined to date contain eight exons separated by unusually short introns, and the consensus splice donor and acceptor sites are strictly conserved between species (Ip *et al.*, 2000; Joseph, 1994). The entire coding sequence of SHBG spans 3.2kb of genomic DNA in the human (Hammond *et al.*, 1989) and 3 kb and 2.5 kb in the rat and rabbit genes respectively (Ip *et al.*, 2000). Species-specific repetitive elements exist within intron 5 of the rat gene (Joseph *et al.*, 1988), and within intron 6 of the human *SHBG* gene (Hammond *et al.*, 1989). These repetitive elements are absent from the rabbit gene and accounts for the smaller size of the *SHBG* gene in this species (Ip *et al.*, 2000). In both human and rat *SHBG* genes, *alu*-like repetitive elements are also present within the 5'sequence flanking exon 1. Interestingly, these repetitive sequences in the *SHBG* genes precede exons which are subject to alternative splicing, and it has been suggested that these repetitive elements may influence alternative mRNA splicing (Hammond *et al.*, 1989; Joseph *et al.*, 1988). In both the human and rat *SHBG* genes, additional alternative exon 1 sequences have been located 1.5kb and 15 kb upstream of the exon 1 coding for the signal for secretion respectively (Hammond *et al.*, 1989, Joseph; 1994) (Figure 1.2), and the differential usage of these exon 1 sequences is likely under distinct tissue-specific and hormonal regulation.

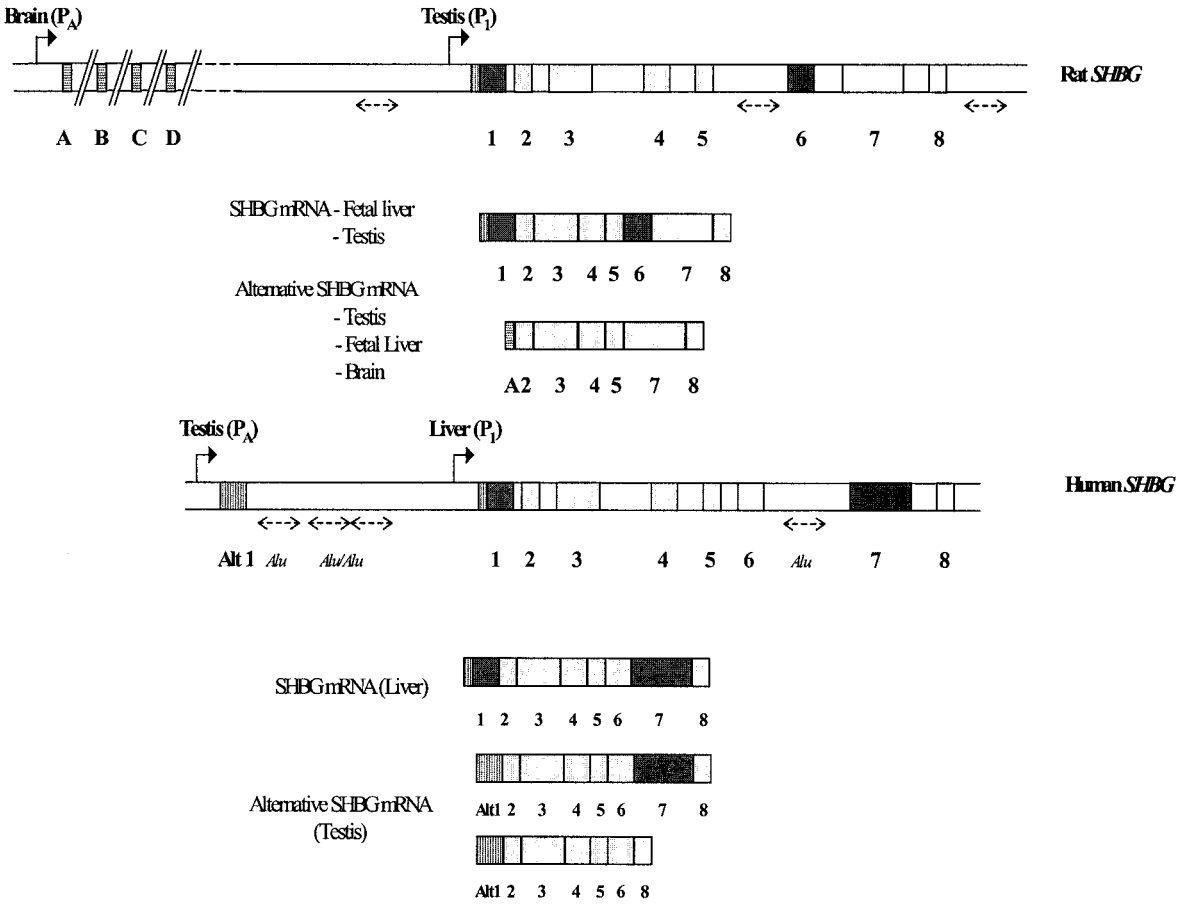

Figure 1.2 Comparison of the structural organization of the rat and human *SHBG* genes and major mRNA products. The eight exons that encode secreted SHBG/ABP are indicated numerically. The transcription start sites in liver (P_1) and testes (P_A) are shown as arrows. Alternative first exons utilized in human testis and rat brain are indicated as Alt 1, and A-D respectively. Differentially utilized exons that contain SHBG coding sequence are shaded. Species-specific repetitive elements that precede these differentially spliced exons are shown as (↔). Modified from Hammond and Bocchinfuso, 1996.

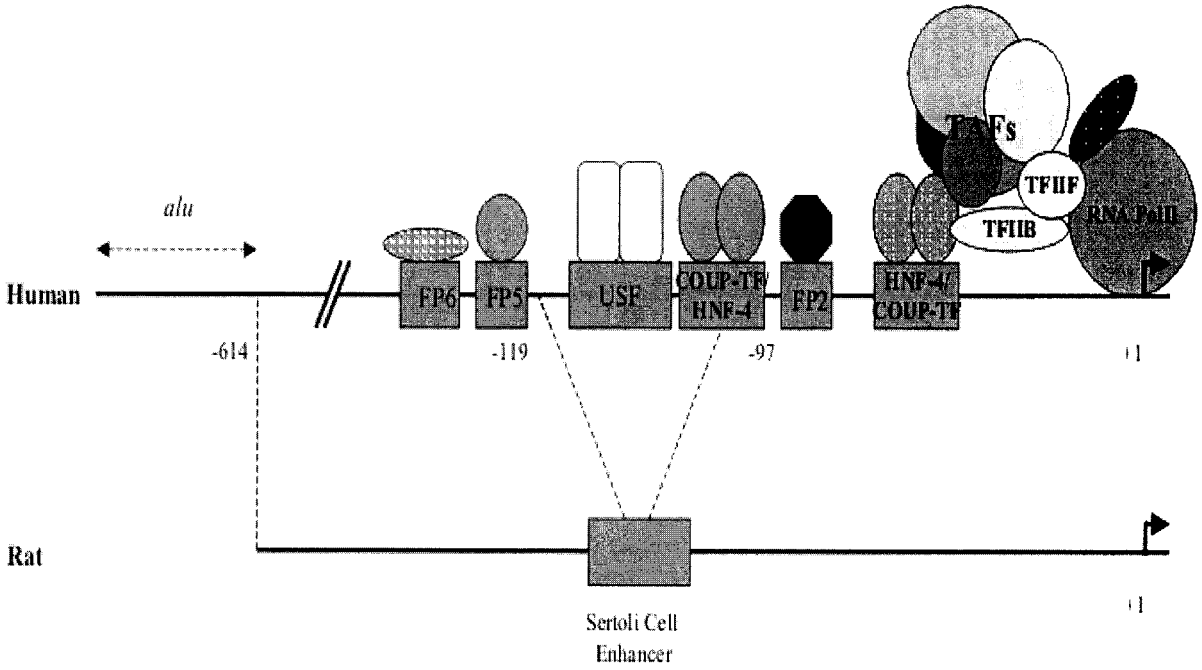

Figure 1.3 Structural comparisons of the organization of human and rat *SHBG* proximal promoters. Footprinted regions (FP's) in the human *SHBG* promoter are numbered according to Jänne and Hammond, 1998. Homology in the promoter sequences diverges 600 bp upstream of the transcription start site in the liver (+1), at which point species-specific repetitive elements are found. A 23 nucleotide region (nts -119 to –97) present in the human promoter is missing in the rat promoter. The additional sequence present in the human promoter disrupts a Sertoli cell enhancer element in present in the rat *SHBG* promoter. Two HNF-4/COUP-TF binding sites (FP 1 and FP 3) are found within the first 100 bp of *SHBG* promoter sequence, and binding of HNF-4 to a FP 1 appears to recruit the basal transcriptional machinery, and activate *SHBG* expression in hepatocytes. The 0.8 kb of promoter sequence shown in this figure are sufficient for human *SHBG* expression in mouse liver, but is incapable of driving expression in the testis (Jänne *et al.*, 1998).

1.6.2 Transcription Units

1.6.2.1 Liver Specific Transcription Unit

At least two transcription units have been identified in the human and rat *SHBG* genes (Figure 1.2), and expression of SHBG from each of these units appears to be regulated by distinct developmental, tissue-specific and hormonal cues. Expression of *SHBG* transcripts coding for secreted SHBG is regulated from a proximal promoter unit (P1) located upstream of exon 1 (figure 1.2). Comparison of the 5' flanking sequences upstream of exon 1 in the human, rat, and rabbit *SHBG* genes revealed that the *SHBG* proximal promoter does not contain a TATA-box sequence, and lacks traditional CAAT box or initiator elements commonly present in eukaryotic gene promoters (Joseph *et al.*, 1988; Hammond *et al.*, 1989; Ip *et al.*, 2000).

Comparison of the rat and human P1 promoters reveals significant sequence similarity within the first 600 bp upstream of the transcription start site, at which point the sequences diverge into species-specific repetitive elements (Jänne and Hammond, 1998). One major difference in the P1 promoter sequences between species is that 23 additional bp are present in the human *SHBG* promoter (-119 to –97) in a region which encompasses a DNaseI footprinted region (Janne and Hammond, 1998), and this contains a hepatocyte nuclear factor-4 (HNF-4) and chicken ovalbumin upstream promoter-transcription factor (COUP-TF) half site, as well as a putative binding site for the Upstream Stimulatory Factors 1-2 (USF-1,2). This difference in promoter structure (Figure 1.3) has been suggested to account for phylogenetic differences in hepatic and testicular SHBG expression, or its response to external stimuli. This is supported by the fact that the additional sequence in the human promoter disrupts a sequence in the rat promoter (-114 bp to –65 bp relative to the transcription start site in the liver) that binds Sertoli cell nuclear proteins and acts as a Sertoli cell-specific transcriptional enhancer (Fenstermacher and Joseph, 1997), and the observation that the liver-specific human *SHBG* promoter is inactive in the testis of transgenic mice (Jänne *et al.*, 1998).

The regulation of hepatic human *SHBG* transcription appears to depend primarily on the presence of a liver-specific HNF-4 binding site at –30 bp, a location where TATA-binding protein (TBP) normally binds a TATA box sequence. Binding of HNF-4 to this site replaces the need for TBP, and appears to be responsible for recruiting the basal

transcriptional machinery (Jänne and Hammond, 1998). Whereas HNF-4 acting through footprint 1 (FP1) is a strong activator of hepatic *SHBG* transcriptional activation, the ubiquitous COUP-TF antagonizes this activation, and suggests that the relative ratio of these two factors is critical for tissue-specific expression (Jänne and Hammond, 1998). Evidence for the importance of HNF-4 in regulating human *SHBG* expression in tissues is strengthened by parallels between the tissue distribution of HNF-4 expression and the human *SHBG* transgene in mice. Specifically, both HNF-4 and SHBG are expressed in the hepatocytes of the liver, the tubular epithelium of the kidney, and the intestine (Jänne *et al.*, 1998; Drewes *et al.*, 1996).

An HNF-4/COUP-TF binding site at FP3 in the human *SHBG* proximal promoter displays an equal binding affinity to HNF-4 and COUP-TF, and shows a weak responsiveness to overexpression of HNF-4 in liver cells (Jänne and Hammond, 1998). This site does not appear to contribute significantly to basal transcriptional activity, but may provide additional regulation in response to external stimuli. For example, levels of serum SHBG in humans are regulated to a large extent by metabolic status (see below). This is intriguing because fatty acids (Dhe-Paganon *et al.*, 2002) and fatty acyl thioesters have been reported to be activating ligands for HNF-4 (Hertz *et al.*, 1998).

1.6.2.2 Alternative *SHBG* transcripts

In addition to the mRNA that encodes SHBG in the liver, *SHBG* transcripts containing an alternative exon 1 sequence have been identified in fetal rat liver, and adult rat brain and testis (Sullivan *et al.*, 1991; Joseph, 1994), as well as human testis (Hammond *et al.*, 1989; Gershagen *et al.*, 1989). Multiple alternative exon 1 sequences have been located in the rat *SHBG* gene (Joseph, 1994), and are present in *SHBG* transcripts in the brain (Wang *et al.*, 1990). These alternative exon 1 sequences replace the exon 1 sequence coding for the signal for secretion of plasma SHBG or testicular ABP. However, these alternative exon 1 sequences show no similarity between species, and the functional significance of transcripts that contain alternative exon 1 sequences is largely unknown. In a single report (Joseph *et al.*, 1996), COS-7 cells transfected with a rat ABP cDNA containing an alternative exon 1 sequence (exon A, figure 1.2), produce an ABP immunoreactive protein that was not secreted but accumulated within the

nucleus. The alternative exon 1 sequence in human SHBG transcripts does not contain a codon for an initiating methionine residue, and it is not clear if these transcripts are translated.

Human *SHBG* transcripts containing the alternative exon 1 sequence are derived from the utilization of an uncharacterized alternative promoter (Hammond and Bocchinfuso, 1996). Unlike SHBG mRNA encoding the serum protein, the resultant RNA products are highly subjected to alternative splicing events, with the most common SHBG splice variants lacking exon 7 in the human transcripts (Hammond *et al.*, 1989), and exon 6 in the rat transcripts (Sullivan *et al.*, 1991). Variant human *SHBG* transcripts lacking exon 7 result in a shift in the major open reading frame which would add 9 novel residues at the carboxy-terminus followed by a premature stop codon (Hammond *et al.*, 1989). Since protein products derived from these alternative transcripts would contain all the structural elements necessary for ligand binding and dimerization (Hildebrand *et al.*, 1995), they may have a function distinct from that of SHBG in the serum which could involve regulation of sex steroid bioavailability at the cellular level. Although these transcripts were first identified in the 1989, their protein products have not been identified in tissues, and their physicochemical and biological properties remains obscure.

1.7 Regulation of Human SHBG Expression

1.7.1 Introduction

The blood levels of human SHBG are highly variable between individuals, and this may reflect the modulation of SHBG expression by a large number of factors, including age, genetics, nutrition and metabolic status. Plasma SHBG levels are primarily determined by its rates of synthesis and secretion by the liver, as well as the biological clearance rate. Studies of the regulation of SHBG in response to treatments have been limited to experiments performed *in vitro* in HepG2 cells, and measurements of changes in serum SHBG levels in response to treatments in clinical studies. Although valuable insight into the regulation of hepatic SHBG expression has been obtained through these approaches, our understanding of how SHBG synthesis by the liver is controlled at the molecular level is limited.

1.7.2 Regulation of SHBG Expression by Sex Steroids

Evidence that SHBG expression in the liver is regulated by sex steroids draws mostly from how SHBG production by HepG2 cells responds to treatments *in vitro*. However, HepG2 cells may not accurately reflect responses of normal hepatocytes because they lack critical mediators of cellular signaling, such as the estrogen receptors (Barkhem *et al.*, 1997) and the Glut2 transporter (Kim and Ahn, 1998). Nevertheless, estradiol (Lee *et al.*, 1987; Plymate *et al.*, 1988; Loukovaara *et al.*, 1995a) and estrogenic isoflavones (Mousavi and Adlercreutz, 1993; Loukovaara *et al.*, 1995b) modestly increase SHBG production and secretion by HepG2 cells. Serum SHBG levels are increased in both men and women upon oral administration of estrogens (Anderson, 1974; van Look and Frolich, 1981; Helgason *et al.*, 1982), and SHBG is increased in a dose-dependent manner in women taking oral contraceptives containing synthetic estrogens (Mandel *et al.*, 1982; Mathur *et al.*, 1985). Moreover, serum SHBG levels in pregnancy are elevated five to ten fold when compared to levels in non-pregnant women (Moore and Bulbrook, 1988), presumably as a result of elevated plasma estrogen concentrations.

Studies looking at the effect of androgens on SHBG secretion *in vivo* and in cell culture models are inconclusive. Male athletes taking anabolic steroids show a 90% decline in serum SHBG levels (Ruokonen *et al.*, 1985). Moreover, serum SHBG concentrations are consistently decreased in hyperandrogenic patients (Toscano *et al.*, 1992), and are increased in men with testicular insufficiency (Anderson, 1974). However, when treated with androgens *in vitro*, the response of SHBG secretion in HepG2 is inconsistent, with some studies demonstrating no effect (Rosner *et al.*, 1984), and others showing an increased secretion (Lee *et al.*, 1987; Plymate *et al.*, 1988). Further, in castrated monkeys treated with testosterone, SHBG levels in the blood decreased coincident with an increase in SHBG mRNA in the liver (Kottler *et al.*, 1990). Because androgen concentrations in the blood correlate poorly with serum SHBG levels under most physiological and pathological conditions, a direct role for androgens in the repression of SHBG production *in vivo* has been questioned (Toscano *et al.*, 1992).

Although adminstration of exogenous androgens and estrogens have clear effects on serum SHBG levels in clinical studies *in vivo*, evidence that this involves direct effects

on *SHBG* transcription in the liver is lacking, and other factors may be involved. For instance, the metabolic clearance rate (MCR) of thyroxine-binding globulin (TBG) is decreased with increasing sialylation, a process which is up-regulated by estrogens (Ain *et al.*, 1987). Based on differences in carbohydrate composition between SHBG in males and females (Tardivel-Lacombe and Degrelle, 1991; Danzo *et al.*, 1991), it is therefore conceivable that steroid dependent changes in SHBG glycosylation may influence the clearance rate of the protein (Joseph, 1994).

1.7.3 SHBG Regulation by Thyroid hormone

A direct relationship between serum SHBG levels and thyroid hormone status was one of the first clinical observations showing that SHBG levels are under hormonal control (Anderson, 1974). Changes in thyroid hormone status during development influences SHBG levels in the blood, and the rapid increases in serum SHBG levels during the first days of postnatal life are suggested to result from an increase in thyroid hormone activity at this time (Leger *et al.*, 1990). In support of this, serum SHBG levels are a sensitive marker for abnormalities in thyroid function. They are decreased in hypothyroidism and are elevated in cases of thyrotoxicosis (Anderson, 1974), and are a useful diagnostic parameter to differentiate between patients with thyrotoxicosis and thyroid hormone resistance where SHBG levels are normal. Although SHBG mRNA and SHBG secretion from HepG2 cells are increased by thyroid hormone (Rosner *et al.*, 1984; Mercier-Bodard and Baulieu, 1986) the mechanism responsible for this regulation is not known.

1.7.4 SHBG Regulation by Peptide hormones

Insulin-like growth factor-I (IGF-I) has been proposed to be an important regulator of SHBG levels in humans (von Schoultz and Carlström, 1989). Changes in serum SHBG levels in development correlate better with IGF-I than steroid hormones (Toscano *et al.*, 1992) and a significant negative correlation between serum levels of SHBG and IGF-I has been demonstrated (Rudd *et al.*, 1986). Further, SHBG production by HepG2 cells is down-regulated by IGF-I (Singh *et al.*, 1990).

Other peptide hormones influence hepatic SHBG production, and associations between elevated serum prolactin and decreased serum SHBG levels have been reported. For example, serum SHBG are decreased in hyperprolactinemic patients (Vermeulen *et al.*, 1982), and return to normal in these patients upon treatment (Lobo and Kletzky, 1983). In cell culture, prolactin decreased SHBG production and secretion by HepG2 cells (Plymate *et al.*, 1988). As well, several clinical observations suggest a negative regulation of serum SHBG levels by growth hormone. For example, serum SHBG levels are reduced by 40% in patients with acromegaly (De Moor *et al.*, 1972), and are two-fold higher in children with growth hormone deficiency than in age-matched controls. Treatment with growth hormone significantly reduces SHBG levels in these patients (Belgorosky *et al.*, 1987).

1.7.5 Regulation of SHBG Expression by Insulin

It is believed that the hyperandrogenic state characteristic of the polycystic ovarian sydrome (PCOS) is a primary cause of decreased serum SHBG levels (Plymate *et al.*, 1981). However, hyperinsulinemia is common among these patients, and the hyperinsulinemic insulin resistance of women with PCOS exerts a causal and direct effect on the hyperandrogenism (Nestler and Strauss III, 1991) and reduced SHBG levels (Nestler *et al.*, 1991). Moreover, unlike the effects of androgens on SHBG production *in vitro*, studies performed in HepG2 cells have demonstrated a direct effect of insulin on decreasing the levels of SHBG mRNA in these cells, and the amounts of SHBG they secrete into the media (Crave *et al.*, 1995; Plymate *et al.*, 1988).

In humans, SHBG levels are negatively correlated with fasting insulin levels in healthy women (Preziosi *et al.*, 1993), and are significantly decreased in patients with insulin resistance (Pugeat *et al.*, 1991). Moreover, low SHBG levels are an early predictor for the predisposition to premature cardiovascular disease and non-insulin dependent (Type II) diabetes mellitus (Ibañez *et al.*, 1997; Ibañez *et al.*, 1998; Lapidus *et al.*, 1986; Lindstedt *et al.*, 1991). Changes in insulin secretion may also explain the association between SHBG and metabolic status (see section 1.7.7). Obesity increases pancreatic insulin secretion and ovarian androgen production, and is associated with significantly reduced serum SHBG levels (Peiris *et al.*, 1989). As well, it is well

Regulators of *SHBG* Expression	Effect on *SHBG* Expression	Reference
Hormonal Regulators		
Androgens	Increase (*in vitro*)	Plymate *et al.*, 1988
	No effect (*in vitro*)	Rosner *et al.*, 1984
Hyperandrogenism	Decrease	Toscano *et al.*, 1992
Estrogens	Increase (*in vitro*)	Plymate *et al.*, 1988
	Increase (*in vivo*)	Mandel *et al.*, 1982
Thyroid hormone	Increase (*in vivo*)	Anderson, 1974
	Increase (*in vitro*)	Plymate *et al.*, 1988
Dietary and Metabolic Factors		
Metabolic Status		
Obesity	Decrease	Apter *et al.*, 1984,
Anorexia nervosa	Increase	Estour *et al.*, 1986
Hyperinsulinemia	Decrease	Pugeat *et al.*, 1991
Dietary Factors		
Dietary phytoestrogens	Increase (*in vitro*)	Mousavi and Adlercreutz, 1993
	Increase (*in vivo*)	Adlercreutz *et al.*, 1987
Dietary fibre	Increase	Adlercreutz *et al.*, 1987
	Decrease	Goldin *et al.*, 1994
Protein intake	Decrease	Longcope *et al.*, 1987, 2000
	Increase	Vermeulen *et al.*, 1996
Carbohydrate	No effect	Longcope *et al.*, 2000
Dietary lipid	Decrease	Reed *et al.*, 1987

Table 1.1 Steroidal, hormonal and metabolic regulators of *SHBG* expression.

established that insulin stimulates ovarian androgen production in girls at puberty (Rosenfield, 1990) and produces a hyperandrogenic state that may further reduce serum SHBG levels in pre-menopausal women (Nestler *et al.*, 1991). However, the contribution of sex steroids to the regulation of SHBG in obese men and women is questioned by the observations of low SHBG levels in obese post-menopausal women (O'Dea *et al.*, 1979), and in obese men with low serum testosterone levels (Pasquali *et al.*, 1995). Despite a large body of literature suggesting an important role for insulin in the regulation of serum SHBG levels in normal and pathological conditions, the mechanism by which insulin regulates SHBG expression is not known.

1.7.6 SHBG and Diet

Studies addressing the effect of dietary constituents on SHBG levels in serum have been conflicting, and this is probably due to the different subject groups used. Perhaps the most significant effect of diet on SHBG production is the effect of the intake of dietary phytoestrogenic compounds. Increased intake of dietary phytoestrogens (Adlercreutz *et al.*, 1987) and isoflavones in post-menopausal women results in modest increases in serum SHBG levels (Duncan *et al.*, 1999; Pino *et al.*, 2000), and this increase in SHBG levels is consistent with an estrogenic response on hepatic SHBG production. Dietary fiber intake is generally associated with increased SHBG levels (Adlercreutz *et al.*, 1987; Longcope *et al.*, 2000), whereas other reports show that increased fiber intake decreases SHBG levels (Goldin *et al.*, 1994).

The effects of dietary protein intake are also inconsistent, with studies demonstrating that increased protein intake is associated with increased SHBG levels (Vermeulen *et al.*, 1996), whereas most studies show a negative association with protein intake (Longcope *et al.*, 1987; Longcope *et al.*, 2000; Anderson *et al.*, 1987). It is known that protein intake increases insulin levels (Rabinowitz *et al.*, 1966), and it has been suggested that increased insulin levels in response to protein intake are responsible for the decreased SHBG (Longcope *et al.*, 2000). It is surprising that there does not appear to be significant effect of carbohydrate intake on SHBG levels (Longcope *et al.*, 2000) because carbohydrate intake is a stimulus for insulin secretion, and this should affect SHBG levels. Dietary lipid intake is negatively correlated with SHBG levels (Longcope

et al., 2000), and decreases in SHBG levels are observed in normal men fed a high fat diet, while a low fat diet increases SHBG levels (Reed *et al.*, 1987).

1.7.7 SHBG and Metabolic Status

Body mass index (BMI) is one of the most consistent clinically reported determinants of SHBG levels in individuals (Glass *et al.*, 1977; Plymate *et al.*, 1981; Apter *et al.*, 1984). Serum SHBG levels are high in patients with anorexia nervosa (Estour *et al.*, 1986), and can be lowered by increasing caloric intake (Barbe *et al.*, 1993). Moreover, a very low calorie diet fed to patients with polycystic ovarian syndrome (PCOS) increase SHBG levels (Franks *et al.*, 1991). Serum SHBG levels are low in obese men, women and children (Plymate *et al.*, 1981; Glass *et al.*, 1977; Apter *et al.*, 1984), and are increased by weight loss (O'Dea *et al.*, 1979; Enriori *et al.*, 1986). This observation is of interest because girls with precocious menarche have lower SHBG levels than girls with later menarche and there is an inverse relationship between BMI and age at menarche (Apter *et al.*, 1984). At the present time it is not known whether levels of serum SHBG are modulated by dietary constituents, or are regulated in response to changes in levels of metabolic regulators in these conditions.

1.7.8 Genetic Polymorphisms

The physiological importance of SHBG expression is underscored by the fact that despite millions of human serum SHBG samples analyzed worldwide, only a single case of an apparent SHBG deficiency has been described (Ahrentsen *et al.*, 1982). It is also unclear whether this report documents subjects with a total lack of SHBG because the sensitivity of the assay used to measure serum SHBG was only 10 nM (Ahrentsen *et al.*, 1982), and serum SHBG levels below this are often detected in clinical samples using more sensitive assays (Hammond *et al.*, 1985). The underlying cause of very low serum SHBG levels, especially in normal weight individuals is unknown, but low SHBG levels are inherited within families (Gross and Horton, 1971), and SHBG levels are more similar within groups of brothers, than among non-brothers (Meikle *et al.*, 1982).

Studies of polymorphic variations in the human *SHBG* gene have been limited. The first characterized variation explains an alteration in SHBG size and isoelectric properties (Van Baelen, 1992). The genetic basis of this first variation in SHBG coding sequence is a single nucleotide substitution (G to A) polymorphism in exon 8 of the human SHBG gene that causes an aspartic acid to asparagine amino acid substitution at amino acid 327 (Power *et al.*, 1992). This variation disrupts *Hinf*I and *BbsI* restriction endonuclease sites within exon 8, and introduces an novel N-linked glycosylation site in the extreme C-terminus of the protein. The resultant disruption of restriction endonuclease sites in exon 8, and changes in the electrophoretic properties of the variant protein associated with an additional carbohydrate chain have facilitated population screening for this single nucleotide polymorphism (SNP). Using a polymerase chain reaction (PCR)-based restriction fragment length polymorphism (RFLP) screening procedure, Cousin *et al.*, 1998 demonstrated that approximately 8% of the population are carriers of this SNP. The SHBG encoded by this genetic variant has no change in steroid binding characteristics (Power *et al.*, 1992), but the additional carbohydrate chain results in a modest increase in the plasma half-life of the protein when injected into rabbits, and may increase its accumulation in the blood (Cousin *et al.*, 1998). Although the occurrence of this polymorphism in individuals does not appear to be clearly associated with any disease state (Cousin *et al.*, 1998), a single report has suggested a link with the estrogen-dependence of breast cancer (Becchis *et al.*, 1999).

An additional polymorphism in the human *SHBG* gene was demonstrated as a *Msp*I RFLP with an allele frequency of 0.04 in the French-Canadian population (Vohl *et al.*, 1994). However, there is no information about the localization of the polymorphism in the human *SHBG* sequence, and its effect on SHBG production or function are unknown.

1.8 Objectives of the Present Study

The processes that regulate SHBG levels in the blood, as well as the expression and sequestration of SHBG by other tissues and cell types are poorly understood. However, these processes are critical in the modulation of sex steroid hormone action at various levels, and changes in the concentrations of SHBG in the blood during

development and as a result of genetic, hormonal, and dietary factors, have obvious implications on sex steroid hormone action and their involvement in the etiology of disease processes. Although there is ample evidence that SHBG gene expression is regulated by various stimuli and conditions, the molecular mechanisms responsible for this remain obscure. As well, the emergence of novel SHBG mRNA products and protein isoforms with functions distinct from the serum protein will provide new insight into the function of SHBG in tissues such as the brain and testis.

This study aims to characterize the molecular mechanisms that control human SHBG expression. The working hypothesis that guided these studies is that the regulation of human SHBG levels in the blood, as well as the regulation and production of novel SHBG isoforms within specific cell types in the brain and testicular cells will together act to provide intricate modulation of sex steroid hormone action. Moreover, these studies aim to clarify the genetic determinants that regulate levels of SHBG in the blood and within sex steroid responsive tissues in normal and pathological conditions. This regulation of SHBG is critical to provide an appropriate physiological response to these hormones. A combination of *in vitro* and *in vivo* approaches were used to address the following objectives:

1) The identification of regions in the human *SHBG* gene that are important in mediating the a) tissue-specific expression, b) developmental regulation, and c) the sexual dimorphic expression of human *SHBG* transgenes in the liver and kidney of mice.
2) The identification and characterization of human SHBG isoforms in the testes of transgenic mice.
3) The identification and characterization of *cis* acting elements within an 800 bp region of the human *SHBG* promoter that influence human *SHBG* expression *in vitro*.
4) The determination how the *SHBG* gene responds to metabolic status, and the identification of regions of the *SHBG* gene that regulate this response.
5) Identification and characterization of variations in the coding sequence of the human *SHBG* gene which are involved in the etiology and pathology of several reproductive conditions.

1.9 References

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CHAPTER 2

EXPRESSION AND REGULATION OF HUMAN SEX HORMONE-BINDING GLOBULIN TRANSGENES IN MICE DURING DEVELOPMENT

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2.1 Introduction

The biological activities of testosterone and estradiol are regulated by sex hormone-binding globulin (SHBG), a plasma glycoprotein that influences the access of sex steroids to target tissues (Hammond, 1995). Hepatocytes are the main source of plasma SHBG (Khan *et al.*, 1981; Jänne *et al.*, 1998), and changes in the expression of the gene (*SHBG*) encoding plasma SHBG in the liver may influence sex steroid hormone-dependent processes during fetal and postnatal development. Little is known about *SHBG* expression during fetal life in mammals, but it occurs transiently in fetal rat livers at a time when sex steroids influence the growth and development of reproductive tissues (Sullivan *et al.*, 1991; Wilson, 1978, Bardin and Catterall, 1981; vom Saal *et al.*, 1997) and the expression of specific genes that may have long lasting consequences persisting throughout life (vom Saal *et al.*, 1997; Joseph *et al.*, 1987). Rodents do not express *SHBG* in the liver postnatally (Sullivan *et al.*, 1991; Joseph *et al.*, 1987), and only trace amounts of SHBG from gonadal sources can be detected in the blood of adult rats (Gunsalus *et al.*, 1978). In other mammals, including humans, changes in plasma SHBG levels occur at puberty (Apter *et al.*, 1984), and abnormal plasma SHBG levels are associated with human diseases caused by inappropriate sex steroid hormone exposure (Anderson, 1974).

Expression of *SHBG* in Sertoli cells gives rise to the testicular form of SHBG known as the androgen-binding protein (ABP), which is secreted into the lumen of seminiferous tubules and is believed to promote sperm maturation in the male reproductive tract (Joseph, 1994). In addition to a messenger RNA (mRNA) for ABP, several differentially spliced *SHBG* transcripts originating from an alternative upstream promoter, have been identified in human testis (Hammond *et al.*, 1989). These alternative *SHBG* transcripts lack the coding sequence for the signal polypeptide required for SHBG or ABP secretion, and they accumulate in a spermatogenic stage-dependent manner within testicular cells of mice carrying and expressing human 11kb (kilobase) *SHBG* transgenes (Jänne *et al.*, 1998).

Hepatocytes and Sertoli cells are major sites of *SHBG* expression, but *SHBG* transcripts have been identified in several other tissues (Larrea *et al.*, 1993; Wang *et al.*, 1990, Misao *et al.*, 1997) including hamster kidneys (Cates and Damassa, 1997) and the kidneys of mice expressing human *SHBG* transgenes (Jänne *et al.*, 1998). In these mice,

expression of the transgenes results in the production and secretion of appreciable amounts of immunoreactive human SHBG, which concentrates at the luminal surface of epithelial cells lining the proximal convoluted tubules (Jänne *et al.*, 1998). The SHBG excreted in their urine retains its steroid-binding properties (Jänne *et al.*, 1998), but the physiological role of *SHBG* expression in the mouse and hamster kidney, and whether it is expressed in human kidney, remains to be determined.

To learn more about the tissue-specific expression and regulation of human *SHBG*, two regions of the human *SHBG* locus were introduced as transgenes into the mouse genome, and their activities have been monitored during fetal and postnatal development. The smallest of these transgenes spans 4.3 kb of human *SHBG* and comprise the entire coding sequence for the SHBG precursor polypeptide, as well as ~0.8 kb of promoter sequence containing a number of *cis*-active elements that may regulate liver and kidney specific expression (Jänne and Hammond, 1998). Several other lines of *SHBG* transgenic mice that carry a much larger (~11 kb) region of human *SHBG* were also studied because these transgenes include an alternative promoter sequence used in the testis of mature animals (Jänne *et al.*, 1998). Moreover, we reasoned that any differences in the spatial and temporal patterns of expression or hormonal regulation of these two different human *SHBG* transcription units might reveal the location of important regulatory sequences.

2.2 Materials and Methods

2.2.1 Animals and treatments

Transgenic mouse lines containing approximately 11 kb (*SHBG*11-a and *SHBG*11-b) or 4.3 kb (*SHBG*4-a and *SHBG*4-b) regions of the human *SHBG* locus have been characterized and genotyped previously (Jänne *et al.*, 1998). Animals were housed under standard conditions and provided with food and water *ad libitum*. At defined ages, or after endocrine manipulations, animals were sacrificed to obtain tissue samples for RNA analysis and blood for serum preparation. All procedures were approved by the Animal Care Committee of the University of Western Ontario (London, Canada).

2.2.2 *In situ* hybridization

Sense and antisense human SHBG riboprobes were transcribed using commercially available reagents (Promega) in the presence of [^{35}S]UTP (Dupont Canada, Mississauga, Canada) from a 0.7 kb 5' *Eco*RI fragment of a human SHBG cDNA in a pT3/T7mp18 vector (Hammond *et al.*, 1987). The protocol for *in situ* hybridization with ^{35}S-labeled riboprobes was performed as described previously (Scrocchi *et al.*, 1993). Briefly, rehydrated tissue sections (4 μm) were treated sequentially with phosphate-buffered saline (PBS) containing 0.2 % Triton X-100, and then incubated with 10 μg proteinase K/ml in 50 mM Tris, 50 mM ethylenediaminetetraacetic acid (EDTA) at 37°C for 30 min, followed by two washes in 2 x SSC (1 x SSC = 0.15 M NaCl and 0.015 M sodium citrate). After postfixation in PBS containing 4 % paraformaldehyde for 30 min, sections were washed in PBS and treated with 0.1 M triethanolamine containing 2.6 mM acetic anhydride at room temperature for 10 min. Sections were then dehydrated through increasing concentrations of ethanol (70 % - 100 %) and air-dried.

Sections were prehybridized in a solution containing 50 % formamide in 2 x SSC for 2 h at 42 C, followed by hybridization with the ^{35}S-labeled riboprobe (1 x 10^6 cpm/100 μl buffer/slide) in a buffer containing 50 % formamide, 2 x SSC, 1 x Denhardt's reagent, 0.1 mg/ml salmon sperm DNA, 0.5 mg/ml yeast tRNA, 0.1 % sodium dodecylsulphate (SDS), 100 mM dithiothreitol (DTT), and 10 % dextran sulfate at 55°C for 20 h. After non-hybridized riboprobes were removed by washing with 50 % formamide and 10 mM DTT in 2 x SSC at 65°C for 25 min, sections were then incubated with 30 μg ribonuclease A/ml 2 x SSC for 30 min at 37°C, followed by washes in 50 % formamide at 65°C for 30 min, 2 x SSC at 30°C for 20 min, and 0.1 x SSC for 20 min. To detect hybridized probes, slides were first subjected to autoradiography by overnight exposure to x-ray film (DuPont), and were then coated with NTB-2 emulsion (Eastman Kodak, Rochester, NY), stored for 1 week at 4 C, and developed in D19 developer (Eastman Kodak). Sections were counter-stained with Harris' hematoxylin to identify the cellular location of silver grains.

2.2.3 Western blot analysis

Diluted mouse serum samples were heat-denatured in loading buffer and subjected

to discontinuous SDS-polyacrylamide gel electrophoresis (SDS-PAGE) with 4% and 10% polyacrylamide in the stacking and resolving gels, respectively. Proteins in the gel were transferred (Towbin *et al.*, 1979) to Hybond ECL nitrocellulose membranes (Amersham Biosciences, Baie d'Urfé, Canada). The membranes were pre-incubated in a 5% skim milk solution, and then incubated overnight at 4°C with primary antisera diluted in TBS-T (10 mM Tris-HCl, pH 7.5, 150 mM NaCl, 0.05% Tween 20) containing 1% skim milk powder. The blots were then washed several times in TBS-T to remove excess antibody, and specific antibody-antigen complexes were identified using a second antibody (horseradish peroxidase-labeled donkey anti-rabbit IgG) and chemiluminescent substrates (Invitrogen, Burlington, Canada) by exposure to x-ray film. The primary antisera used were raised in rabbits against pure human SHBG (Hammond *et al.*, 1985) or against mouse SHBG expressed as a glutathione-S-transferase fusion protein in *E. coli* by using a pGex2T expression vector (Amersham Biosciences). The mouse SHBG was released from the GST-fusion protein by thrombin cleavage and purified by glutathione-Sepharose chromatography for use as an immunogen, as recommended by Pharmacia Biotech.

2.2.4 Serum SHBG and testosterone measurements

The concentrations of human SHBG in transgenic mouse serum were determined using a saturation ligand-binding assay (Hammond and Lähteenmäki, 1983). In this assay, endogenous steroids were first removed from serum samples by dilution (1:100) in a dextran-coated charcoal (DCC) suspension and incubation (30 min) at room temperature. Samples were then further diluted (1:10-1:20) and incubated (1 h) at room temperature with 10 nM 5α-[^{3}H]dihydrotestosterone (Amersham Biosciences) followed by an additional incubation (30 min) at 0 C. Non-specific binding was estimated in the presence of a 400 molar excess of non-radioactive 5α-dihydrotestosterone. Free ligand was removed by incubation (10 min) with an ice cold DCC slurry and separation by centrifugation. Supernatants containing SHBG bound ligand were taken for radioactivity measurements to determine serum SHBG concentrations by assuming one steroid ligand bound per molecule of SHBG (Hammond and Lähteenmäki, 1983). Serum testosterone concentrations were determined using a radioimmunoassay kit (Orion Diagnostica, Oulunsalo, Finland).

2.2.5 Tissue RNA analysis

Total RNA isolated from mouse tissues using TRIzol reagent (Invitrogen) was separated by electrophoresis on a 1% agarose gel in the presence of formaldehyde, and transferred to a Zeta Probe nylon membrane (BioRad, Mississauga, Canada). Membranes were hybridized with either a ^{32}P-labeled human SHBG cDNA (556 bp 3' *Eco*RI fragment) or a ^{32}P-labeled mouse SHBG cDNA (535 bp 3' *Eco*RI fragment) in a solution containing 0.5 M sodium phosphate, pH 7.3 and 7 % SDS at 65°C for 18 h. This was followed by high-stringency washing with a 40 mM sodium phosphate buffer containing 1 % SDS at 65°C, and exposure to x-ray film. The blots were then stripped of hybridized ^{32}P-labeled SHBG probes by two 20 min incubations in 0.1 x SSC, 0.5 % SDS at 95°C. After overnight exposure to x-ray film to ensure complete removal of probe, membranes were re-probed with a ^{32}P-labeled cDNA for 18S ribosomal RNA as a control for loading and transfer. The relative intensities of radiographic signals obtained for human *SHBG* and 18S transcripts were compared by densitometry.

We also used total RNA isolated from brain and testis to further analyze the human *SHBG* transcripts present in these tissues. To accomplish this, reverse transcription (RT) was performed at 42°C for 50 min using 3 μg of total RNA and 200 U of Superscript II together with an oligo-dT primer and reagents provided by Invitrogen. An aliquot (1 μl) of the RT product was amplified in a 20 μl reaction in the presence of 1 U Taq polymerase, 0.05 mM $MgCl_2$, 1.25 μM of each dNTP and 0.2 μM of each oligonucleotide primer. For this purpose, we used an oligonucleotide corresponding to a 5' sequence (5'-GCGGTTCAAAGGCTCCC) in the *SHBG* alternative exon 1, or a primer specific to a sequence in exon 1 (5'-GAGTTGTCTGAGCCGCCG), together with a reverse primer complementary to a sequence (5'-TGGCTTCTGTTCAGGGCC) within exon 8 of the human *SHBG* gene (Hammond *et al.*, 1989). The PCR was performed for 40 cycles at 94°C for 30 min; 65°C for 30 sec and 72°C for 1 min. PCR products were cloned using the PCR Blunt II TOPO Cloning kit (Invitrogen), and plasmids containing PCR products were sequenced.

2.3 Results

2.3.1 Expression of human *SHBG* transgenes in fetal mice

The tissue distribution of human *SHBG* transcripts in an *SHBG*11-b fetal transgenic mouse was assessed at 17.5 days *post coitum* (dpc) by *in situ* hybridization, and this demonstrated that *SHBG* transcripts are present in the fetal gut as well as the liver, but are absent in the kidney (Figure 2.1). Examination of the *in situ* hybridization of the antisense complementary RNA probe under high power (x 63) revealed that the signal was distributed throughout the liver, while in the gut it was confined to the epithelial cells of the duodenum (not shown).

The relative abundance of *SHBG* transcripts in wild-type and transgenic fetal mouse livers was examined during late gestation by Northern blotting (Figure 2.2), and was compared with the levels of mouse and human SHBG in serum samples taken from late fetal mice at the same gestational ages (Figure 2.2). In liver extracts of *SHBG* transgenic mice, the major *SHBG* transcript is approximately 1.6 kb in size (Figure 2.2). An additional transcript of approximately 2.5 kb is also detected using human and mouse SHBG cDNA probes, and this is believed to represent a partially processed transcript (Hammond *et al.*, 1989). These data indicate that expression of murine *SHBG* declines in the fetal liver from day 16 of gestation to birth together with a corresponding decline in amounts of SHBG in the blood of the wild-type neonatal mice. Differences were observed in the relative amounts of mouse SHBG present in serum from wild type and *SHBG* transgenic mice at 17dpc (Figure 2.2) and this was confirmed in a repeat experiment. It is therefore possible that expression of *SHBG* transgenes influences expression of mouse *SHBG* in the liver. More importantly, the expression of both the *SHBG*11 and *SHBG*4 transgenes in the fetal liver increases between day 17 of gestation and term (Figure 2.2 A), with greater amounts of both human *SHBG* transcripts in the liver, and human SHBG in the blood of the neonatal mice expressing the 11kb *SHBG* transgene (Figure 2.2 B). Interestingly, human SHBG is present in the serum of *SHBG*4-a fetal mice at 17 dpc (Figure 2.2 B) while human SHBG mRNA is undetectable in the liver at this stage (Figure 2.2 A), indicating that the transgene is probably expressed in the fetus at some earlier stage of gestation. Although the antiserum against mouse SHBG appears to cross-react partially with human SHBG (see Figure 2.2 B,

*shbg*11-b d0 sample), there is a clear size difference between human and mouse SHBG that allows the specific recognition of mouse SHBG in serum samples from fetal *SHBG* transgenic mice (Figure 2.2 B). This size difference can be attributed to the fact that mouse SHBG has three sites for N-glycosylation whereas human SHBG has only two N-linked carbohydrate chains (Hammond, 1993).

2.3.2 Postnatal ontogeny and sexual dimorphic expression of human *SHBG* transgenes in the liver

At the dilutions of serum used for saturation analysis, SHBG was undetectable in samples from age-matched wild-type mice, and our measurements of human SHBG in transgenic mouse serum samples were therefore unaffected by any other steroid-binding protein (eg. alpha-fetoprotein) that may have been present at different stages of mouse development. Moreover, as the liver is the major site of plasma SHBG biosynthesis (Jänne *et al.*, 1998), measurements of human SHBG concentrations in blood samples provides a convenient means of monitoring the hepatic expression of different *SHBG* transgenes in relatively large numbers of transgenic animals.

The serum levels of human SHBG in male and female *SHBG*11-a transgenic mice increased markedly within the first ten days after birth. In the male mice, the levels increased progressively thereafter until they reached a plateau at about day 40 (Figure 2.3 A). By contrast, serum SHBG levels in the female mice did not change appreciably after ten days of age. However, a three-fold difference was apparent in human SHBG serum levels in male and female *SHBG*11-a transgenic mice by the time they reach sexual maturity at day 40 (Figure 2.3 A). This sex difference was confirmed in another line (*SHBG*11-b) of mice carrying an 11 kb *SHBG* transgene, and the serum concentrations of human SHBG in sexually mature male *SHBG*11-b mice were about 2.5 times greater than in their female counterparts (figure 2.3 B).

Serum concentrations of human SHBG in male and female *SHBG*4-a transgenic mice were similar and increased progressively from birth through to day 60, after which they appeared to decline slightly (Figure 2.3 B). There was also no sex difference in the serum levels of human SHBG in a separate line (*SHBG*4-b) of mice carrying a 4.3 kb human *SHBG*

transgene (data not shown). Thus, additional sequences present in the 11 kb human *SHBG* transgenes must account for their sexual dimorphic expression. These sequences also appear to repress the expression of the transgenes in the livers of *SHBG*11-a female mice at about the time they reach sexual maturity (Figure 2.3 A) because this does not occur in female mice carrying a 4.3 kb *SHBG* transgene (Figure 2.3 B).

The ontogenic changes and gender difference in serum SHBG levels observed in these mice were also reflected in corresponding differences in the relative abundance of human SHBG mRNA in the liver (data not shown)

2.3.3 Relationship between serum SHBG and testosterone levels in human *SHBG* transgenic mice

The presence of human SHBG in the blood of *SHBG* transgenic mice results in levels of serum testosterone that are 10-100 times higher than in wild-type mice of the same age (Selmanoff *et al.*, 1977). At birth, serum testosterone levels were much higher in *SHBG*11-a and *SHBG*4-a male transgenic mice (Fig. 2.4, panels A and C, respectively) compared with those in their female littermates (Fig. 2.4, panels B and D, respectively). In the male mice, the serum testosterone levels decreased to very low levels after birth and remained low until the animals were 30 days of age, after which they fluctuated considerably from 35 nmol/l to 1.75 μmol/l. Serum testosterone levels in *SHBG*11-a and *SHBG*4-a female transgenic mice were generally very low in animals before weaning, but increased by 20 to 30 days of age with the onset of sexual maturation. However, there was considerable variation in serum testosterone levels between sexually mature animals (Figure 2.4, panels B and D, respectively). Despite the wide variations in serum testosterone levels in mature male and female mice, these levels were generally much higher than those measured in their wild-type counterparts at 90 days (Jänne *et al.*, 1998). As expected, correlations were observed between serum SHBG and testosterone levels in male ($r = 0.52$ $p<0.001$) and female ($r=0.49$ $p<0.001$) *SHBG* transgenic mice.

2.3.4 Postnatal ontogeny of human *SHBG* expression in the testis of transgenic mice

Human *SHBG* transcripts are undetectable in the testes of mice containing 4.3 kb

SHBG transgenes, and in *SHBG*11-a and *SHBG*11-b transgenic mouse testes they are almost exclusively derived from an alternative upstream promoter (Jänne *et al.*, 1998). These alternative human *SHBG* transcripts in the testis are obviously under different regulatory control than SHBG mRNA in the liver and kidney. Furthermore, they were readily detectable at 10 days of age in the testes of *SHBG*11-a mice, and increased rapidly in abundance to levels seen in sexually mature animals by 30 days of age (Figure 2.5). Compared with the changes in serum testosterone levels in the same animals during the first 20 days of life (Figure 2.4, panel A), it is apparent that the human *SHBG* transcripts in the testes accumulate (Figure 2.5) well before any appreciable increases in serum testosterone (Figure 2.4, panel A).

2.3.5 Ontogeny and regulation of human *SHBG* expression in the kidneys of transgenic mice

Human *SHBG* transgenes are expressed in the epithelial cells of the proximal renal tubules, and immunoreactive human SHBG appears to be secreted into the renal tubules (Jänne *et al.*, 1998). In the kidneys of male and female transgenic mice, human SHBG mRNA levels accumulated (at ~20 days of age) and reached mature levels earlier (at ~40 days of age) in *SHBG*11-a mice (Figure 2.6 B), when compared to *SHBG*4-a mice (Figure 2.6 A). In the *SHBG*4-a mice, SHBG mRNA increased in abundance at 30 days of age, and did not reach peak levels until at least 60 days of age. The expression of both *SHBG*11-a and *SHBG*4-a transgenes in the kidney was also clearly sexually dimorphic, with much higher SHBG mRNA levels in the kidneys of male mice (Figure 2.6, panels A and B). Expression of human *SHBG* transgenes in the kidney was surprising since it has not been reported to be a site of expression in humans, but this may be relevant because we have demonstrated that the mouse kidney is a site of expression of the endogenous *ABP* gene (Figure 2.6, panel C)

The marked increases in renal SHBG mRNA in male transgenic mice occurred at about the same time as the increases in serum testosterone during sexual maturation (Figure 2.4, panels A and C). When this was considered together with the differences in human SHBG mRNA abundance in kidneys of male and female *SHBG* transgenic mice (Figure 2.6),

we reasoned that the expression of human *SHBG* transgenes in the mouse kidney might be androgen-dependent. This was confirmed by first demonstrating a clear reduction in the abundance of SHBG mRNA in the kidneys of male *SHBG*4-a (Figure 2.7 A and 2.7 C panel i) and *SHBG*11-a (Fig. 2.7 B panel i) mice after castration. Densitometric analysis of these Northern blots revealed that after castration the relative abundance of SHBG mRNA was 19-56% of the level in intact control mice depending on their age (Figure 2.7 A). By contrast, castration had no effect on the relative abundance of human SHBG mRNA in the livers of the transgenic mice (Figure 2.7A) or on SHBG levels in their serum (data not shown). Additional evidence that human *SHBG* expression in the mouse kidney is regulated by androgens was obtained by treating castrated male (Figure 2.7 B panel ii and 2.7 C panel i) and female (Figure 2.7 C panel ii) *SHBG* transgenic mice daily with 100 μg 5α DHT given subcutaneously in sesame oil (vehicle) for up to 5 days. In castrated male mice, the relative abundance of human SHBG mRNA in the kidneys increased after only 2 days of treatment (Fig. 2.7 B panel ii) and increased further after 5 days of treatment (Figure 2.7 B panel ii and 2.7 C panel i). Furthermore, in ovariectomized female *SHBG*4-a mice, the same treatment with 5α DHT for 5 days resulted in a marked increase the amount of SHBG mRNA in the kidney (Figure 2.7 C panel ii).

2.3.6 Expression of human *SHBG* in the female transgenic mouse brain

The regional distribution of human SHBG mRNA in the brains of female *SHBG*4-a and *SHBG*11-a transgenic mice was characterized by *in situ* hybridization. This revealed that low levels of *SHBG* transcripts are present in the *SHBG*4 transgenic mouse brain, and are primarily localized to the cerebral and piriform cortex, with lower levels of expression in the dentate gyrus of the hippocampus (Figure 2.8 panel iv, v). By contrast, human SHBG mRNA in the *SHBG*11 transgenic mouse brain was more widely distributed, and more abundant when intensities of dark field micrographs are compared. As was seen in the *SHBG*4 brain, *SHBG* transcripts in the *SHBG*11 brain are found in the cerebral and piriform cortex, and very high levels of transcript are present in the hippocampus. In addition, SHBG mRNA was found in the caudate putamen, substantia nigra, hypothalamus, amygdala, and Purkinje cells of the cerebellum, as well as in cells of the pons of the *SHBG*11-a brain

(Figure 2.8 A, panels vii, viii, ix). No hybridization was observed in the brains of age-matched female wild type mice (Figure 2.8 A, panels i-iii).

The nature of the transcripts present in the *SHBG*11-a brain were characterized by reverse transcriptase-polymerase chain reaction (RT-PCR) using forward primers specific to sequences in exon 1, or the alternative exon 1 sequence present in the 11 kb human *SHBG* transgene. This analysis revealed that SHBG mRNA in the brain is derived from two distinct transcription units, and give rise to transcripts containing either the exon 1 sequence (Figure 2.9 A, lanes 3,4), or the alternative exon 1 (Figure 2.9 A, lanes 1,2). Interestingly, *SHBG* cDNA amplified with primers specific to the alternative exon 1 sequence resulted in multiple products in brain and testis samples. When these products were cloned and sequenced, the approximately 1.1 kb band was found to contain the alternative exon 1 sequence, followed by the sequences of exons 2-8, while the middle band (0.9 kb) contained the alternative exon 1 followed by the sequences of exons 2-6, and 8. The smallest product, amplified only in brain samples, was found to contain the alternative exon 1 sequence, and exons 2-5 and 8.

A maternal phenotype characterized by high rates of infanticide and maternal neglect coincides with the presence of alternative *SHBG* transcripts in the brains of *SHBG*11 female mice (Table 2.1). Importantly, the phenotype observed in the *SHBG*11 mothers does not appear to be a result of high levels of circulating SHBG, because both *SHBG*4 and *SHBG*11 mice have very high levels of human SHBG in their serum (Jänne *et al.*, 1998).

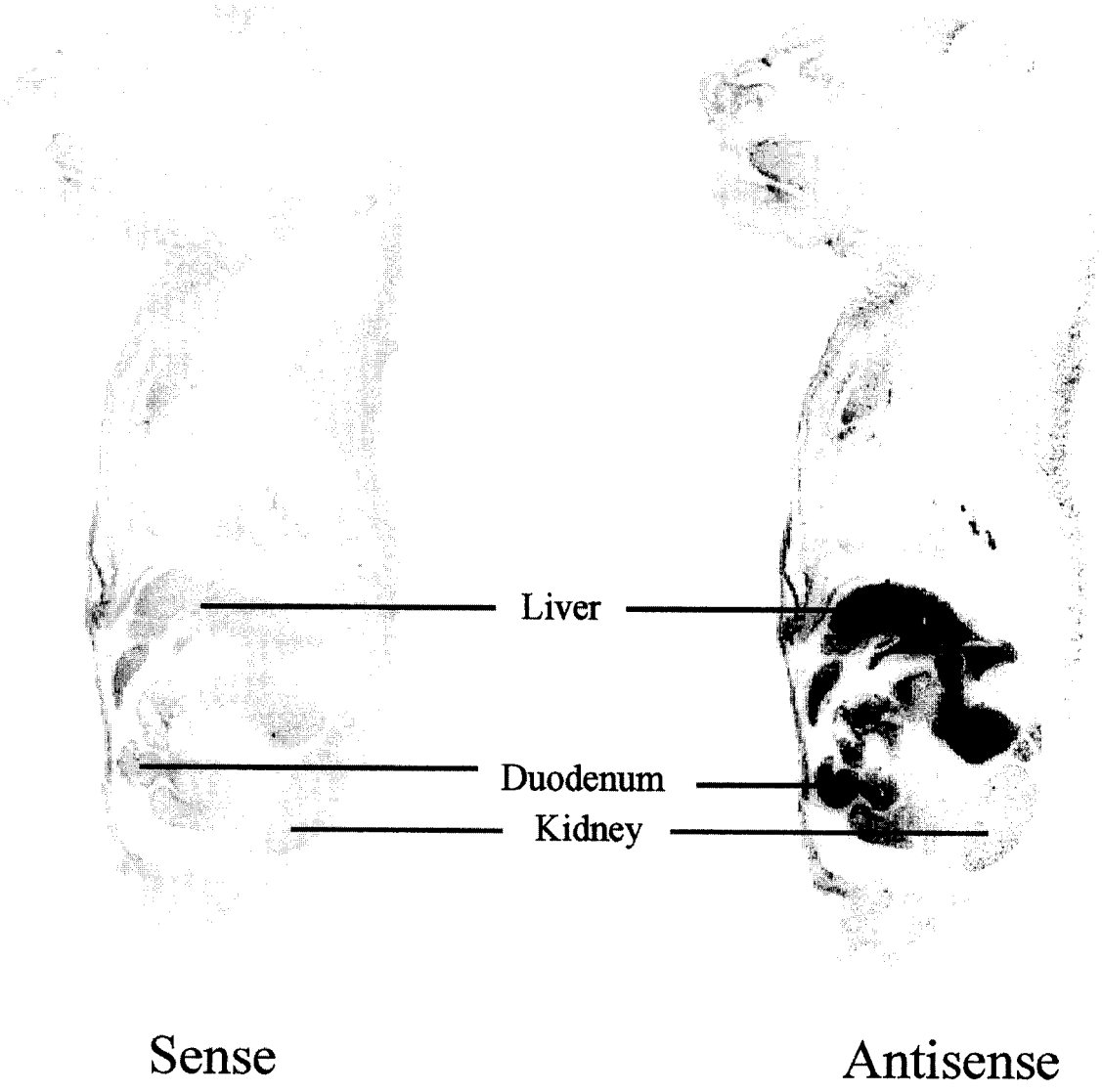

Figure 2.1 Autoradiogram of *in situ* hybridization of a 17.5 days post coital *SHBG*11-b transgenic mouse fetus using ^{35}S-labeled sense and antisense human SHBG cRNA ribroprobes.

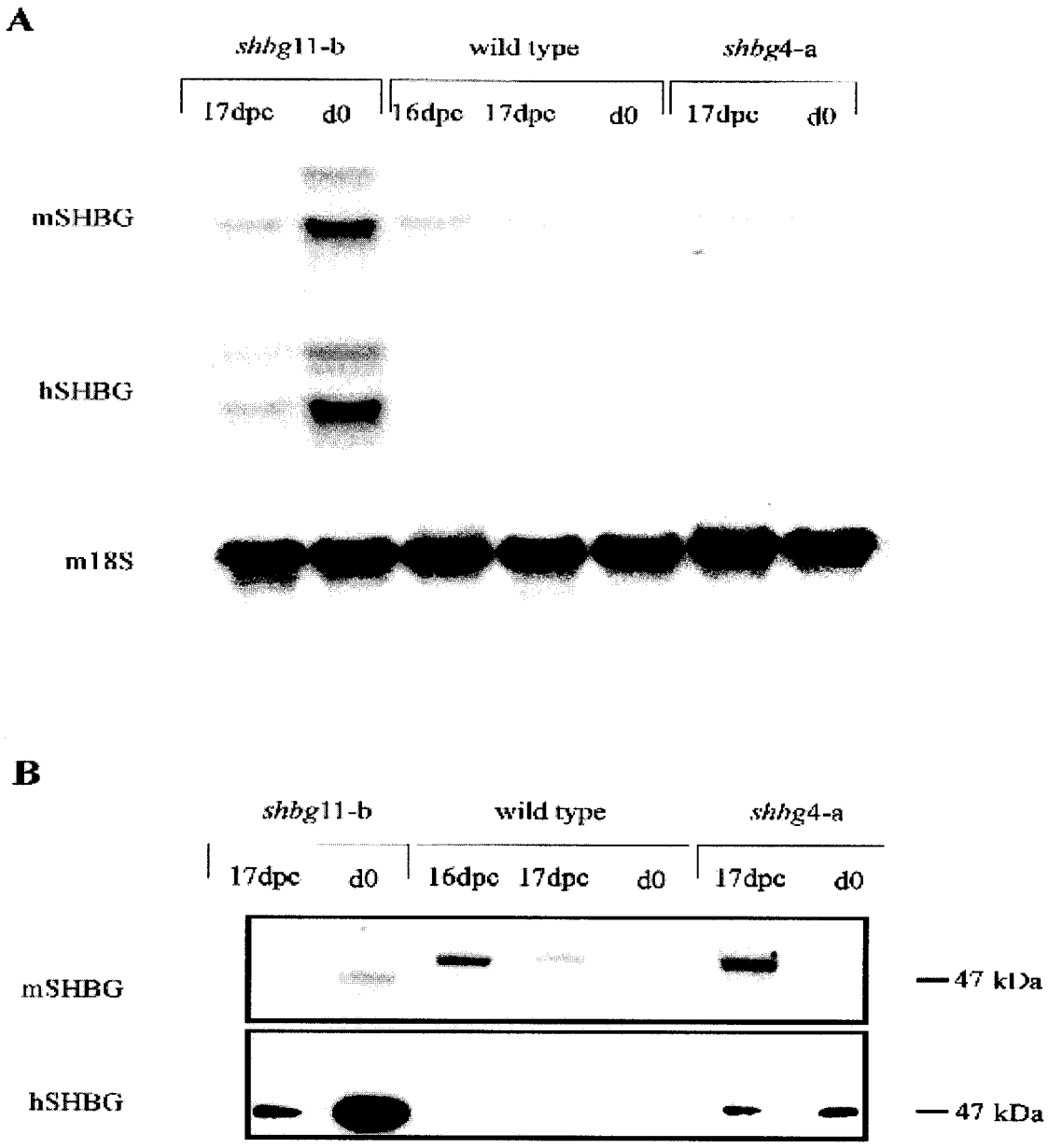

Figure 2.2 Ontogeny of endogenous mouse *SHBG* and human *SHBG* transcripts in fetal and neonatal liver (A). Total liver RNA (30 μg) was separated on an agarose gel in the presence of formaldehyde, transferred to a nylon membrane, and probed with ^{32}P-labeled 3' mouse SHBG cDNA (mSHBG). After exposure to a phosphor imaging screen, the membrane was stripped and re-probed with ^{32}P-labeled 3' human SHBG cDNA (hSHBG). The membrane was stripped after exposure to a phosphor imaging screen and was then re-probed with ^{32}P-labeled cDNA for mouse 18S ribosomal RNA. Serum levels of mouse SHBG and human SHBG in fetal and neonatal mice were estimated by western blotting (B). Mouse serum was diluted 1:30 or 1:100 for immunoblotting with antisera against mouse SHBG or human SHBG respectively. The position of the 47 kDa molecular size marker is shown on the right of the blots.

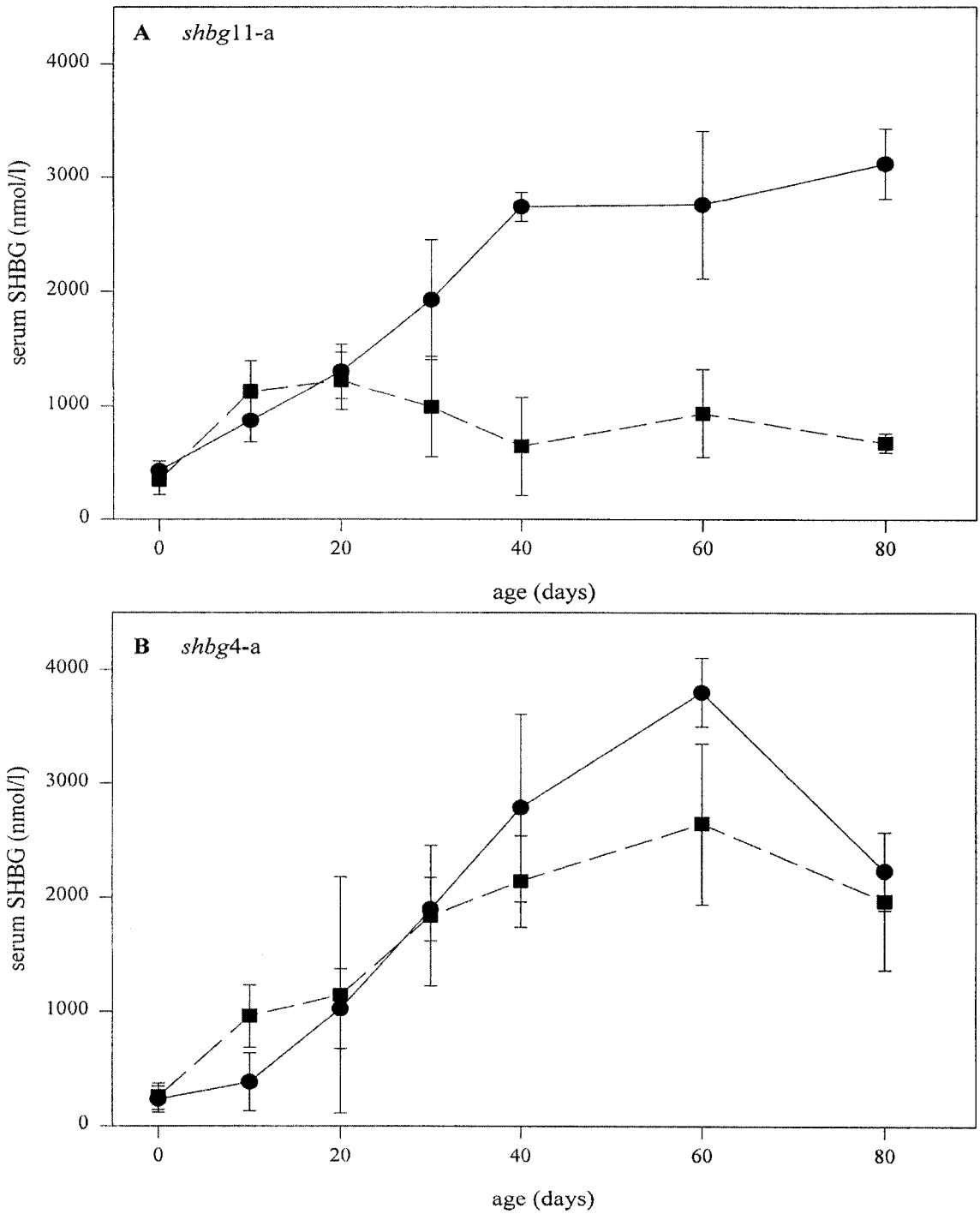

Figure 2.3 Ontogeny of human SHBG in the serum of *SHBG*11-a transgenic mice (panel A) and *SHBG*4-a transgenic mice (panel B). Means ± SEM of measurements in male (●) and female (■) mice are shown. At each time point, duplicate serum SHBG measurements from 3-4 animals were analyzed.

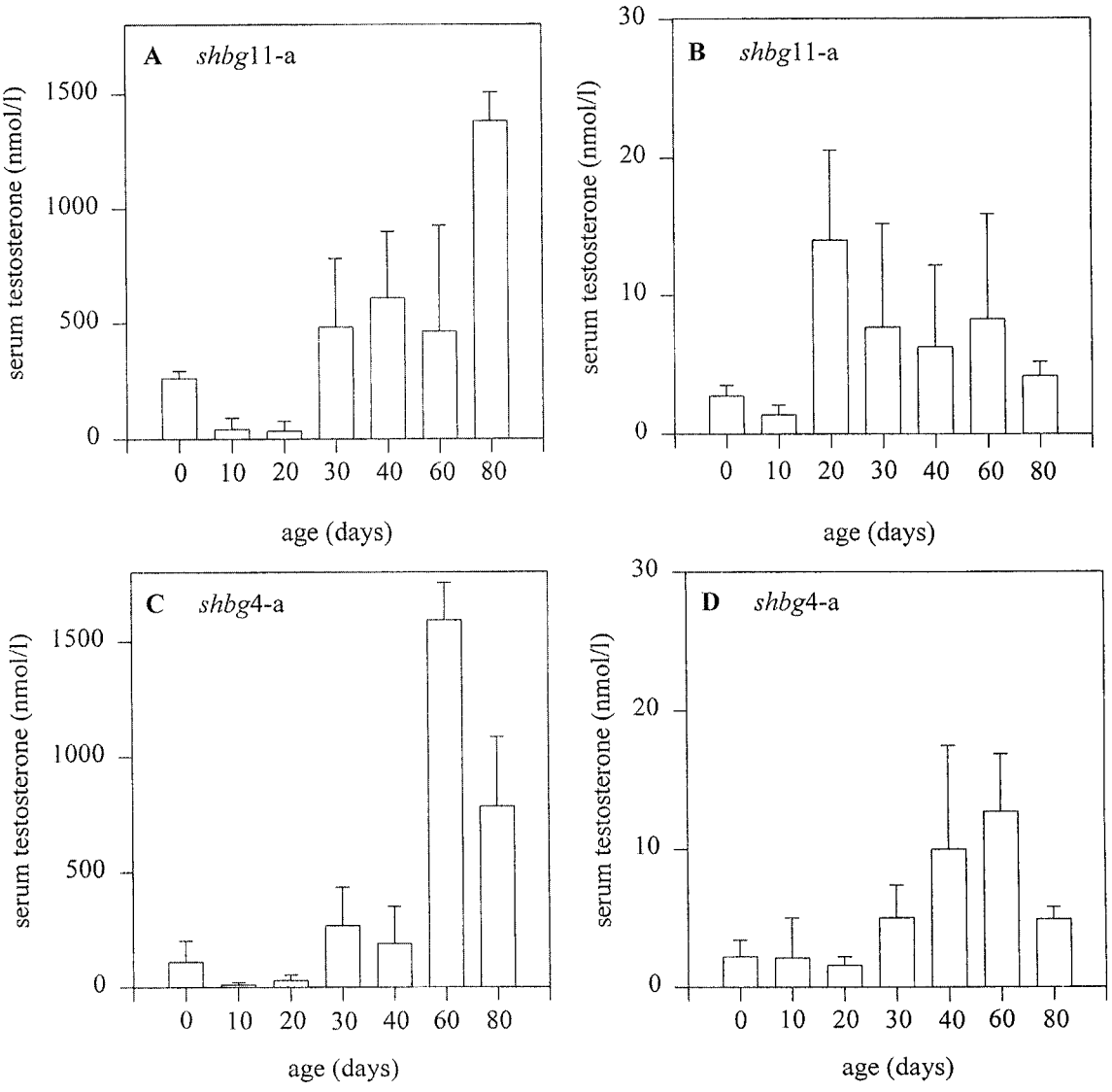

Figure 2.4 Serum concentrations of testosterone in male and female *SHBG*11-a transgenic mice (panels A and B, respectively) and male and female *SHBG*4-a mice (C and D, respectively). The values are presented as means ± SEM from duplicate measurements of 3-4 animals at each time point.

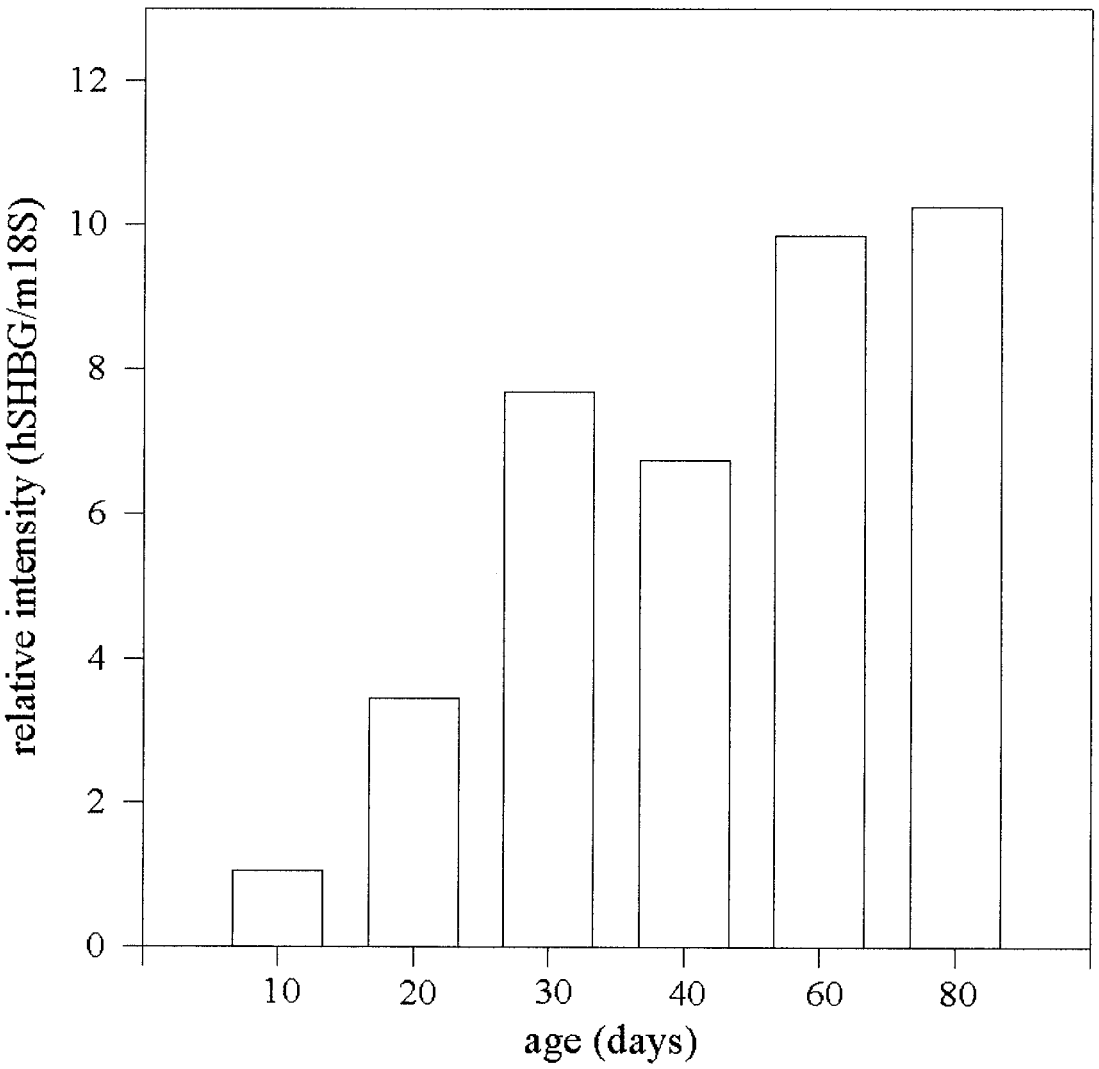

Figure 2.5 Ontogeny of human *SHBG* transcripts in the *SHBG*11-a transgenic mouse testis. The relative abundance of human *SHBG* transcripts with respect to 18S RNA was determined by densitometry and is presented as the mean of two animals at each time point.

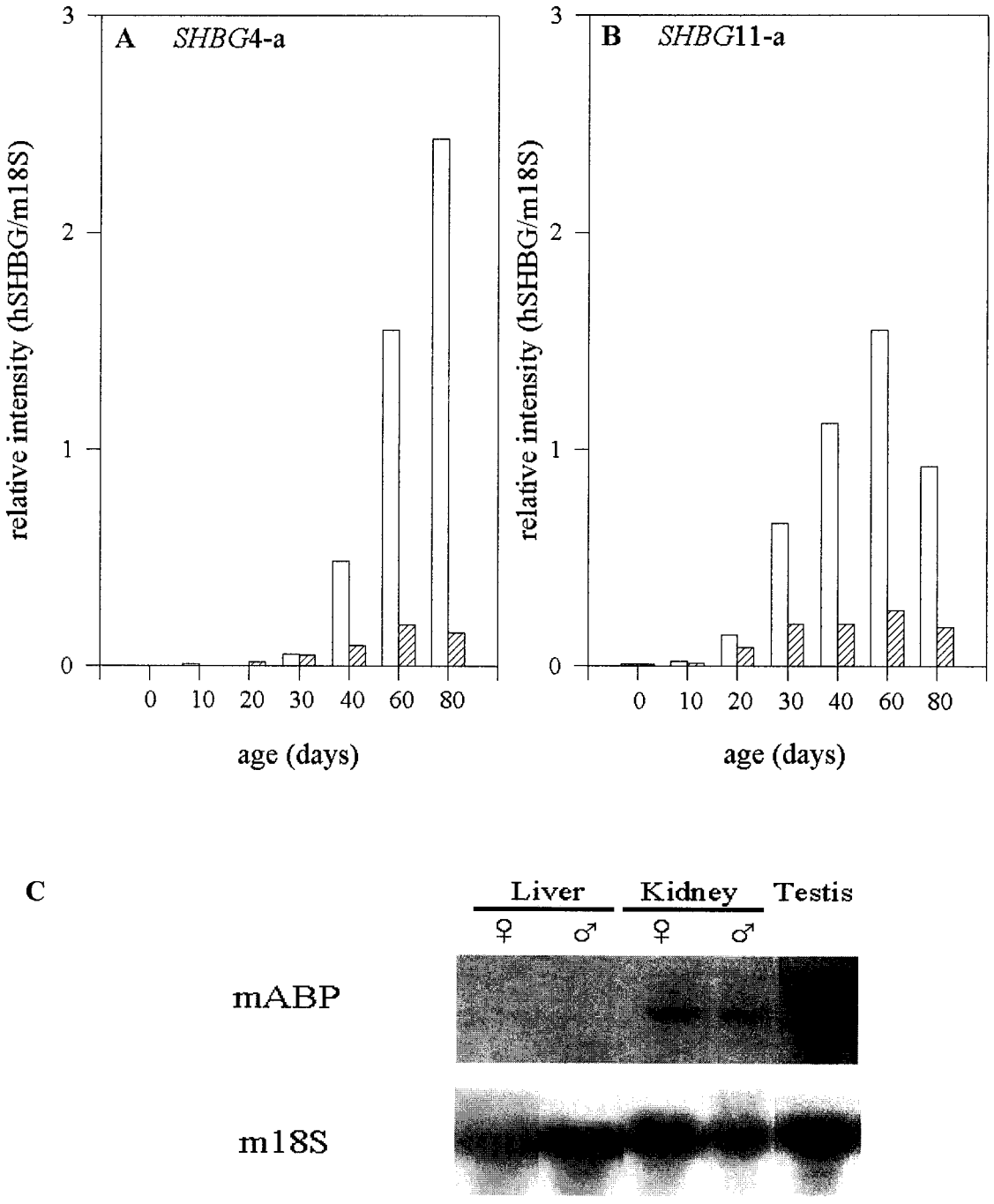

Figure 2.6 Ontogeny of human *SHBG* gene expression in kidney of *SHBG*4-a (A) and *SHBG*11-a (B) transgenic mice and expression of the mouse *ABP* gene in the kidney (C). The relative abundance of human SHBG mRNA with respect to 18S RNA is presented as the mean from two animals at each time point. Samples from male kidneys are shown in the open bars; female kidneys in hatched bars. C. Northern blot demonstrating low levels of endogenous expression of the mouse *ABP* gene in the kidney when compared to that in the testis. No ABP mRNA was detected in liver RNA from adult male or female mice.

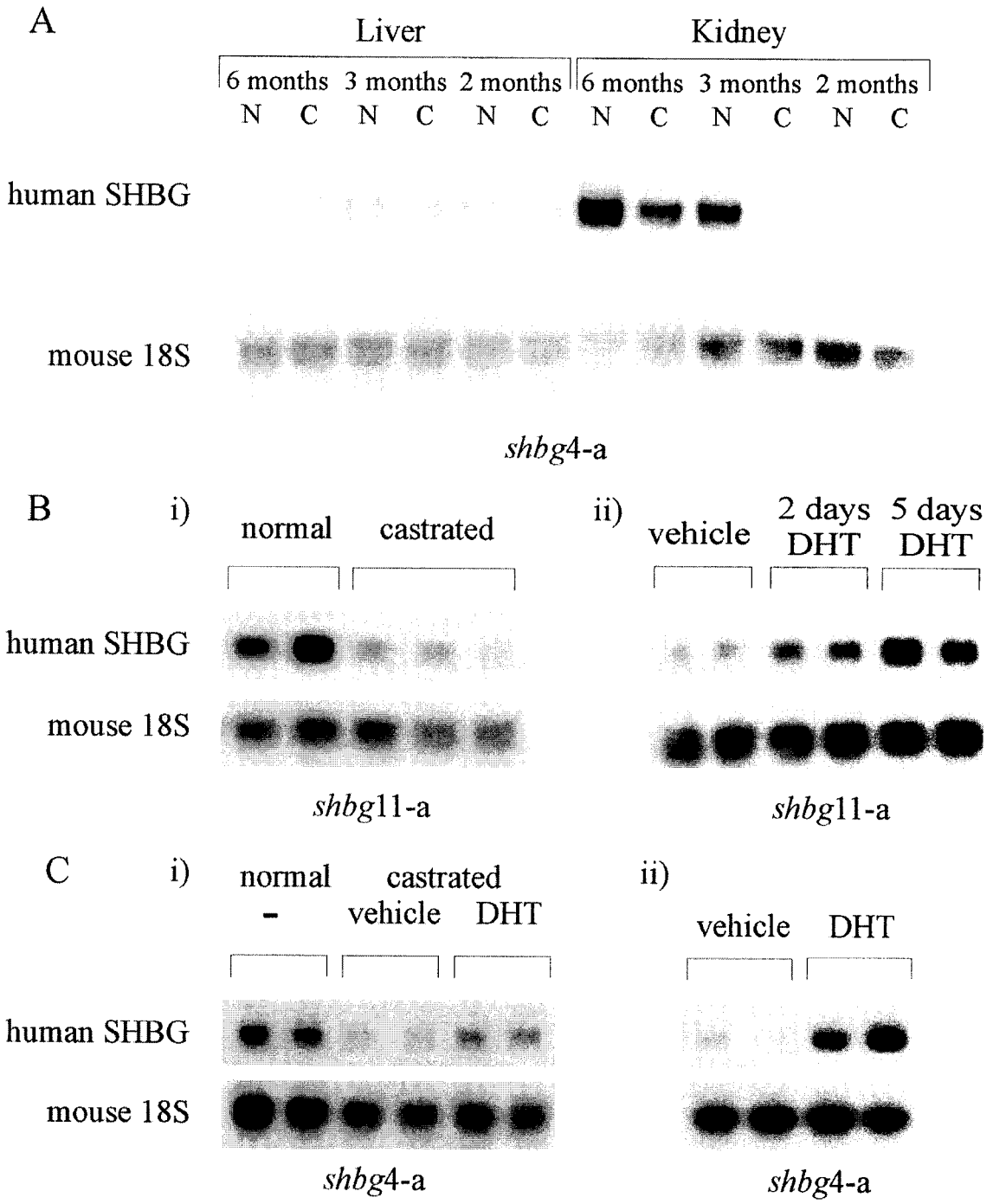

Figure 2.7 Northern blots showing the relative abundance of human SHBG mRNA in liver and kidney of normal (N) and castrated (C) male mice at different ages (A); the effect of castration (i) and androgen treatment (ii) in male *SHBG*11-a mice (B) and the effects of androgen treatment in castrated male (i) and ovariectomized female (ii) *SHBG*4-a mice (C).

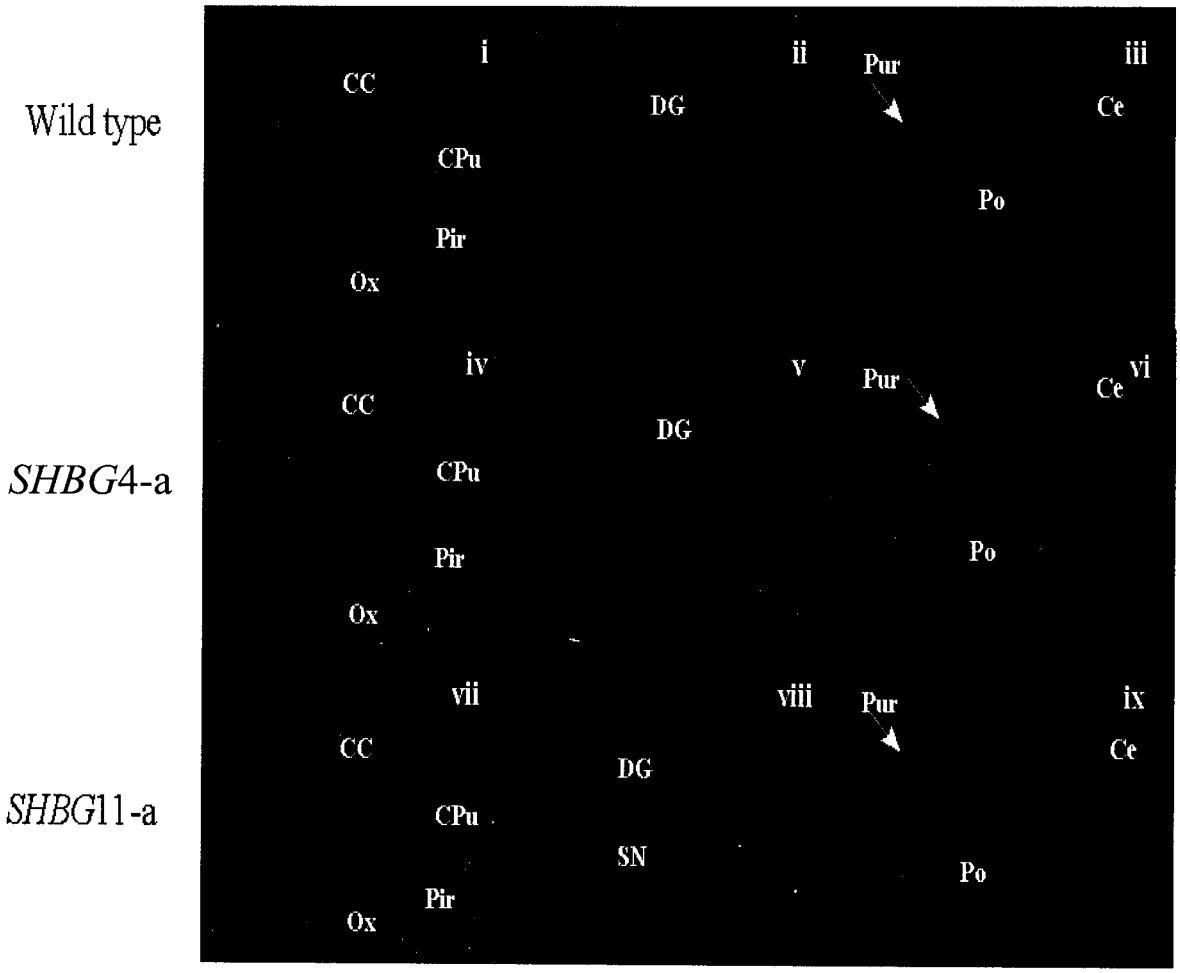

Figure 2.8 *In situ* hybridization of wild type (panels i-iii), *SHBG*4-a (panels iv-vi) and *SHBG*11-a (panels vii-ix) female mouse brains using ^{35}S-labeled antisense human SHBG cRNA riboprobes. (CC – Corpus Callosum, CPu – Caudate Putamen, Ce – cerebellum, DG – Dentate Gyrus, Ox – Optic chiasm, Pir – Piriform cortex, Po – Pons, Pur – Purkinje cells, SN – Substantia Nigra.

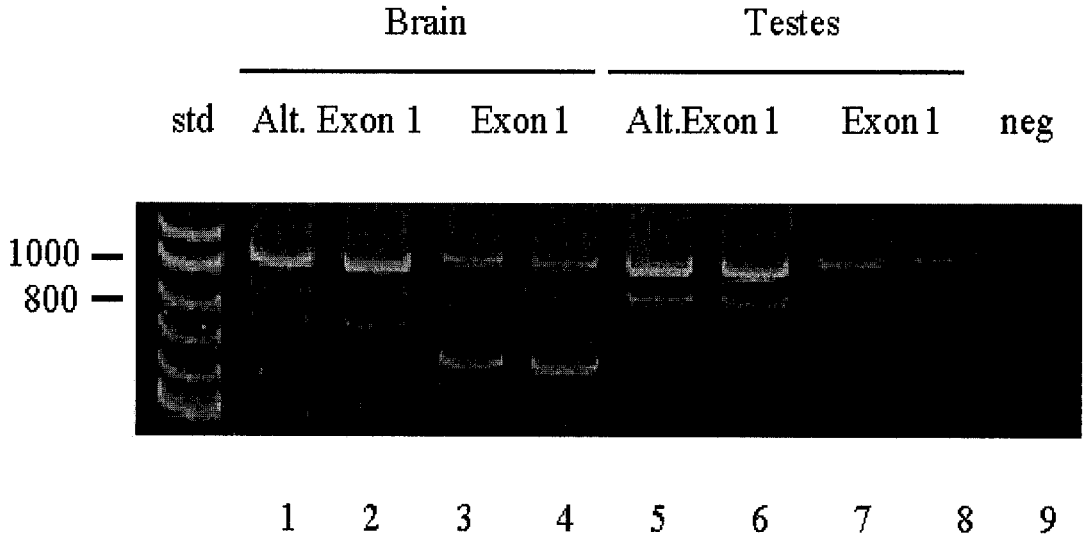

Figure 2.9 Characterization of human *SHBG* transcripts present in *SHBG*11-a brain and testes. An RT-PCR reaction with primers corresponding to human *SHBG* alternative exon 1 and exon 8 sequences resulted in amplification of three products of 1.13 kb, 0.93 kb and 0.79 kb when total RNA extracts from 11 kb human *SHBG* transgenic brain were used as templates (lanes 1, 2), and two products of 1.13 kb and 0.93 kb using RNA isolated from testes (lanes 5, 6). RT-PCR reactions using exon 1 and exon 8 specific primers resulted in the amplification of two products of 1.15 kb and approximately 0.64 kb in brain samples, and a single product of 1.15 kb in testes samples. Std – 100 bp DNA standard, Neg – water control.

Transgenic Mouse Line	Total Number of Litters	Litter Size	Percentage Dead Litters
*SHBG*11-a	32	6.8 +/- 2.0	13
*SHBG*11-b	36	5.3 +/- 2.1	22
*SHBG*11-c	22	6.1 +/- 1.5	48
*SHBG*4-a	41	6.5 +/- 3.0	2

Table 2.1 Transgenic mothers expressing an 11 kb human *SHBG* transgene display abnormal maternal behaviour. The percentage of dead litters reflects the number of litters born to transgenic mothers that were dead within 3 days following birth as a result of maternal neglect or infanticide.

2.4 Discussion

The transcription unit responsible for the production of plasma SHBG is contained within a 4.3 kb human genomic DNA fragment that, when introduced into the mouse genome, is expressed in the hepatocytes and proximal renal tubules of mature animals (Jänne *et al.*, 1998). Within this portion of the human *SHBG* locus, the eight exons that encode the SHBG precursor polypeptide are preceded by ~0.8 kb of promoter sequence which is apparently sufficient to direct transcription in these tissues. A larger 11 kb region of the human *SHBG* locus has also been used to generate several lines of transgenic mice in which *SHBG* transcripts are found in the testis and brain, as well as the liver and kidney of mature animals. By examining the expression of this type of transgene in fetal mice, we have now demonstrated that the human *SHBG* gene is also expressed in the developing duodenum as well as the fetal liver. This is of interest because of our recent finding that HNF-4 plays an important role in regulating the activity of the human *SHBG* proximal promoter (Jänne and Hammond, 1998), and because HNF-4 is expressed in the same tissues in which we have observed expression of the human *SHBG* transgenes in mice, i.e. the liver, kidney and epithelial cells lining the gut (Sladek, 1993). We have also now obtained some indication that there are temporal differences between the expression of the endogenous mouse *SHBG* and the human *SHBG* transgenes in the liver during late fetal and neonatal life. In this context we have demonstrated that endogenous *SHBG* expression occurs in the fetal mouse liver at about the same gestational age as previously reported in fetal rat liver (Sullivan *et al.*, 1991), and is reduced to undetectable levels by term. By contrast, the human *SHBG* transgene expression increased during late gestation. Although human *SHBG* transcripts were undetectable at day 17 of fetal life in *SHBG*4-a mice, the presence of human SHBG in the serum suggests that this transgene might be expressed in the liver or some other tissue even earlier in gestation. Although the ~0.8kb of promoter sequence in the 4.3kb transgene is sufficient for expression in the fetal liver, additional upstream sequences therefore most likely influence its temporal expression. There are obvious differences in the rodent and human *SHBG* proximal promoters, and it will be of interest to determine how these differences may contribute to species specific differences in the way this gene is expressed in the liver throughout development.

The mouse and rat *SHBG* genes are transcriptionally silent in the liver postnatally (Sullivan *et al.*, 1991; Joseph *et al.*, 1987), but the hepatic production of human SHBG in transgenic mice increases steadily after birth. What is remarkable, however, is that the hepatic biosynthesis of SHBG in female mice expressing the 11kb *SHBG* transgene shows no increase after the onset of sexual maturation. By contrast, serum concentrations of human SHBG continue to increase in mice containing the 4.3kb *SHBG* transgene until about 60 days regardless of their gender. Thus ovarian steroids probably act through upstream regulatory sequences present in the 11kb *SHBG* transgenes to repress a post-pubertal increase in hepatic SHBG production. This was unexpected because the plasma levels of SHBG in humans are also sexually dimorphic after puberty, with levels falling much more markedly in males when compared to females (Apter *et al.*, 1984; Belgorosky and Rivarola, 1986). Although this decrease in young men has been attributed to an androgen-dependent decrease in the biosynthesis of plasma SHBG (el-Awady *et al.*, 1989), this explanation remains questionable because orchidectomy has no effect on serum SHBG levels (Leinonen *et al.*, 1979). Castration of male *SHBG* transgenic mice also had no effect on SHBG mRNA levels in the liver, and this further excludes the possibility that the sexual dimorphism in 11kb *SHBG* transgene expression is due to the much higher plasma levels of testosterone in male mice.

It is widely accepted that SHBG reduces the MCR of sex steroids that interact with its binding site (Siiteri *et al.*, 1982), and this probably contributes to the much higher serum concentrations of testosterone in SHBG mice when compared to wild-type mice (Selmanoff *et al.*, 1977). It also explains why there are good correlations between plasma SHBG and testosterone levels in both the male and female *SHBG* transgenic animals. This was not observed previously in transgenic mice that over-express a rat *SHBG* transgene in the testis and have detectable levels of rat ABP in their blood (Joseph *et al.*, 1997), but this is probably due to the much lower levels of serum rat ABP in these mice and its much lower affinity for sex steroids when compared to human SHBG (Westphal, 1986). Increased serum testosterone levels could result, in part, from an up-regulation of testicular androgen biosynthesis in response to decreased sex steroid bioavailability at the level of the hypothalamic pituitary axis. In support of this, the steroid-binding capacity of SHBG is much higher than the concentration of testosterone in serum, and this would tend to maintain

a low free testosterone concentration in the blood. Surprisingly, the presence of very high concentrations of human SHBG in the blood of these mice throughout postnatal development does not appear to have appreciable effects on their sexual development or fertility.

Human SHBG transcripts cannot be detected by Northern blotting in the testis of mice carrying a 4.3kb human *SHBG* transgene (Jänne *et al.*, 1998). In the latter study we also found that a majority of transcripts in the testis of *SHBG*11 transgenic mice originate from an alternative promoter that is not present in the 4.3 kb *SHBG* transgenes. These transcripts in the testis of *SHBG*11-a transgenic mice contain an alternative exon 1 sequence (Hammond *et al.*, 1989), and their presence in testicular cells is tightly regulated throughout the spermatogenic cycle (Jänne *et al.*, 1998). Although the biological significance of these transcripts is not known, they may encode an N-terminally truncated form of SHBG that remains within cells (Tsvetnitsky *et al.*, 1998). These transcripts within the mouse testis increased in abundance starting on about day 10 and reach a plateau by day 30. It will be of interest to determine how their expression is regulated. From the present study it appears that androgens may not be involved in this process because these alternative human *SHBG* transcripts begin to accumulate within the mouse testis well before any significant increases in serum testosterone levels.

Expression of human *SHBG* transgenes in the mouse kidney was unexpected, but we have obtained evidence that the mouse *SHBG* gene is also expressed in the kidney (Figure 2.6 C). It was therefore of interest to examine how the human *SHBG* transgenes are regulated in the kidney, and our present results indicate that the levels of human SHBG mRNA in the kidney do not increase until animals undergo sexual maturation. At this time, there is a marked increase in human SHBG mRNA abundance in the kidney, particularly in male animals, and this appears to occur earlier in animals expressing the 11kb *SHBG* transgenes. The sexual dimorphism in human SHBG mRNA abundance in the kidneys indicated that gonadal steroids, and in particular androgens, might be responsible for increasing the expression of *SHBG* in this tissue at puberty. This was confirmed by castrating male mice and observing a marked decrease in kidney SHBG mRNA abundance which could be reversed by treatment with 5α-dihydrotestosterone. This androgen dependence was also demonstrated in the kidneys of female mice. Furthermore, this effect

of androgens was observed in both male and female *SHBG*4 mice, and the *cis*-elements that mediate this must therefore be located within this region of the human *SHBG* gene. We do not know what role SHBG gene expression plays in the mouse kidney, and whether our observations in transgenic mice recapitulate the situation in the human kidney, but the kidney is very sensitive to sex steroids and some renal diseases are known to be sex hormone-dependent (Grossman *et al.*, 1991).

In the rat, ABP mRNA is present in all regions of the brain by Northern blot analysis, and immunoreactive ABP can be detected in the hypothalamic median eminence, as well as the supraoptic and periventricular nuclei (Wang *et al.*, 1990). Human SHBG mRNA in the brains of transgenic mice carrying a 4 kb human *SHBG* transgene is localized in the cerebral cortex and piriform cortex, with lower levels in the dentate gyrus of the hippocampus. A broader distribution of human SHBG transcripts is found in the brains of *SHBG*11 mice, with accumulations in regions including the cerebral and piriform cortex, caudate putamen, the hippocampus, amygdala, substantia nigra, hypothalamus, pons and the Purkinje cells of the cerebellum. This demonstrates that additional sequences in the 11 kb human *SHBG* transgene, including elements flanking an alternative exon 1 sequence, are necessary for expression in specific regions of the brain. Human SHBG mRNA in the *SHBG*11 mouse brain consists of both transcripts containing the exon 1 sequence required for production of the secreted protein, as well as differentially spliced *SHBG* transcripts containing an alternative exon 1 sequence that lacks an in-frame AUG codon. Although the functions of proteins derived from *SHBG* trancripts containing an alternative exon 1 sequence are unknown, the fact that they lack a coding sequence for the secretion signal polypeptide suggests that their protein products would remain within the cell or discrete cellular compartments, and may exert a role in the regulation of sex steroid bioavailablity at the cellular level. Observations that estrogen receptor α or β mRNA (Shughrue *et al.*, 1997) and immunoreactivity (Warrier Mitra *et al.*, 2003; Azcoitia *et al.*, 1999) are present in the same regions of the brain that contain SHBG mRNA support a role for SHBG in regulating sex steroid-dependent processes within the same cell types in the brain. Moreover, a phenotype of maternal neglect and infanticide observed in transgenic mice expressing an 11 kb human *SHBG* transgene is very similar to a phenotype observed in mice that lack either the estrogen

receptor α (Ogawa *et al.*, 1998) or α and β (Ogawa *et al.*, 2000). Transgenic mice that express a 4 kb human *SHBG* transgene do not display this phenotype. This is important because both *SHBG*4 and *SHBG*11 mice have similar levels of human SHBG in their blood, and it is therefore likely that the maternal phenotype in *SHBG*11 mothers is due to the presence of SHBG isoforms in the brain, and that these SHBG isoforms may compete with nuclear receptors for sex steroid ligand. It is also possible that SHBG is produced and secreted from some cell types within the brain because SHBG transcripts are also present in the brains of mice containing a 4 kb human *SHBG* transgene, and this may have some other function. In support of this, human SHBG injected into the brains of female mice stimulates sexual receptivity (Caldwell *et al.*, 2000; Caldwell *et al.*, 2002), presumably through interaction with SHBG binding sites identified in the medial preoptic area and medial basal hypothalamus (Caldwell, 2001). The function of SHBG produced in the brain may therefore exert a broader role in regulating sex steroid hormone action.

In summary, an analysis of mice expressing human *SHBG* transgenes has provided new insight into the spatial and temporal expression of the human *SHBG* in an *in vivo* context, and has revealed the location of elements within the gene that might be involved in its hormonal regulation. These mice therefore not only provide a model system for studying the hormonal control of human *SHBG*, but may provide insight into the function of this protein.

2.5 References

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CHAPTER 3

A HUMAN SEX HORMONE-BINDING GLOBULIN ISOFORM ACCUMULATES IN THE ACROSOME DURING SPERMATOGENESIS

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3.1 Introduction

Mammalian genes encoding sex hormone binding globulin (*SHBG*) contain at least two transcription units (Hammond *et al.*, 1989; Joseph, 1994). In humans, the transcription unit responsible for the production of plasma SHBG by hepatocytes consists of eight exons that span approximately 3.2 kb on chromosome 17 (Hammond *et al.*, 1989; Bérubé *et al.*, 1990), and is under the control of a promoter sequence that contains several well defined binding sites for liver enriched transcription factors (Jänne and Hammond, 1998; Hogeveen *et al.*, 2001). The human *SHBG* gene contains a second transcription unit that consists of an alternative exon 1 sequence which replaces the exon 1 sequence present in the SHBG mRNA found in the liver. As a consequence, these differentially spliced *SHBG* transcripts lack the secretion signal sequence associated with the plasma SHBG precursor polypeptide.

The expression of the *SHBG* gene in the testis has been studied extensively in the rat (Joseph, 1994). In this species, the *SHBG* gene is expressed in Sertoli cells (Reventos *et al.*, 1988; Hall *et al.*, 1990) and encodes the SHBG homologue that is generally known as the testicular androgen binding protein (ABP). The ABP produced by rat Sertoli cells is secreted into the lumen of seminiferous tubules where it is thought to serve primarily as a carrier of testosterone throughout the male reproductive tract (Joseph, 1994). Although *SHBG* transcripts are present in the human testis (Hammond *et al.*, 1989), virtually nothing is known about their function or how they are regulated, and evidence that they encode a precursor polypepide containing a leader sequence for secretion is lacking. In fact, all the available evidence suggests that the human testis contains several alternative *SHBG* transcripts comprising a non-coding alternative exon 1 sequence and some of them also lack exon 7 sequences (Hammond *et al.*, 1989; Gershagen *et al.*, 1989). Differentially-spliced human *SHBG* transcripts lacking exon 7 sequences have also been identified in several other tissues (Misao *et al.*, 1997; Misao *et al.*, 1998), but their 5' sequences have not been characterized. Like the human *SHBG* gene, the rat *SHBG* gene produces transcripts that consist of alternative exon 1 sequences, and these have been identified in the rat brain (Wang *et al.*, 1990) and the fetal rat liver (Sullivan *et al.*, 1993). There is no obvious sequence similarity between the alternative exon 1 sequences associated with various *SHBG* transcripts in different species, but there is evidence they encode SHBG isoforms comprising sub-

cellular localization signals within a unique amino-terminal sequence (Joseph *et al.*, 1996).

To study the tissue-specific expression of various human *SHBG* transcripts, we have produced several lines of transgenic mice (Jänne *et al.*, 1998) containing either a 4 kb or an 11 kb human *SHBG* transgene. The 4 kb transgene comprises the eight exons encoding plasma SHBG (Hammond *et al.*, 1989) and 0.8 kb of 5'-flanking DNA that includes the promoter utilized in the liver (Jänne and Hammond, 1998), whereas the 11 kb human *SHBG* transgene contains an additional 5'-flanking DNA sequence that includes an alternative exon 1 sequence associated with the *SHBG* transcripts present in the human testis (Hammond *et al.*, 1989; Gershagen *et al.*, 1989). We have shown previously that only the 11 kb human *SHBG* transgene is expressed in the mouse testis, as evidenced by the presence of human *SHBG* transcripts in the seminiferous epithilium (Jänne *et al.*, 1998). These studies also indicated that the human *SHBG* transcripts accumulate in a spermatogenic cycle stage-dependent manner in this location, but the cell type in which they were located could not be clearly identified and their protein products eluded detection (Jänne *et al.*, 1998). We have therefore re-examined this issue, and have found that the majority of human *SHBG* transcripts in the testis of these mice comprise the alternative exon 1 sequence associated with SHBG cDNAs from a human testis library (Hammond *et al.*, 1989). Furthermore, these alternatively-spliced human *SHBG* transcripts are confined to testicular germ cells and an immunoreactive human SHBG isoform accumulates in the acrosome of developing spermatids and immature sperm in the transgenic mice. We have also obtained direct evidence that this acrosomal SHBG isoform binds steroids, and is also present in human sperm.

3.2 Materials and Methods

3.2.1 Animals

Transgenic mice containing 11 kb (lines *SHBG* 11-a and *SHBG* 11-b) or 4 kb (lines *SHBG* 4-a and *SHBG* 4-b) regions of human *SHBG* gene have been characterized previously (Jänne *et al.*, 1998; Jänne *et al.*, 1999). Animals were housed under standard conditions and provided with food and water *ad libitum*. At approximately 10 weeks of age, mice were sacrificed for the isolation of Sertoli cells and/or germ cells for protein and RNA analysis

(see below). For immunohistochemistry, mice were perfused with PBS followed by 4% paraformaldehyde (Jänne *et al.*, 1998). All procedures were approved by the Animal Use Subcommittee of the University Council on Animal Care (University of Western Ontario).

3.2.2 Immunohistochemistry

The anti-human SHBG antibodies used for immunohistochemistry were purified from a rabbit anti-serum by immunoaffinity chromatography using an N-hydroxysuccinimide-activated HiTrap column coupled to purified human SHBG (Avvakumov *et al.*, 2000), according to instructions provided by Amersham Pharmacia Biotech (Mississauga, Canada). Testes from perfused mice (see above) were further fixed in 4% paraformaldehyde at 4°C for 24 h, and subsequently dehydrated with a series of ethanol solutions and embedded in paraffin. The paraffin sections were de-waxed and incubated at high power in a microwave oven for 10 min in citrate buffer, pH 9.9. The sections were then cooled at room temperature for 20 min, and treated with a 0.03% hydrogen peroxide solution for 7 min, prior to incubation (overnight at 4 C) with affinity-purified rabbit antibodies against human SHBG. The immunoreactive human SHBG was detected using the EnVision+ System, HRP (DAB) from DAKO Corporation (Carpinteria, CA).

Sperm from transgenic mice (Jänne *et al.*, 1998) were spread on slides, fixed for 5 min in 4% paraformaldehyde, and washed with PBS. After incubation in citrate buffer, pH 9.9, for 30 min at room temperature, the sections were treated with 0.03% hydrogen peroxide solution for 7 min at room temperature. The sections were incubated with the affinity-purified antibodies against human SHBG overnight at 4 C, and immunoreactivity was detected using the EnVision+ System (DAKO Corporation).

3.2.3 Sertoli cell and germ cell isolation

A mixed population of Sertoli cells and germ cells was isolated from the testes of wild type and transgenic mice (Weiss *et al.*, 1997). Briefly, the testes were excised and washed in Dulbecco's Modified Eagle's Media (DMEM)/NUT mix F-12 culture medium (Invitrogen Canada Inc., Burlington, Canada) supplemented with penicillin, streptomycin and

amphotericin (Weiss *et al.*, 1997). The testes were then decapsulated and incubated in a collagenase solution (0.9 mg/ml) at 33°C for 10 min with agitation. After centrifugation, the pellets were resuspended in 50 ml of the same culture medium and allowed to sediment for 15 min. This was repeated three times. A second incubation with collagenase (0.9 mg/ml) was then performed at 33°C for 10 min. After centrifugation, the pellets were washed twice with PBS and frozen for RNA and protein extraction (see below).

Germ cells were isolated separately from the mouse testes using an established method (Weiss *et al.*, 1997). Briefly, testes were excised and washed with PBS supplemented with penicillin, streptomycin and amphotericin (Weiss *et al.*, 1997), decapsulated and minced for 5 min in the PBS solution. The medium was removed and the remaining testicular fragments were digested in PBS containing trypsin (80 mg/ml) at 33°C for 10 min. The reaction was stopped by adding 25 mg/ml trypsin inhibitor, and the resulting solution was treated with deoxyribonuclease I (0.4 mg/ml) at room temperature for 5 min. The isolated tubules were subjected to several rounds of mincing and filtration, as described previously (Weiss *et al.*, 1997; Selva *et al.*, 2000). After centrifugation, the pellet was resuspended in 15 ml DMEM/NUT mix F-12 culture medium (Invitrogen) supplemented with 10% fetal bovine serum and incubated in a tissue culture flask for 5 h. The supernatant containing germ cells without Sertoli cells was recovered and centrifuged. After two washes with PBS, the pellet was frozen in aliquots for protein or RNA extraction.

3.2.4 Western Blot Analysis

Soluble protein was extracted from mixed populations of Sertoli cells and germ cells and from isolated germ cells (see above) with 50 mM Tris-HCl, pH 7.5, 150 mM NaCl, 1% Nonidet P-40, 0.5% sodium deoxycholate, 0.1% SDS at 4°C for 12 h. Human donor sperm samples were centrifuged (350 x g for 10 min) to fractionate seminal plasma and sperm, and sperm samples were either washed in HTF culture medium (Irvine Scientific, Santa Ana, CA) or purified by Percoll density gradient centrifugation prior to extraction with 0.25 M Tris-HCl, pH 8.0, by sonication in a water bath and three freeze-thaw cycles. Samples were heat denatured in loading buffer and subjected to discontinuous SDS-polyacrylamide gel electrophoresis (SDS-PAGE) with 4% and 10% polyacrylamide in the stacking and resolving

gels, respectively. Proteins in the gel were transferred (Towbin *et al.*, 1979) to Hybond ECL nitrocellulose membranes (Amersham Pharmacia Biotech). The membranes were first blocked for 1 h in PBS containing 0.01% Tween-20 and 5% skim milk, and were then incubated overnight at 4°C with primary antibodies against human SHBG (DAKO Corporation; kindly provided by Dr. Francina Munell) in the same buffer. The blots were then washed three times in PBS containing 0.01 % Tween-20 for 15 min to remove excess antibody, and specific antibody-antigen complexes were identified using a horseradish peroxidase-labeled donkey anti-rabbit IgG and chemiluminescent substrates (Pierce, Rockford, Il) by exposure to x-ray film.

To assess the influence of glycosylation on the electrophoretic mobility of immunoreactive human SHBG in different samples (i.e., diluted serum and protein extracts from isolated testicular cells) we performed a similar western blotting experiment. For this purpose, protein was extracted from isolated testicular cells with 0.25 M Tris-HCl, pH 8.0, by sonication in a water bath and three freeze-thaw cycles, and cell debris was removed by centrifugation. Samples were then treated with N-glycosidase F (Roche Diagnostics, Laval, Quebec) at 37°C overnight, as recommended by the enzyme supplier, prior to analysis by SDS-PAGE and western blotting, as described above.

3.2.5 RNA analysis

Total RNA was extracted from mouse testicular cells and liver using TRIzol reagent (Invitrogen); separated by electrophoresis on a 1% agarose gel in the presence of formaldehyde, and transfered to a Zeta-Probe nylon membrane (Bio-rad Laboratories Inc., Mississauga, Canada). The membrane was hybridized with various ^{32}P-labeled human SHBG cDNAs: i.e., the 3'- *Eco*RI fragment spanning exons 6-8 (Hammond *et al.*, 1987); the *SHBG* exon 1 sequence encoding the leader sequence for secretion of SHBG, and the *SHBG* alternative exon 1 sequence (Hammond *et al.*, 1989). In addition, cDNAs for mouse vimentin and transition protein 1 were used as markers for somatic cells and germ cells, respectively. In some experiments, a mouse SHBG cDNA corresponding to exon 6-8 sequences was used as a probe, and a cDNA for 18 S ribosomal RNA was also used as an additional control for RNA loading and transfer (Jänne *et al.*, 1999).

We also used the total RNA from isolated germ cells to further analyze the human *SHBG* transcripts. To accomplish this, reverse transcription (RT) was performed at 42°C for 50 min using 3 μg of total RNA and 200 U of Superscript II together with an oligo-dT primer and reagents provided by Invitrogen. An aliquot (1 μl) of the RT product was amplified in a 20 μl reaction in the presence of 1 U Taq polymerase, 0.05 mM $MgCl_2$, 1.25 μM of each dNTP and 0.2 μM of each oligonucleotide primer. For this purpose, we used an oligonucleotide corresponding to a 5' sequence (5'-GCGGTTCAAAGGCTCCC) in the *SHBG* alternative exon 1 and a reverse primer complementary to a sequence (5'-TGGCTTCTGTTCAGGGCC) within exon 8 of the human *SHBG* gene (Hammond *et al.*, 1989). The PCR was performed for 40 cycles at 94°C for 30 min; 65°C for 30 sec and 72°C for 1 min. A mouse transition protein 1 cDNA was amplified by RT-PCR under the same conditions using as two specific primers (5'-CCAGCCGCAAGCTAAAGACTCATGC and 5'- AGCTCATTGCCGCATCACAAGTGGG) to control for the integrity and relative amounts of germ cell mRNA in the samples. The PCR products were resolved by electrophoresis in a 1% agarose gel, and purified using the GenElute Gel Extraction Kit (Sigma-Aldrich Canada Ltd, Oakville, Canada). They were then cloned using the Zero Blunt TOPO PCR Cloning Kit (Invitrogen), and plasmids containing PCR products were sequenced.

3.2.6 SHBG-steroid binding assays

To determine whether the immunoreactive SHBG extracted from testicular cells binds steroids we used a saturation ligand binding assay (Westphal, 1986). In this assay, the endogenous steroids were first removed from protein extracts (25 μg/μl) by dilution (1:3) in a dextran-coated charcoal (DCC) suspension and incubation for 30 min at room temperature. After centrifugation to remove the DCC, aliquots of the supernatants were further diluted (1:3) and incubated at room temperature for 1h with 10 nM [^{3}H] 5α-dihydrotestosterone (PerkinElmer Life Sciences, Boston, MA) followed by an additional incubation (30 min) at 0 C. Nonspecific binding was estimated in the presence of excess unlabeled 5α-dihydrotestosterone (DHT). Free ligand was removed by incubation (10 min) with an ice-

cold DCC suspension and, following separation of the DCC by centrifugation, the supernatant containing SHBG-bound ligand was taken for radioactivity measurements (Hammond and Lähteenmäki, 1983). A similar protocol was used to determine the steroid-binding properties of the immunoreactive SHBG extracted from the acrosome, and involved a Scatchard plot (Scatchard, 1949) to determine the affinity constant and a competitive binding assay to determine steroid-binding specificity, as described previously (Hammond and Lähteenmäki, 1983).

3.3 Results

3.3.1 The 11 kb human *SHBG* transgene is expressed in germ cells of the mouse testis

A single Northern blot was used to measure the relative abundance of human *SHBG* transcripts in mixed populations of Sertoli cells and germ cells, as well as a pure population of germ cells isolated from the testes of wild type mice and mice containing 4 kb or 11 kb human *SHBG* transgenes (Figure 3.1). When a human SHBG cDNA that recognizes exon 6-8 sequences was used as probe, human *SHBG* transcripts were only detected in RNA extracts of testicular cells from mice containing the 11kb human *SHBG* transgene. The use of mouse vimentin (a marker of somatic cells) and transition protein 1 (a germ cell specific marker) cDNAs allowed us to demonstrate the purity of the germ cells isolated from the testes of transgenic mice (Figure 3.1, lanes 7 and 8). These data indicate that similar numbers of germ cells were present in all samples, as evidenced by the presence of similar amounts of transition protein 1 mRNA. Therefore, since the relative abundance of human *SHBG* transcripts in the germ cell only preparation was similar to that in the mixed population of Sertoli cells and germ cells, this suggested that the *SHBG* transcripts in the testis of 11 kb human *SHBG* transgenic mice cannot be derived from Sertoli cells.

To further characterize the human *SHBG* transcripts that accumulate in testicular germ cells, a Northern blot of total RNA extracts of germ cells, isolated from transgenic mice containing either the 4 kb or 11 kb human *SHBG* transgenes, was performed using exon 1- and alternative exon 1-specific cDNA probes. As a control, we also included a similar amount of total RNA from an 11 kb human *SHBG* transgenic mouse liver (Figure 3.2). When comparing the ratios of signals obtained using these exon 1-specific cDNAs with those

obtained using a cDNA corresponding to human *SHBG* exon 6-8 sequences (Figure 3.2), it is again apparent that *SHBG* transcripts are only present in germ cells from the 11 kb human *SHBG* transgenic mice. As expected, the *SHBG* transcript in the liver RNA sample comprises predominantly the exon 1 sequence containing the translation initiation codon for the SHBG precursor polypeptide and the leader sequence for secretion. By contrast, the *SHBG* transcript in the germ cells from mice containing the 11 kb human *SHBG* transgene, can only be detected using the cDNAs that recognize the alternative exon 1 sequence and sequences corresponding to exons 6-8.

The human alternative *SHBG* transcripts were also analysed in germ cells from transgenic mice by a RT-PCR with specific primers for human *SHBG* alternative exon 1 and human *SHBG* exon 8, and mouse transition protein 1 specific primers were again used in a RT-PCR as a positive control for the presence of intact mRNA species. Human *SHBG* transcripts were detected only in the germs cells of the 11 kb human *SHBG* transgenic mice, while mouse transition protein 1 was amplified in all samples including those from 4 kb human *SHBG* transgenic mice and wild type mice (Figure 3.3). When the two differently sized RT-PCR products amplified using human *SHBG* specific primers were cloned and sequenced, the approximately 1.1 kb RT-PCR product (Figure 3.3) was found to contain the alternative exon 1 sequence followed by the sequences of exons 2 - 8, while the smaller and less abundant RT-PCR product (approximately 0.9 kb) contained the alternative exon 1 sequence followed by the sequences of exons 2-6 and 8.

Expression of the endogenous mouse *SHBG* gene was examined in the testicular cell preparations from wild-type mice by Northern blotting, and mouse vimentin and transition protein 1 cDNAs were again used to monitor for the presence of somatic cells and germ cells, respectively (Figure 3.4). When we used a cDNA corresponding to mouse *SHBG* exon 6-8 sequences, murine *SHBG* transcripts were only detected in the mixed population of Sertoli cells. Although similar levels of transition protein 1 mRNA levels were detected in the two different isolated testicular cell preparations, murine SHBG transcripts could not be detected in the purified germ cells (Figure 3.4). These data therefore indicate that the mouse *SHBG* gene is expressed in Sertoli cells rather than in germ cells.

3.3.2 Immunoreactive human *SHBG* accumulates in the acrosome during spermiogenesis in mice expressing the 11 kb human *SHBG* transgene

The immunoaffinity-purified rabbit antibodies against human SHBG do not detect antigens in the testes of wild type mice, and this illustrates the specificity of the immunoreactivity observed at low power magnification (10X) in the sections of testes from transgenic mice containing the 4 kb or 11 kb human *SHBG* transgenes (Figure 3.5 A). In these sections, similar amounts of immunoreactive human SHBG are present in the interstitial compartment of both transgenic mouse lines, irrespective of the size of the human *SHBG* transgene. Because we have been unable to detect any human SHBG transcripts in the testes of mice containing the 4 kb human *SHBG* transgene, and because the plasma levels of human SHBG are similar in these two lines of transgenic mice (Jänne *et al.*, 1998), the immunoreactivity in the interstitial compartment reflects the sequestration of SHBG from the plasma. In contrast to previous studies, in which we were not able to detect any immunoreactive human SHBG within the seminiferous tubules of mice containing human *SHBG* transgenes (Jänne *et al.*, 1998), microwave pretreatment of histology sections in a high pH buffer most likely exposed human SHBG epitopes within the seminiferous tubules of the 11kb human *SHBG* transgenic mouse testis (Figure 3.5 A). At higher magnification, this immunoreactive human SHBG could be detected within the acrosome of spermatids during spermiogenesis only in the seminiferous tubules of transgenic mice containing the 11 kb human *SHBG* transgene (Figure 3.5 B). Moreover, this immunoreactivity could be detected in the acrosome as soon as it begins to form on spermatids (stage VII of spermatogenesis) and persists in the acrosome as it develops throughout the elongation stages (stages IX-XII) of spermiogenesis (Figure 3.5 B).

To determine whether the immunoreactive human SHBG remains within the acrosome after sperm are released into the male reproductive tract, we also performed immunohistochemistry on sperm isolated from the epididymis of transgenic mice containing either the 4 kb or 11 kb human *SHBG* transgenes (Figure 3.5 C), and this clearly shows that immunoreactive human SHBG is only present in the acrosome of sperm from the 11 kb human *SHBG* transgenic mice (Figure 3.5 C).

3.3.3 Biochemical characteristics of the immunoreactive human SHBG extracted from testicular cells of transgenic mice and human sperm

To determine the molecular size of the immunoreactive SHBG within the acrosome of 11 kb human *SHBG* transgenic mouse germ cells, we used western blotting to examine protein extracts isolated from testicular cells, i.e., a mixed population of Sertoli cells and germ cells or isolated germ cells. The lack of any immunoreactive molecules in the protein extract of testicular cells from wild type mice confirms the specificity of the anti-human SHBG antibodies used for this purpose (Figure 3.6). In addition, there was no immunoreactivity in the protein extract of isolated testicular cells from mice containing a 4 kb human *SHBG* transgene (Figure 3.6), despite the fact that the testes of these animals contain appreciable amounts of immunoreactivity in the interstitial cell compartment (Figure 3.5 A). Thus, the approximately 45 kDa immunoreactive protein observed in the isolated testicular cell extracts of mice containing the 11 kb transgene cannot be accounted for by contamination of SHBG from blood or the interstitial cell compartment.

In preliminary Western blotting experiments of the testicular protein extracts, we noticed that the apparent molecular size of the immunoreactive human SHBG in these samples was slightly smaller than that in a serum sample from the same animals, and that its electrophoretic heterogeneity was different from that associated with human SHBG purified from serum. To explore this further, we compared the electrophoretic behaviour of immunoreactive SHBG in serum and testicular cell extracts from the 11 kb human *SHBG* transgenic mice. For this experiment, aliquots of the samples were also treated with N-glycosidase F to remove N-linked oligosaccharides prior to western blot analysis (Figure 3.7). This confirmed that the apparent molecular size of immunoreactive human SHBG in the testicular protein extract (44 - 46 kDa) is smaller and electrophoretically more heterogeneous than the major electrophoretic isoform of SHBG in serum (50-51 kDa). However, treatment with N-glycosidase F clearly reduces the electrophoretic heterogeneity of immunoreactive SHBG in both samples, and results in similarly sized immunoreactive products of about 43 kDa for deglycosylated serum SHBG, and 42 kDa for deglycosylated acrosomal SHBG (Figure 3.7).

The steroid-binding properties of the immunoreactive human SHBG in the protein extracts from isolated testicular cells were also examined using a steroid-binding capacity assay. Our experiments indicated that specific binding could only be detected in the extracts from mice containing the 11 kb human *SHBG* transgene and that the highest levels were present in one particular line (*SHBG*11-b) of these mice (Jänne *et al.*, 1998). We therefore prepared protein extracts of mixed populations of Sertoli cells and germ cells from these mice to study the steroid binding characteristics of the SHBG extracted from the acrosome. A Scatchard analysis using [^{3}H]DHT as labeled ligand indicates that the affinity constant of the acrosomal SHBG is essentially the same as SHBG in serum (Figure 3.8). Furthermore, a competition analysis with other SHBG ligands indicates that testosterone and estradiol compete equally as effectively for the binding of [^{3}H]DHT to the acrosomal SHBG, as compared to serum SHBG (Table 3.1).

Our finding that expression of alternative human *SHBG* transcripts in the testis of transgenic mice results in accumulation of an SHBG isoform in the acrosome of epididymal sperm led us to determine whether SHBG is also present in human sperm. To accomplish this, we compared the electrophoretic mobility of human SHBG in a serum sample with human SHBG in seminal plasma, unwashed sperm in seminal plasma, washed sperm, and sperm that had been purified by Percoll® density gradient centrifugation (Figure 3.9). This Western blot demonstrates that the apparent molecular size (M_r) of the immunoreactive SHBG in human sperm is about 5 kDa smaller than SHBG in either blood or seminal plasma. Furthermore the M_r of immunoreactive SHBG in human sperm is similar to that of the immunoreactive SHBG extracted from the testicular cells of 11kb human *SHBG* transgenic mice, but is less heterogeneous with respect to its electrophoretic mobility (Figure 3.9).

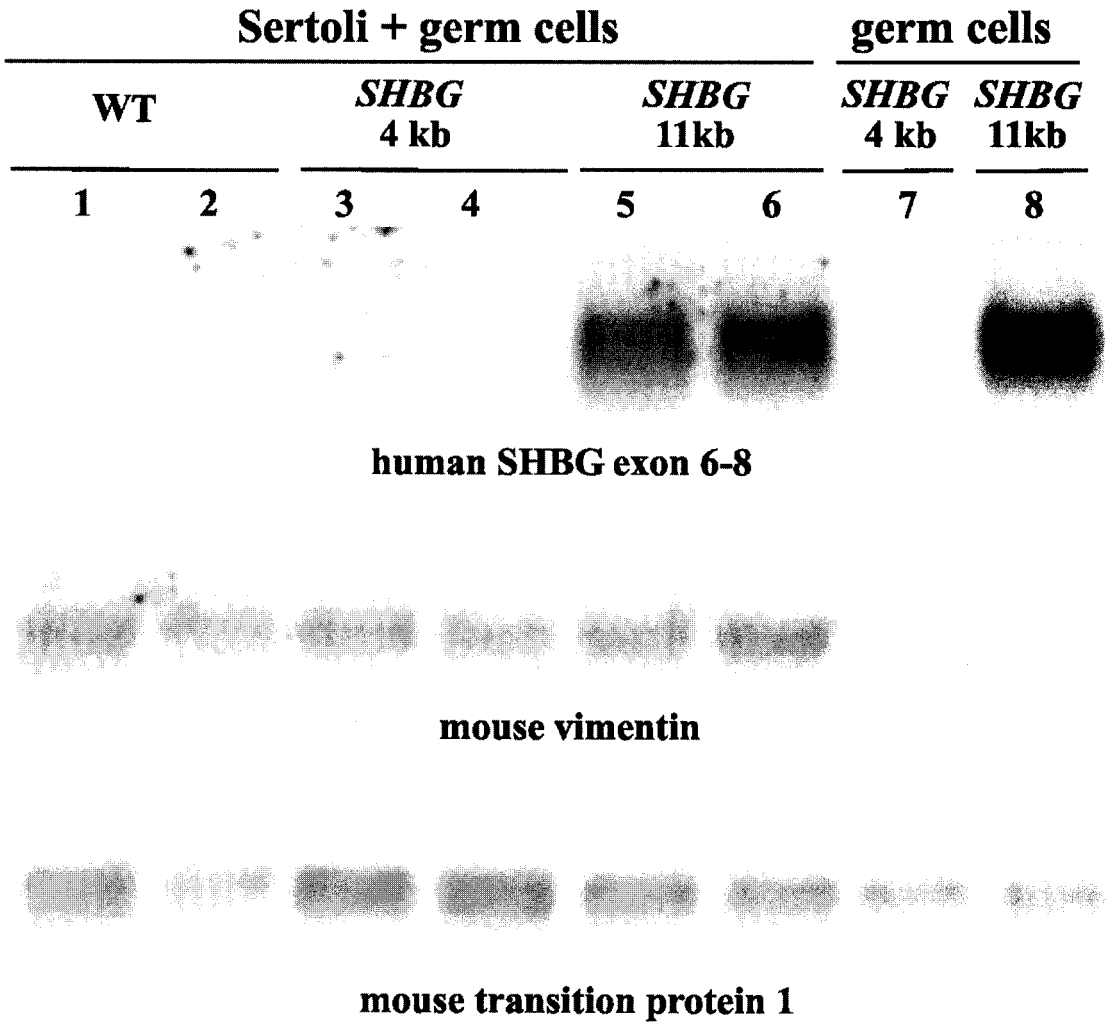

Figure 3.1 Human *SHBG* transcripts accumulate in testicular cells from 11 kb human *SHBG* transgenic mice. Human SHBG transcripts were only detected in total RNA extracted from testicular cells of 11 kb human *SHBG* transgenic mice on a northern blot (lanes 5,6 and 8). Mouse vimentin (marker of somatic cells) mRNA was detected in the mixed populations of Sertoli cells and germ cells from wild type and transgenic mice (lanes 1 to 6), but was not detected in isolated germ cells samples from transgenic mice (lanes 7 and 8). Mouse transition protein 1 (marker of germ cells) expression was detected in all samples from wild type and transgenic mice (lanes 1 to 8). Based on similar amounts of transition protein 1 mRNA in all samples, it appears that the relative abundance of *SHBG* transcripts is greater in the germ cells (lane 8) of 11 kb human *SHBG* transgenic mice than in the mixed population of Sertoli cells and germ cells (lanes 5 and 6) from these animals.

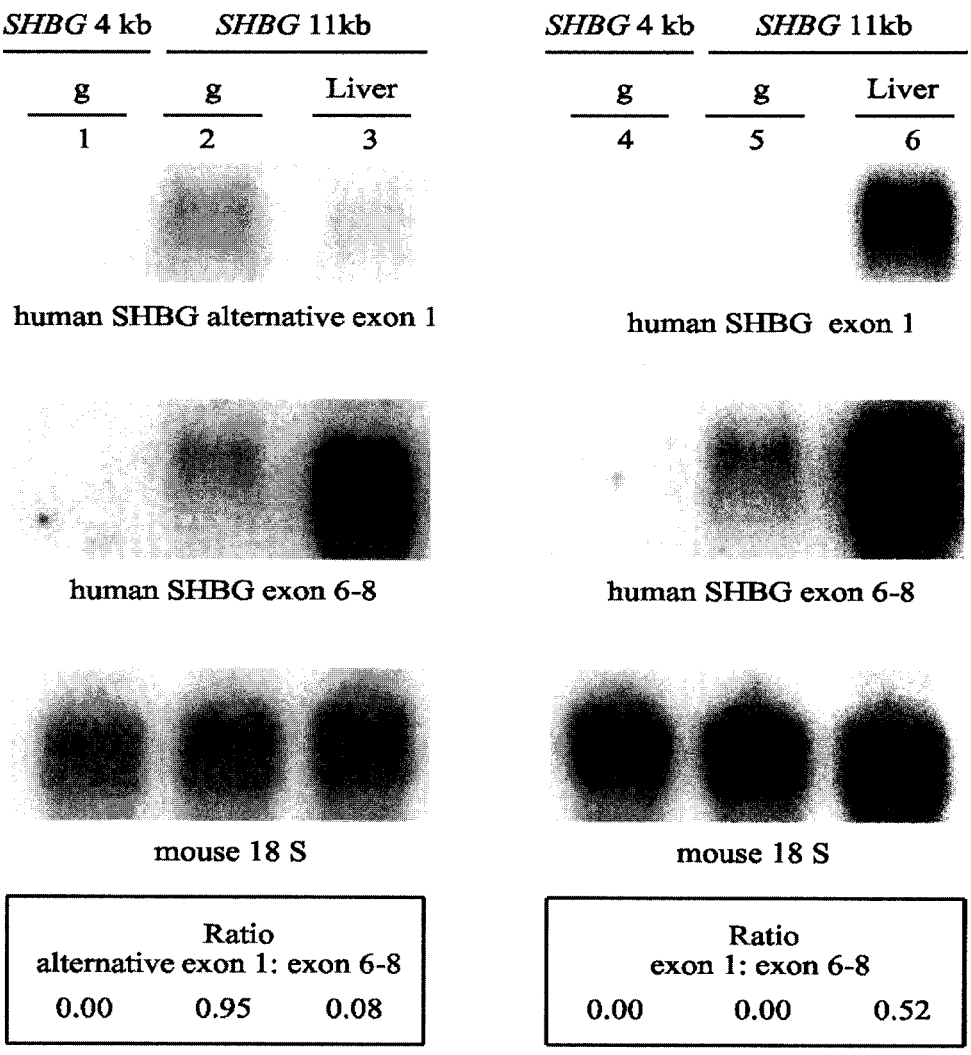

Figure 3.2 Human *SHBG* expression occurs in the germ cells of 11 kb human *SHBG* transgenic mice, and the resulting human *SHBG* transcripts contain an alternative exon 1 sequence. Human *SHBG* transcripts were only detected with a human SHBG cDNA (exon 6-8 sequences) in total RNA extracts from germ cells (lanes 2 and 5) and liver (lanes 3 and 6) of 11 kb human *SHBG* transgenic mice. Human *SHBG* transcripts containing the exon 1 sequence could only be detected in the liver RNA extracts (lane 6). When the same samples were examined using a probe for the alternative human *SHBG* exon 1 sequence, the intensity of signals obtained for RNA from the germ cells of 11 kb human *SHBG* transgenic mice were similar to those obtained using the human SHBG cDNA that recognizes human *SHBG* exon 6-8 sequences (lane 2). By contrast, when these two probes were used to examine the RNA extract from the liver (lane 3), the signal obtained using the alternative human *SHBG* exon 1 sequence was almost undetectable and this is most apparent when the ratios of these signals is compared (see below blots). A cDNA for mouse 18 S RNA was used to demonstrate that similar amounts of total RNA were present in all samples.

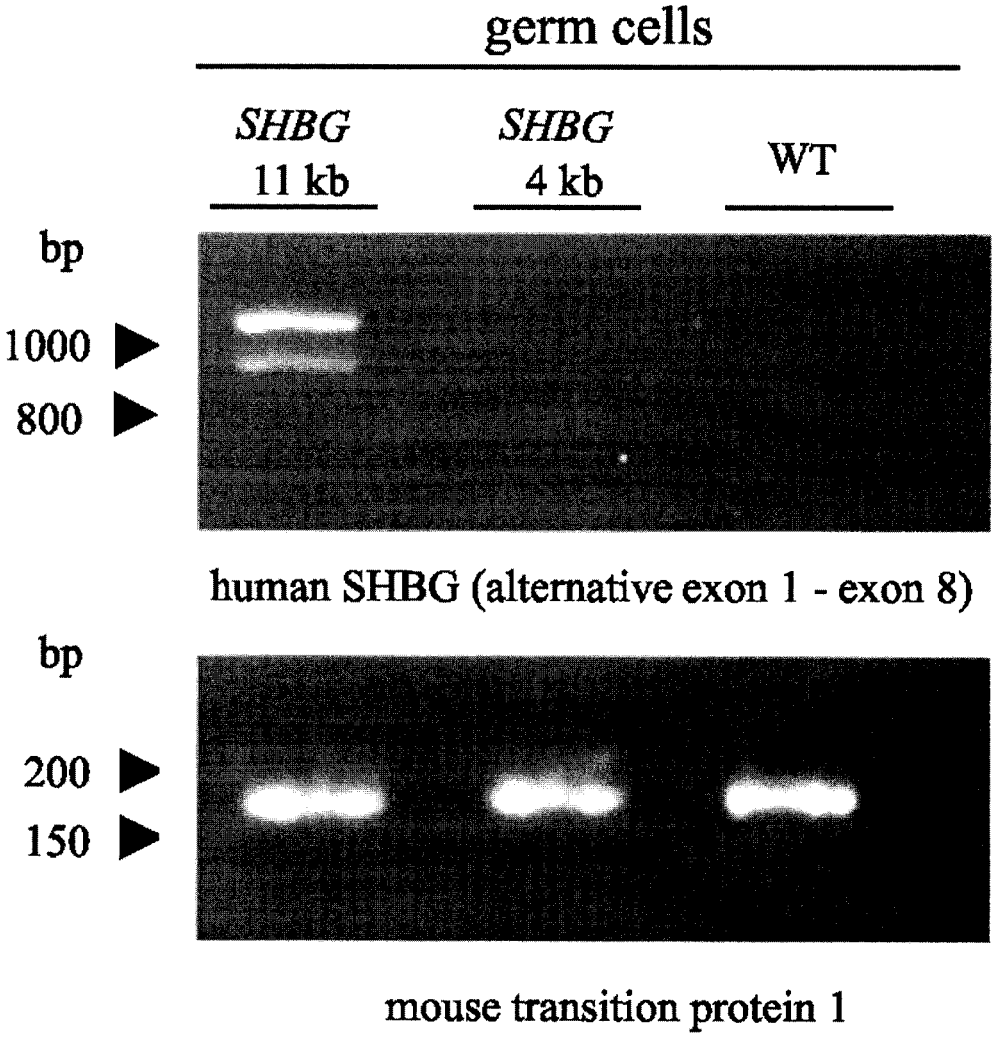

Figure 3.3 Two human *SHBG* transcripts containing alternative exon 1 sequences are present in total RNA extracts of 11 kb human *SHBG* transgenic mouse germ cells. An RT-PCR with oligonucleotide primers corresponding to human *SHBG* alternative exon 1 and exon 8 sequences resulted in two products of 1.13 kb and 0.93 kb when total RNA extracts from 11 kb human *SHBG* transgenic mouse germ cells was used as template. No products were obtained when similar amounts of testicular germ cell RNA extracts (as defined by the RT-PCR of mouse transition protein 1) were used from wild-type (WT) or 4 kb human *SHBG* transgenic mice.

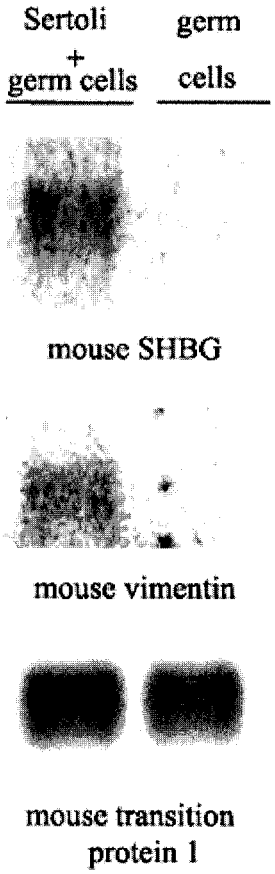

Figure 3.4 Mouse *SHBG* transcripts are present in Sertoli cells but are absent in germ cells. A mouse *SHBG* transcript of approximately 1.6 kb is present only in the total RNA extract of the mixed population of Sertoli cells and germ cells isolated from a wild-type (WT) mouse testis. A mouse vimentin cDNA was used as a monitor somatic cells and vimentin mRNA was absent in the isolated germ cell RNA extract. However, the presence of mouse transition protein 1 mRNA in the total RNA from both types of testicular cell extract indicated that similar amounts of germ cells were present in both samples.

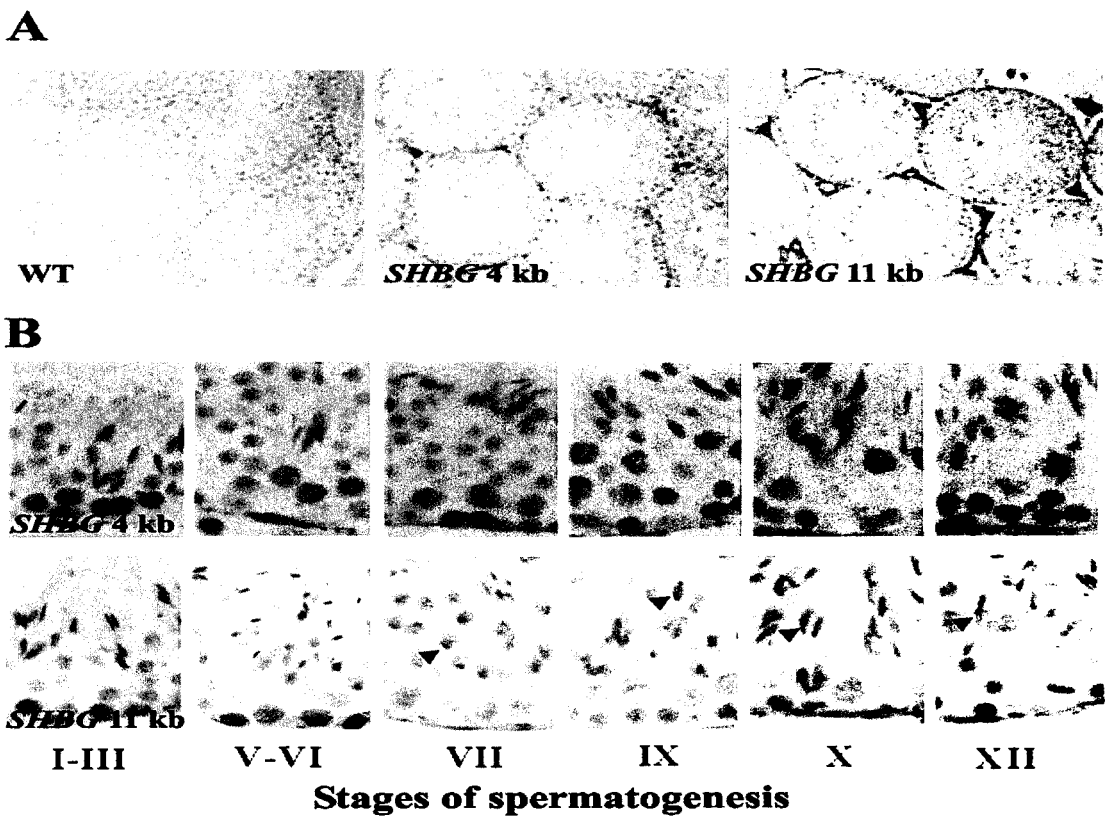

Figure 3.5 Immunoreactive human SHBG is present in the acrosome of germ cells within the 11 kb human *SHBG* transgenic mouse testis. Panel A. Immunoreactive human SHBG (brown stain) is present in the interstitial compartment of the testis from 4 kb and 11 kb human SHBG transgenic mice (10X lens magnification) while the testis of wild-type (WT) mice was completely devoid of any immunoreactivity. Panel B. At higher magnification (60X), immunorective human SHBG (brown stain) can be detected only within the seminiferous tubules of 11 kb human *SHBG* transgenic mice, and it can be seen to accumulate in the acrosome (arrowheads) of the germ cells during stages VII- XII of spermatogenesis. Panel C. Immunoreactive human SHBG (brown stain) is specifically located in the acrosome (arrowhead) of epididymal spermatozoa taken from 11 kb human *SHBG* transgenic mice (100X).

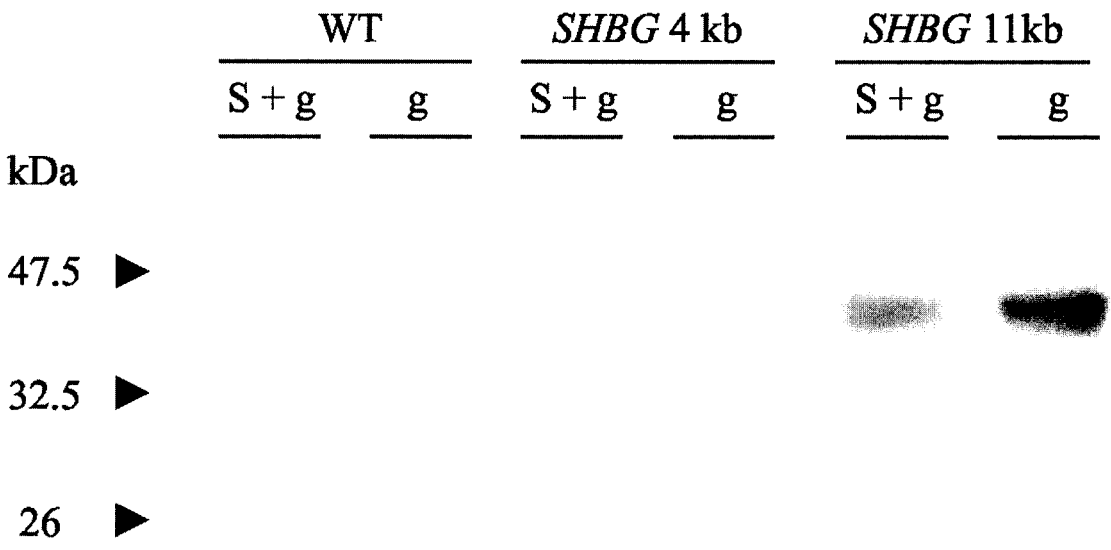

Figure 3.6 Immunoreactive human SHBG is present in testicular cell extracts from 11 kb human *SHBG* transgenic mice. An immunoreactive protein of approximately 47 kDa was detected by western blotting in protein extracts from a mixed population of Sertoli cells and germ cells (S + g) and from the isolated germ cells (g) of 11 kb human *SHBG* transgenic mice. When similar amounts of these cell extracts (50 μg) from wild-type (WT) and 4 kb human *SHBG* transgenic mice were examined in the same way no immunoreactive human SHBG was detectable. The positions of protein size markers is shown on the left.

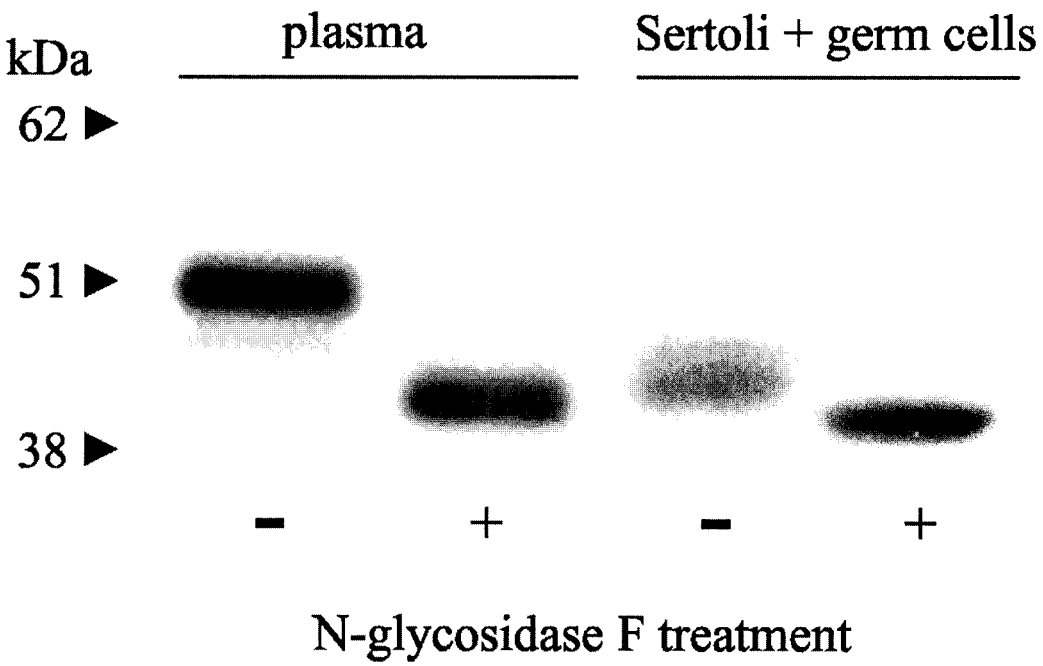

Figure 3.7 Electrophoretic variants of plasma and acrosomal human SHBG from 11 kb human *SHBG* transgenic mice reflect differences in glycosylation. The western blot shows differences in the electrophoretic mobility of plasma SHBG and acrosomal SHBG extracted from a mixed population of Sertoli cells and germ cells isolated from (S + g) before (-) and after (+) treatment of N-glycosidase F to remove N-linked oligosaccharides. The positions of protein size markers is shown on the left.

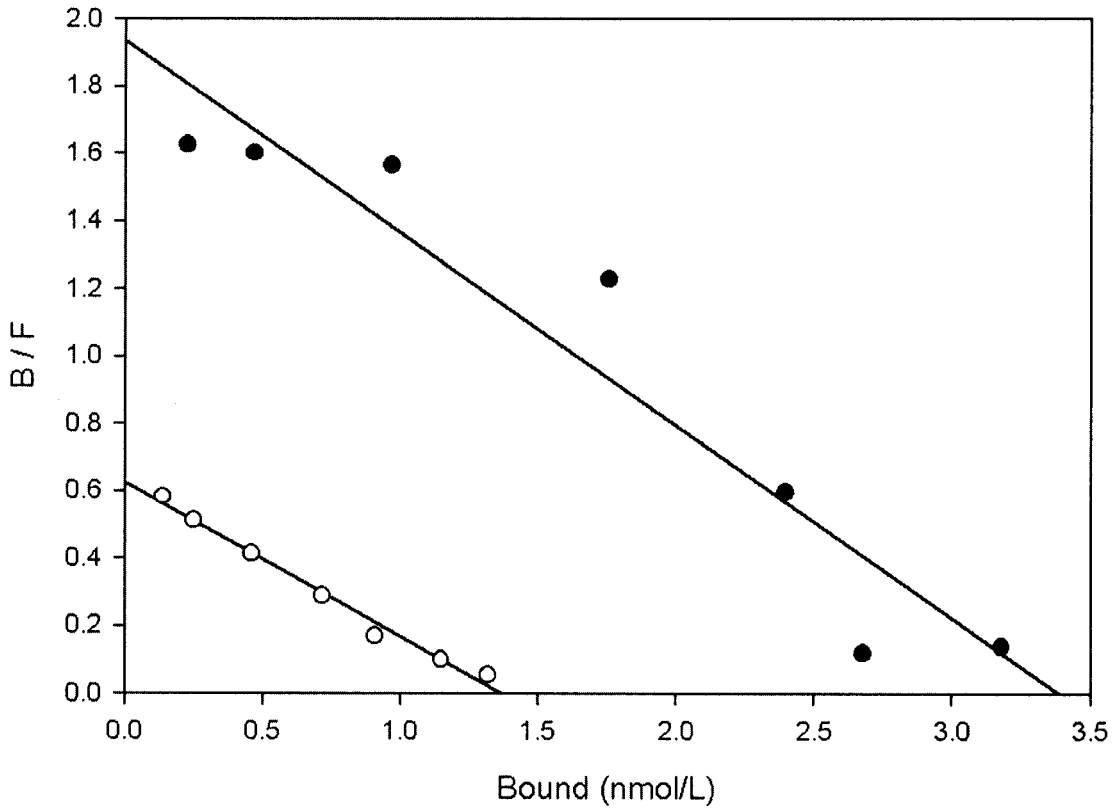

Figure 3.8 Scatchard plots indicate that the steroid-binding affinities of plasma SHBG (closed circles) and acrosomal SHBG (open circles) from 11 kb human *SHBG* transgenic mice are very similar. The steroid-binding affinities of plasma SHBG (K_d = 1.74 nM) and acrosomal SHBG (K_d = 2.25 nM) were determined using [^{3}H]DHT as labeled ligand. The bound over free [^{3}H]DHT ratio (B/F) was plotted against the amount of [^{3}H]DHT bound specifically to SHBG.

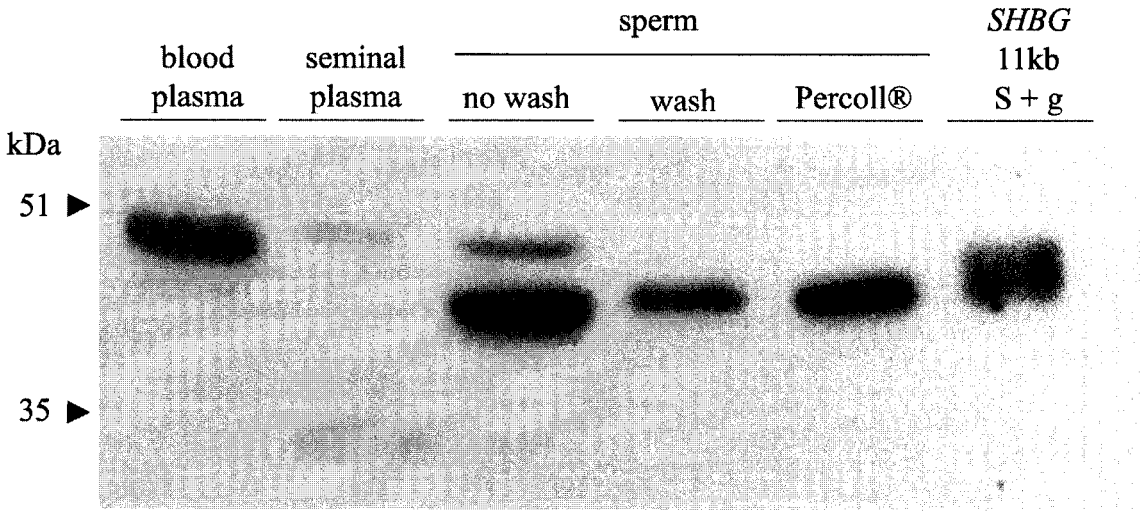

Figure 3.9 Western blot demonstrating the presence of an immunoreactive human SHBG isoform in human sperm samples that differs in size from SHBG blood plasma or seminal plasma. For comparison, an extract of Sertoli cells and germ cells (S + g) from 11 kb human *SHBG* transgenic mice has been analyzed next to an extract of Percoll -purified human sperm. The positions of molecular size markers in kDa are shown on the left.

SHBG Isoform	Relative Binding Affinity*		
	DHT	Testosterone	Estradiol
Serum	100	30.4	2.1
Acrosomal	100	33.5	2.9

Table 3.1 Comparison of the relative binding affinity of steroid ligands for serum and acrosomal SHBG isoforms. *Determined as a ratio of the concentration of steroid competitor resulting in 50% reduction in specific binding of [^{3}H] DHT to the concentration of DHT required to produce the same effect multiplied by 100.

3.4 Discussion

Based on extensive studies of ABP production by the rat testis (Joseph, 1994), it is generally assumed that the presence of an SHBG-like protein in the epididymis of other mammalian species is the result of *SHBG* gene expression in Sertoli cells (Westphal, 1986). In rats, the transcription unit responsible for plasma SHBG production by the fetal liver is also expressed in the testis of sexually mature animals (Joseph, 1994), with the highest levels of expression occurring in Sertoli cells during sexual development (Reventos *et al.*, 1988; Joseph *et al.*, 1987). In contrast, the 4 kb human *SHBG* transcription unit that is expressed in the liver of transgenic mice is not expressed in the testis (Jänne *et al.*, 1998), despite the fact that the corresponding rat *SHBG* transcription unit with a similar 5' regulatory region is expressed strongly as a transgene in the mouse testis (Reventos *et al.*, 1993). These observations provided an indication that there are species-specific differences in the way the *SHBG* gene is expressed in the testis.

Our results confirm this because they clearly show that the human and rodent *SHBG* genes are expressed in different cell types within the testis. Like the rat, expression of the mouse *SHBG* gene appears to be confined to Sertoli cells. However, expression of human *SHBG* transgenes in the mouse testis occurs in germ cells, and the resulting transcripts comprise an alternative exon 1 sequence identical to that present in several SHBG cDNAs isolated from a human testis library (Hammond *et al.*, 1989). Thus, cell-type specific differences in *SHBG* expression in the testis of different species is likely due to the utilization of different transcription units under the control of distinct promoter sequences.

In the germ cells, the human *SHBG* gene appears to be under the control of a promoter flanking the alternative exon 1 sequence, and 4 kb human *SHBG* transgenes are not expressed in the mouse testis because they lack this alternative exon 1 and its flanking regulatory sequences. Essentially nothing is known about how this promoter is controlled, but our previous studies have shown that human *SHBG* transcripts within the seminiferous epithelium of our transgenic mice begin to increase in abundance at stage V of spermatogenesis, and the highest levels are found between stages VII and IX (Jänne *et al.*, 1998). This corresponds well with the stage (VII) at which the proacrosomal vesicles form (Russell *et al.*, 1990), and when we first detect immunoreactive human SHBG in the germ

cells. The identification of germ cells as the cell type in which the alternative *SHBG* transcripts are located will now allow us to select an appropriate cell type for studies of how the promoter that controls their expression is regulated.

Differentially-spliced *SHBG* transcripts have been identified in several human tissues, and in many cases these transcripts lack exon 7 sequences (Hammond *et al.*, 1989; Misao *et al.*, 1997; Misao *et al.*, 1998). Analysis of human *SHBG* transcripts in the germ cells of our transgenic mice by RT-PCR also indicate that some alternative exon 1 containing human *SHBG* transcripts lack exon 7 sequences. This type of alternatively-spliced transcript would result in a premature termination of the open-reading frame encoding an SHBG isoform, and this type of product would most likely fold abnormally and undergo rapid degradation, as shown for a human SHBG variant encoded by an abnormal allele with a premature stop codon in exon 8 (Hogeveen *et al.*, 2002). In addition, if human *SHBG* transcripts lacking exon 7 sequences produce a carboxy-terminally truncated form of SHBG, this protein would lack consensus sites for N-glycosylation and our western blot analysis indicates that the immunoreactive human SHBG in the mouse germ cells is N-glycosylated. We therefore conclude that the human SHBG isoform we have identified in the acrosome cannot be the product of a transcript lacking exon 7.

Our data also indicate that the most abundant human *SHBG* transcript in the mouse germ cells comprises the alternative exon 1 sequence followed by a sequence corresponding to human *SHBG* exons 2-8 (Hammond *et al.*, 1989). The 5'-end of this alternative exon 1 sequence has not yet been identified, but preliminary primer extension analysis (unpublished), and comparisons with the published human *SHBG* sequence (Hammond *et al.*, 1989), indicates that the complete alternative exon 1 sequence lacks an in frame AUG codon. This might imply that the first conventional translation initiation codon is the AUG codon for Met30 in the mature SHBG protein sequence, which is located in exon 2 (Hammond *et al.*, 1989). However, since the size of the acrosomal SHBG isoform after de-glycosylation is within 1-2 kDa of the size of serum SHBG treated in the same way, it is unlikely that its amino-terminus corresponds to Met 30 in SHBG because the deglycosylated acrosomal SHBG isoform would then be at least 3 kDa smaller than the deglycosylated serum SHBG. Furthermore, the acrosomal SHBG isoform accumulates in the proacrosomal

vesicle, which forms from granules that originate from the Golgi apparatus, and it must therefore comprise a leader sequence that is removed as the nascent protein undergoes translocation through the rough endoplasmic reticulum. Thus, we conclude that translation of the major alternative human *SHBG* transcript in testicular germ cells must start from a non-conventional translation initiation codon as part of an internal ribosome entry site (Vagner *et al.*, 2001), and that the precursor polypeptide it encodes comprises a novel amino-terminal leader sequence.

The electrophoretic mobility of acrosomal SHBG is more heterogeneous than SHBG in serum when analysed by SDS-PAGE, and our data indicate that this is due to a difference in the extent of N-glycosylation. This might also be attributed to the fact that spermatids at all stages of development were used for the extraction of acrosomal SHBG, and the fact that the carbohydrate composition of glycoproteins in the acrosome changes throughout spermiogenesis in a species-specific manner (Martinez-Menarguez *et al.*, 1992). Although the functional significance of N-linked carbohydrates within the carboxy-terminal domain of SHBG remains obscure, it has been shown previously that the glycosylation has no influence on its steroid-binding activity (Bocchinfuso *et al.*, 1992). However, one particular N-glycosylation site is invariably conserved across a wide variety of mammalian species, and is likely to be functionally important (Hammond, 1993). It could for instance influence the ability of SHBG to interact with other proteins on the surface of specific cell types (Avvakumov *et al.*, 1988), and this might be relevant to its function in the acrosome.

Given the morphological differences in the testes of humans and rodents, and the fact that rodents lack SHBG in the blood, it is not surprising that there are differences in the way the human and rodent *SHBG* genes are expressed in the testis. Previous studies have shown that the overexpression of a rat *SHBG* transgene in the Sertoli cells of the mouse testis results in an increase in germ cell apoptosis (Selva *et al.*, 2000), but this does not occur in 11 kb human *SHBG* transgenic mice in which the transgene is expressed within the germ cells. Furthermore, there are marked differences in the levels of *SHBG* gene expression in the testis of mice and rats (Wang *et al.*, 1989), and attempts to demonstrate that human Sertoli cells secrete a protein with steroid-binding properties similar to SHBG have not been successful (Santiemma *et al.*, 1992). In this context, there is also no reason to assume that SHBG gene

products function similarly in the human and rodent testis. Based on the observation that human SHBG accumulates in the acrosome of sperm in our transgenic mice, we examined human sperm samples and confirmed that an SHBG isoform, which can be distinguished from plasma SHBG on the basis of its electrophoretic mobility, is also present in ejaculated human sperm. The SHBG extracted from human sperm is slightly smaller and less heterogeneous than that extracted from transgenic mouse testicular cells, and this might be due to more uniform glycosylation of the SHBG in ejaculated human sperm when compared to the SHBG extracted from mixed populations of germ cells at different stages of maturation (Martinez-Menarguez *et al.*, 1992). These data lead us to conclude that the expression of the human *SHBG* transgene in mice mimics the situation in the human testis, and that production of an SHBG isoform in germ cells must also result in its accumulation in the acrosome of human sperm.

Male mice expressing the 11 kb human *SHBG* transgene in their testes are reproductively normal (Jänne *et al.*, 1998), and this suggests the presence of human SHBG in the acrosome has no deleterious effects on sperm function in this animal model. Any positive effects that it might have on either sperm viability or capacity for fertilization would be difficult to assess in these animals. It may also not be relevant to the human situation because of species-specific differences in sperm maturation events, sperm-egg recognition and fertilization. Why human SHBG might accumulate in the acrosome therefore remains to be determined and analysis of the content of SHBG in various normal and abnormal human sperm samples may shed some light on this. In this context, the fact that acrosomal SHBG retains the capacity to bind steroids is of particular interest, and raises the question of what steroid ligands are bound to human SHBG in this location. Thus, in addition to providing new information about human *SHBG* gene expression in the testis, our studies provide the first evidence that alternative human *SHBG* transcripts encode an SHBG isoform that is distinct from plasma SHBG, and which might serve a novel function unrelated to extracellular steroid transport.

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CHAPTER 4

HUMAN SEX HORMONE-BINDING GLOBULIN PROMOTER ACTIVITY IS INFLUENCED BY A $(TAAAA)_n$ REPEAT ELEMENT WITHIN AN *alu* SEQUENCE

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4.1 Introduction

Plasma sex hormone binding globulin (SHBG) binds testosterone and estradiol with high affinity and selectivity, and regulates the access of these sex steroids to their target tissues (Siiteri *et al.*, 1982). Hepatocytes are the primary site of plasma SHBG biosynthesis, and changes in the blood levels of SHBG are influenced by hormonal, as well as metabolic and nutritional status (Hammond, 1990). Low serum SHBG levels are commonly found in women with polycystic ovarian syndrome (PCOS) and disorders characterised by androgen excess (Anderson, 1974), and have been reported to be a prognostic indicator for the onset of Type II diabetes and cardiovascular disease (Lim *et al.*, 1999; Skafar *et al.*, 1997). Low serum levels of SHBG are also inherited within families (Meikle *et al.*, 1982; Jaquish *et al.*, 1997), and associations between abnormal serum SHBG levels and disease processes may therefore be obscured by genetic differences that contribute to variations in human *SHBG* gene expression.

We have recently characterised two binding sites for HNF-4 and COUP-TF within the human *SHBG* proximal promoter which influence its transcriptional activity in human HepG2 hepatoblastoma cells (Jänne and Hammond, 1998). In particular, binding of HNF-4 to a TA rich sequence close to the transcription start site in liver cells appears to substitute for the TATA-binding protein in the initiation of transcription (Jänne and Hammond, 1998). The possible function of the second HNF-4 binding site in the *SHBG* proximal promoter is not as clear, but it may contribute to phylogenetic differences in the temporal and tissue specific expression of *SHBG* because it lies within a region that is absent in the corresponding region of *SHBG* genes in other mammalian species (Jänne and Hammond, 1998). Apart from this obvious difference in *SHBG* promoter sequences between species, the human and rodent *SHBG* promoters show a remarkable degree of sequence conservation, which only begins to diverge at the boundary of several *alu* sequences located at about –700 nts from the human *SHBG* transcription start site (Jänne and Hammond, 1998). This likely represents a functional boundary within the promoter sequence because human *SHBG* gene sequences containing only 803 nts of promoter sequence are expressed in a spatially and temporally appropriate manner when introduced as transgenes into the mouse genome (Jänne

et al., 1998; Jänne *et al.*, 1999). Although the significance of repetitive elements within promoter sequences is unclear, they may contain nuclear factor binding sites that contribute to the regulation of transcription (Sharan *et al.*, 1999; Norris *et al.*, 1995; Vansant and Reynolds, 1995).

By characterising the upstream region of the human *SHBG* promoter, we have now found that a $(TAAAA)_6$ repeat within an *alu* sequence binds a 46 kDa liver enriched nuclear protein, and acts to silence transcription. More importantly, the number of TAAAA repeats at this location is highly variable between individuals within the general population, and the transcriptional activity of the *SHBG* promoter and the binding of nuclear protein to this element are both directly related to the number of TAAAA repeats.

4.2 Materials and Methods

4.2.1 *In Vitro* footprinting

In vitro footprinting templates of human *SHBG* promoter upstream regions were produced by digesting a human *SHBG* fragment (Hammond *et al.*, 1989) with *Xho*I and *Xba*I. This released a 504 base pair (bp) region of the *SHBG* promoter corresponding to -803/-299 nucleotides (nt) relative to the transcription start site in the liver (Jänne and Hammond, 1998). This fragment was further digested with *Hae*III or *Hin*fI, and the products (-803/-656 nt *Xho*I-*Hae*III, -735/-587 nt *Hin*fI-*Hin*fI, -587/-362 nt *Hin*fI-*Hin*fI, -541/-298 nt *Hae*III-*Xba*I) were cloned into the *Eco*RV site of pBluescript (Stratagene, LaJolla, CA) in the correct orientation to permit labelling of the sense strand after digestion with *Hin*dIII. The *Hin*dIII-digested constructs were end-labeled with the Klenow fragment of DNA Polymerase I in the presence of $[\alpha\text{-}^{32}P]$dCTP and purified using a NICK™ column (Amersham Pharmacia Biotech, Inc., Baie d'Urfé, Québec). Radiolabeled probes were released from the plasmids by digestion with *Eco*RI and purified by 6% polyacrylamide gel electrophoresis (PAGE). The DNaseI footprinting reactions with mouse liver nuclear extracts were carried out as described previously (Jänne and Hammond, 1998). Hydroxy-radical footprinting with the same extracts was also performed using the -803/-656 nt *Xho*I-*Hae*III region of the human *SHBG* promoter (Tullius *et al.*, 1987; O'Halloran *et al.*, 1989). Mouse liver nuclear extracts were used in these and other experiments to characterize and study the regulation of human

SHBG promoter activity because human *SHBG* transgenes are expressed efficiently in mouse hepatocytes postnatally (Jänne *et al.*, 1998; Jänne *et al.*, 1999).

4.2.2 Reporter plasmids

Human *SHBG* promoter deletion constructs were generated by amplifying a region from an *SHBG* fragment (Hammond *et al.*, 1989) in a polymerase chain reaction (PCR). This was done using a common reverse primer containing an *Xba*I site (-299), and forward primers (containing an *Xho*I site) corresponding to various positions in the 5′ promoter region (Table 4.1). These PCR products were digested with *Xho*I and *Xba*I, and then subcloned into a pSP72 vector (Promega Corp., Madison WI) containing the –299/+60 nt region of the human *SHBG* proximal promoter. The entire promoter sequences were then excised with *Hin*dIII and *Xho*I and inserted into a pGL2 Basic luciferase reporter plasmid (Promega).

Site-directed mutagenesis was accomplished using a pSELECT vector containing the –803/-299 nt region of the human *SHBG* promoter, according to the Altered Sites manual (Promega). Mutated sequences in pSELECT were removed by *Xho*I and *Xba*I digestion and subcloned into the pSP72 vector containing the –299/+60 nt region of the human *SHBG* promoter, and the entire promoter sequences were then inserted into pGL2 Basic, as described above. All PCR-generated and mutated sequences were confirmed by DNA sequencing using a commercially available kit (Amersham Pharmacia Biotech).

4.2.3 Cell culture and transfection

All cell culture reagents were from Life Technologies, Inc. (Burlington, Canada). Human HepG2 hepatoblastoma cells were maintained in DMEM supplemented with 10% FBS, and were transiently transfected with human *SHBG* promoter-pGL2 reporter constructs (1.2 μg) and pCMV LacZ (0.2 μg) using LipofectAMINE® reagent (Jänne and Hammond, 1998). Cells were harvested 48 h following transfection, and cell extracts were prepared by three cycles of freezing and thawing for analysis of luciferase and β-Galactosidase activity. Luciferase units were divided by readings obtained from a β-Galactosidase assay to correct for efficiency of transfection.

4.2.4 Electrophoretic Mobility Shift Assay (EMSAs)

Mouse liver nuclear protein extracts (4 μg) were prepared (Sierra, 1990), and then incubated in EMSA buffer (2.5 mM HEPES pH 7.6, 0.5 mM EDTA, 0.5 mM DTT, 50 mM NaCl, 6.25% glycerol and 3 μg poly dIdC) on ice for 10 min in the presence or absence of double-stranded competitor oligonucleotides (Table 4.2). The corresponding end-labeled oligonucleotide probes were then added, and the binding reaction was allowed to proceed for 15 min at room temperature. For antibody supershift assays, radiolabeled probe was incubated with nuclear extract on ice for 15 min prior to addition of either normal rabbit serum, or an antiserum specific to SP1 (Santa Cruz Biotechnology, Inc., Santa Cruz, California). Reactions were then incubated for 15 min at room temperature before electrophoresis. Nuclear proteins bound to radiolabeled oligonucleotides were separated from free probe by 5% PAGE, and the gel was dried and exposed to Biomax MR film (Eastman Kodak Company, Rochester, NY) against an intensifying screen at –80°C.

4.2.5 Analysis of nuclear factor binding to TAAAA repeats by UV crosslinking

Binding reactions of nuclear protein extracts to double-stranded oligonucleotides comprising various TAAAA repeats (Table 4.2) were carried out under the same conditions used for EMSAs. After a 15 min incubation at room temperature, samples were exposed to UV light (302 nm) at a distance of 10 cm for 15 min at 0°C. Equal volumes of sodium dodecylsulfate (SDS) loading buffer were added and samples were heated at 95°C for 5 min and subjected to SDS-PAGE with 4% and 10% acrylamide in the stacking and resolving gels, respectively. Gels were dried and exposed to X-ray film, as described above.

(A) Description	PCR Oligonucleotides for Promoter Deletions
-299 reverse	5′GAGGTCTAGAAACAGTCCTCCCCTGCGT
-696 forward	5′TGAACTCGAGGGGTCAGGGAGTGGGTGA
-670 forward	5′GAACTCGAGCTAGACTGTTTAGGCCCTGT
-617 forward	5′TGAACTCGAGGGGGGCAGGTCCCTTCTTAAT
-552 forward	5′TGAACTCGAGGCCTCAGAGGCCTGGGAAAAG
-509 forward	5′TGAACTCGAGAGATGCCAGGCACTGTGCCTG
-458 forward	5′TGAACTCGAGACCCCGTAAAGTATTATTTTC

(B) Description	Mutagenic Oligonucleotides
Δ6TAAAA	5′GAAATCACCCACTCCCTGACCCGACAGAGTCTTGCTCTATCACCC
4xTAAAA	5′GAAATCACCCACTCCCTGACCCA(TTTTA)$_4$GACAGAGTCTTGCTCTATCACCC
5xTAAAA	5′GAAATCACCCACTCCCTGACCCA(TTTTA)$_5$GACAGAGTCTTGCTCTATCACCC
7xTAAAA	5′GAAATCACCCACTCCCTGACCCA(TTTTA)$_7$GACAGAGTCTTGCTCTATCACCC
8xTAAAA	5′GAAATCACCCACTCCCTGACCCA(TTTTA)$_8$GACAGAGTCTTGCTCTATCACCC
9xTAAAA	5′GAAATCACCCACTCCCTGACCCA(TTTTA)$_9$GACAGAGTCTTGCTCTATCACCC
10xTAAAA	5′GAAATCACCCACTCCCTGACCCA(TTTTA)$_{10}$GACAGAGTCTTGCTCTATCACCC
mutation of SP1 (-536/-510)	5′GGTCTGAACTCCT**AA**CCCAGTCTTTT

Table 4.1 Sequences of oligonucleotides used for (A) deleting or (B) mutating human *SHBG* promoter sequence in the context of *luciferase* reporter gene constructs. Underlined sequences denote restriction enzyme recognition sites used for creation of human SHBG promoter constructs. Δ6TAAAA oligonucleotide designed to remove the TAAAA sequence. Mutations introduced into the FP12 SP1 binding site (-536/-510) (in bold) were designed based upon a previous mutation in an SP1 consensus sequence (Xie *et al.*, 1997).

Description	EMSA Oligonucleotides
6xTAAAA	5′gTC(TAAAA)$_6$TGGGT 3′ AG(ATTTT)$_6$ACCCAg
7xTAAAA	5′gTC(TAAAA)$_7$TGGGT 3′ AG(ATTTT)$_7$ACCCAg
8xTAAAA	5′gTC(TAAAA)$_8$TGGGT 3′ AG(ATTTT)$_8$ACCCAg
9xTAAAA	5′gTC(TAAAA)$_9$TGGGT 3′ AG(ATTTT)$_9$ACCCAg
10xTAAAA	5′gTC(TAAAA)$_{10}$TGGGT 3′ AG(ATTTT)$_{10}$ACCCAg
FP12	5′gAAAAGACTGGGGGAGGAGTTCAGAC 3′ TTTTCTGACCCCCTCCTCAAGTCTGg
FP12 mutant	5′GAAAAGACTGGG**TT**AGGAGTTCAGAC 3′ TTTTCTGACCC**AA**TCCTCAAGTCTGg
SP1	5′GCTGAGGGCGGGACCGCATC 3′CGACTCCCGCCCTGGCGTAG
HNF-4/COUP-TF	5′tcgaCTGGGCAGGGGTCAAGGGTCAGTGCCC 3′ GACCCGTCCCCAGTTCCCAGTCACGGGagct

Table 4.2 Sequences of oligonucleotides used in electrophoretic mobility shift assays (EMSAs). Lowercase letters indicate additional sequences that were filled using the Klenow fragment of DNA polymerase I and [α^{32}P]dCTP (Sierra, 1990). Mutations (in bold) introduced into FP12 (-536/-510) were based upon a previous mutation that disrupts an SP1 recognition sequence (Xie *et al.*, 1997). Double-stranded oligonucleotides representing SP1 (Prince and Rigby, 1991) and HNF-4/COUP-TF (Jänne and Hammond, 1998) binding sites were used as reported by others.

4.2.6 Southwestern blot analysis

Nuclear proteins extracted from MSC-1 mouse Sertoli cells and human HeLa cervical cancer cells (Dignam *et al.*, 1983), as well as mouse liver (Sierra, 1990), were fractionated by SDS-PAGE and transferred by electrophoresis onto a Hybond™ECL™ nitrocellulose membrane (Amersham Pharmacia Biotech). The membrane was washed three times for 45 min in a buffer containing 10 mM Tris pH 7.5, 150 mM NaCl, 10% glycerol, 2.5% Nonidet P-40, 5% milk powder, and 0.1 mM DTT. It was then rinsed twice in binding buffer (10 mM Tris pH 7.5, 40 mM NaCl, 1 mM EDTA, 1 mM DTT, 8% glycerol, and 0.125% milk powder). A radiolabeled double-stranded oligonucleotide (250,000 cpm/mL) spanning the $(TAAAA)_6$ sequence in the *SHBG* upstream promoter (Table 4.2) was added to the binding solution containing 5 mM $MgCl_2$ and 5 μg/mL poly dIdC, and was incubated with the membrane for 16 h at room temperature. The membrane was then washed in 50 mM Tris pH 7.5, 150 mM NaCl and exposed to Biomax MR film, as described above.

4.2.7 Analysis of the $(TAAAA)_n$ repeat in the SHBG promoter

Genomic DNA was isolated from peripheral blood leukocytes of healthy human volunteers (Sambrook *et al.*, 1989). Amplification of the TAAAA repeat region was accomplished using PCR with a forward primer (5′GCTTGAACTCGAGAGGCAG) within an *alu* sequence in the human *SHBG* promoter (Hammond *et al.*, 1989), and a reverse primer (5′CAGGGCCTAAACAGTCTAGCAGT) corresponding to a sequence at –651/-673 nt within the upstream promoter sequence. Amplified products were separated by 10% PAGE followed by staining with ethidium bromide. The number of TAAAA repeats was quantified by cloning PCR products in the pCR®–BluntII-TOPO® vector (Invitrogen Corp., Carlsbad, CA) for sequencing. Serum samples from the same individuals were also taken for measurements of SHBG concentrations using a commercially available immunoradiometric assay kit (ORION Diagnostica, Oulunsalo, Finland).

4.3 Results

4.3.1 Identification of nuclear factor-binding sites within the upstream region of the human *SHBG* promoter

Putative binding sites for nuclear proteins in the human *SHBG* upstream promoter were demonstrated by *in vitro* footprinting using mouse liver nuclear extracts (Figure 4.1). One of these footprints (FP17) contains a sequence (5′-TGATAGAGCAAGAC), which resembles a CEBPβ binding site when examined by homology searches using MatInspector (Quandt *et al.*, 1995) release 2.1. In addition, a sequence (5′-GGGGGAGGAGT) within FP12 (nt-527/-517) resembles a consensus SP1 binding site (5′-GGGGCGGGGC/T) by analysis using the TRANSFAC database (Wingender *et al.*, 2000). A region containing six TAAAA repeats, which is resistant to DNaseI digestion in the presence or absence of nuclear protein, lies between FP16 and FP17 (Figure 4.1), and the boundary of the footprint within the TAAAA repeat region was confirmed by hydroxy-radical footprinting with a mouse liver nuclear protein extract (Figure 4.2).

Interaction between SP1 and a double-stranded oligonucleotide that included FP12 (nt-536/-510) was confirmed by EMSA (Figure 4.3 A). The specificity of this interaction was demonstrated by inhibition of DNA:protein complexes using an oligonucleotide containing a known SP-1 binding site (Table 4.2). In addition, the major DNA:protein complex formed in an EMSA reaction was supershifted with an SP1 specific antibody (Figure 4.3 B).

4.3.2 Sequences in the human *SHBG* upstream promoter silence transcription

The activity associated with the nuclear factor binding sites identified by DNaseI footprinting (represented by boxes in Figure 4.4 A) was investigated by adding them sequentially to a human *SHBG* proximal promoter-luciferase reporter gene construct (Jänne and Hammond, 1998). These constructs were used for transcriptional activity measurements in a human hepatoblastoma cell line (HepG2) that produces SHBG (Khan *et al.*, 1981). This revealed that upstream sequences markedly reduce transcriptional activity when compared to the activity of the proximal promoter (Figure 4.4 A). In particular, addition of sequences that include FP16, as well as a putative CEBPβ-binding site within FP17 and the intervening

$(TAAAA)_6$ sequence resulted in progressive and significant reductions of promoter activity (Figure 4.4 A). The reduction of transcription by sequences at the 5′ boundary of the upstream promoter appeared to be associated specifically with the $(TAAAA)_6$ sequence because a 6-fold increase in transcription was observed when it was removed from the full-length promoter (Figure 4.4 B). It was also noted that mutation of the SP1 binding site (Table 4.2) within FP12, which prevents its interaction with liver nuclear extracts in an EMSA (data not shown), also resulted in a similar increase in the transcriptional activity of the full-length promoter (Figure 4.4 B). However, recovery of the transcriptional activity of the full-length promoter was not further enhanced by removal of TAAAA pentanucleotide repeat in combination with a mutant SP1 binding site within FP12 (Figure 4.4 B). It is also important to note that the presence of FP12 appears to have a negative effect on transcription only in the context of upstream sequence within the full-length promoter (Figure 4.4 A).

4.3.3 Nuclear factor binding to a $(TAAAA)_6$ sequence in the human *SHBG* promoter

When tested in an EMSA, a radiolabeled oligonucleotide comprising the $(TAAAA)_6$ sequence interacted with nuclear factors extracted from mouse liver, and could be competed for by unlabeled oligonucleotides spanning this repeat region, but not with an unrelated DNA sequence (Figure 4.5). To further characterise the nuclear factor(s) binding at this pentanucleotide repeat, a radiolabeled $(TAAAA)_6$ repeat oligonucleotide sequence (Table 4.2) was used as a probe for a southwestern blot (Figure 4.6). The radiolabeled probe recognized a nuclear protein with an apparent molecular weight of 46 kDa, which is enriched in mouse liver nuclear extracts when compared to nuclear extracts from mouse MSC-1 Sertoli cells or HeLa cells (Figure 4.6). By contrast, a nuclear protein with a molecular weight of approximately 67 kDa, was recognized in all nuclear extracts, and likely represents a ubiquitous nuclear factor which binds to this sequence (Figure 4.6).

4.3.3 The number of TAAAA repeats in the human *SHBG* upstream promoter varies and influences its activity

In view of reports that the number of TAAAA repeats in the promoters of other human genes varies among individuals (Durocher *et al.*, 1998), we amplified the region of

the *SHBG* upstream promoter containing the TAAAA repeat sequence from eight healthy male volunteers. The sizes of the PCR products indicated that the number of TAAAA repeats is highly variable both within and between individuals (Figure 4.7 A), and by sequencing them we demonstrated that the repeat number ranges from 6 to 10 (Figure 4.7 B). The serum *SHBG* concentrations in these individuals (with respect to their $(TAAAA)_n$ allele genotypes) were as follows: 13 nmol/L (6/10), 14 nmol/L (6/6), 15 nmol/L (7/7), 25 nmol/L (7/8), 24 nmol/L (6/8), 24 nmol/L (6/7), 15 nmol/L (6/8) 23 nmol/L (9/7). Although there was no obvious relationship between serum SHBG concentrations and the number of TAAAA repeats on each allele, an individual with two $(TAAAA)_6$ containing *SHBG* alleles had a relatively low serum SHBG level. However, the number of individuals we have studied is small, and no attempt was made to control for factors that might otherwise influence serum SHBG levels, such as body mass index.

The influence of this polymorphism on transcription was tested in the context of the full-length human *SHBG* promoter. We found that the activity of human *SHBG* promoter-luciferase reporter constructs in HepG2 cells increased 5 fold as the number of TAAAA repeats was increased from six to seven. As additional repeat sequences were added, little if any further increases in promoter activity were observed (Figure 4.8). These increases in promoter activity associated with increasing repeats is strikingly similar to the increase observed when the $(TAAAA)_6$ sequence is removed from the full-length promoter (Figure 4.4 B). Taken together, these data suggest that the silencing activity is associated specifically with the presence of only six TAAAA repeats. This was confirmed by performing similar experiments using full-length *SHBG* promoter sequences containing only four or five TAAAA repeats, which also provided approximately 5-fold higher levels of transcriptional activity when compared to the promoter sequence containing six repeats (Figure 4.8).

4.3.5 Nuclear factor binding to various TAAAA repeats

To further examine nuclear factor binding in relation to the number of TAAAA repeats, we performed a UV crosslinking experiment with radiolabeled oligonucleotides representing the various numbers (6-10) of TAAAA repeats observed in the general population (Figure 4.7), in the presence of mouse liver nuclear protein extracts (Figure 4.9).

This clearly demonstrated that the radiolabeled oligonucleotide containing six TAAAA repeats preferentially binds nuclear proteins, and the major complexes formed were 55-60 kDa in size (Figure 4.9). Furthermore, the relative abundance of these complexes declined markedly when oligonucleotides containing more than six repeats were used, and this is consistent with the marked increase in transcriptional activity of promoters containing more than six TAAAA repeats (Figure 4.8). The specificity of the complex formed using the TAAAA repeat oligonucleotide sequence was demonstrated by competition with unlabeled oligonucleotide, and the lack of complex formation in the presence of nuclear protein without exposure to UV light. In addition, exposure of radiolabeled oligonucleotides to UV did not result in the formation of any high molecular weight complexes in the absence of nuclear protein (data not shown). Taking into account the size of the radiolabeled probe (approximately 10 kDa), the size of the major UV crosslinked complexes is consistent with a complex between the oligonucleotide containing the six TAAAA repeats and the 46 kDa liver enriched protein identified as the TAAAA binding protein by southwestern blotting (Figure 4.6).

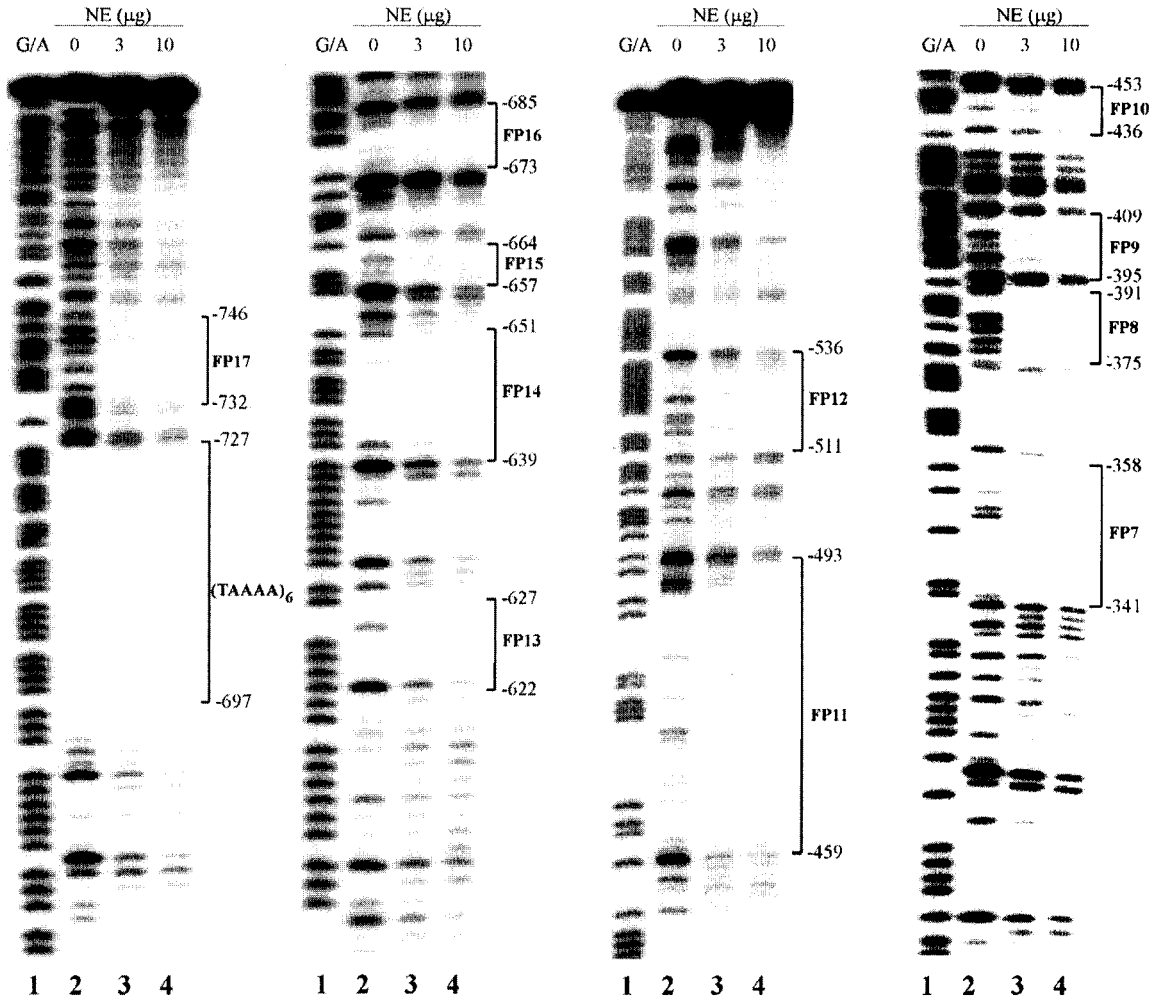

Figure 4.1 DNaseI *in vitro* footprinting of putative transcriptional regulatory elements in the upstream region of the human *SHBG* promoter. End-labeled fragments of the upstream region of the human *SHBG* promoter were incubated with 0 μg (lane 2), 3 μg (lane 3), or 10 μg (lane 4) of adult mouse liver nuclear extract (NE) and subjected to digestion with DNaseI. Digested fragments were purified and analysed by denaturing polyacrylamide gel electrophoresis. A Maxam Gilbert (G/A) sequencing reaction (lane 1) was run as a size marker to define the boundaries of the footprinted regions (FP7-FP17, shown by brackets). The numbering of these footprints extends from those identified previously in the *SHBG* proximal promoter (Jänne and Hammond, 1998). A bracket is also used to define the boundary of a $(TAAAA)_6$ repeat element which could not be digested by DNaseI in the absence (Lane 2) or presence (Lanes 3 and 4) of nuclear protein.

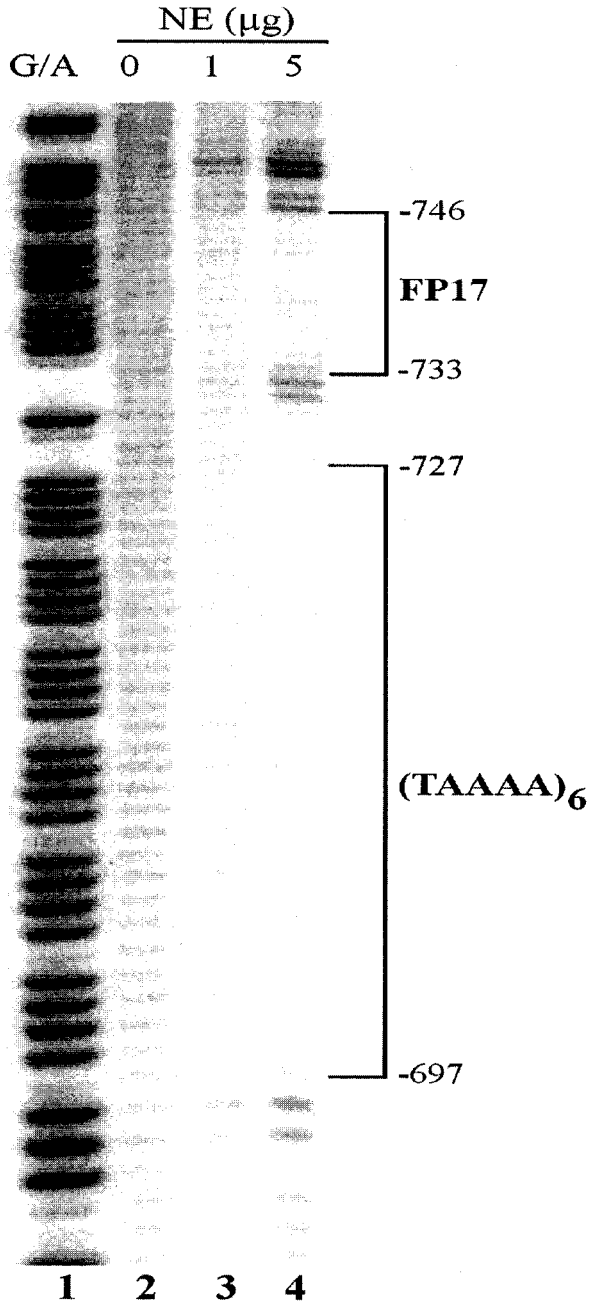

Figure 4.2 Hydroxy-radical footprinting of the TAAAA repeat within the upstream region of the human *SHBG* promoter. A radiolabeled –803/-656 fragment of the human *SHBG* promoter was incubated in the absence (lane 2) or presence of 1μg (lane 3) or 5 μg (lane 4) of mouse liver nuclear protein extract (NE), and was then subjected to hydroxy-radical DNA cleavage. The products were purified and separated on a denaturing 8% polyacrylamide gel, alongside the products of a Maxam-Gilbert (G/A) sequencing reaction as size marker (lane 1). The $(TAAAA)_6$ region and FP17 were protected from cleavage, and are shown in brackets.

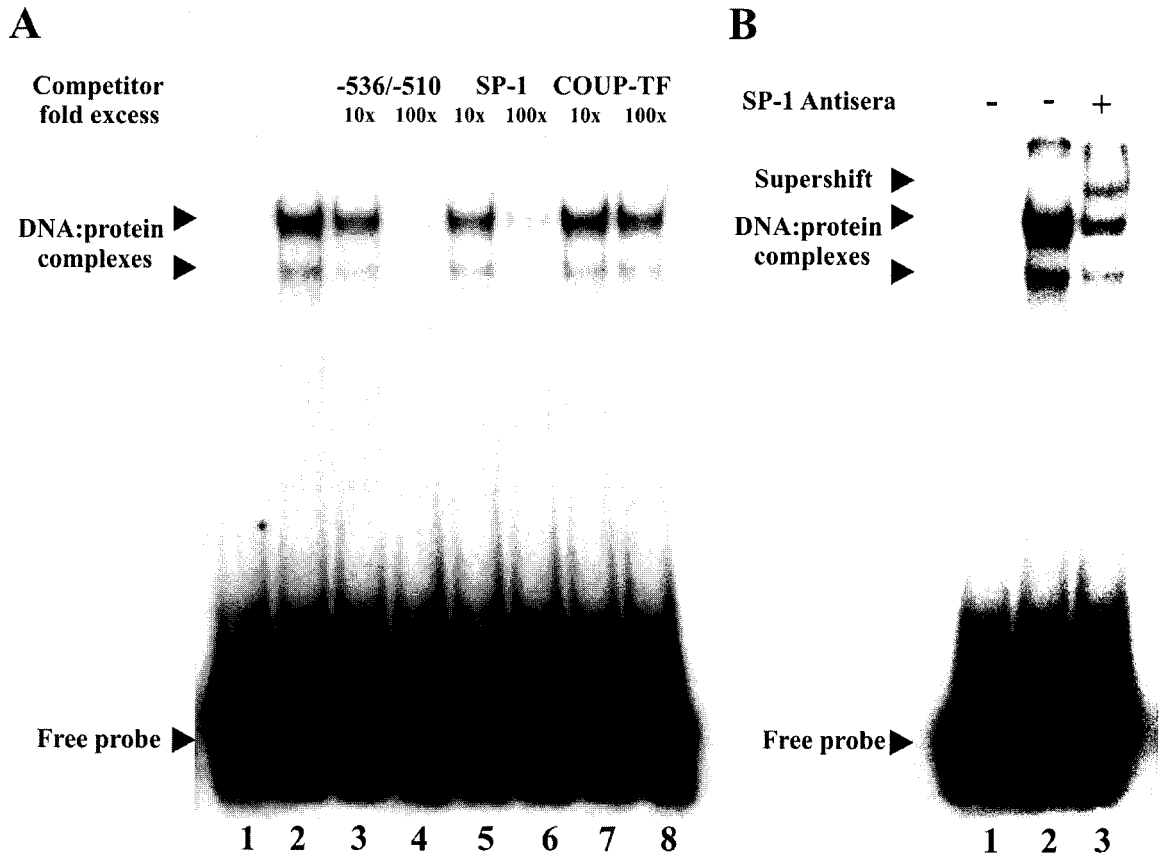

Figure 4.3 A footprinted region (FP12) in the human *SHBG* promoter (-536/-510) is an SP1 nuclear factor binding site. A, End-labeled double-stranded oligonucleotides spanning FP12 in the human *SHBG* promoter were incubated with 2 μg of adult mouse liver nuclear extract in the absence (lane 2) or presence of excess unlabeled competitor oligonucleotides (lanes 3-8). Free probe was separated from DNA:protein complexes by non-denaturing polyacrylamide gel electrophoresis. An unlabeled oligonucleotide which includes the HNF4/COUP-TF binding site in FP3 of the human *SHBG* promoter (Jänne and Hammond, 1998) was used to demonstrate specificity. B, Antibody supershift assays were performed by adding normal rabbit serum (lane 2), or antisera against SP1 (lane 3) during incubations of double-stranded end-labeled oligonucleotides with mouse liver nuclear proteins. Lane 1 is without nuclear protein.

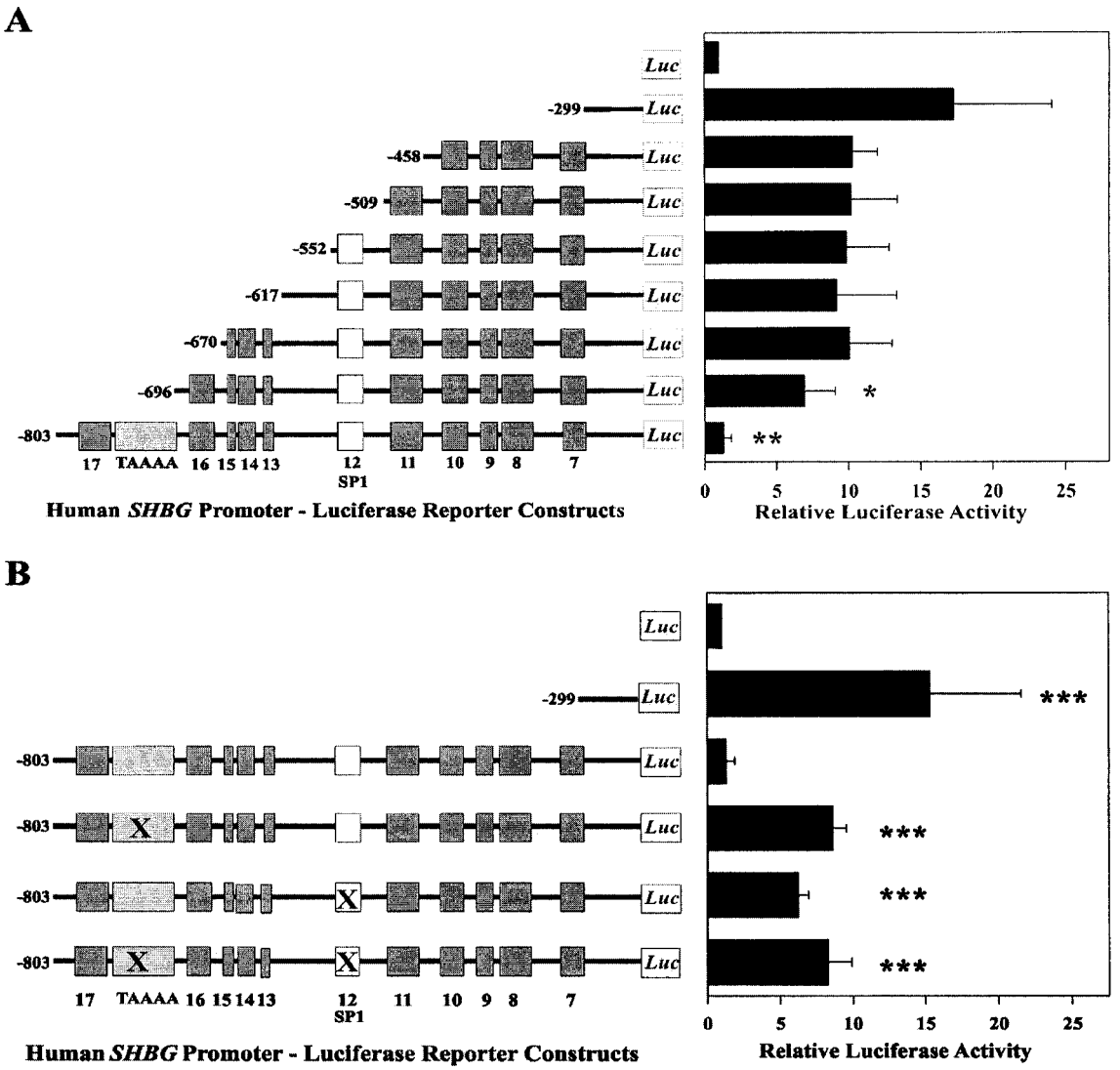

Figure 4.4 Analysis of human *SHBG* promoter activity in HepG2 cells. A, A series of human *SHBG* promoter deletion constructs was generated and transiently transfected into HepG2 cells. Regions of the human *SHBG* promoter protected from DNaseI digestion by mouse liver nuclear proteins (represented as boxes) were sequentially removed, and the effect of these nuclear factor binding sites on promoter activity was assessed. B, The transcriptional silencing activity of the $(TAAAA)_6$ repeat appears to require the presence of an SP1 site within FP12. Mutation of FP12 to prevent SP1-binding (X) relieves the transcriptional silencing of the $(TAAAA)_6$ repeat. The relative activities of the human *SHBG* reporter constructs are expressed relative to that of the promoterless pGL2 Basic luciferase reporter plasmid (*Luc*). In panel A, statistically significant differences were * = $p<0.05$; ** = $p<0.001$ when compared to the activity of the –299/+60 human *SHBG* promoter. In panel B, statistically significant differences were *** = $p<0.001$ when compared to the full-length (-803/+60) human *SHBG* promoter. Data are represented as means + S.E.M from at least 3 experiments performed in triplicate.

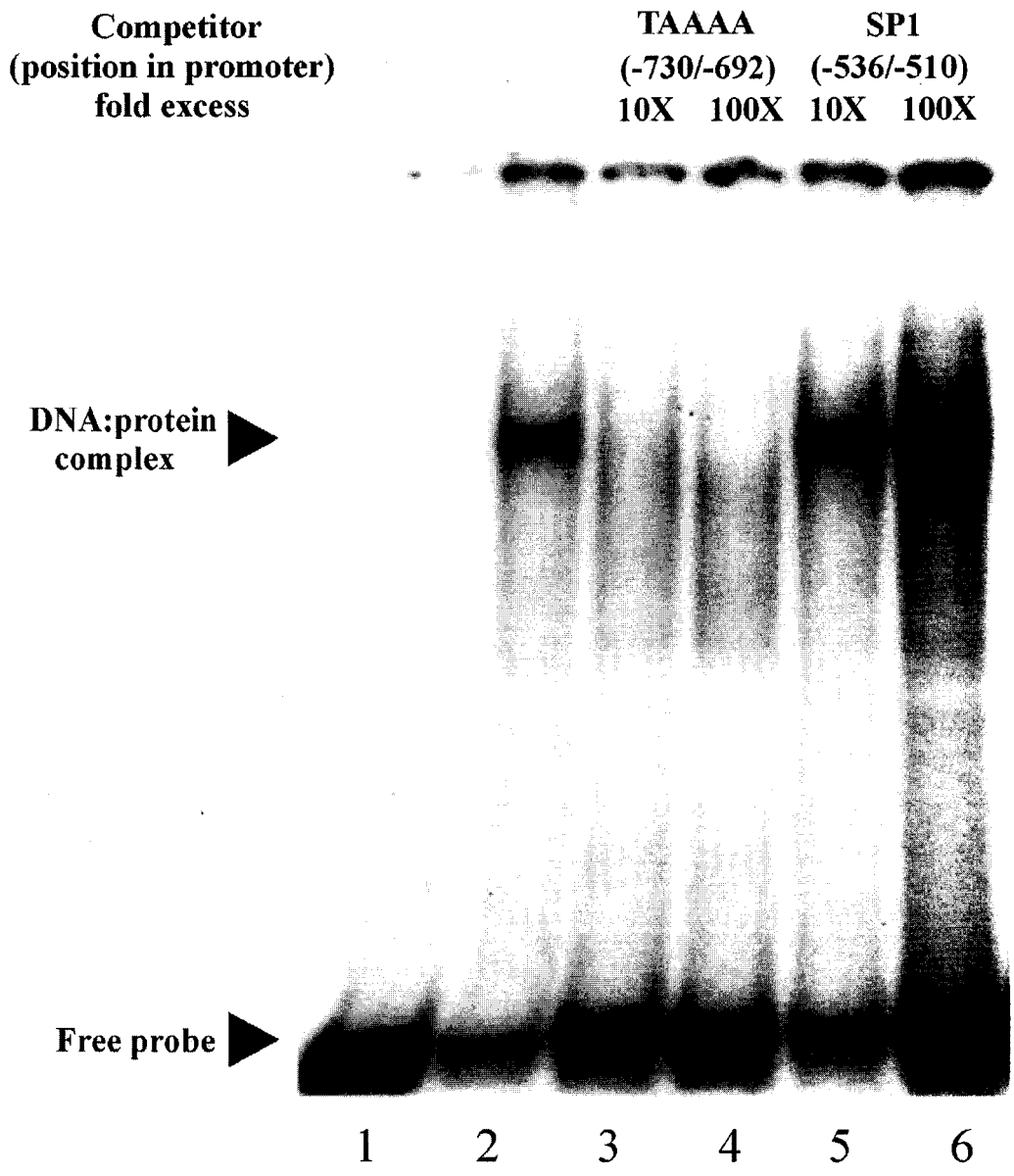

Figure 4.5 A $(TAAAA)_6$ repeat in the human *SHBG* promoter interacts with a protein from mouse liver nuclear extract. Radiolabeled oligonucleotides spanning the $(TAAAA)_6$ repeat in the human *SHBG* promoter were incubated with 4 μg of adult mouse liver nuclear extract in the absence (lane 2) or presence of excess unlabeled competitor oligonucleotides (lanes 3-6). Free probe was separated from DNA:protein complexes by non-denaturing polyacrylamide gel electrophoresis. Lane 1 represents the radiolabeled probe in the absence of nuclear protein. A double-stranded oligonucleotide spanning the SP1 site in the human *SHBG* promoter (FP12, -536/-510) was used as an unrelated competitor to demonstrate specificity.

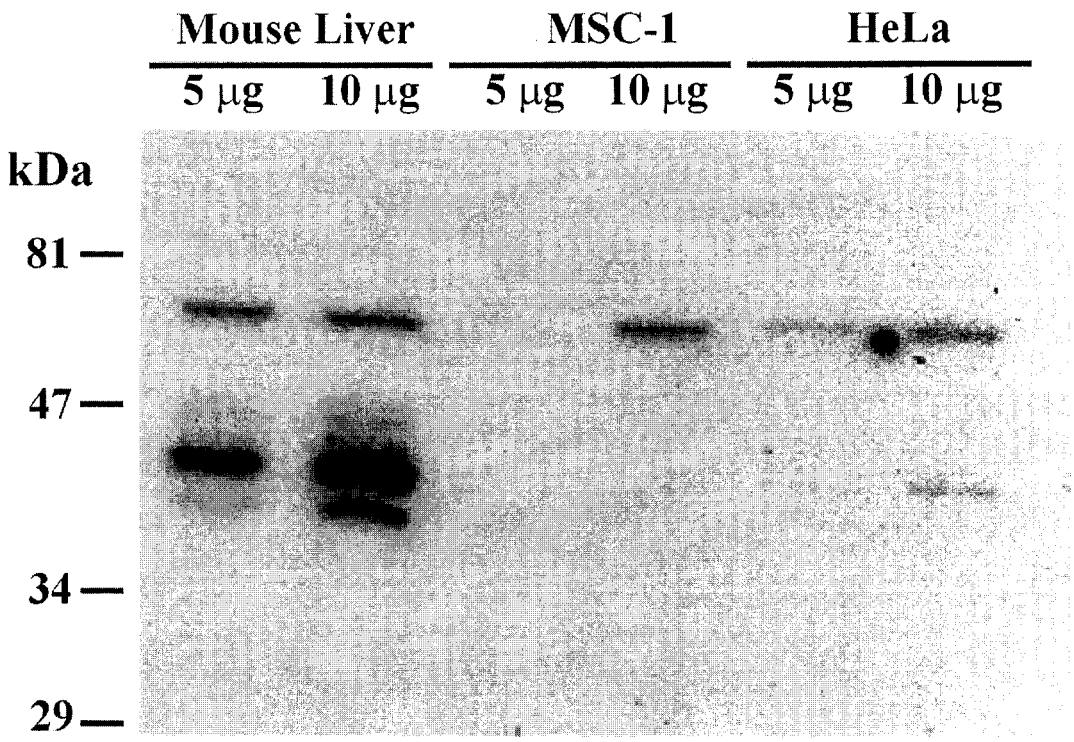

Figure 4.6 Southwestern blotting of nuclear factors binding to the $(TAAAA)_6$ repeat element. Nuclear proteins from adult mouse liver, a mouse sertoli cell line (MSC-1), and HeLa cells were separated on a denaturing SDS-PAGE gel, followed by transfer to a nitrocellulose membrane. The blot was incubated with a radiolabeled double-stranded oligonucleotide spanning the six TAAAA pentanucleotide repeats in the 5′ region of the human *SHBG* promoter. Migration of standards of known molecular mass is shown on the left.

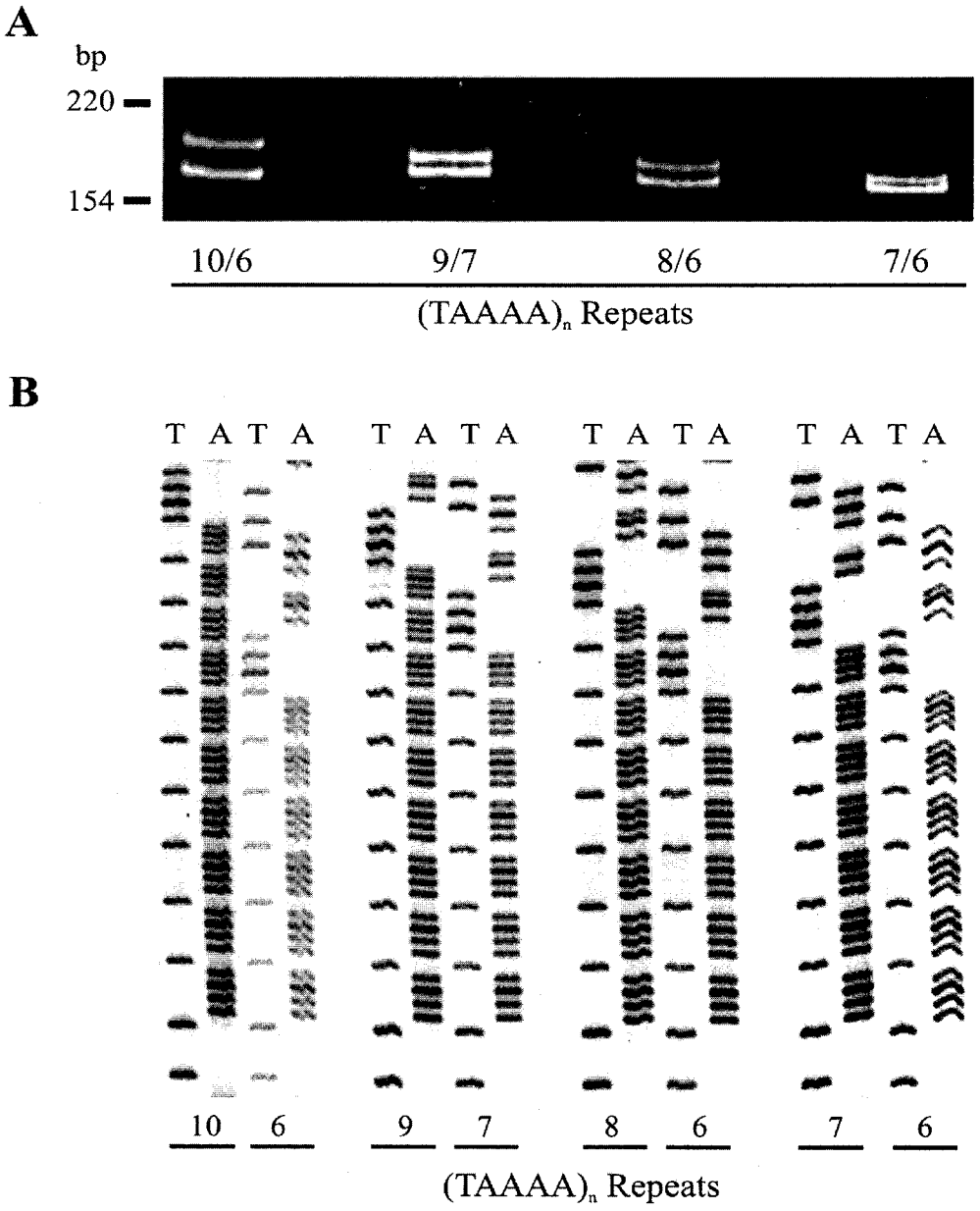

Figure 4.7 Demonstration of a $(TAAAA)_n$ polymorphism in the human *SHBG* promoter. A, Illustrates the degree of polymorphism in the TAAAA repeat element in the 5′ region of the human *SHBG* promoter within DNA samples from four individuals. This was accomplished by PCR amplification of this sequence using a forward primer within the *alu* sequence together with a reverse primer within the human *SHBG* promoter, and gives rise to an amplified product of 160 bp in samples with six repeats. B, The number of TAAAA repeats in *SHBG* alleles was confirmed by sequencing the resultant PCR products (T/A reactions are shown) obtained from 4 individuals, (panel A). The sequence of the allele containing 7 repeats on the right of the figure appears to contain an additional polymorphism in the A lane but repeat sequencing has indicated that this is an artifact due to compression of the sequencing products.

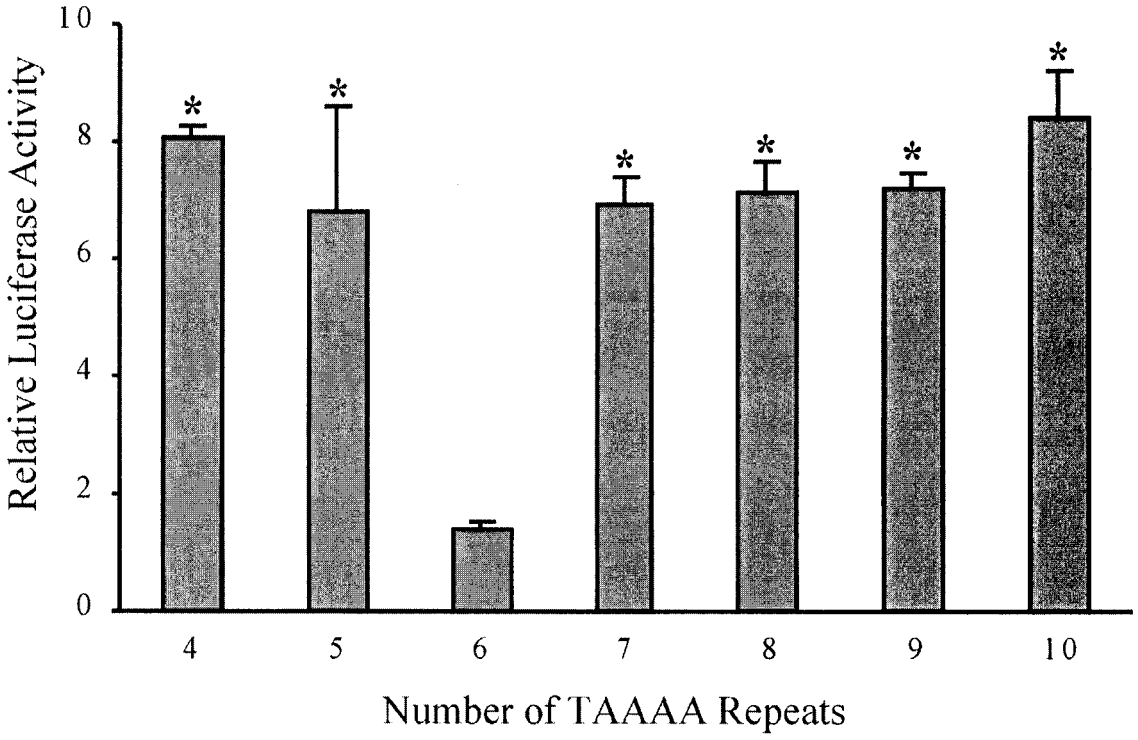

Figure 4.8 Functionality of a $(TAAAA)_n$ polymorphism in the human *SHBG* promoter. HepG2 cells were transiently transfected with human *SHBG* promoter-*luciferase* reporter vectors containing varying numbers of TAAAA repeat elements corresponding to that observed in the general population. Transfection efficiency was corrected by co-transfection with pCMVLacZ control vector and measuring the resultant β-galactosidase activity. The activities of the human *SHBG* reporter constructs containing the TAAAA repeat polymorphism are expressed relative to that of the promoterless pGL2 Basic luciferase reporter plasmid. * Indicates a statistically significant ($p<0.001$) difference when compared to the activity of a human SHBG promoter containing a $(TAAAA)_6$ repeat. Data are represented as means + S.E.M from at least 3 experiments performed in triplicate.

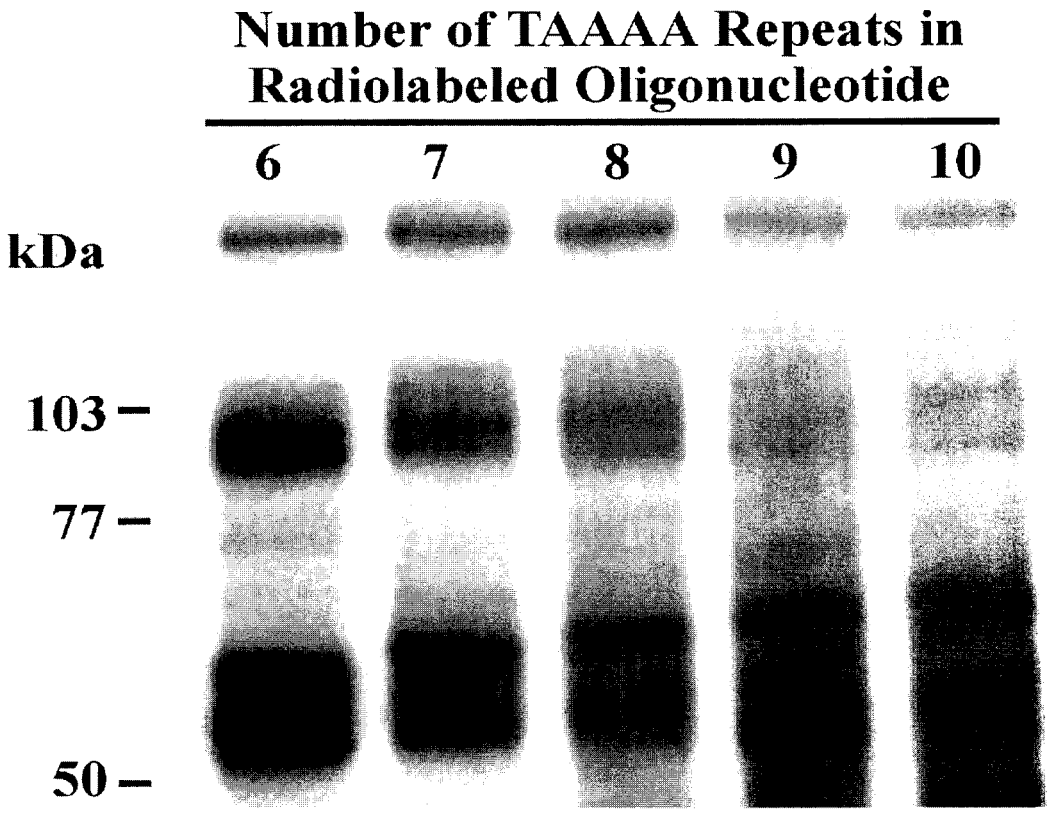

Figure 4.9 Nuclear factor binding to the TAAAA repeat element is dependent on repeat number. UV crosslinking of adult mouse liver nuclear proteins to radiolabeled probes spanning the TAAAA repeat element in the 5′ region of the human *SHBG* promoter demonstrating differential nuclear factor binding to different numbers of TAAAA repeat elements. The migration of standards of known molecular size is shown on the left. No DNA:protein complexes were observed in the absence of UV light in the presence of nuclear protein, or in the presence of UV light in the absence of nuclear protein (data not shown).

4.4 Discussion

Blood levels of SHBG in humans are highly variable even between healthy individuals (Meikle *et al.*, 1982). To determine if this variability can be explained by a genetic polymorphism, we sequenced the first 300 nts of the human *SHBG* promoter from normal individuals and patients with various reproductive and endocrine disorders in a separate study, but found no significant deviations between sequences. By contrast, our studies of human *SHBG* promoter activity in HepG2 hepatoblastoma cells alerted our attention to a $(TAAAA)_6$ repeat located within an *alu-Sx* sequence close to the 5' boundary of the transcription unit expressed in hepatocytes (Jänne *et al.*, 1998). This particular pentanucleotide repeat has been found to vary in number within the human cholesterol side-chain cleavage enzyme *CYP11A1* promoter (Durocher *et al.*, 1998), and there is a strong association between alleles with this polymorphism and the total serum testosterone levels in patients with PCOS and hyperandrogenism (Gharani *et al.*, 1997). Since this $(TAAAA)_6$ repeat appeared to have a silencing effect on *SHBG* promoter activity, we focussed our attention on the possible factor(s) that might bind to it, and how it might function in concert with other elements in the upstream region of the human *SHBG* promoter to influence transcription.

The upstream region of the human *SHBG* promoter silences transcription in HepG2 cells, and most of this activity is associated with a region between FP16 and FP17, which includes the $(TAAAA)_6$ repeat element. We have demonstrated that the $(TAAAA)_6$ repeat is responsible for this silencing activity by removing it, or by increasing the number of repeats according to the number observed in the general population. It is also clear that this activity is dependent on downstream promoter elements, and appears to involve an SP1 nuclear factor-binding site within FP12. Although SP1 is generally considered to be an activator of transcription, it can participate in transcriptional silencing through interactions with other transcriptional regulators in a context dependent manner (Talianidis *et al.*, 1995; Shou *et al.*, 1998). This might explain why FP12 only appears to regulate *SHBG* promoter activity in association with upstream elements (FP16-FP17) within the promoter. While our EMSA supershift data indicate that SP1 binds to FP12, we also observed that a second protein complex forms with a FP12 oligonucleotide, and this complex does not appear to be

supershifted with an SP1-specific antiserum. It is therefore possible that FP12 interacts with another SP1-related factor, such as SP3 (Hagen *et al.*, 1992; Hagen *et al.*, 1994), and this might be relevant because SP3 often acts as a negative regulator of transcription (De Luca *et al.*, 1996; Ammanamanchi and Brattain, 2001). Furthermore, competition between SP3 and other SP1-related factors for a common site within promoter sequences alters their transcriptional activity (Hagen *et al.*, 1994).

Variations in the number of polynucleotide repeats within several other promoters have been reported to modulate transcription (Yamada *et al.*, 2000; Beutler *et al.*, 1998), and have been linked to disease states (Gharani *et al.*, 1997; Yamada *et al.*, 2000; Beutler *et al.*, 1998). Although the number of $(TAAAA)_n$ repeats in the *CYP11A1* promoter is closely associated with serum testosterone levels, and might therefore reflect variations in the expression of the gene (Gharani *et al.*, 1997), it is not known whether this is due to an effect at the level of transcription. Differences in the number of an inverted $(TTTTA)_n$ repeat in the apolipoprotein(a) gene (*APO(a)*) promoter have also been associated with individual variations in plasma Lp(a) levels (Mooser *et al.*, 1995; Valenti *et al.*, 1999). Furthermore, an *APO(a)* promoter containing nine TTTTA repeats is associated with low plasma Lp(a) levels, and has a five-fold lower transcriptional activity in HepG2 cells, when compared to a promoter sequence containing eight TTTTA repeats from an individual with relatively high plasma levels of Lp(a) (Wade *et al.*, 1993). The $(TAAAA)_n$ repeat found within the 5′ region of the human *SHBG* promoter occurs frequently within the human genome (Kim and Crow, 1998) and is a common feature of repetitive elements such as *alu* sequences (Millar *et al.*, 2000) and LINE elements (Kim and Crow, 1998). Close inspection of the pentanucleotide repeats in the *CYP11A1* and *APO(a)* promoters by RepeatMasker (**http://www.genome.washington.edu/uwgc/analysistools/repeatmasker.htm**) indicates that they also flank *Sp/q* and *Sg* subfamilies of human *alu* sequences, respectively, and differences in the number of these repeats between individuals likely reflect an inherent instability of *alu* repetitive elements (Mighell *et al.*, 1997).

Unlike the $(TA)_n$ dinucleotide repeat in the UDP-glucuronosyltransferase 1 (*UGT1A1*) promoter, where increasing numbers of repeats are associated with decreased promoter activity (Beutler *et al.*, 1998), the activity of the human *SHBG* promoter is not

linear with respect to TAAAA repeat number. Differences in the number of TTTTA repeats in the *APO(a)* promoter influence its transcriptional activity, but this has not been studied in any great detail (Wade *et al.*, 1993). In particular, there is no information concerning the identity of proteins that might interact with these types of repeats, or how they influence gene transcription. In experiments presented here, silencing of the human *SHBG* promoter was only observed in the presence of six TAAAA repeats, and this correlated with the preferential binding of a liver-enriched 46 kDa nuclear factor to the $(TAAAA)_6$ repeat element. Although alleles containing less than six TAAAA repeats were not observed in the limited group of individuals we examined, the activities of *SHBG* promoters containing 4 or 5 TAAAA repeats also lacked the silencing properties associated with the presence of six repeats. These data suggest that the 46 kDa factor that binds six TAAAA repeats with high affinity acts in concert with downstream elements within the human *SHBG* promoter to alter its transcriptional activity.

In summary, a $(TAAAA)_n$ repeat polymorphism within the human *SHBG* promoter has a marked effect on its transcriptional activity *in vitro* in HepG2 cells. This could contribute to individual differences in plasma SHBG levels and thereby influence the access of sex steroids to their target tissues. Furthermore, variations in the number of TAAAA repeats within regulatory regions of other human genes may contribute to inter-individual differences in gene expression. The genomic DNA samples we examined were from healthy male volunteers of various ethnic backgrounds, and the number of TAAAA repeat elements ranged from 6-10 in this limited group of subjects. Although there was no obvious relationship between the number of TAAAA repeat elements and the serum SHBG concentrations, the number of individuals examined is too small to draw any conclusions, especially as almost all of them were all bi-allelic for different numbers of TAAAA repeats. It is, however, important to appreciate that the ability of the $(TAAAA)_n$ polymorphism to influence the transcriptional activity of the human *SHBG* promoter as naked DNA in transient transfection experiments may not necessarily reflect its activity in the context of genomic DNA within a chromatin structure. It will therefore be important to conduct a carefully controlled clinical study to determine whether this polymorphism is associated with differences in plasma SHBG levels, and to determine whether specific alleles are associated

with sex steroid hormone dependent diseases and/or correlate with responses to various hormone treatments that influence plasma SHBG levels.

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CHAPTER 5

DIETARY REGULATION OF HUMAN *SHBG* EXPRESSION

5.1 Introduction

Sex hormone-binding globulin (SHBG) binds androgens and estradiol with high affinity and high selectivity, and regulates the bioavailability and transport of these sex steroids to target tissues (Hammond, 1995). Hepatocytes of the liver are the major source of circulating SHBG in human plasma (Khan *et al.*, 1981), and the hepatic production and secretion of SHBG appears to be regulated by a variety of hormonal and metabolic factors including sex steroids (Anderson, 1974), thyroid hormone (Sarne *et al.*, 1988), and dietary components (Longcope *et al.*, 2000). Moreover, low SHBG levels are reported to be an early indicator of the onset of Type II diabetes (Lindstedt *et al.*, 1991) and cardiovascular disease (Lapidus *et al.*, 1986).

A large body of clinical evidence demonstrates that *SHBG* expression is regulated by metabolic factors. Levels of SHBG in the blood are highly correlated with metabolic status, and a significant negative correlation exists between serum SHBG levels and body mass index (Glass *et al.*, 1977; Plymate *et al.*, 1981; Apter *et al.*, 1984). Low serum SHBG levels are associated with obesity (Glass *et al.*, 1977; Plymate *et al.*, 1981; Apter *et al.*, 1984), whereas they are elevated in patients with anorexia nervosa (Estour *et al.*, 1986) and can be reduced by increasing caloric intake (Barbe *et al.*, 1993). In humans, there is a significant negative correlation between fasting insulin levels and SHBG in healthy women (Preziosi *et al.*, 1993). Serum SHBG levels are also low in patients with polycystic ovarian syndrome and its associated hyperinsulinemic insulin resistance (Pugeat *et al.*, 1991). Treatment of the human HepG2 cell line with insulin results in decreased levels of SHBG mRNA, and a reduction in the amount of SHBG secreted into the media (Crave *et al.*, 1995; Plymate *et al.*, 1988). However, this hepatoblastoma cell line may not accurately reflect the situation in hepatocytes *in vivo*, and it is not known whether these effects of insulin occur at the level of *SHBG* gene transcription.

Comparison of the human and rat *SHBG* sequences flanking the exon 1 encoding the secretion signal polypeptide reveals high similarity within the first 600 bp of promoter sequence, after which sequences diverge into species specific repetitive elements (Jänne and Hammond, 1998). The *cis*-acting elements that regulate human *SHBG* promoter activity in HepG2 cells have been characterized in some detail (Jänne and Hammond; 1998, Hogeveen

et al., 2001), and the ~0.8 kb of promoter sequence present in a 4 kb human *SHBG* transgene is sufficient for high levels of *SHBG* expression in mouse hepatocytes (Jänne *et al.*, 1998; Jänne *et al.*, 1999). In particular, binding of the liver-specific hepatocyte nuclear factor-4 (HNF-4) to a TA rich region near the transcription start site in the liver appears to be responsible for recruiting the basal transcriptional machinery in place of TBP, and directing *SHBG* expression in HepG2 cells (Jänne and Hammond, 1998). One major difference between the *SHBG* promoters in these species is a region within the human promoter encompassing two DNaseI footprinted regions that is absent in the rat promoter (Jänne and Hammond, 1998). Within this sequence, an HNF-4 site is flanked by an E-box like motif that is a putative binding site for the ubiquitous USF (Figure 1.3), a transcription factor that has been argued to function as part of a glucose response element (GlRE) that regulates gene expression in response to glucose (Lefrancois-Martinez *et al.*, 1995; Vaulont and Kahn, 1994). Although the role of USF in regulating transcriptional responses to diet has been questioned (Kaytor *et al.*, 1997), other nuclear proteins binding to E-box like sites may be involved in the regulation of gene expression (Hasegawa *et al.*, 1999; Yamashita *et al.*, 2001). Interestingly, the particular arrangement of HNF-4 and USF binding sites in the human *SHBG* promoter has been shown to function as an element important in the regulation of expression of metabolic genes in response to insulin and glucose (Diaz Guerra *et al.*, 1993). Furthermore, it has been suggested that the additional sequences in the human *SHBG* promoter may be important for the postnatal expression of *SHBG* in the liver, or be involved in the regulation of *SHBG* expression in response to external stimuli.

To study the effects of diet and metabolic cues on the regulation of human *SHBG* expression, we have used transgenic mice expressing human *SHBG* transgenes to examine their response to different dietary and metabolic interventions. We have found that levels of SHBG in the blood and mRNA in the liver are highly regulated by diet, and these effects on SHBG expression are not mediated through a USF-site.

5.2 Materials and Methods

5.2.1 Electrophoretic mobility shift assays

Mouse liver nuclear protein extracts (4 μg) were incubated in EMSA buffer (2.5 mM

HEPES, pH 7.6, 0.5 mM EDTA, 0.5 mM DTT, 50 mM NaCl, 6.25% glycerol and 3 μg poly dIdC) on ice for 10 min in the presence or absence of double-stranded competitor oligonucleotides. The corresponding end-labeled oligonucleotide probes were then added, and the binding reaction was allowed to proceed for 15 min at room temperature. Nuclear proteins bound to radiolabeled oligonucleotides were separated from free probe by 6 % PAGE in 0.5 x TBE, and the gel was dried and exposed to Biomax MR film (Eastman Kodak Company, Rochester, NY) against an intensifying screen at –80°C. For antibody supershift experiments, nuclear extracts were incubated as above on ice for 10 min before, and 15 min after addition of the radiolabeled oligonucleotide. Aliquots (1 μl) of antiserum or normal rabbit serum were then added and the complexes were further incubated at room temperature for 15 min. Protein-DNA complexes were separated from free probe by native 6 % PAGE, and the gel was dried and exposed to x-ray film as described above.

Footprint 4 EMSA oligos: 5′ TCGACTGATAGCTGAGTCTTGTGACTGGGCCCCTC
3′ GACTATCGACTCAGAACACTGACCCGGGGAGAGCT

MLP USF oligos 5′ TCGACGATCTGTAGGCCACGTGACCGGC
3′ GCTAGACATCCGGTGCACTGGCCGAGCT

5.2.2 Production of transgenic mice

An *Xho*I restriction endonuclease site was introduced into the pSELECT vector by ligating a *Bgl*II-digested *Bgl*II-*Xho*I-*Bgl*II self-complementary double-stranded oligonucleotide (5' CCC *AGA TCT* CTC GAG *AGA TCT* GGG) (*Bgl*II sites in italics, *Xho*I site underlined) into the *Bam*HI site of pSELECT (Promega Corp., Madison, WI). A 4 kb *Xho*I genomic human *SHBG* construct was cloned into the *Xho*I site of the pSELECT-*Xho*I vector for removal of the footprint 4 (FP4) region in the human *SHBG* promoter (Jänne and Hammond, 1998) by site-directed mutagenesis according to the Promega "*Altered Sites*" protocol. Briefly, removal of the FP4 site was accomplished by annealing a mutagenic oligonucleotide (5'CTT GAC CCC TGC CCA GGG GCT CAC CCC TCT GGG GAT CAA T) together with a vector-specific ampicillin resistance repair primer to single-stranded pSELECT-*Xho*I containing the 4 kb *hSHBG Xho*I fragment. Second strand DNA synthesis and ligation was completed by incubation with T4 DNA polymerase in the presence of T4

DNA Ligase for 4 hours at 37 C. The BMH71-18 mut S mismatch repair-deficient *E. coli* strain was transformed with products from the synthesis reaction by the heat-shock method (Hanahan, 1985) and bacterial cells were grown in LB-media containing 125 μg/mL ampicillin to select for successfully mutated pSELECT vector. Plasmid DNA was isolated from the ampicillin-resistant culture by the alkaline-lysis method (Birnboim and Doly, 1979) and was used to transform *E. coli* JM107 bacterial cells. Removal of the FP 4 sequence from the human *SHBG* promoter was confirmed by DNA sequencing (Sanger *et al.*, 1977).

Mice were obtained from in-house breeding stocks and housed in a pathogen-free facility. The one-cell embryos for microinjections were obtained by superovulating CBA X C57BL/6 hybrid females with pregnant mare serum and hCG (Sigma Chemical Co. Mississauga, Canada) and mating them with CBA X C57BL/6 males (Hogan *et al.*, 1994). The same hybrid cross was used to propagate transgenic mouse lines. Female CD-1 mice were used to produce pseudo-pregnant recipients by mating with vasectomized males. Genomic DNA was isolated from toe biopsies of newborns by incubating in 25 mM NaOH, 0.2 mM EDTA for 20 min at 95 C. Samples were then neutralized by addition of an equal volume of 40 mM Tris-Cl pH 8.0. One microlitre of this DNA preparation was used in a PCR reaction with PCR Supermix HiFidelity (Invitrogen) and 10 pmol of human *SHBG* primers spanning exon 7 (Fwd: 5' GGA AGA ATT CGG CCA CAG GCA GTA GGC, Rev: 5' GGT TGA ATT CCG CCT CCC TTG AGC TG).

5.2.3 Animal treatments

Animals were housed under standard conditions and provided with food and water *ad libitum*, unless otherwise mentioned. Transgenic mice containing human *SHBG* transgenes were maintained on a basal diet containing 53 % carbohydrate (PicoLab Mouse Diet™ 20), and where indicated were fed an isocaloric high carbohydrate diet (70 % carbohydrate, TD 98029, Harlan-Teclad). For fasting and re-feeding studies, food was removed for a period of 24 hours, followed either by a 24 hr refeeding period for one group of animals, or sacrifice by CO_2 intoxication. Control mice had free access to food during this time. At the end of each experiment, mice were sacrificed by CO_2 intoxication, and tissues were collected for RNA analysis. Diabetes in mature mice was induced by a single

interperitoneal injection of 200 mg/kg streptozotocin (Sigma) in a 50 mM citrate buffer, pH 4.5, and diabetes in these animals was confirmed by an elevated blood glucose level (>400 mg/dl) measured by Fast-Take glucose strips (LifeScan Inc., Milpitas, CA). Transgenic mice containing 4 kb or 11 kb regions of the human *SHBG* locus have been genotyped and characterized previously (Jänne *et al.*, 1998, Jänne *et al.*, 1999). All procedures were approved by the animal care committee of the University of Western Ontario (London, Canada)

5.2.4 Tissue RNA analysis

Total RNA (5 μg) extracted from mouse tissues using TRIzol reagent (Invitrogen) was separated by electrophoresis on a 1% agarose gel in the presence of formaldehyde and transferred to a ZetaProbe GT nylon membrane (Bio-Rad). Membranes were hybridized with a ^{32}P-labeled human SHBG 3' cDNA overnight at 65°C in a buffer containing 0.5 M sodium phosphate and 7 % SDS, followed by high stringency washing and exposure to a phosphor-imaging screen. Blots were then stripped of the hybridized SHBG cDNA probe, and membranes were re-probed with a ^{32}P-labeled cDNA for 18S ribosomal RNA as a control for loading and transfer efficiency. The relative intensities of radiographic signals obtained for human SHBG and 18S transcripts were compared by densitometry.

5.2.5 Serum SHBG and insulin measurements

The concentrations of human SHBG in transgenic mouse serum were determined using a saturation ligand binding assay (Hammond and Lähteenmäki, 1983) as described in section 2.2.4. Briefly, endogenous steroids were removed from serum samples by dilution (1:100) in a dextran-coated charcoal suspension and an incubation (20 min) at room temperature. Samples were then further diluted (1:10) and incubated for one hour at room temperature in the presence of 10 nM [^{3}H]5α-dihydrotestosterone (DHT) (Amersham Pharmacia Biotech) followed by an additional 30 min incubation at 0 C. Non-specific binding was estimated in the presence of a 400-fold molar excess of non-radioactive 5α-DHT. Free ligand was removed by incubation (10 min) with an ice-cold dextran-coated charcoal slurry and separation by centrifugation. Supernatants containing SHBG-bound

ligand were taken for radioactivity measurements to determine relative serum SHBG levels.

Insulin levels in the serum of mice were measured by the 1-2-3 Ultrasensitive Mouse Insulin EIA kit (ALPCO Diagnostics, Windham, NH).

5.3 Results

5.3.1 A USF-binding site (FP 4) in human *SHBG* proximal promoter

An E-box-like motif protected from DNaseI digestion by mouse liver nuclear extract (FP 4) lies immediately upstream of an HNF-4 site in the human *SHBG* promoter (Jänne and Hammond, 1998). This region is of particular interest in light of the observation that this particular arrangement of nuclear factor binding sites is important for the regulation of the L-type pyruvate gene in response to glucose (Diaz Guerra *et al.*, 1993; Jänne and Hammond, 1998). Comparison of the FP 4 sequence with known transcription factor binding sites by MatInspector (Quandt *et al.*, 1995) reveals a significant homology to a binding site for the basic-helix-loop-helix transcription factor USF. To examine nuclear protein interaction with this cis-element in more detail, we performed electrophoretic mobility shift assay (EMSA) using mouse liver nuclear extracts and radiolabeled double-stranded oligonucleotides spanning this footprinted region (Figure 5.1). Specific DNA-protein interactions were observed with this region, and could be competed for by unlabeled FP 4 oligonucleotide (Figure 5.1). Furthermore, specific protein binding to this region could be competed for by a consensus USF binding site found in the adenovirus major late promoter (Potter *et al.*, 1991). Antisera specific to USF-1 or USF-2 were used to confirm the identity of the nuclear proteins binding to FP 4 in the human *SHBG* promoter. Incubation of antisera against either USF-1 or USF-2 in an EMSA reaction resulted in a partial supershift of the radiolabeled DNA-protein complex, and when used together, a complete supershift was observed (Figure 5.1).

5.3.2 Transgenic mice expressing a 4 kb human *SHBG* transgene lacking the FP 4 site

The human and rat *SHBG* promoters share significant sequence similarity within the first 600 bp, at which point they diverge into species-specific repetitive elements (Jänne and Hammond, 1998). However, 23 additional nucleotides are present in the human *SHBG*

promoter, and this sequence encompasses an HNF-4 half site and a USF site at FP 4 (Figure 1.3, Figure 5.1). It has been suggested that these differences in the human and rodent *SHBG* promoter sequences could explain the phylogenetic differences in temporal and tissue-specific expression of *SHBG* in postnatal life (Jänne and Hammond, 1998). To address this possibility, we selectively removed a 20 bp region encompassing the FP 4 USF site by site-directed mutagenesis in the context of a 4 kb human *SHBG* transgene used previously to generate several lines of transgenic mice that have been characterized previously (Jänne *et al.,* 1998; Jänne *et al.*, 1999).

Surprisingly, removal of the USF binding site at FP 4 in the human *SHBG* promoter had little effect on the postnatal expression of *SHBG*, as these mice had very high levels of human SHBG in their serum in adulthood, and showed a tissue-specific expression similar to that of *SHBG*4 mice (Jänne *et al.*, 1999) by Northern blot analysis (Figure 5.2). In particular, human SHBG mRNA is present in the liver and kidneys of *SHBG*4-FP4$^-$ mice, but is not detectable in the brain, or spleen (Figure 5.2). Interestingly, SHBG mRNA was detected in total RNA isolated from the testes of *SHBG*4-FP4$^-$ mice (Figure 5.2).

5.3.3 Effect of a high carbohydrate diet on human *SHBG* expression

Human SHBG levels in the serum of male and female *SHBG*11-a transgenic mice decreased significantly when fed a high carbohydrate diet (70 %) containing sucrose as the source of carbohydrates (Figure 5.3 A, B). Striking changes in human SHBG levels were observed in both male and female mice of both *SHBG*4 and *SHBG*11 animals. This decrease in serum SHBG could be reversed by switching the diet back to a basal diet, and the effect is reproducible over several diet phases (Figure 5.3 A, B). Significant changes in serum SHBG levels could be detected 3 days following a switch of the diets (Figure 5.3 B, 21 - 23 days). Since the half-life of human SHBG in the rhesus monkey is on the order of 33 hours (Loncope *et al.*, 1992), and 38 hours in rabbits (Cousin *et al.*, 1998), this indicates that changes in human *SHBG* expression in response to diet occur rapidly in these mice. The effect of the high carbohydrate diet was specific to hepatic *SHBG* expression as human SHBG mRNA in the liver was decreased approximately 4-fold in animals fed the high carbohydrate diet, whereas SHBG mRNA in the kidney was unaffected by the nature of the

diet (Figure 5.3 C). Further, the USF site at FP 4 does not function as a part of a putative glucose response element in the context of human *SHBG* transgenes since similar changes in serum levels of human SHBG were observed in *SHBG*4-FP4$^-$ mice (Figure 5.3 D). Blood glucose levels were lower (103 ± 11 mg/dl vs. 137 ± 2 mg/dl, $p < 0.05$) and insulin levels were elevated (0.98 ± 0.12 ng/ml vs. 0.53 ± 0.03 ng/ml, $p<0.05$) in animals fed a high carbohydrate diet, when compared to animals on the basal diet.

Whereas starch was the source of carbohydrate in the basal diet, carbohydrates were provided in the form of sucrose in the high carbohydrate diet. To address whether the dramatic decrease in serum levels of human SHBG is dependent on the source of carbohydrates, female *SHBG*11-a animals were switched from a basal diet to a high carbohydrate diet (78 %) containing glucose, sucrose, or corn starch as the source of carbohydrates. All three of these diets resulted in decreases in serum levels of human SHBG, but the sucrose diet had a more profound effect that was statistically significant when compared to either the glucose or corn starch diets (Figure 5.4).

Interestingly, the induction of diabetes in 60 day old male *SHBG*11-a transgenic mice resulted in a 60 % reduction in SHBG levels within one week of streptozotocin treatment (Figure 5.5), at a time when SHBG levels in these mice have stabilized (Jänne *et al.*, 1999). A high carbohydrate diet fed to diabetic *SHBG*11-a mice with blood glucose levels > 400 mg/dl had no further effect on decreasing the levels of human SHBG in the serum (Figure 5.5).

5.3.4 Effect of fasting and refeeding on hepatic expression of human *SHBG*

Human SHBG mRNA in the livers of *SHBG*4-a mice increased when these animals were fasted for 24 hours (Figure 5.6 A). When animals were re-fed for 24 hours following the period of fasting, a decrease in SHBG mRNA levels below that of control animals was observed (Figure 5.6 A). The increase in hepatic *SHBG* expression was independent of insulin, as a similar increase in SHBG mRNA during fasting was observed in streptozotocin-induced diabetic mice (Figure 5.7), although the decrease in mRNA levels upon refeeding appears to be compromised. Furthermore, the USF binding site at FP 4 does not play a role in regulating changes in hepatic *SHBG* expression in response to fasting/refeeding because

similar changes in SHBG mRNA were observed in the livers of *SHBG*4-FP4^{-} mice in response to fasting and refeeding (Figure 5.6 B).

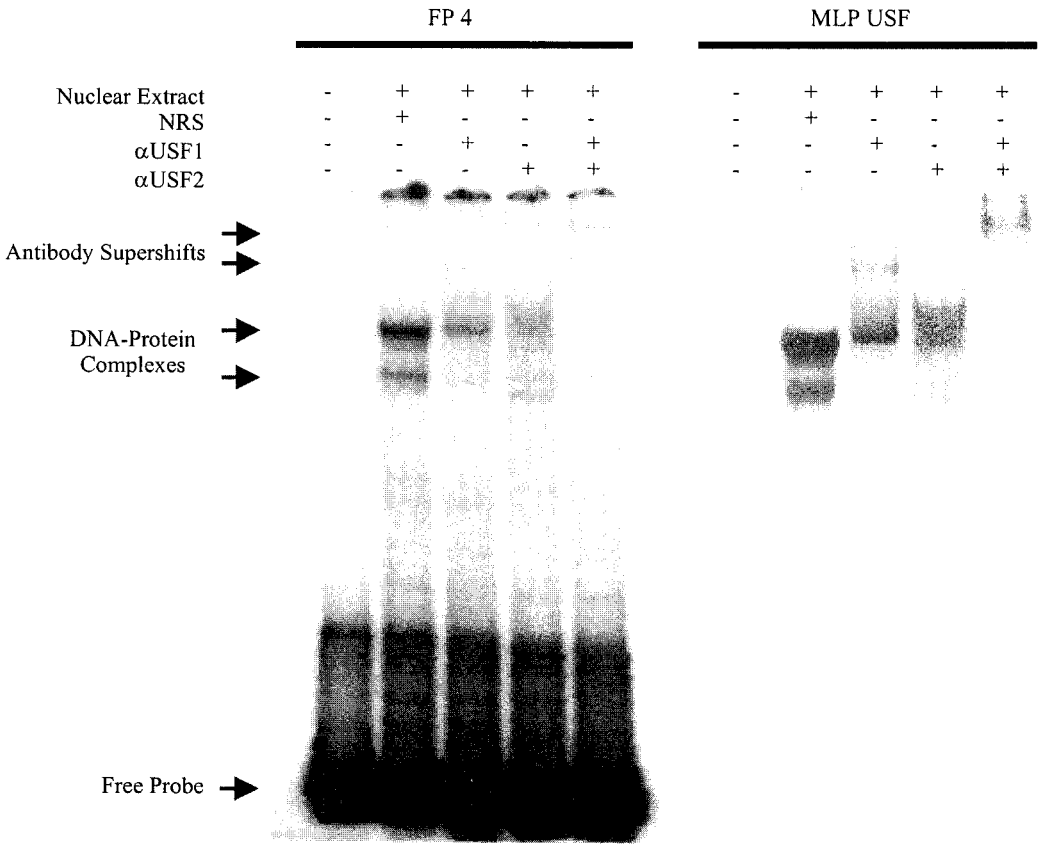

Figure 5.1 USF proteins bind to footprint 4 (FP 4) in the human *SHBG* proximal promoter. Electrophoretic mobility shift analysis reveals similar DNA-nuclear protein complexes with radiolabeled oligonucleotides corresponding to FP 4 in the human *SHBG* proximal promoter (Jänne and Hammond, 1998), and the USF site (5'-TCGACGATCTGTAGGCCACGTGACCGGC with E-box underlined) in the adenovirus major late promoter (MLP). Incubation of bound nuclear proteins with antisera raised against either USF1 or USF2 resulted in antibody supershifts. When both antibodies were used together, a complete supershift of mouse liver nuclear protein-DNA complexes was observed with both human *SHBG* FP4 and adenovirus MLP USF radiolabeled probes. NRS = normal rabbit serum.

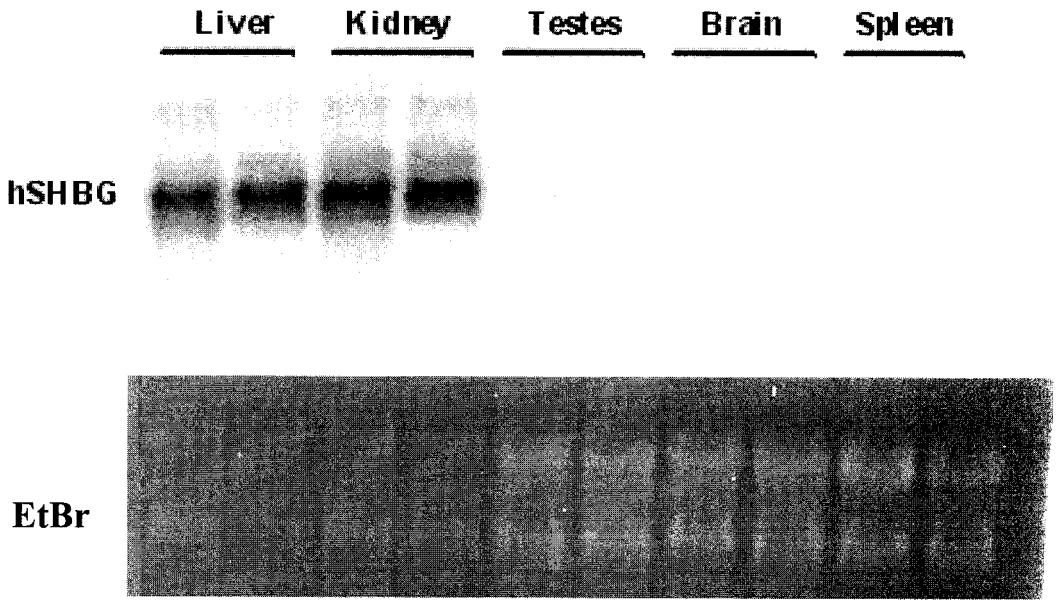

Figure 5.2 Tissue distribution of human SHBG mRNA in *SHBG*4-FP4$^-$ male mice. Total RNA isolated from the liver, kidney (5 μg), testes (15 μg), brain and spleen (20 μg) of *SHBG*4-FP4$^-$ male mice was separated on an agarose gel in the presence of formaldehyde, transferred to a nylon membrane, and probed with ^{32}P-labeled 3' human SHBG cDNA. Shown below the blot is the ethidium bromide stained gel demonstrating appropriate RNA loading.

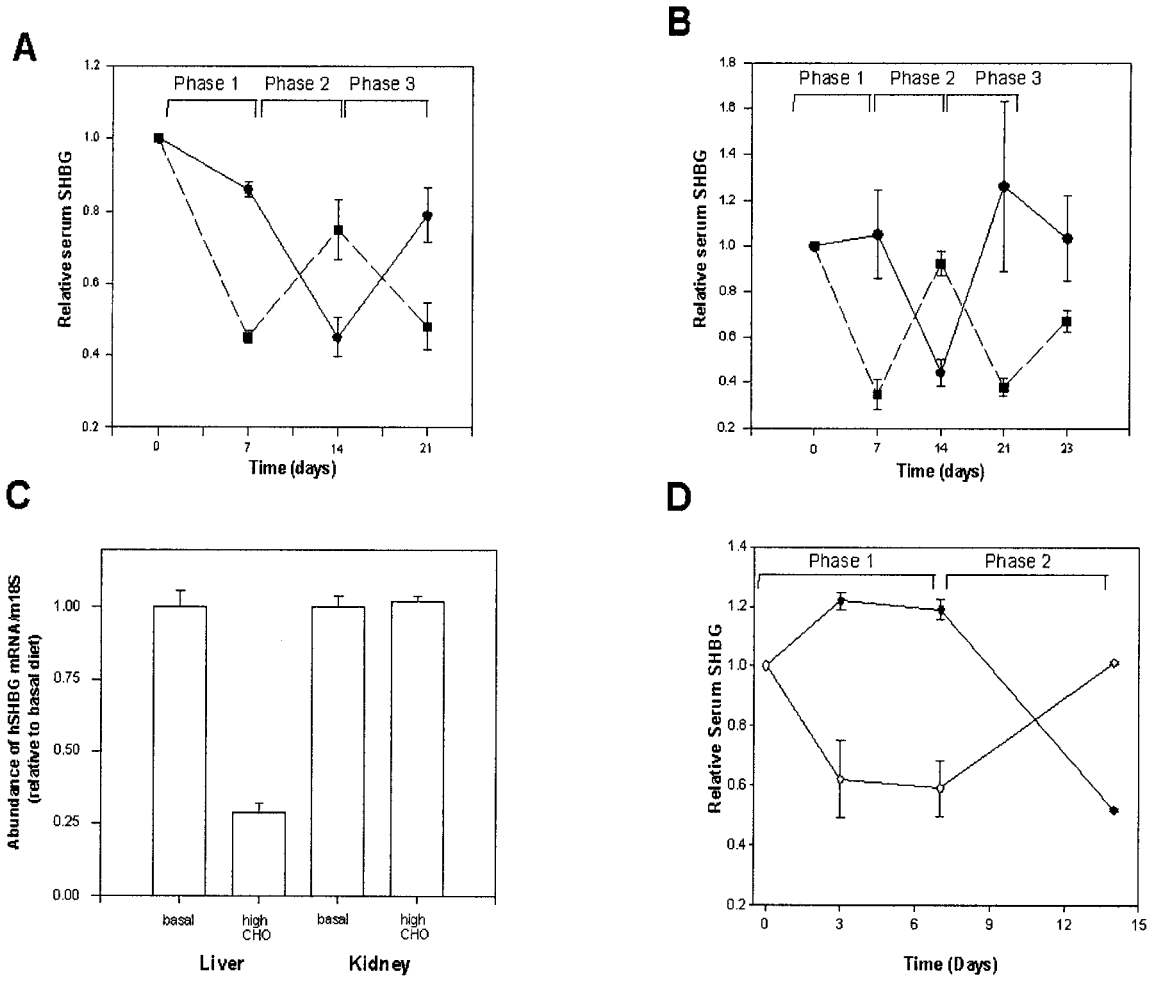

Figure 5.3 Changes in human SHBG levels in serum (A, B) and SHBG mRNA levels in liver and kidney (C) of male (A) and female mice (B) *SHBG*11-a mice after feeding with a high carbohydrate diet. A blood sample was taken from mice (n=3 per group) at the beginning of the experiment to establish base-line serum SHBG levels (expressed as 1.0). Mice were then treated initially with either a basal diet (solid line) or a high carbohydrate diet (dashed line) for seven days (Phase 1), at which point another blood sample was collected and the diets were switched. This treatment and blood sampling protocol was repeated twice (Phases 2 and 3). The serum SHBG levels after each treatment phase were expressed relative to the base-line levels. At the end of the experiment, livers and kidneys were taken for analysis of human SHBG mRNA content. The relative amounts of human SHBG mRNA in tissues from mice treated with the high carbohydrate diet at the end of the last treatment phase were expressed relative those in animals treated with the basal diet. D) Changes in human SHBG serum levels of *SHBG*4-FP4$^-$ male after feeding a high carbohydrate diet. A blood sample was taken from mice (n=3 per group) at the beginning of the experiment to establish base-line serum SHBG levels. Mice were then treated initially with either a basal diet (●) or a high carbohydrate diet (○) for seven days (Phase 1), at which point another blood sample was collected and the diets were switched. Data points are shown as means ± SEM from duplicate measurements of 3 animals per group.

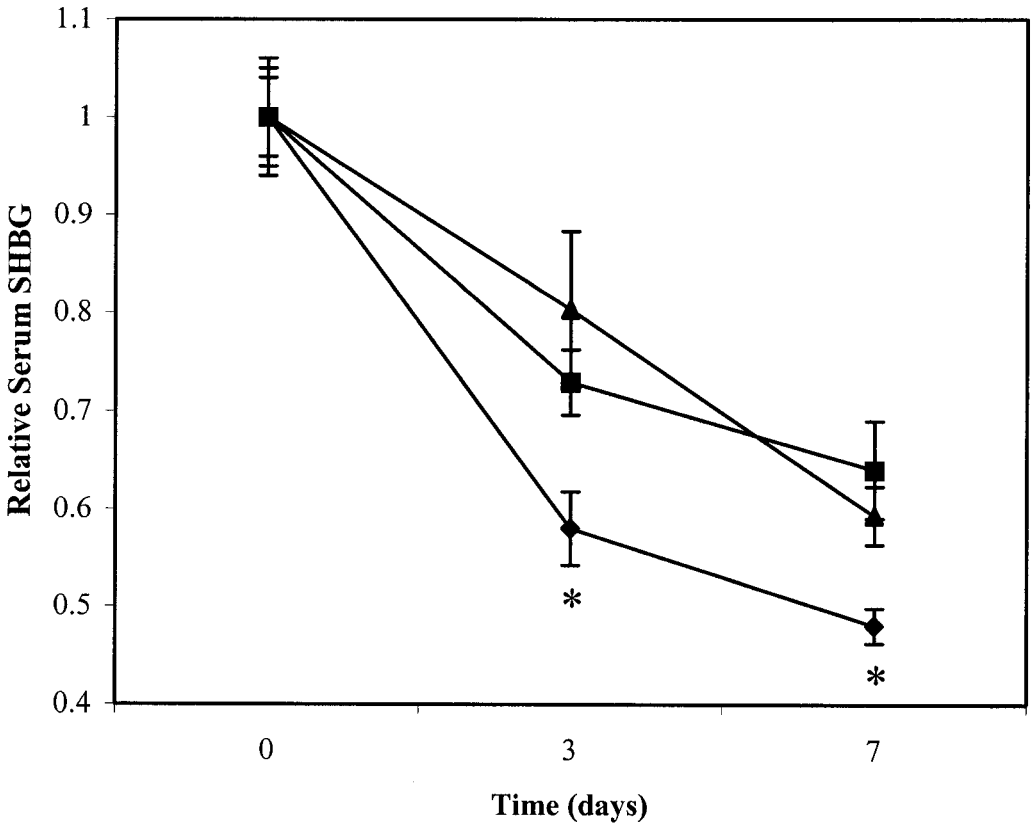

Figure 5.4 Effect of different carbohydrates on serum SHBG levels in mice fed a high carbohydrate diet. At the beginning of the experiment, a blood sample was taken from human *SHBG*11-a female mice (60 d) fed a basal diet (53 % carbohydrate) to establish base-line serum SHBG levels (expressed as 1.0). Mice were then treated (n=4 per group) with a high carbohydrate diet (78 %) containing corn starch (■), glucose (▲), or sucrose (◆) as the source of carbohydrate. Blood samples were taken at 3 and 7 days after switching the diet. The serum SHBG levels at each time point are expressed relative to the base-line levels. Relative serum SHBG levels are expressed as means +/- SEM of duplicate serum SHBG measurements for 4 animals. * = statistically significant ($p<0.05$) when compared to serum SHBG levels from mice fed the high carbohydrate diet containing corn starch or glucose.

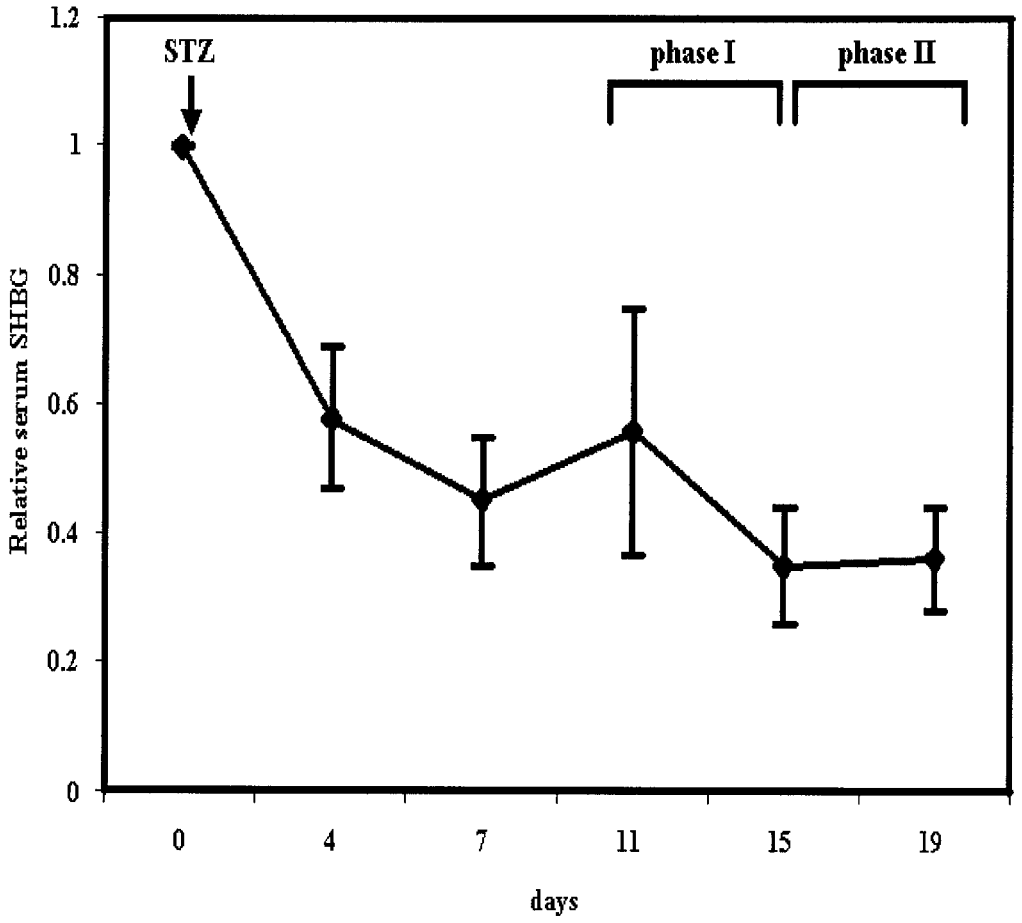

Figure 5.5 Effect of dietary manipulation on levels of human SHBG levels in the blood in STZ-induced 11kb h*SHBG* diabetic mice fed a high carbohydrate diet. Blood samples were taken prior to STZ treatment to establish a baseline serum SHBG level (represented as 1.0 at time 0), and at 4, 7 and 11 days post-treatment. Induction of diabetes by streptozotocin was confirmed by measurement of blood glucose levels greater than 600 mg/dL. At 11 days post treatment the diet was switched from the basal diet to a high carbohydrate diet (phase I) for five days. At this time another blood sample was collected, and animals were switched to the basal diet (phase II). Serum SHBG levels are represented relative to baseline measurements and are expressed as means +/- SEM from duplicate measurements from 3 animals.

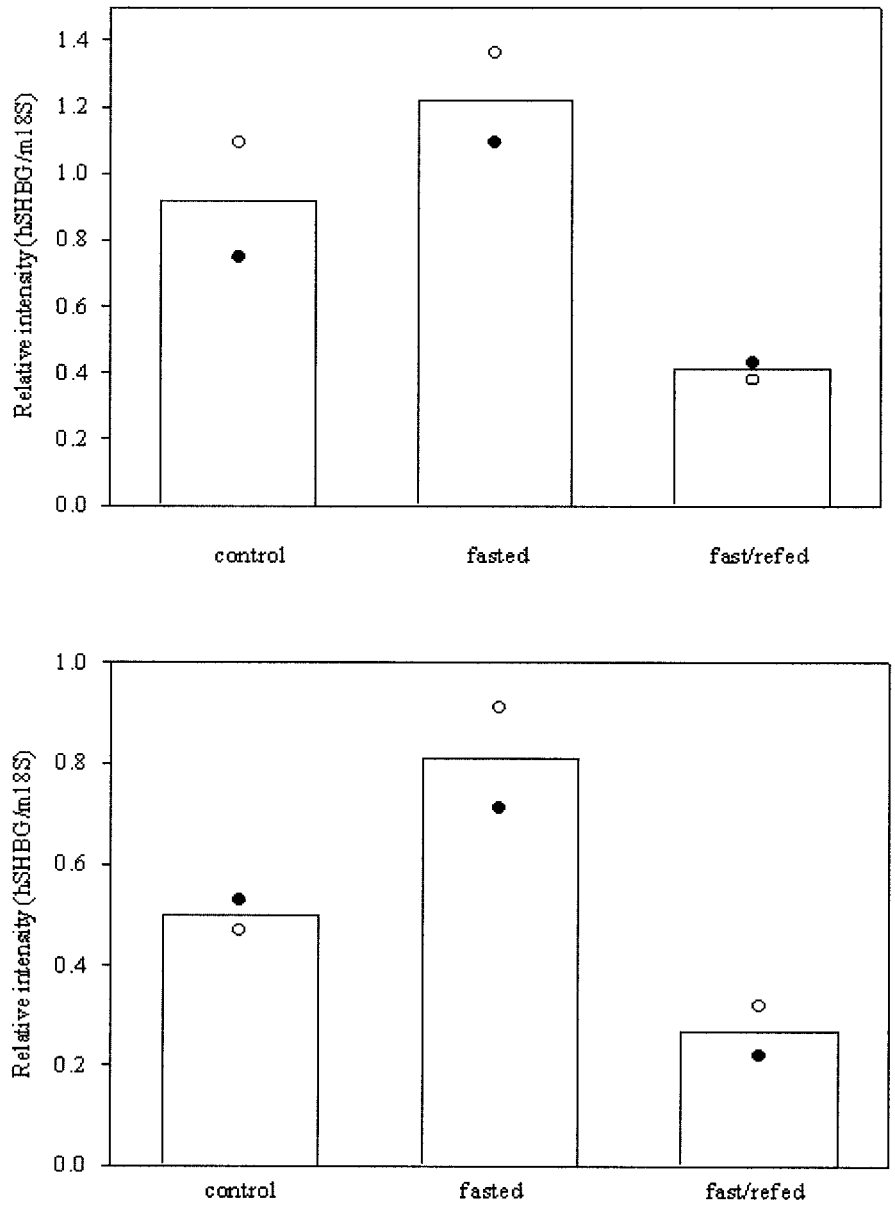

Figure 5.6 Changes in human SHBG mRNA levels in the livers of transgenic mice after a fasting and refeeding treatment. *SHBG*4-a (A), or *SHBG*4-FP4$^-$ (B) transgenic mice were either maintained with food (control - 2 animals), fasted for 24 hours (fast - 2 animals), or fasted for 24 hours followed by a 24 hour refeeding period (fast/refed - 2 animals). Animals were sacrificed at the end of the treatment period and livers were taken for RNA isolation and Northern blot analysis. Levels of human SHBG mRNA were quantified by densitometry and were corrected for loading and transfer efficiency by dividing by the signal obtained from hybridization with m18S RNA. Dots represent relative SHBG mRNA from a single animal.

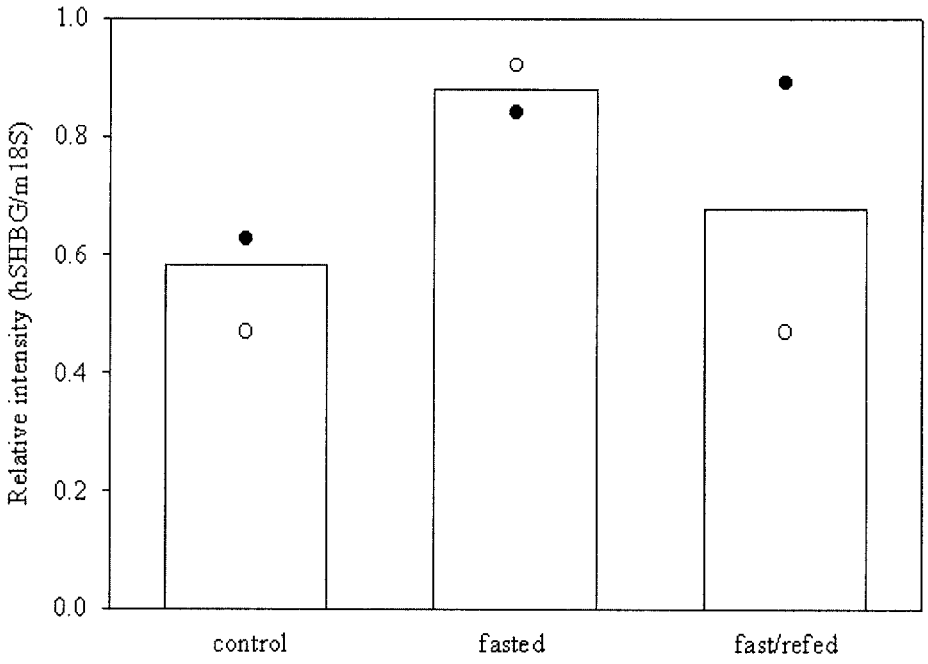

Figure 5.7 Effect of fasting and refeeding on SHBG mRNA in diabetic *SHBG*11-a mice. Streptozotocin treated diabetic (blood glucose > 400 mg/dl) *SHBG*11-a mice were either maintained with food (control - 2 animals), fasted for 24 hours (fast - 2 animals), or fasted for 24 hours followed by a 24 hour refeeding period (fast/refed - 2 animals). Animals were sacrificed at the end of the treatment period and livers were taken for RNA isolation and Northern blot analysis. Levels of human SHBG mRNA were quantified by densitometry and were corrected for loading and transfer efficiency by dividing by the signal obtained from hybridization with m18S RNA. Dots represent relative SHBG mRNA from a single animal.

5.4 Discussion

While the *SHBG* gene in the rodent liver is transcriptionally silent postnatally, the human gene continues to be active throughout life. Moreover, the regulation of hepatic SHBG production in humans is tightly regulated by a variety of factors, and abnormal SHBG levels in the blood are associated with a number of pathological conditions including diabetes and cardiovascular disease. It is believed that phylogenetic differences in hepatic SHBG production during development, and the regulation of SHBG levels in the blood postnatally in humans is a result of differences in regulatory sequences in *SHBG* promoters between species.

The FP4/USF binding site in the human *SHBG* promoter is absent in the rodent *SHBG* promoters (Jänne and Hammond, 1998), and it has been suggested that this difference in the regulatory regions may explain why humans express *SHBG* in the liver postnatally, while rodents do not. However, a 4 kb human *SHBG* transgene lacking the FP 4 sequence is expressed in a similar manner as *SHBG*4 mice characterized previously (Jänne *et al.*, 1998; Jänne *et al.*, 1999), and human SHBG mRNA is present in the livers and kidneys of adult *SHBG4*-FP4$^-$ mice. Interestingly, while SHBG mRNA is undetectable in the testes of transgenic mice containing a 4 kb human *SHBG* transgene, low levels of *SHBG* transcripts were observed in total RNA isolated from the testes of *SHBG*4-FP4$^-$ mice. This is intriguing since the 23 additional nucleotides present in the human *SHBG* promoter disrupts a Sertoli cell enhancer element in the rat *SHBG* promoter (Fenstermacher and Joseph, 1997). It is therefore possible that the additional sequence in the human *SHBG* promoter explains why the gene is not active in the Sertoli cells in the testes of human *SHBG* transgenic mice (Jänne *et al.*, 1998; Selva *et al.*, 2002). The complete removal of the FP 4/USF site in the human *SHBG*4-FP4$^-$ transgene may therefore relieve part of the testicular repression of this transcription unit in the Sertoli cells. It will therefore be important to identify the localization of human *SHBG* transcripts and protein in the testes of these mice, and determine if they accumulate in the same testicular cell types as transcripts in the testes of *SHBG*11 mice. Although the *SHBG*4-FP4$^-$ mice specifically lack a footprinted region corresponding to FP 4 (Jänne and Hammond, 1998), the rodent *SHBG* promoter also lacks an adjacent HNF-4/COUP-TF half site at FP 3 which is still present in the *SHBG*4-FP4$^-$

transgene. It will therefore be of interest to determine if removal of the entire additional nucleotides in the human promoter will further alter the temporal or tissue-specific expression of human *SHBG* in transgenic mice.

Serum levels of SHBG in humans are highly regulated by metabolic factors, and specific dietary components have been shown to alter SHBG levels in humans (Longcope *et al.*, 2000). Serum SHBG levels are negatively correlated with fasting insulin levels in healthy women (Preziosi *et al.*, 1993), and are significantly decreased in patients with insulin resistance (Pugeat *et al.*, 1991). Moreover, low SHBG levels are an early predictor for the predisposition to premature cardiovascular disease and non-insulin dependent (Type II) diabetes mellitus (Ibañez *et al.*, 1997; Ibañez *et al.*, 1998; Lapidus *et al.*, 1986; Lindstedt *et al.*, 1991). Further, insulin treatment decreases the levels of SHBG mRNA in HepG2 cells, and reduces the amount of SHBG that is secreted into the media (Plymate *et al.*, 1988; Crave *et al.*, 1995). However, it is interesting that several reports have demonstrated decreased serum SHBG levels in young patients with Type 1 diabetes and microalbuminuria (Holly *et al.*, 1992; Rudberg and Persson, 1995; Barkai and Tombacz, 2001). The effect of insulin on the regulation of *SHBG* expression in humans therefore appears contradictory, as an insulin resistant state would be expected to result in increased rather than decreased SHBG levels, and levels should be increased in patients with insulin-dependent diabetes. The dietary and metabolic regulation of hepatic *SHBG* expression appears to be complex, and the involvement of insulin in terms of any direct correlation is not always true, and other factors that may be involved in this regulation, such as glucose, are often overlooked.

Insulin and glucose exert independent effects on gene transcription (Vaulont and Kahn, 1994; Vaulont *et al.*, 2000), and because variations in the rate of insulin secretion are primarily under dietary control *in vivo*, the insulin-dependent effects of changes in diet are difficult to distinguish from effects due to dietary components. Furthermore, effects of glucose on *SHBG* gene expression in HepG2 cells cannot be assessed because they lack expression of critical glucose transporters and are unresponsive to glucose (Kim and Anh, 1994). Therefore, as a first step in addressing the mechanism of regulation of *SHBG* gene expression in response to dietary and metabolic factors, this study employed dietary and metabolic manipulations in mice expressing human *SHBG* transgenes. To dissociate the

effects of insulin and diet on modulating *SHBG* expression in human *SHBG* transgenic mice, dietary and metabolic interventions were also performed in animals with compromised pancreatic β-cell function.

Levels of human SHBG in the serum of transgenic mice expressing human *SHBG* transgenes responded dramatically to increasing the carbohydrate composition of the diet, and these changes in serum SHBG levels were accompanied by four fold reductions in hepatic SHBG mRNA. Fructose can replace glucose in the transcriptional activation of the L-type pyruvate kinase gene, and the maximal activation of gene expression associated with fructose occurs more rapidly than with glucose (Munnich *et al.*, 1987). It was therefore interesting that the decrease in serum levels of human SHBG were more pronounced, and occurred more rapidly in a high carbohydrate diet containing sucrose when compared to diets containing glucose or corn starch.

Decreases in serum levels of human SHBG in mice fed a high carbohydrate diet occurred coincident with a decrease in blood glucose levels, and an increase in serum insulin levels. Nevertheless, while peripheral blood glucose levels decreased, the hepatic exposure to glucose in mice fed a high carbohydrate diet is undoubtedly increased. It is unclear from these experiments whether insulin or glucose is responsible for changes in hepatic *SHBG* expression in response to a high carbohydrate diet. We therefore performed similar experiments in streptozotocin-induced diabetic mice expressing human *SHBG* transgenes. In mice with compromised pancreatic β cell function, human SHBG levels in the serum decreased dramatically coincident with increases in blood glucose greater than 400 mg/dl. This decrease in serum levels of human SHBG is similar to what is seen in *SHBG* transgenic mice fed a high carbohydrate diet. Although there was no additional decrease in SHBG levels in diabetic animals fed a high carbohydrate diet, the elevated blood glucose levels in these mice may have already resulted in a maximal repression of hepatic *SHBG* expression. It was somewhat surprising that serum SHBG levels decreased 60 % within one week after streptozotocin treatment because insulin decreases SHBG mRNA and protein levels in HepG2 cells (Plymate *et al.*, 1988, Crave *et al.*, 1995), and hyperinsulinemic insulin resistance is associated with low SHBG levels in humans (Pugeat *et al.*, 1991). However, SHBG levels have been shown to be decreased in patients with type 1 diabetes and

microalbuminuria (Holly *et al.*, 1992; Rudberg and Persson, 1995; Barkai and Tombacz, 2001). Although the negative correlations associated with insulin and SHBG levels in humans are certainly relevant, these data suggest that blood glucose may exert an additional effect on hepatic *SHBG* expression. In further support of a role for glucose in the regulation of *SHBG* expression, SHBG mRNA levels in the liver increased in mice fasted for 24 hours, and this was also seen in streptozotocin-induced diabetic animals.

In humans, the level of caloric intake can regulate hepatic *SHBG* expression. Obesity is associated with low SHBG levels and levels can be increased by decreasing caloric intake (Franks *et al.*, 1991). Further, patients with anorexia nervosa have increased SHBG levels (Estour *et al.*, 1986) that can be lowered by increasing caloric intake (Barbe *et al.*, 1993). It was interesting then that human SHBG mRNA levels were increased in the liver when transgenic animals fasted for 24 hours, and were decreased upon a 24 hr refeeding period.

Serum levels of human SHBG in *SHBG*4-FP4$^-$ mice respond identically to *SHBG*4 mice when fed a high carbohydrate diet, or are subjected to a fasting and refeeding regimen. Therefore it does not appear that a USF binding site in the human *SHBG* promoter functions as part of a glucose response element. However, similar to the HNF-4 site in the L-type pyruvate kinase gene promoter (Diaz Guerra *et al.*, 1993), it is possible that the HNF-4 site contained in the additional nucleotides in the human promoter contributes to the regulation of gene expression in response to diet, and it may be necessary to remove this region as well. It will therefore be important to further characterize this and other elements in the human *SHBG* gene that are responsible for mediating the effects of dietary and metabolic interventions on human *SHBG* expression in the liver.

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CHAPTER 6

HUMAN SEX HORMONE-BINDING GLOBULIN VARIANTS ASSOCIATED WITH HYPERANDROGENISM AND OVARIAN DYSFUNCTION

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6.1 Introduction

Human sex hormone binding globulin (SHBG) transports testosterone and estradiol in the blood (Anderson, 1974; Hammond, 1990). Blood concentrations of SHBG are a major determinant of the metabolic clearance of these sex steroids and their access to target tissues (Siiteri *et al.*, 1982), and their measurements provide a means of estimating the amounts of circulating nonprotein-bound or "free" sex steroids (Selby, 1990). Abnormally low serum SHBG levels are frequently found in women with polycystic ovarian disease (PCOS) and contribute to hyperandrogenic symptoms such as hirsutism and acne (Anderson, 1974). Serum SHBG levels are also reduced in patients with Type 2 diabetes and coronary heart disease (Lindstedt *et al.*, 1991; Lapidus *et al.*, 1986). The reason that SHBG levels are low in serum samples from many of these individuals is unclear, but it has been reported that SHBG deficiencies are inherited (Ahrentsen *et al.*, 1982; Meikle *et al.*, 1982).

Human SHBG is a homodimeric plasma glycoprotein encoded by a 4 kb gene spanning 8 exons on the short arm (p12→p13) of chromosome 17 (Hammond *et al.*, 1989; Bérubé *et al.*, 1990). It is produced by hepatocytes (Khan *et al.*, 1981; Jänne *et al.*, 1998) under the control of various hormonal and metabolic regulators (Rosner *et al.*, 1984; Plymate *et al.*, 1988). Exon 1 of human *SHBG* contains the coding sequence for the secretion signal polypeptide, while exons 2-8 encode two contiguous LG domains (Hammond *et al.*, 1989; Grishkovskaya *et al.*, 2000). The amino-terminal LG domain encoded by exons 2-4 contains the steroid-binding site (Grishkovskaya *et al.*, 2000), the dimer interface (Avvakumov *et al.*, 2001), and several cation-binding sites (Avvakumov *et al.*, 2000). The functional significance of the carboxy-terminal LG domain of SHBG is less well established, but removal of only a few amino acids from the carboxy-terminus of the rat orthologue of human SHBG, which is generally known as the androgen-binding protein (ABP), prevents secretion of a functional protein when expressed in mammalian cells (Joseph and Lawrence, 1993). This domain also normally contains two sites for N-glycosylation, one of which is invariably conserved in SHBG molecules across a wide range of mammalian species (Hammond, 1993).

A single nucleotide polymorphism (SNP) within the human *SHBG* coding sequence is responsible for a common electrophoretic variant with an additional N-glycosylation

consensus sequence within the carboxy-terminal LG domain (Power *et al.*, 1992). An additional carbohydrate chain at this position has no impact on the steroid-binding activity of SHBG, but it may reduce the plasma clearance of SHBG and account for a modest accumulation of the variant protein in the blood (Cousin *et al.*, 1998). More recently, we have identified a polymorphic $(TAAAA)_n$ repeat in the human *SHBG* promoter that influences its transcriptional activity (Hogeveen *et al.*, 2001), but it remains to be determined whether this pentanucleotide repeat contributes to the differences in plasma SHBG levels between individuals. In this report, we have identified two sequence variations in the coding region of human *SHBG* that account for unusually low serum SHBG levels in a patient with severe hyperandrogenism. Furthermore, at least one of these sequence variations is associated with hyperandrogenic states and ovarian dysfunction in several other patients.

6.2 Materials and Methods

6.2.1 Patients and clinical samples

The proband was a 27 year old patient from the north of France. Her body weight was normal and she had a history of regular menstrual cycles. She reported a mild hirsutism that increased dramatically during pregnancy, and this was associated with other symptoms of virilism including deepening of the voice and enlargement of the clitoris. Her pregnancy was otherwise normal and she delivered twin girls with no signs of virilization. Two years after this pregnancy, hirsutism was scored between 14 and 16 according to the scoring system of Ferriman and Gallwey (Ferriman and Gallwey, 1961), but other symptoms of virilism were no longer apparent. Blood samples were taken at this time, and serum total testosterone, androstenedione, 17-hydroxyprogesterone and dehydroepiandrosterone (DHEAS) levels were within the ranges for normal women. In contrast, serum SHBG was undetectable using a routine immunoassay. She was then placed under treatment with 50 mg cyproterone acetate and 2 mg estradiol taken orally 21 days each month. Under this treatment, additional blood samples were taken for more detailed studies of serum SHBG levels and for genetic analysis. Blood samples were also taken from the proband's father and sister for DNA sequence analysis and serum SHBG measurements. At the time of blood sampling, her father was under treatment for hypertension and her sister was taking an oral contraceptive containing

20 μg ethinyl estradiol and 150 μg gestodene.

Blood samples were also taken from the following groups of women for DNA sequence analysis and serum SHBG measurements: Group I; 88 non-obese fertile women with regular menstrual cycles (i.e., normal controls). Group II; 132 normal weight women (body mass index <25 kg/m^2) with symptoms of hirsutism, as defined by Ferriman and Gallwey (Ferriman and Gallwey, 1961). Group III; 93 overweight women (body mass index >25 kg/m^2) with symptoms of hirsutism. Group IV; 69 women with no evidence of androgen excess, but with irregular menstrual cycles due to a variety of clinical problems including obesity, anorexia nervosa, precocious menopause, and hyper-prolactinemia. Group V; 53 women with 21-hydroxylase deficiency. Group VI; 47 women with a variety of other endocrine disorders. Approximately 5% of the controls and patients studied were of North African origin, while the remainder were French Caucasian.

6.2.2 SHBG and testosterone assays

Two different immunoradiometric assays (SHBG-RIACT kit from CisBio International, Gif-sur-Yvette, France, and an SHBG IRMA kit from ORION Diagnostica, Oulunsalo, Finland) were used to determine SHBG concentrations in serum samples, and these gave similar results. The values reported are those obtained using the SHBG-RIACT kit. The method used to measure the very low steroid binding capacity of SHBG in the proband serum, and its binding affinity by Scatchard analysis, was based on an established ligand-binding assay (Nisula and Dunn, 1979) except that an anti-SHBG mononclonal antibody-Sepharose (kindly provided by Dr. Catherine Grenot, INSERM U 329) was used instead of concanavalin A-Sepharose. A conventional steroid binding capacity assay (Hammond and Lähteenmäki, 1983) and a time-resolved immunofluorometric assay (Niemi *et al.*, 1988) were also used to measure SHBG levels in serum samples and cell culture medium. Western blotting was used to examine the electrophoretic properties of SHBG in serum (Cousin *et al.*, 1998), as well as in culture medium samples and cell extracts (Bocchinfuso and Hammond, 1994). In order to obtain sufficient SHBG from the proband serum for western blot analysis, the SHBG in serum samples was first isolated by immuno-affinity adsorption (Cousin *et al.*, 1998).

Total serum testosterone levels (normal female reference range: 0.42-1.49 nM) and the amounts of non-SHBG-bound testosterone (normal female reference range: 62-236 pM) were determined in serum samples by radioimmunoassay (Déchaud *et al.*, 1989). The serum distribution of testosterone was measured using the centrifugal ultrafiltration/dialysis method (Siiteri *et al.*, 1982).

6.2.3 Screening for *SHBG* sequence variations

Two primer pairs were used to amplify the entire coding sequence for the SHBG precursor polypeptide from the proband's genomic DNA. The 5' amplicon primer set comprised a 5' untranslated region-specific oligonucleotide sequence; 5'-GAGTTGTCTGAGCCGCCG-3', and an oligonucleotide complementary to an intron 4 sequence; 5'-AGCCACCCAGCAGTGCTT-3'. The 3' amplicon primer set comprised an intron 3-specific oligonucleotide; 5'-ACAGGAAGGTGGCAGAAA-3', and an oligonucleotide complementary to a region 3' to the polyadenylation site in exon 8; 5'-GCCTGGTACATTGCTAG-3'. The PCR products were inserted into the pCRBlunt II-TOPO vector (Invitrogen Life Technologies, San Diego, California, USA) and sequenced. Exon sequences that deviated from the consensus *SHBG* sequence (Genbank Accession #M 31651) were re-amplified in a PCR using exon-specific primer pairs (Power *et al.*, 1992) and sequenced.

6.2.4 Expression of SHBG in CHO cells

We used a pRc/CMV vector (Invitrogen) to express wild type and mutated SHBG cDNAs in CHO cells, as described previously (Bocchinfuso and Hammond, 1994). At near confluence, the transfected cells were washed with PBS, and cultured for a further 2 days in serum-free DMEM (Invitrogen Life Technologies). The SHBG content of cell culture medium was examined by Western blot analysis (section 2.2.3). In addition, we also measure the SHBG content of the CHO cells grown in this way by Western blotting (Section 2.2.3). To accomplish this, soluble proteins were extracted from the CHO cells by homogenization in 40 mM Tris, pH 7.5, 1 mM EDTA, 150mM NaCl, while insoluble protein was extracted from the resulting pellet using 10mM Tris, pH 8, 100mM EDTA containing

0.5 % sodium-dodecyl-sulphate.

Consensus and variant *SHBG* alleles were also inserted as 4 kbp *Hin*dIII - *Xba*I genomic DNA cassettes in pRc/CMV. The *Hin*dIII site was introduced within the 5' untranslated sequence 36 bp from the translation initiation codon in exon 1, while the *Xba*I site was introduced after 68bp of non coding sequence 3' from the stop codon within exon 8. These constructs were transfected into mouse BW-1 hepatoma cells (Szpirer and Szpirer, 1975) and human HepG2 cells using LIPOFECTAMINE reagent, as recommended by Invitrogen Life Technologies, and pools of stable transformants were obtained by culture under G418 selection (Bocchinfuso and Hammond, 1994). Cell culture medium was harvested from near confluent cultures grown in serum-free medium for 2 days for western blotting. In addition, the transfected BW-1 cells were also harvested from near confluent cultures grown in αMEM containing 10% fetal bovine serum for RNA extraction using TRIzol solution (Invitrogen Life Technologies) for reverse transcriptase-PCR and northern blot (Bocchinfuso *et al.*, 1991) analyses of *SHBG* transcripts. The primer sequences used for the reverse transcriptase-PCR analyses were as follows: Exon 3 forward primer, 5'-GACCCAGAGGGAGTGATTT-3'; Exon 4 forward primer, 5'-GAGGGGGACTCTGTG-3' ; Exon 5 reverse primer, 5'-GAATTCTGCCTGAGT-3'.

In addition, the rates of SHBG secretion from pools of BW-1 cells that constitutively express the *Msp*I variant or consensus alleles were assessed by growing replicate (n=3) cultures to near confluence, and then removing the culture medium, washing the cells once with phosphate buffered saline, and then replacing the medium. Samples of culture medium were then removed at timed intervals over 6 hours for SHBG measurements using an ultra-sensitive, time-resolved immunofluorometric assay (Niemi *et al.*, 1988). At the end of this experiment, cells were harvested for RNA extraction to assess the relative amounts of *SHBG* transcripts by northern blotting (Bocchinfuso *et al.*, 1991).

6.3 Results

6.3.1 Identification of two different coding sequence variations within the *SHBG* alleles of a single individual

We measured the proband's serum SHBG levels by two different immunoassays and

conventional steroid-binding capacity assays. In each case, the values obtained (0.5 - 1 nmol/L) were approximately an order of magnitude lower than those found in other hyperandrogenic patients, and about 50 times lower than those in normal age-matched women (30-69 nmol/L). These results were even more remarkable because the proband was being treated at the time of blood sampling with an oral estrogen and anti-androgen formulation that would normally be expected to increase serum SHBG levels (see Materials and Methods for details). Overlapping genomic fragments spanning the entire transcription unit encoding SHBG (Hammond *et al.*, 1989; Jänne *et al.*, 1998) were therefore PCR-amplified using the proband's DNA (Figure 6.1 a). Sequence analysis of the PCR products revealed two alleles based on the presence or absence of a SNP (P_1) within exon 4 (Figure 6.1 b). This occurs on one allele (Figure 6.1b), and converts the codon (CCG) for P156 into a leucine codon (CTG). On the other allele, there is a single nucleotide deletion (P_2) within exon 8 (Figure 6.1 b). The transcription units of both *SHBG* alleles were completely sequenced and no other deviations from the published human *SHBG* sequences (Hammond *et al.*, 1989; Gershagen *et al.*, 1989) were noted. This analysis also included the 800 bp *SHBG* promoter sequence (GenBank accession number M31651).

6.3.2 Screening for the *Msp*I polymorphism.

The SNP within exon 4 disrupts an *Msp*I site (CCGG) at the boundary between exon 4 and intron 4 (CC/TGGT, intron splice junction underlined) in the consensus *SHBG* sequence (Hammond *et al.*, 1989). We therefore established a PCR-based assay to screen for this *Msp*I polymorphism within the proband's family, as well as in 88 healthy control women and several groups of patients with various reproductive and/or endocrine disorders (see Table 6.1 for details). The proband's father and sister do not carry the *Msp*I polymorphism (Figure 6.2), and this allowed us to deduce that her deceased mother was a heterozygous carrier of this allele (see also below). The *Msp*I polymorphism was not present in any of the

Group	n	BMI kg/m^2	SHBG nmol/L	*Msp*I polymorphism
I. normal women	88	21.1 ± 2.1	55 ± 29	0
II. non-obese/hirsute	132	21.3 ± 2.0	39 ± 22	3
III. obese/hirsute	93	31.8 ± 5.9	25 ± 17	0
IV. ovarian dysfunction	69	28.0 ± 8.7	37 ± 30	1
V. 21 hydroxylase deficiency	53	24.6 ± 6.8	39 ± 17	0
VI. endocrine diseases	47	24.3 ± 5.2	42 ± 24	0

Table 6.1
Details of normal women and various patient groups screened for the *Msp*I polymorphism showing numbers of subjects in each group screened, mean ± SD body mass index (BMI) and serum SHBG levels and number of *Msp*I polymorphism carriers identified.

control subjects tested, but four other heterozygous carriers of this *Msp*I polymorphism were identified amongst the patients tested and these individuals were French Caucasians. Three of them were within Group II (non-obese/hirsute); two of whom had PCOS (Figure 6.2; patients CV and DC) and one with idiopathic hirsutism and was taking l-thyroxine (150 μg daily) after a surgically removed thyroid cancer (not shown). The other carrier of this *Msp*I polymorphism (Figure 6.2; patient CS) was identified within a group of women with ovarian dysfunction (Table 6.1, Group IV). Her BMI was normal, but she suffered from a precocious menopause at age 19.

Both PCOS patients with the *Msp*I polymorphism in Group II were of normal weight and had serum SHBG levels (17 - 27 nmol/L) that were below the normal female reference range (30 - 69 nmol/L). The serum SHBG level (35 nmol/L) in the other patient from Group II was in the low normal range, but this is a low level when considering she was under treatment with thyroid hormone (Anderson, 1974). The two patients with PCOS had total serum testosterone (1.75 nM for patient CV and 1.90 nM for patient DC) and non-SHBG-bound testosterone levels (288 pM for patient CV and 576 pM for patient DC) that were above the normal range. Although the serum testosterone measurements in the two other patients with an *Msp*I polymorphism were within normal ranges (see Materials and Methods) these values may have been influenced by their unusual clinical conditions and/or treatments.

6.3.3 Screening for the single nucleotide deletion in exon 8.

The single nucleotide deletion (P_2) within exon 8 causes a frame shift within the codon for E326, and introduces a novel carboxyl-terminal coding sequence (S-L-P-P-L-F-A) followed by a stop codon (Figure 6.3 a). Although this results in the loss of a *Bbs*I site, the development of a screening assay was complicated because this occurs in close proximity to a relatively common SNP (P_3), which introduces an additional consensus site for N-glycosylation (Figure 6.3 a) due to a D327N substitution (Power *et al.*, 1992). Nevertheless, discrimination between P_3 and P_2 could be accomplished by separate digestions of PCR products spanning this region with *Bbs*I and *Hin*fI (Figure 6.3 b). In subjects with either the P_2 or P_3 sequence variations, a *Bbs*I site in the consensus *SHBG* sequence is disrupted (Figure 6.3 b). However, alleles containing P_3 also lack the *Hin*fI site present in the consensus

sequence, which is preserved in the allele containing P_2 (Figure 6.3 b).

This analysis demonstrated that the proband and her sister inherited the single nucleotide deletion (P_2) in exon 8 from their father (Figure 6.3 b), and confirms that the proband's *SHBG* allele containing the *Msp*I polymorphism was inherited from her deceased mother. We failed to identify other carriers of P_2 in the 482 women (Table 6.1) who had been tested for the *Msp*I polymorphism. Therefore, although P_2 is clearly transmitted within the pedigree we have examined, it remains to be determined whether it exists within other patient groups characterized by low plasma levels of SHBG, or in other ethnic groups.

6.3.4 Biochemical properties of the proband's SHBG and its influence on the serum distribution of testosterone

The small amount of SHBG in the proband's serum was semi-purified by immunoaffinity adsorption, and a western blot indicated that the molecular sizes of its major electrophoretic isoforms are essentially normal (Figure 6.4 a). However, it lacks a minor electrophoretic isoform (see bold arrowhead in Figure 6.4 a) that corresponds to SHBG monomers in which the N-glycosylation sites are partially utilized (Bocchinfuso *et al.*, 1992).

The steroid-binding properties of the SHBG isolated from the proband's serum were also examined by Scatchard analysis, and were very similar to SHBG in normal male and female serum samples (Figure 6.4 b). Thus, the SHBG in the proband's blood is capable of binding steroid, and we assessed its impact on the serum distribution of testosterone (Figure 6.4 c). This indicated that even the small amount of SHBG in the proband's blood exerts some influence on the serum distribution of testosterone (Figure 6.4 c). However, the percentage of non-protein-bound or "free" testosterone in the proband's serum (9.2%) is very much higher than in a normal control (Figure 6.4 c), or in normal women (1.4%) of similar age with a mean serum SHBG concentration of 47 nM (Hammond *et al.*, 1984). As a result, the concentration of free testosterone (99 pM) in the proband's serum far exceeds the female reference range (6 - 22 pM) based on an average percentage free testosterone of 1.5%, and this undoubtedly accounts for her hyperandrogenic symptoms. The amount of non-SHBG-bound testosterone (i.e., albumin bound and free) in the proband's serum (697 pM) was also measured in an independent assay (Déchaud *et al.*, 1989), and also greatly exceeds

the normal reference range (62-236 pM). Moreover, when compared with the total testosterone concentration in the proband's serum (1.1 nM), these results confirm our estimates of the distribution of testosterone in the proband's serum: i.e. 9.2% free, 58.4% albumin-bound, and 32.4% SHBG-bound.

6.3.5 Production and secretion of SHBG variants encoded by the proband's *SHBG* alleles

To determine whether the very low SHBG levels in serum samples from the proband are due to a defect in the production or secretion of the SHBG P156L variant and/or the carboxy-terminally truncated SHBG, we first expressed the variant alleles as cDNAs in CHO cells, and performed a western blot analysis of the resulting culture medium and transfected cell extracts. This indicated that the SHBG P156L variant is produced and secreted by CHO cells (Figure 6.5 a, lanes 1 and 2), and that its steroid-binding characteristics are normal (Figure 6.5 b). However, the relative abundance of the two major electrophoretic isoforms associated with SHBG P156L and normal SHBG differ (Figure 6.5 a, lane 1 vs. lane 2), with the low mobility isoform being predominant in SHBG P156L. A similar pattern is also seen when comparing the partially glycosylated monomers of SHBG P156L and SHBG in the insoluble cell extracts (Figure 6.5 a, lane 7 vs. lane 8). By contrast, expression of a cDNA containing the single nucleotide deletion found in exon 8 resulted in no SHBG in the culture medium (Figure 6.5 a, lane 3), and only trace amounts of an approximately 34 kDa immunoreactive truncation product within the insoluble extract of the transfected cells (Figure 6.5 a, lane 9). In addition, since there was no evidence of a truncated form of SHBG on the western blot of SHBG isolated from the proband's serum (Figure 6.4 a), the very small amount of SHBG in the proband's serum must represent the product of the allele encoding the SHBG P156L variant.

6.3.6 Effect of the *Msp*I polymorphism on *SHBG* expression.

Apart from the *Msp*I polymorphism (P_1), the allele encoding the SHBG P156L variant showed no other deviations from the consensus *SHBG* sequence that might contribute to a reduction in its expression. This sequence analysis also included the 800 bp proximal promoter responsible for human *SHBG* expression in the liver (Jänne *et al.*, 1998). Since the

*Msp*I polymorphism is located close to the exon 4: intron 4 boundary, we performed an experiment to determine whether it influences the splicing or relative abundance of *SHBG* transcripts, or their translation in hepatocyte derived cell lines. This was done by introducing the sequences of the P_1 variant or consensus *SHBG* into BW-1 mouse hepatoma cells and human HepG2 hepatoblastoma cells. These genomic fragments span the translation start site for the SHBG precursor polypeptide and the polyadenylation sequence within exon 8 of human *SHBG* (Hammond *et al.*, 1989; Jänne *et al.*, 1998), and were expressed constitutively under the control of a CMV promoter. Reverse transcriptase-PCR and northern blot analysis of SHBG mRNA from stable transformants obtained after neomycin selection, indicated that cells producing SHBG P156L contained only appropriately spliced *SHBG* transcripts, as did cells expressing the consensus *SHBG* sequences (Figure 6.6 a). The relative abundance of *Msp*I variant and consensus *SHBG* transcripts in these cells was also essentially identical, but a western blot indicated that the variant sequence consistently resulted in less SHBG P156L in the culture medium of these cells (data not shown). As in the case of the SHBG produced by CHO cells (Figure 6.5 a), the electrophoretic isoform with reduced mobility associated with the SHBG P156L variant was always relatively more abundant than that in the normal protein, irrespective of the liver cell line in which it is expressed (data not shown). This is also consistent with the unique electrophoretic profile of the SHBG isolated from the proband's serum (Figure 6.4 a), and these data indicated that the SHBG P156L variant is glycosylated abnormally and that its production and/or secretion might be reduced when compared to the SHBG produced by the consensus *SHBG* allele. We confirmed this by comparing the secretion rate of the SHBG P156L variant and the normal SHBG protein from BW-1 cells transfected with either the variant or consensus human *SHBG* sequences under the control of a CMV promoter. The relative abundance of *SHBG* transcripts in these cells was similar (Figure 6.6 b), while the amount of SHBG P156L in the culture medium, and the rate at which it accumulated, are clearly much reduced when compared to the SHBG produced by the consensus *SHBG* sequence (Figure 6.6 c).

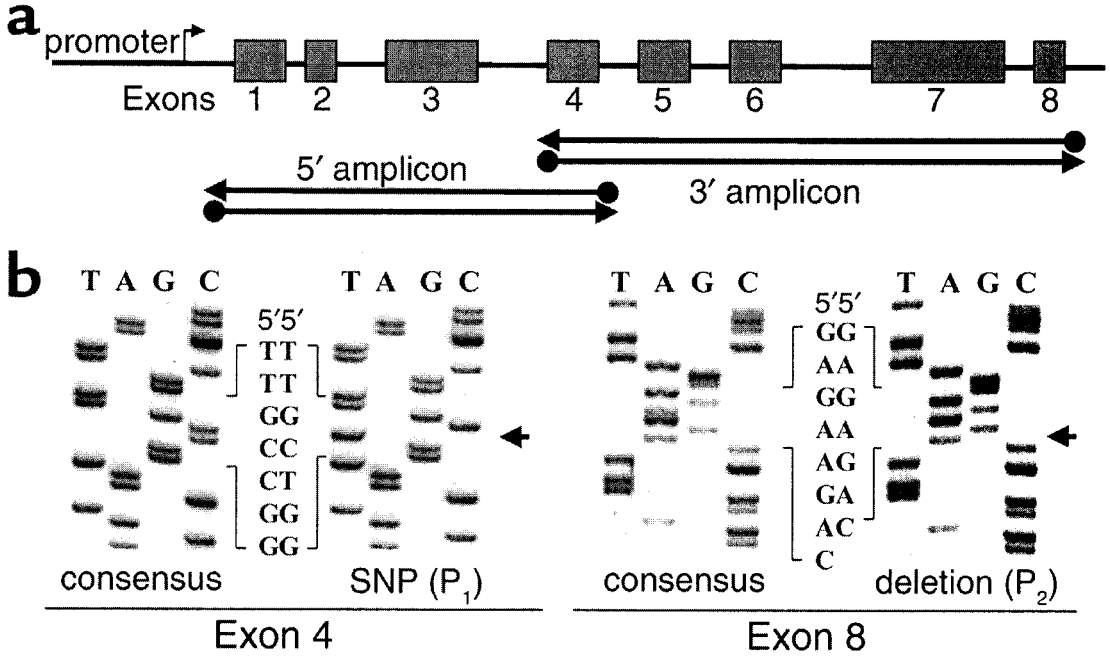

Figure 6.1 Identification of *SHBG* sequence variations in a DNA sample from the proband. **(a)** Schematic representation of the 4.3 kb genomic fragment comprising the human *SHBG* transcription unit showing the transcription start site (arrow), and the positions of exons in relation to the two overlapping amplicons generated for sequence analysis. **(b)** The sequence variations in exons 4 and 8 within separate *SHBG* alleles from the proband are compared with the consensus *SHBG* sequences on the proband's other allele. The single nucleotide polymorphism (SNP) in exon 4 is designated as P_1, while the single nucleotide deletion in exon 8 is designated as P_2. Separate alleles were identified based on the presence or absence of P_1 within the exon 4 sequences of the overlapping 5' and 3' amplicons shown in panel A. Bold arrowheads indicate the positions of sequence variations.

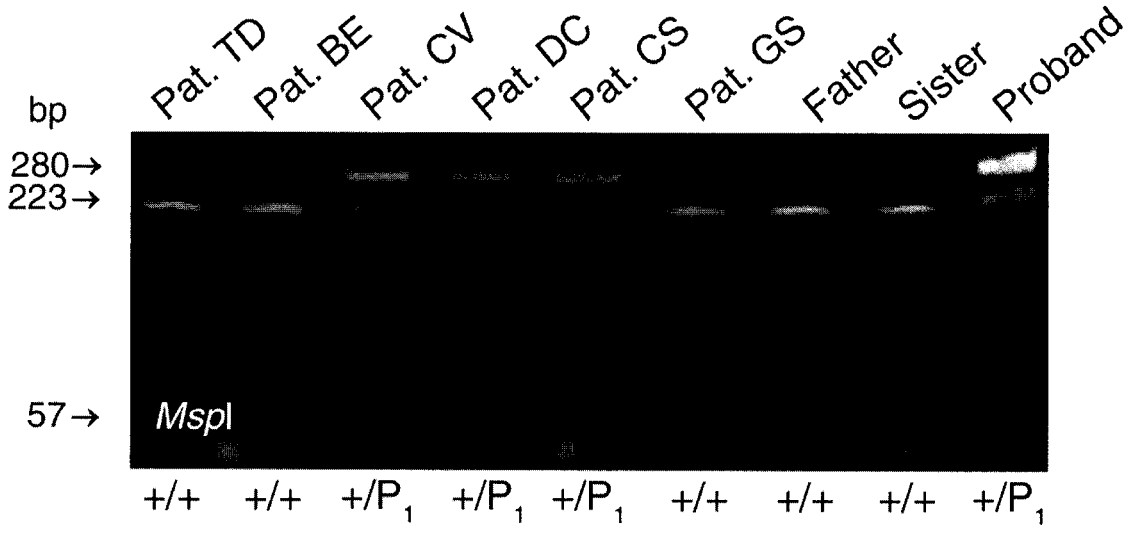

Figure 6.2 Identification of a single nucleotide polymorphism (P_1) which disrupts an *Msp*I site within the exon 4:intron 4 boundary of human *SHBG* in DNA samples from the proband and female patients with reproductive disorders. Ethidium bromide-stained polyacrylamide electrophoresis gel of PCR-amplified exon 4 sequences digested with *Msp*I. The enzyme fails to completely digest PCR-products from the variant allele in samples from the proband and three other patients (Pats. CV, DC and CS). By contrast, PCR-products (sizes in base pairs shown on the left) from the proband's father and sister, and three patients (Pats. TD, BE and GS) are digested completely by *Msp*I. Genotypes are: homozygous consensus sequence (+/+); heterozygous carriers of the *Msp*I SNP (+/P_1).

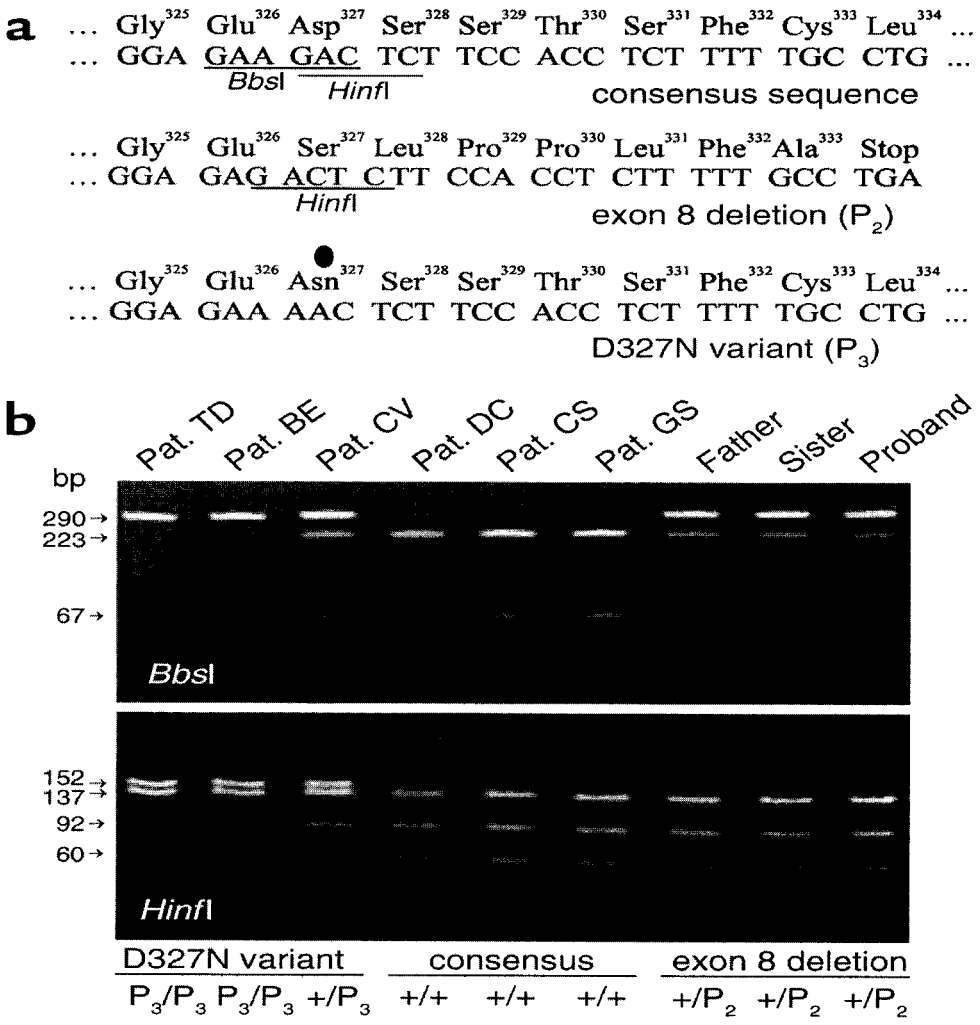

Figure 6.3 Identification of a single nucleotide deletion (P_2) in exon 8 of the human *SHBG* in DNA samples from the proband and her family members. **(a)** P_2 disrupts a *Bbs*I site in the consensus *SHBG* sequence, and causes a frame shift in codon E326 that introduces a novel sequenced followed by a stop codon. A common SNP (P_3) also disrupts this *Bbs*I site, as well as a *Hin*fI site which is preserved in P_2. Solid oval indicates the position of an additional N-glycosylation site introduced by the D327N substitution caused by P_3. **(b)** Ethidium bromide-stained polyacrylamide electrophoresis gel of PCR-amplified exon 8 sequences digested with either *Bbs*I or *Hin*fI. Approximately half of the PCR products from the proband and from her father and sister are resistant to *Bbs*I digestion but are completely digested with *Hin*fI. By contrast, patients who are homozygous (Pats. TD and BE) or heterozygous (Pat. CV) for P_3 show either complete or partial resistance to *Bbs*I and *Hin*fI digestion, respectively. The PCR products (sizes in base pairs shown on the left) from patients (Pats. DC,CS, and GS) in which the consensus exon 8 sequence is present are digested completely by *Bbs*I and *Hin*fI. Genotypes are: homozygous consensus sequence (+/+); heterozygous carriers of the single nucleotide deletion (P_2) in exon 8 (+/P_2); homozygous carriers of P_3 (P_3/P_3); heterozygous carriers of P_3 (+/P_3).

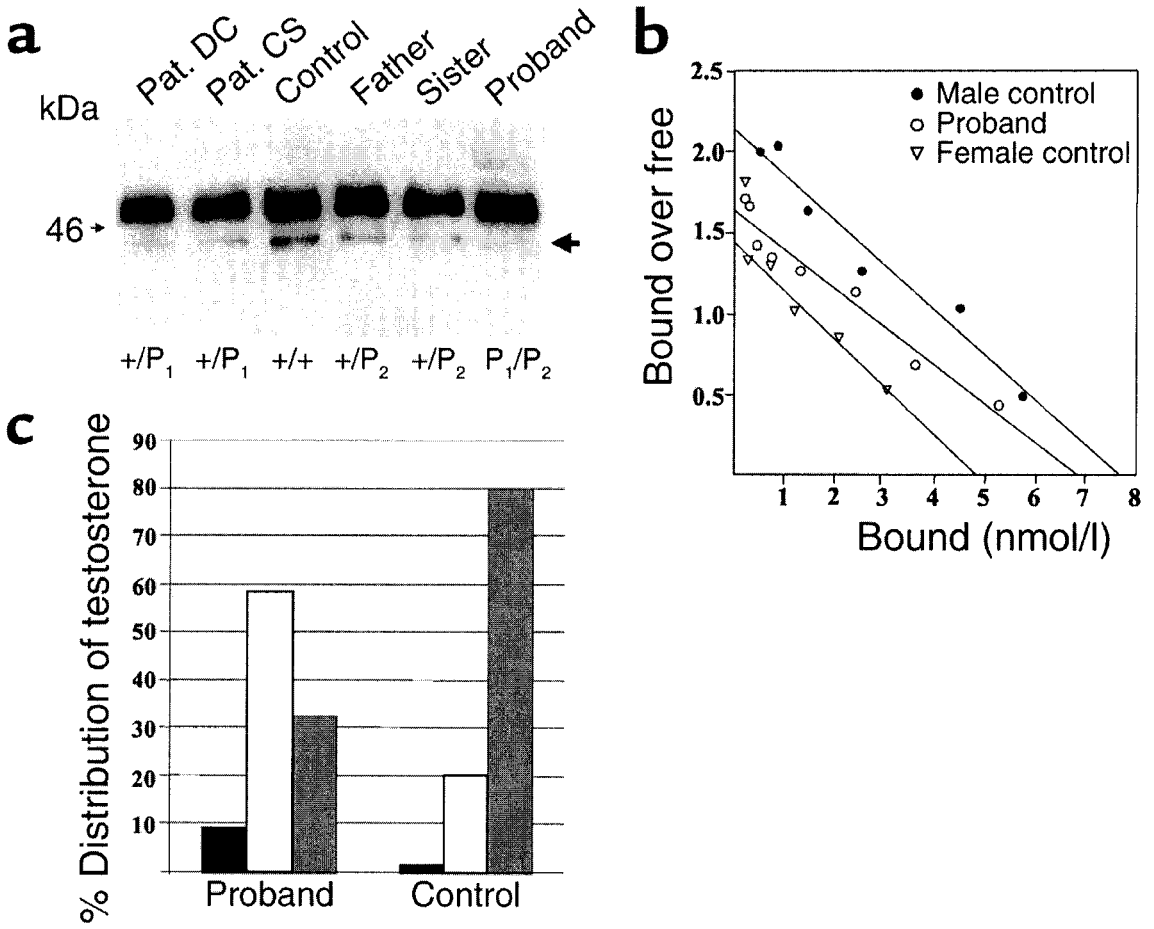

Figure 6.4 Biochemical properties of SHBG in serum from the proband and her family members, and from a normal female control and two patients (Pat. DC and Pat. CS) identified as heterozygous carriers of the *Msp*I polymorphism (P_1). **(a)** Western blot of SHBG immuno-precipitated from serum samples. Genotypes are: homozygous consensus sequence (+/+); heterozygous carriers of the *Msp*I SNP (+/P_1); heterozygous carriers of single nucleotide deletion in exon 8 (+/P_2); proband heterozygous for P_1 and P_2 ($P1/P_2$). **(b)** Scatchard plots obtained for SHBG isolated from the proband (K_d = 4.1 nM), and from male (K_d = 3.6 nM) and female (K_d = 3.3 nM) controls, using [^{3}H]DHT as labeled ligand (see Methods for details). **(c)** Distribution of testosterone between the SHBG-bound (grey bars), albumin-bound (white bars) and non-protein-bound or "free" (black bars) fractions in serum from the proband and a normal female control.

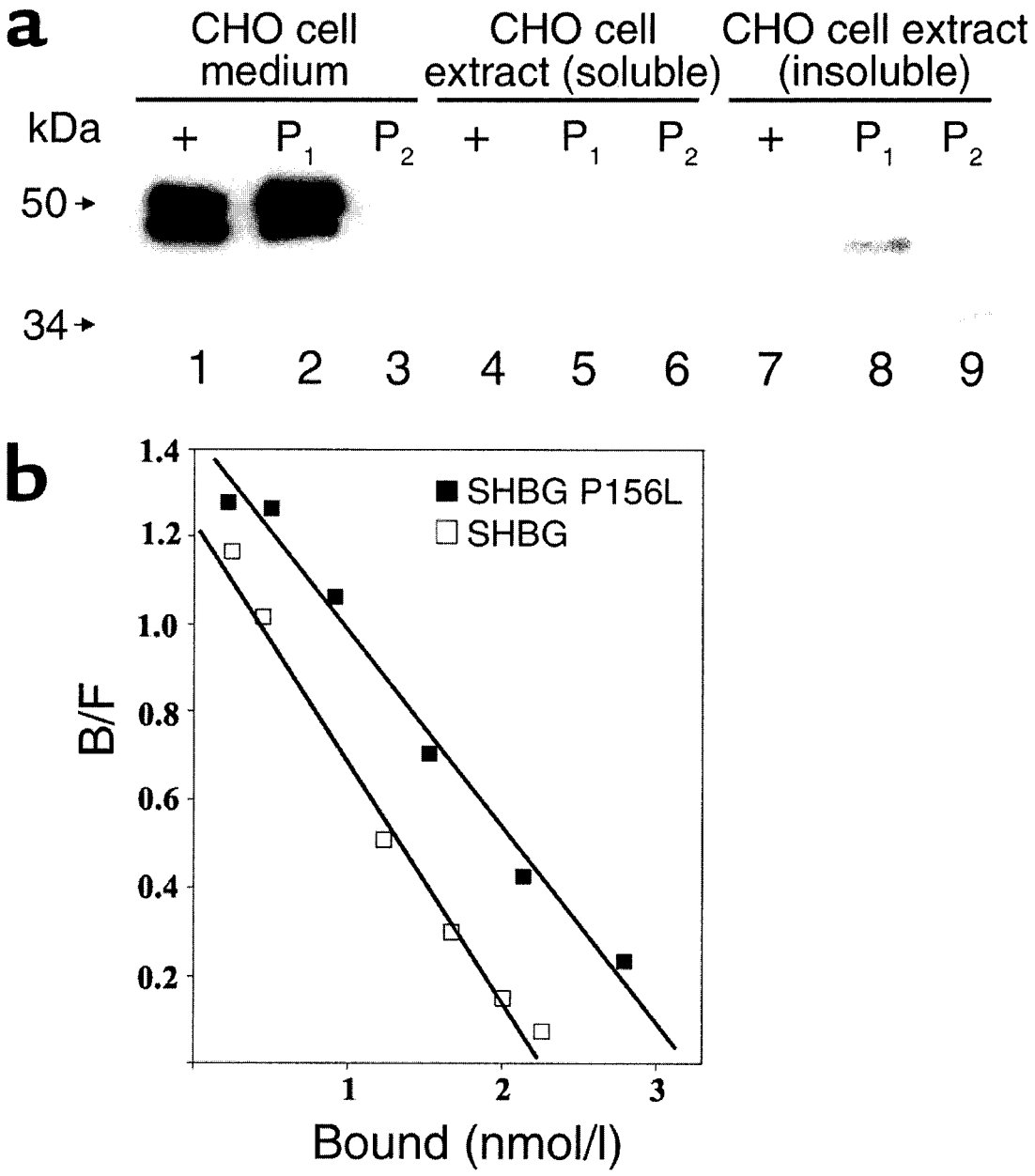

Figure 6.5 Comparison of the electrophoretic properties and steroid-binding characteristics of the products of the consensus and variant human SHBG cDNA sequences expressed in Chinese hamster ovary (CHO) cells. **(a)** Western blot of culture media and cell extracts from CHO cells transfected for stable expression of consensus (+) and variant (P_1 or P_2) SHBG cDNA sequences. P_1; the *Msp*I polymorphism. P_2; the single nucleotide deletion in exon 8. Lanes are numbered under the blot. **(b)** Scatchard plots for human SHBG (K_d = 1.8 nM) and the SHBG P156L variant (K_d = 2.2 nM) produced by CHO cells.

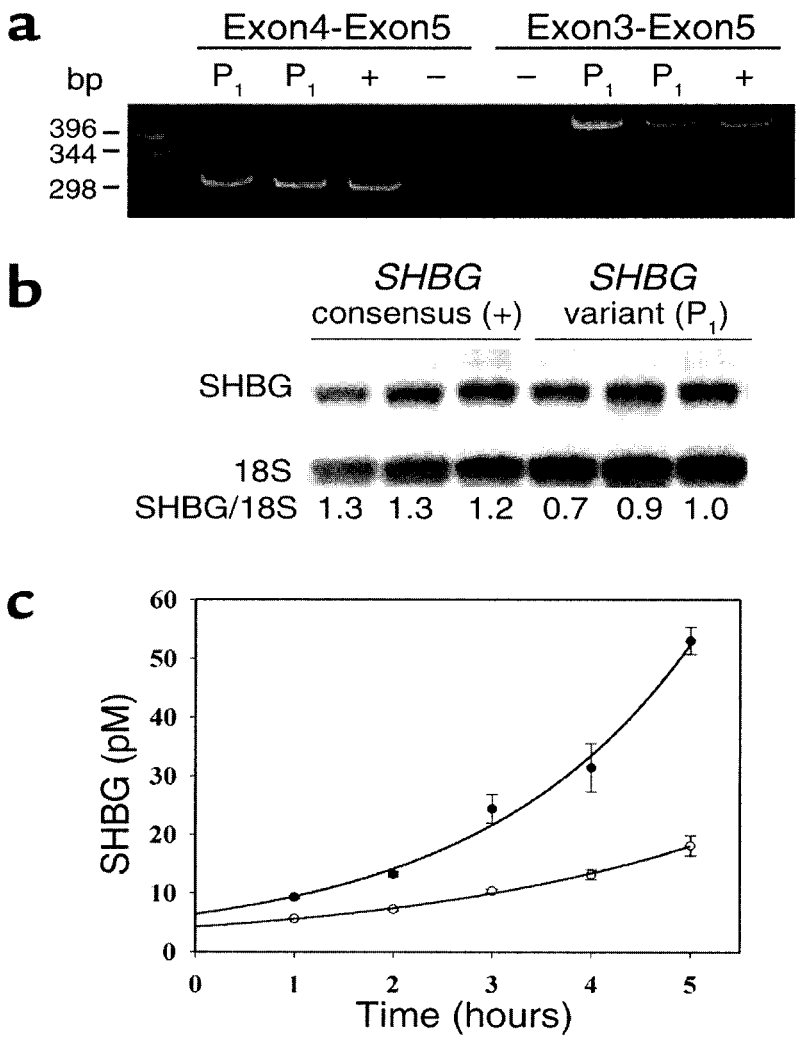

Figure 6.6 Expression of the *Msp*I variant *SHBG* allele (P_1) and the consensus *SHBG* allele (+) under the control of a CMV promoter in mouse BW-1 cells. **(a)** Ethidium bromide-stained polyacrylamide electrophoresis gel of reverse transcriptase-PCR amplification products obtained using primers that span the splice junctions between exons 4-5 and exons 3-5. Positions of DNA size markers are shown on the left. Water control for PCR (-). **(b)** Northern blot of RNA extracted from replicate transfections of BW-1 cells with the expression vector (pRc/CMV) containing either the *SHBG* consensus or variant (P_1) genomic sequences probed with a human SHBG cDNA, and a cDNA for 18S RNA as a control for loading and transfer efficiency (Bocchinfuso *et al.*, 1991). Signals obtained using a phosphorimager were quantified using ImageQuant software (Molecular Dynamics, Sunnyvale, California, USA), and SHBG:18S ratios are shown below the northern blots. **(c)** Rate of SHBG secretion by near confluent cultures containing similar amounts of SHBG transcripts, as shown in (b). Data points are the means ± SEM of duplicate measurements of SHBG levels in culture medium from replicate cultures of cells (n=3) expressing the consensus *SHBG* allele (closed circles) or the *Msp*I variant allele (open circles).

6.4 Discussion

We have identified sequence variations within the coding regions of separate *SHBG* alleles from a female patient who presented with severe hyperandrogenism during pregnancy. The level of SHBG in a blood sample taken two years after her pregnancy was exceptionally low, and we suspected a genetic abnormality might account for this. In an earlier report of a familial SHBG deficiency (Ahrentsen *et al.*, 1982), the proband also suffered from symptoms (hirsutism and amenorrhea) consistent with androgen excess and ovarian dysfunction. Although the sensitivity of the assay used to measure serum SHBG in this previous report was only 10 nM (Ahrentsen *et al.*, 1982), SHBG was also undetectable in serum from her sister during mid-gestation (Ahrentsen *et al.*, 1982), at a time when serum SHBG levels are usually in excess of 100 nM (Anderson, 1974). However, serum SHBG levels below 10 nM are occasionally detected in patient samples using more sensitive immunoassay techniques, and it is therefore unclear whether the subjects investigated in this earlier report actually suffered from a total lack of SHBG.

The serum SHBG levels in the proband we identified were remarkably low, despite the fact that she had a normal body-mass index and was under an estrogenic/antiandrogenic treatment which normally increase serum SHBG levels markedly. It is therefore likely that plasma SHBG levels must have also been very low during her pregnancy, and this would explain the severe hyperandrogenic symptoms she experienced at that time. What is also remarkable is that the twin girls she delivered showed no signs of virilization at birth. However, given the unusual maternal hormonal environment under which these infants developed, and the effects that exposures to endocrine active substances may have on health issues during later life (McLachlan, 2001), clinical monitoring of such infants is advisable.

The discovery of two novel mutations in the coding regions of separate SHBG alleles within a single patient is remarkable. The Mendelian transmission of the two variant *SHBG* alleles within the proband's family is unambiguous, and she inherited the allele containing a deletion in exon 8 from her father, as did her sister. Her father's serum SHBG level (52 nmol/L) was within the normal reference range for men (9-54 nmol/L), but he was under treatment for hypertension and we cannot exclude the possibility that his medication influences SHBG production or its plasma half-life. Her sister's serum SHBG level (30

nmol/L) was only just within the normal female reference range, but at the time of blood sampling she was taking an oral contraceptive formulation that increases serum SHBG levels (Kuhl *et al.*, 1995). This would be consistent with our findings that a cDNA representing the variant allele she inherited from her father is incapable of producing plasma SHBG in CHO cells. It is also in line with previous observations that carboxyl-terminal truncation mutants of rat ABP are not secreted when expressed in mammalian cells (Joseph and Lawrence, 1993). However, mutant mRNAs that contain a frame-shift are known to undergo rapid degradation via a nonsense-mediated decay pathway (Byers, 2002). Although we have only detected this particular variant *SHBG* allele in the proband's immediate family, it is a null allele and it will be important to screen other populations to determine whether individuals homozygous for this allele exist.

The proband must have inherited the allele containing the *Msp*I polymorphism from her mother who died prematurely due to unfortunate circumstances. There are 15 *Msp*I sites in human *SHBG* (Genbank Accession #M 31651), and the mutation we have identified most likely accounts for an *Msp*I restriction fragment length polymorphism (RFLP) that has an allele frequency of 0.04 in the French-Canadian population (Vohl *et al.*, 1994). No link has been made between this RFLP and serum SHBG levels, and no homozygotes have been reported, but the size of this RFLP is consistent with a polymorphism in the *Msp*I site at the exon 4:intron 4 boundary. It is also of interest that many French-Canadians originate from the same region in France as does the proband. Given the prevalence of this *Msp*I RFLP in the French-Canadian population, it will be important to confirm that this RFLP is due to the *Msp*I polymorphism we have identified, because this may reveal homozygous carriers, and provide more information about the clinical phenotype associated with this SNP.

The most intriguing question we faced is why the proband's serum SHBG levels were much lower than we expected given that the allele containing the *Msp*I polymorphism appears to be expressed relatively normally when examined as a transgene *in vitro*. If we assume that the allele containing the deletion in exon 8 fails to contribute to the formation of plasma SHBG as our expression studies imply, we would have expected to find at least 10 nM of the SHBG P156L variant in the proband's serum unless its production is abnormally low. We therefore first examined the possibility that the *Msp*I polymorphism in

some way interferes with the processing of nascent *SHBG* transcripts due to its proximity to an exon-intron consensus splice site, or reduces the accumulation of *SHBG* transcripts within cells. The results of our experiments indicate that neither of these possibilities appear to explain why the proband's serum SHBG levels are about an order of magnitude lower than expected.

One consistent observation we made is that the relative abundance of the electrophoretic isoforms associated with the SHBG P156L variant differs from that of those associated with normal SHBG, and this can be attributed to differences in the utilization of the two sites for N-glycosylation within the carboxy-terminal domain of the molecule (Bocchinfuso *et al.*, 1992). Since this amino acid substitution occurs within the amino-terminal laminin G-like domain of SHBG, it must somehow exert a long-range effect on post-translational modifications within the carboxy-terminal region of the molecule; this is feasible since we have observed similar effects when other amino acids are substituted within the amino-terminus of SHBG (Avvakumov *et al.*, 2001). Thus, the P156L substitution appears to influence the degree of N-glycosylation of SHBG, due presumably to subtle differences in the folding and post-translational processing of the nascent polypeptide.

The P156L substitution occurs within a phylogenetically conserved region of SHBG (Hammond, 1993) adjacent to residues located within the homodimer interface (Avvakumov *et al.*, 2001). It also lies within an inter-β strand loop region that contains a coordination ligand (A160) for the calcium atom that plays an important role in maintaining the structural stability of the SHBG homodimer (Grishkovskaya *et al.*, 2000; Bocchinfuso and Hammond, 1994). The substitution of a highly conserved proline at position 156 (Hammond, 1993) might therefore be expected to influence the secondary structure of SHBG and might explain subtle differences in its post-translational modification. It might also influence its rate of production/secretion or clearance from the blood circulation. Our observation that the SHBG P156L variant is secreted at a much reduced rate when compared to that of normal SHBG from mouse liver cells, despite the fact the levels of transcripts encoding them are similar in these cells, suggests that the variant protein is not processed efficiently during secretion. This is also consistent with the observation that greater amounts of immunoreactive SHBG are detected in the insoluble fraction of CHO cells expressing the SHBG P156L variant when

compared to the wild-type protein.

The genetic screening methods reported here will help identify individuals in whom genetic variants contribute to low serum SHBG levels, as opposed to others in whom serum SHBG levels are reduced because of some hormonal or metabolic factor that regulates its production or metabolism. Our studies so far have focused on clinical disorders affecting women, and it will be important to determine whether the genetic variations in *SHBG* we have identified are also linked to clinical conditions in men. In particular, it will be important to determine whether they are associated with reproductive disorders in men because SHBG is expressed in the human testis and may play a role in spermatogenesis (Hammond *et al.*, 1989; Joseph, 1994). Finally, the use of these genetic screening methods should enable us to define more accurately how serum SHBG levels might be associated with other diseases. In addition, the presence of abnormally low plasma levels of SHBG in individuals who carry the *SHBG* variants described in this report will undoubtedly lead to increased exposures to free testosterone and estradiol throughout their lifetimes, and may contribute to the etiology of sex steroid-dependent cancers of the prostate and breast.

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CHAPTER 7

CONCLUSIONS

7.1 Introduction

Changes in serum SHBG levels during development, or in response to dietary intake, as well as changes due to pathological conditions, influence the bioavailability of sex steroids and their actions in responsive tissues. Abnormal exposure to sex steroids has negative effects on fertility and contributes to the etiology of several diseases. It is therefore important to understand the factors contributing to the exposure of target tissues to sex steroids, and the molecular mechanisms that regulate their activity. Thus, the objectives of this thesis were to characterize the genetic components that regulate SHBG levels in the blood, as well as the production of novel SHBG isoforms that may act together with the plasma protein to maintain an appropriate exposure to sex steroids in responsive tissues including the brain and testis. Transgenic mice containing either 4 kb or 11 kb regions of the human *SHBG* locus were used in this study to gain insight into the genetic elements required for the regulation of *SHBG* expression during development, as well as under various endocrine, dietary and metabolic interventions. The regulatory elements within the human *SHBG* upstream promoter that regulate transcription in HepG2 cells were also characterized, and three novel human *SHBG* sequence variations were found.

7.2 Developmental and tissue-specific regulation of human *SHBG* expression

7.2.1 Developmental expression of the human *SHBG* gene

SHBG mRNA and immunoreactive protein are produced in fetal rat hepatocytes (Sullivan *et al.*, 1991; Becchis *et al.*, 1996), and in the livers of transgenic mice expressing human *SHBG* transgenes (Jänne *et al.*, 1999). Although expression of *SHBG* in rat (Sullivan *et al.*, 1991) and mouse (Jänne *et al.*, 1999) liver is undetectable at birth, SHBG is present in the serum of these rodents during fetal life. Although SHBG is present in serum of the human fetus, (Abramovich *et al.*, 1978), there is no direct evidence that *SHBG* is expressed in the fetal human liver. However, our results from transgenic mice expressing human *SHBG* transgenes show that it is expressed in late gestation, and gives rise to appreciable levels of human SHBG in the serum during fetal life (Jänne *et al.*, 1999). Within the first days following birth, there are rapid increases in SHBG levels in humans (August *et al.*, 1969), and in the serum of transgenic mice expressing human *SHBG* transgenes (Jänne *et al.*,

1999). Serum SHBG levels are sexually dimorphic in adult life in humans (Apter *et al.*, 1984) and in transgenic mice expressing 11kb *SHBG* transgenes, but not in mice containing a 4 kb region of the human *SHBG* locus. This demonstrates that additional sequences present in the 11kb transgene respond to physiological events at puberty that alter SHBG levels in these mice, and result in a two fold higher level of SHBG in males than in female transgenic mice. In adult humans however, serum SHBG levels are two-fold higher in women than in men. The sexual dimorphism in serum SHBG levels in 11kb human *SHBG* transgenic mice is opposite of that seen in humans, and this may be explained by differences in the complement of regulatory pathways in these species, or regions of the human *SHBG* gene not present in the 11k genomic construct. In addition, our studies demonstrate that the human *SHBG* gene is expressed in the epithelial cells of the duodenum during fetal life in mice containing an 11 kb human *SHBG* transgene (Jänne *et al.*, 1999). It is not known if the *SHBG* gene is expressed in the human intestine, but SHBG produced in this tissue may be physiologically relevant because the intestine is a target tissue for sex steroid hormone action (Thomas *et al.*, 1993).

While the *SHBG* gene in rodent liver is transcriptionally silent postnatally, transgenic mice containing and expressing human *SHBG* transgenes have very high levels of SHBG mRNA in the liver, and micromolar levels of human SHBG in the blood (Jänne *et al.*, 1998). The 0.8 kb of 5' flanking sequence in this transgene contains several binding sites for liver-specific transcription factors (Jänne and Hammond, 1998; Hogeveen *et al.*, 2001), and is sufficient for the expression of the transgene in mouse hepatocytes (Jänne *et al.*, 1998), and a hepatocyte-derived cell line (Jänne and Hammond, 1998). Phylogenetic differences in the hepatic expression of the *SHBG* gene postnatally are believed to result from differences in the promoter sequence flanking exon 1. Comparison of the human and rat *SHBG* promoters reveals significant sequence similarity within the first 600 bp upstream of the transcription start site in the liver, at which point the sequences diverge into species-specific repetitive elements (Jänne and Hammond, 1998). One major difference between the two promoters is a region of 23 additional nucleotides present in the human promoter (-119 to –97 relative to the transcription start site in the liver) that contains binding sites for USF (section 5.3.1) and the liver-specific transcription factor, HNF-4 (Jänne and Hammond, 1998), and it has

been suggested that this additional sequence might be important for postnatal expression of the *SHBG* gene in the human liver. However, this region does not appear to be necessary for the hepatic expression of *SHBG* postnatally because the rabbit *SHBG* promoter also lacks this region, but continues to express *SHBG* in the liver throughout development (Ip *et al.*, 2000). In addition, transgenic mice containing a 4 kb human *SHBG* transgene that lack part of this additional promoter sequence encompassing the USF binding site (*SHBG*4-FP4$^-$) maintain expression of human *SHBG* in the liver postnatally. This region may however be important for maintaining species-specific regulatory functions in other tissues (section 7.2.3).

7.2.2 Regulation of *SHBG* expression in kidney

The expression of human *SHBG* in the kidneys of transgenic mice was unexpected, but the hamster *SHBG* gene is expressed in the kidney (Cates and Damassa, 1997), and low levels of endogenous SHBG mRNA are present in mouse kidney (Section 2.3.5). It is not yet known if the *SHBG* gene is expressed in human kidney, but SHBG produced in the kidney may be part of a mechanism by which androgens are recycled back into the circulation, especially in species that lack SHBG in the blood. This function of SHBG in the kidney may be similar to the role of the vitamin D binding protein in the reuptake of vitamin D through its interaction with megalin on the surface of cells lining the renal tubules (Nykjaer *et al.*, 1999). Interestingly, there was a striking sexual dimorphism in SHBG mRNA levels in the kidneys of both 4 kb and 11 kb human *SHBG* transgenic mice, with a marked increase in SHBG mRNA levels in the kidneys of male animals during sexual development. The increase in SHBG mRNA occurred coincident with increases in serum testosterone levels at puberty, and unlike the sexual dimorphism in the liver, the sex difference in the kidney is directly a result of androgen regulation (Jänne *et al.*, 1999). These data also showed that genomic sequences present within the 4 kb human *SHBG* transgene are sufficient for androgen-dependent sexual dimorphism in kidney. The functional significance of SHBG production by the kidneys of mice is not known, but the kidney is a classic tissue for androgen regulation (Catterall *et al.*, 1986) and SHBG secreted by epithelial cells of the proximal convoluted tubule (Jänne *et al.*, 1998) may exert a regulatory role in this process. However, despite the presence of human SHBG in the kidneys, the expression of

androgen responsive genes in the kidney of transgenic mice are not altered (Jänne and Hammond, unpublished results).

7.2.3 Phenotype of human *SHBG* transgenic mice

Serum testosterone levels were elevated 10-100 fold in transgenic mice expressing human *SHBG* transgenes when compared to their wild-type littermates (Jänne *et al.*, 1999). Although the elevated levels of serum testosterone may result from an increased *de novo* synthesis of testicular androgens in response to decreased steroid bioavailability at the level of the hypothalamic-pituitary axis, it is more plausible that the accumulation of testosterone in the blood of transgenic mice is due to a decreased MCR of steroids as a result of the presence of human SHBG in the plasma. Despite very high levels of circulating human SHBG, and significantly elevated serum testosterone levels, human *SHBG* transgenic mice do not display any obvious phenotype. Although it is surprising that, unlike transgenic mice overexpressing rat ABP in the testes which display increased germ cell apoptosis (Selva *et al.*, 2000) and decreased fertility (Reventos *et al.*, 1993), no such phenotype is observed in 11 kb human *SHBG* transgenic mice that express SHBG isoforms that accumulate in the acrosome of germ cells (Jänne *et al.*, 1998; Selva *et al.*, 2002). However, this may be due to the fact that the presence of serum SHBG may compensate for the presence of testicular SHBG isoforms by increasing testosterone levels in the testis.

7.2.4 Testicular expression of human *SHBG* transgenes

Transgenic mice expressing a 4 kb human *SHBG* transgene do not contain human SHBG mRNA in the testis. By contrast, SHBG mRNA in the human testis (Hammond *et al.*, 1989), and the testes of 11 kb human *SHBG* transgenic comprise an alternative exon 1 sequence (Jänne *et al.*, 1998; Selva *et al.*, 2002). Alternative *SHBG* transcripts in 11 kb human *SHBG* transgenic mouse testis increase in abundance before the onset of puberty (Jänne *et al.*, 1999), and accumulate within germ cells in a spermatogenic stage-dependent manner. It appears, therefore, that the expression of human *SHBG* in the testes of transgenic mice is regulated by signaling events within the seminiferous epithelium.

Although high levels of rat ABP mRNA and protein are produced in the Sertoli cells

of mice expressing a rat *ABP* transgene (Reventos *et al.*, 1993), the testes of mice containing a 4 kb human *SHBG* transgene (*SHBG*4) that comprises a similar amount of regulatory region flanking exon 1 do not contain human SHBG mRNA (Jänne *et al.*, 1998). Unlike ABP mRNA in the rat Sertoli cells that is regulated by a promoter flanking the exon 1 encoding the signal polypeptide necessary for secretion, production of transcripts containing an alternative exon 1 sequence in the human testis, and the testes of 11 kb human *SHBG* transgenic mice are regulated by an uncharacterized promoter upstream of the alternative exon 1. Whereas the rat (Joseph, 1994) and mouse (Selva *et al.*, 2002) express the *ABP* gene exclusively in the Sertoli cells, the human gene is only expressed in the germ cells, and the difference in testicular cell type expression of *SHBG* genes between species is the result of the utilization of distinct promoters. Expression of the rat *ABP* gene in the Sertoli cells is dependent on FSH and testosterone (Joseph *et al.*, 1988; Reventos *et al.*, 1988) and since both rat ABP and human SHBG mRNA accumulate in a spermatogenic stage dependent manner (Jänne *et al.*, 1998; Ritzén *et al.* 1982), it is interesting to speculate that expression of human *SHBG* in the testis is also regulated by these same factors through different promoters.

Differences in the sequence flanking exon 1 in the human and rat promoters likely explains why the human liver-specific promoter is inactive in the testis (Jänne *et al.*, 1998). In particular, a region of 23 additional nucleotides present in the human promoter (Jänne and Hammond, 1998) that disrupts a Sertoli cell enhancer sequence in the rat promoter (Fenstermacher and Joseph, 1997) may be important for species-specific differences in *SHBG* expression in the testis. In support of this, transgenic mice containing a 4 kb human *SHBG* transgene that lacks part of this additional sequence (*SHBG*4-FP4$^-$) express the human *SHBG* gene in the testis (section 5.3.2). Therefore, this region likely acts to repress the transcription of the human gene in the Sertoli cell, and it will be important to identify the localization of human *SHBG* transcripts and protein in the testes of these mice to determine whether they are found in the Sertoli cells, or are retained in the germ cells, as in the testes of *SHBG*11 mice.

The production of *SHBG* transcripts in the germ cells of 11 kb human *SHBG* transgenic mice results in the production of immunoreactive SHBG isoforms that accumulate

within the acrosome of developing spermatids (Selva *et al.*, 2002). These SHBG isoforms are retained throughout sperm maturation, and are present in the acrosome of epididymal sperm. Importantly, the expression of *SHBG* in the testes of transgenic mice containing an 11 kb transgene recapitulates the expression of *SHBG* in human germ cells because SHBG isoforms are also found in the acrosome of ejaculated human sperm (Selva *et al.*, 2002). Unlike human SHBG isoforms in germ cells, rat ABP is secreted from the Sertoli cells into the lumen of the seminiferous tubule, and travels through the male reproductive tract where it is thought to maintain an appropriate androgen environment necessary for spermatogenesis and sperm maturation (Turner *et al.*, 1984; Joseph, 1994). Based on morphological differences in organization of germ cells within rat and human seminiferous tubules, and the fact that rodents lack circulating SHBG, it is not surprising that the human and rodent *SHBG* genes are expressed in different testicular cell types. Although the function of SHBG isoforms within the acrosome is not known, it is fully capable of binding sex steroids, and may be required to modulate sex steroid bioavailability directly at the level of the individual germ cell.

In addition, human SHBG within the acrosome of sperm may exert a function in the regulation of sex steroid activity outside the testis. Ejaculated human spermatozoa contain P450 aromatase mRNA and protein activity (Aquila *et al.*, 2002), and binding sites for 17β-estradiol on membranes from human spermatozoa (Hyne and Boettcher, 1978; Hernandez-Perez *et al.*, 1979) are concentrated in the central portion of the sperm tail (Cheng *et al.*, 1981). Moreover, incubation of human spermatozoa with 17β-estradiol increases sperm motility (Beck *et al.*, 1976; Cheng and Boettcher, 1979) and the presence of human SHBG isoforms within the acrosome may regulate sex steroid action in ejaculated sperm. Further, acrosomal SHBG may function during some later process such as fertilization. Male mice expressing an 11 kb human *SHBG* transgene in the testes are reproductively normal (Jänne *et al.*, 1998), and the presence of acrosomal SHBG isoforms in sperm from these mice does not appear to have any deleterious effect on sperm function. The function of human SHBG isoforms that accumulate within the acrosome remains to be resolved, and it will therefore be of particular interest to determine whether levels of acrosomal SHBG in human sperm samples are correlated with reproductive function.

7.2.5 Human SHBG in the brain

The brain is an important target for sex steroid action, and estrogens in particular are essential for the masculinization of the male brain, male and female sexual behaviours, and the initiation and maintenance of maternal behaviour (McEwan, 1983; Ogawa *et al.*, 2000). Mice that lack the estrogen receptors α and β lack aggressive and reproductive behaviours, and have abnormal maternal behaviours (Ogawa *et al.*, 1998, 2000). Intriguingly, 11kb human *SHBG* transgenic mice also display a similar defect in maternal behaviour that involves a high rate infanticide and maternal neglect when compared to mice expressing a 4 kb transgene that have similar levels of human SHBG in their blood, and this is coincident with the expression of human SHBG mRNA in the brain (Section 2.3.6). By *in situ* hybridization, very little SHBG mRNA is seen in the 4k female mouse brain, but the intensity of hybridization is clearly increased in 11k female mouse brain. Human SHBG mRNA in the *SHBG*11 transgenic mouse brain accumulated within the cerebral cortex, piriform cortex, amygdala, substantia nigra, dentate gyrus of the hippocampus, hypothalamus, and in the Purkinje cells of the cerebellum. All of these regions of the brain also contain the estrogen receptors α or β (Shughrue *et al.*, 1997; Azcoitia *et al.*, 1999), and SHBG within these regions may regulate the activity of sex steroids at the local level.

In the *SHBG*11 transgenic mouse testis, novel SHBG isoforms derived from transcripts containing a non-coding alternative exon 1 retain sex steroid-binding activity (Selva *et al.*, 2002), but they lack the sequence encoding a signal for secretion that is encoded by exon 1 (Hammond *et al.*, 1989). Although it is not known if SHBG isoforms derived from alternative transcripts contain an alternate targeting signal, rat ABP produced from a cDNA containing an alternative exon 1 sequence obtains nuclear localization in COS-7 cells (Joseph *et al.*, 1996). Nevertheless, human SHBG isoforms are retained within cellular compartments (Selva *et al.*, 2002), and would likely act to regulate the intracellular bioavailability of sex steroids. It is therefore possible that intracellular SHBG isoforms in the brains of 11 kb human *SHBG* mice might compromise maternal behaviour through competition with the estrogen receptors for ligand within the same brain structures. The function of SHBG within the brain may thus extend beyond that of regulating sex steroid transport and bioavailability, and could also involve hormonal actions that activate rapid

changes in neural activity. In support of this, SHBG injected into the brain increases sexual receptivity in female mice, suggesting that this protein may exert a role in the regulation of sexual behaviour. This effect only occurred when SHBG was unliganded or coupled to estradiol (Caldwell *et al.*, 2000), but not when bound with DHT (Caldwell *et al.*, 2002). The estradiol-dependent effect of SHBG on stimulating sexual receptivity has been suggested to occur through its interaction with specific SHBG binding sites identified in the medial preoptic area or medial basal hypothalamus (Caldwell, 2001). In support of a role for SHBG in the regulation of sexual behaviour in humans, a female patient who is a compound heterozygote for two variant *SHBG* alles with extremely low serum SHBG levels, complained of libido failure (Hogeveen *et al.*, 2002).

7.3 Regulation of human *SHBG* expression by dietary factors

7.3.1 Introduction

Serum levels of SHBG in humans are highly regulated by metabolic factors, and a large body of clinical studies have demonstrated correlations between insulin and SHBG levels. For example, serum SHBG levels are negatively correlated with fasting insulin levels in healthy women (Preziosi *et al.*, 1993), and are significantly decreased in patients with insulin resistance (Pugeat *et al.*, 1991). Moreover, low SHBG levels are an early predictor for the predisposition to premature cardiovascular disease and non-insulin dependent (Type II) diabetes mellitus (Ibañez *et al.*, 1997; Ibañez *et al.*, 1998; Lapidus *et al.*, 1986; Lindstedt *et al.*, 1991). Furthermore, insulin treatment decreases the levels of SHBG mRNA in HepG2 cells, and reduces the amount of SHBG that is secreted into the media (Plymate *et al.*, 1988; Crave *et al.*, 1995). However, it is not clear whether the negative correlations observed in human patients is a result of a reduction in SHBG production by elevated serum insulin levels, or a consequence of abnormal glucose metabolism. Importantly, the metabolic regulation of hepatic SHBG expression is complex, and the involvement of insulin in terms of any direct correlation is debatable. For instance, several reports have demonstrated decreased serum SHBG levels in young patients with Type 1 diabetes and microalbuminuria (Holly *et al.*, 1992; Rudberg and Persson, 1995; Barkai and Tombacz, 2001).

7.3.2 Dietary regulation of human *SHBG* expression in transgenic mice

Dietary and nutritional factors have been shown to alter SHBG levels (Longcope *et al.*, 2000; Reed *et al.*, 1987; Adlercreutz *et al.*, 1987). However, variations in the rate of insulin secretion are primarily under dietary control *in vivo*, and the insulin-dependent effects of changes in diet are difficult to distinguish from effects due to dietary components. Our results demonstrate that human SHBG levels in transgenic mice are negatively regulated by a high carbohydrate diet. Further, there is a significantly more rapid and more profound decrease in SHBG levels when mice are fed high carbohydrate diets in which sucrose is the source of carbohydrate. This was interesting because fructose can replace glucose in the transcriptional activation of the L-type pyruvate kinase gene, and the maximal activation of gene expression associated with fructose occurs more rapidly than with glucose (Munnich *et al.*, 1987). This suggests that both glucose and fructose may be important dietary regulators of human *SHBG* expression *in vivo*. This is strengthened by the dramatic decreases in serum levels of human SHBG in transgenic mice with elevated blood glucose levels associated with compromised pancreatic β cell function, and the observation that patients with insulin dependent (Type 1) diabetes also have low SHBG levels (Holly *et al.*, 1992; Rudberg and Persson, 1995; Barkai and Tombacz, 2001).

Caloric intake also appears to regulate *SHBG* expression in humans. In anorexic patients, elevated serum SHBG levels (Estour *et al.*, 1986) are decreased when caloric intake is increased (Barbe *et al.*, 1993). As well, low SHBG levels associated with obesity (Glass *et al.*, 1977) are increased by a reduced calorie diet (Franks *et al.*, 1991). It is therefore interesting that the increase in human SHBG mRNA in the livers of transgenic mice fasted for 24 hours decreases significantly following a period of refeeding. Changes in blood glucose are likely to function in this regulation as well, since streptozotocin-induced diabetic animals have a similar increase in levels of human SHBG mRNA in the liver after a period of fasting.

Levels of human SHBG in the serum of *SHBG*4 mice decrease dramatically when fed a high carbohydrate diet, and changes in serum SHBG levels are associated with a four fold reduction in human SHBG mRNA in the liver. Further, the hepatic expression of *SHBG* is increased in *SHBG*4 mice fasted for 24 hours, and there is a striking decrease upon refeeding.

Therefore, the genetic elements necessary for changes in *SHBG* gene expression in response to diet are contained within the 4 kb human *SHBG* transgene. Interestingly, adjacent USF and HNF-4 sites in the human *SHBG* promoter are organized in a similar manner to an element in the L-type pyruvate kinase gene promoter necessary for its transcriptional response to glucose (Jänne and Hammond, 1998; Diaz Guerra *et al.*, 1993). However, the USF site in the human *SHBG* promoter is not required for changes in *SHBG* gene expression in response to dietary manipulations because transgenic mice expressing a 4 kb human *SHBG* transgene lacking this site respond normally to a high carbohydrate diet, as well as to a fasting and refeeding regimen. Although it is not known if the dietary and metabolic effects on hepatic *SHBG* gene expression are due to changes in transcriptional activity of the human *SHBG* promoter, it also contains binding sites for C/EBPβ and SP1 (Hogeveen *et al.*, 2001), which have both been shown to function in the regulation of gene expression in response to diet (Ghosh *et al.*, 2001; Arizmendi *et al.*, 1999; Fukuda *et al.*, 1999). It will therefore be important to identify the elements in the human *SHBG* gene that are responsible for mediating the dietary and metabolic effects on the expression of human *SHBG* in the liver.

7.4 Genetic polymorphisms in the human *SHBG* gene

7.4.1 Introduction

Serum SHBG levels are highly variable between individuals, and some of this variability can be attributed to genetic differences (Meikle *et al.*, 1982, Gross and Horton, 1971). The underlying genetic origins that result in abnormal serum SHBG levels are unknown, but we have identified several novel polymorphisms in the SHBG coding sequence (Hogeveen *et al.*, 2002) and promoter sequence (Hogeveen *et al.*, 2001) that may contribute to low serum SHBG levels.

7.4.2 $(TAAAA)_n$ polymorphism in the human *SHBG* promoter

The 0.8 kb of promoter sequence flanking exon 1 in the human *SHBG* gene is sufficient for temporal and tissue-specific expression of human SHBG in transgenic mice (Jänne *et al.*, 1998; Jänne *et al.*, 1999), and binding sites for liver-specific transcription

NCBI SNP Cluster ID	Observed Alleles	Position in *SHBG*	Estimated Heterozygosity	Effects
not entered	$(TAAAA)_n$	promoter	not available	-5' region of the liver-specific promoter influences transcription (n = 6-10)
rs1799941	G/A	promoter	not available	-Position –7 from transcription start site in the liver
not entered	C/T	exon 1	not available	-R25C amino acid substitution in leader sequence for secretion
rs6260	G/A	exon 1	0.175	-R25H amino acid substitution in leader sequence for secretion
rs6257	T/C	intron 1	not available	-Unknown
rs6258	C/T	exon 4	0.012	-P156L amino acid substitution -Causes decrease in secretion -Associated with hyperandrogenism and ovarian dysfunction
rs6261	T/C	Exon 7	0.019	-Silent variation in codon for S260 (TCT to TCC)
rs6259	G/A	Exon 8	0.14	-D327N substitution introduces additional N-glycosylation site -Increased serum half-life
rs5030659	A/-	Exon 8	not available	-Single nucleotide deletion in codon for E326 results in truncation and no secretion -Null allele -Associated with hyperandrogenism and ovarian dysfunction

Table 7.1 Polymorphisms in the human *SHBG* gene.

factors within this region are important for regulating *SHBG* promoter activity in hepatocytes (Jänne and Hammond, 1998; Hogeveen *et al.*, 2001). We have identified that the number of TAAAA pentanucleotide repeats within an *alu* sequence in the upstream region of the human *SHBG* promoter is highly variable between individuals in the general population, and the number of repeats in this region has a dramatic effect on the transcriptional activity of the human *SHBG* promoter (Hogeveen *et al.*, 2001). Similarly, we have found that a liver-specific transcription factor binds to this repeat sequence in a manner which is dependent on the number of TAAAA repeat elements, and the level of nuclear factor binding at this site correlates to transcriptional activity of the human *SHBG* promoter in HepG2 cells (Hogeveen *et al.*, 2001). A large scale study to determine if this polymorphism has any influence on serum SHBG levels in humans has yet to be performed, but preliminary evidence suggests that a correlation exists between the number of TAAAA repeats and concentrations of SHBG in the blood in hirsute patients (Cousin *et al.*, 2002). This finding may help to explain the large variability in serum SHBG levels between individuals, and may in part explain why low SHBG levels are inherited within families (Gross and Horton, 1971; Meikle *et al.*, 1982).

7.4.3 SHBG coding sequence variations

We have also identified two variations in the human SHBG coding sequence on separate alleles in a single individual with serum SHBG levels almost two orders of magnitude lower (0.5–1 nM) than the normal female reference range (Hogeveen *et al.*, 2002). The patient experienced severe hirsutism and hyperandrogenic symptoms during pregnancy, and despite the presumably high free sex steroid concentration during her pregnancy, delivered twin girls that showed no signs of excessive androgen exposure. Although it is assumed that increases in serum SHBG levels during pregnancy serve to protect against virilization of the female fetus (Anderson, 1974), our findings suggest that SHBG does not exert a major role in this process.

A polymorphism (C to T single nucleotide substitution) at the splice donor site near the exon 4:intron 4 boundary results in a proline to leucine substitution at residue 156. This gives rise to a protein that is differentially glycosylated, but binds sex steroids with a similar affinity as the wild-type protein. Although splicing of the variant SHBG mRNA is not

affected by the SNP, the production and secretion of P156L SHBG is impaired when compared to that of the wild-type protein when expressed as a genomic transgene in the mouse BW-1 hepatocyte cell line. This SNP disrupts an *Msp*I restriction endonuclease site within exon 4, and the PCR-based screening assay developed in this study was used to screen 482 women for the P156L allele. Four individuals were identified as carriers of the P156L allele, 3 of whom were in the non-obese/hirsute grouping and of these two had PCOS. The other patient had premature menopause at the age of 19. This mutation we have identified likely corresponds with a *Msp*I RFLP with an allele frequency of 0.04 in the French-Canadian population (Vohl *et al.*, 1994), and is present with a frequency of 0.023 and 0.015 in the Polish and Nordic populations, respectively (Försti *et al.*, 2002). However, in both of these studies, serum SHBG levels were not measured, nor were any clinical symptoms reported. Since this variation is not uncommon, it will be of interest to find patients who are homozygous for the P156L allele, as we have linked this polymorphism with low SHBG levels together with hyperandrogenism and ovarian dysfunction in patients who are heterozygous for this variation. It will also be necessary to look at possible phenotypes in men who carry this allele because *SHBG* is expressed in the human testis (Hammond *et al.*, 1989; Selva *et al.*, 2002), and may have a novel species-specific role in the human acrosome (Selva *et al.*, 2002).

The other SHBG variant identified on the patient's other *SHBG* allele was single nucleotide deletion in exon 8 (ΔE326), close to a well characterized SNP that introduces an additional site for N-glycosylation (D327N) (Power *et al.*, 1992, Cousin *et al.*, 1998). The nucleotide deletion variant identified in our study results in a shift in the reading frame of the SHBG mRNA, and introduces a novel seven amino acid truncated carboxy-terminal region. This protein is not secreted when expressed as a cDNA in CHO cells (Hogeveen *et al.*, 2002). Both the ΔE326 and D327N alleles disrupt a *Bbs*I restriction endonuclease site, but only the D327N allele also disrupts a *Hinf*I site, and this difference in restriction sites between the two alleles facilitated the development of a PCR-based screening assay. Using this technique, we found that the ΔE326 *SHBG* variant allele is very rare, and was only detected in members of her immediate family. Nevertheless, this SHBG variant represents the first *SHBG* null allele identified.

7.4.4 Other SHBG variants

Several other variations in the *SHBG* gene have been reported (Table 8.1), although the effect of these polymorphisms on the expression of *SHBG*, or the effect of these variants on the function of SHBG have not been studied. Two variations have been found in the leader sequence for secretion within exon 1 (Försti *et al.*, 2002). A single nucleotide substitution (C to T) at nt 2830 (M31651 GenBank) changes Arg to Cys at position 25 in the leader sequence. This mutation was found in only one Polish case of breast cancer, and her daughter, and although both were diagnosed for bilateral breast cancer, both patients were carriers of the *BRCA1* 5382insC mutation (Grzybowska *et al.*, 2000). The other SNP within the coding sequence for the secretion signal is a G to A substitution at nt 2831 (M31651 GenBank) and results in a Arg to His substitution, and has been reported to occur in a frequency of 5-15% in European, Asian, African-American and African Pygmy populations (http://www.genome.wi.mit.edu/cvar_snps). However, it is not known whether these variations in the leader sequence required for SHBG secretion will alter its ability to be secreted.

Other variations in the promoter or coding sequence of SHBG most likely exist in human populations. It is likely that their identification will help explain the wide variability in serum SHBG levels between individuals, and provide additional insight into the functions of SHBG in human health and disease.

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APPENDIX

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> Thank you,
> Sincerely,
>
> Kevin N. Hogeveen
> PhD Candidate
> Department of Physiology and Pharmacology
> University of Western Ontario
> Rm 4088, London Regional Cancer Centre
> 790 Commissioners Road East
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1) Hogeveen, K.N., Talikka, M. and Hammond, G.L. (2001) Human sex hormone-binding globulin promoter is influenced by a (TAAAA)n repeat element within an alu sequence. J Biol Chem 276: 36383-36390.
2) Selva, D.M., Hogeveen, K.N., Seguchi, K., Tekpetey, F. and Hammond, G.L. (2002) A human sex hormone-binding globulin isoform accumulates in the acrosome during spermatogenesis. J Biol Chem 277: 45291-45398.
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PhD Candidate
Department of Physiology and Pharmacology
Subject: FW: Copyright release
Date: Wed, 7 May 2003 08:25:01 -0400
From: "Frazier, Evelyn" <EFRAZIER@ENDO-SOCIETY.ORG>

To: <knhogeve@uwo.ca>

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Greetings Mr. Herman,
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Janne, M., Hogeveen, K.N., Deol, H.K. and Hammond, G.L. (1999) Expression and Regulation of human sex hormone-binding globulin transgenes in mice during development. Endocrinology 140: 4166-4174.
Thank you,
Sincerely

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KEVIN NEIL HOGEVEEN

EMPLOYMENT

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EDUCATION

May 1998-present	Ph.D Candidate, Department of Pharmacology and Toxicology UNIVERSITY OF WESTERN ONTARIO Cancer Research Labs, London Regional Cancer Centre Supervisor: Dr. Geoffrey L Hammond **Project**: Dysregulation of plasma corticosteroid-binding globulin
May 1998	B.Sc. (Hons.), QUEEN'S UNIVERSITY, Kingston, Ontario Faculty of Arts and Science, Department of Biochemistry

TEACHING

1999	Pharmacology 480 Laboratory: Radioligand Binding
1998	Pharmacology 480 Laboratory: Radioligand Binding

SCHOLARSHIPS AND AWARDS

1998- 2003	Canadian Institutes of Health Research Studentship "Dysregulation of plasma corticosteroid-binding globulin production"
1999	Special University Scholarship (University of Western Ontario)
2000	Special University Scholarship (University of Western Ontario)
2001	Special University Scholarship (University of Western Ontario)
2002	Special University Scholarship (University of Western Ontario)
2003	Special University Scholarship (University of Western Ontario)
2003	Harry and Gudrun Sharma Graduate Award in Pharmacology and Toxicology

PROFESSIONAL MEMBERSHIPS

May 2000-present	Student member of The Endocrine Society (www.endo-society.org)

PUBLICATIONS

1. Jänne M, **Hogeveen KN**, Deol HK, Hammond GL. (1999): Expression and regulation of human sex hormone-binding globulin transgenes in mice during development. *Endocrinology*. **140**: 4166-4174.

2. **Hogeveen KN**, Talikka M, Hammond GL. (2001): Human sex hormone-binding globulin promoter activity is influenced by a $(TAAAA)_n$ repeat element within an *Alu* sequence. *J. Biol. Chem.* **276**: 36383-36390.

3. **Hogeveen KN**, Cousin P, Pugeat M, Dewailly D, Soudan B, Hammond GL. (2002): Variations in the human sex hormone binding globulin (*SHBG*) gene associated with hyperandrogenism and ovarian dysfunction. *J. Clin. Invest.* **109**: 973-981.

4. Hammond GL, **Hogeveen KN**. Sex hormone-binding globulin. In: *Encyclopedia of Hormones*. (eds. T. Norman and H Henry) **In Press.**

5. Selva DM, **Hogeveen KN**, Seguchi K, Tekpetey F, Hammond GL (2002) A human sex hormone-binding globulin isoform accumulates in the acrosome during spermatogenesis. *J. Biol. Chem.* 2002 **277:** 45291-45298.

ABSTRACTS

1. **Hogeveen KN**, Janne M, Hammond GL. A hepatocyte-enriched nuclear factor binds to an *alu* TAAAA repeat and modulates transcription from the human *shbg* promoter in HepG2 cells. *81st Annual Meeting of the Endocrine Society, San Diego, California, USA.* June 12 – 15, 1999.

2. **Hogeveen KN**, Hammond GL. A $(TAAAA)_n$ polymorphism in the human sex hormone binding globulin promoter regulates transcription. *82nd Annual Meeting of the Endocrine Society, Toronto, Canada.* June 21 – 24, 2000.

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4. Selva DM, **Hogeveen KN**, Seguchi, K, Hammond GL. Human *SHBG* transgene expression in mouse testis results in accumulation of sex hormone-binding globulin in the acrosome. *84th Annual Meeting of the Endocrine Society, San Francisco, California, USA.* June 19 – 22, 2002.

5. **Hogeveen KN**, Hammond GL Regulation of human sex hormone-binding globulin by metabolic factors. *International Congress on Hormonal Steroid and Hormones and Cancer (11th International Congress on Hormonal Steroids (ICHS)/7th International Congress on Hormones and Cancer (ICHC)*, Fukuoka, Japan, October 21 – 25, 2002