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The Structure, Biosynthesis And Function Of Corticosteroid Binding Globulin

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THE STRUCTURE, BIOSYNTHESIS AND FUNCTION OF CORTICOSTEROID BINDING GLOBULIN

by

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Abstract

Corticosteroid binding globulin (CBG) is the major plasma transport protein for glucocorticoids. Complementary DNAs for rat, mouse, rabbit and squirrel monkey CBG were isolated, sequenced and their deduced primary structures were aligned. Overall, they exhibit 40.9% identity, and their comparison revealed conserved *N*-glycosylation sites and the probable position of the cysteine located within the human CBG steroid binding domain. Rat CBG mRNA is produced primarily by the liver, and in adults, males have two-thirds the CBG mRNA levels of females. During the last third of pregnancy, maternal hepatic CBG mRNA levels are relatively constant while fetal levels are highest at day 15 of gestation and decline to barely detectable levels at term. Concentrations of CBG mRNA are very low at birth, and adult values are attained by puberty. However, serum CBG concentrations do not reach adult levels until 6 weeks of age, and this is probably due to the relatively short half-life of CBG in infants (6.9 h) when compared to adults (14.5 h). Dexamethasone reduces rat serum CBG and hepatic CBG mRNA levels by 4 and >26-fold, respectively, and the latter is the result of reduced CBG gene transcription. Thyroxine tends to increase serum CBG and hepatic CBG mRNA levels, but not CBG gene transcription. Sepsis reduces rat hepatic CBG mRNA levels to 11% of normal, and this is reflected by low serum CBG levels. Rat and human carriers of CBG variants with reduced cortisol binding activity have been identified, and sequence analysis of BioBreeding rat CBG cDNAs and exons of the human CBG gene revealed methionine^{276} to isoleucine^{276} and leucine^{93} to histidine^{93} substitutions in rat and human CBG, respectively. Scatchard analysis of media

obtained from Chinese hamster ovary cells transfected with normal and mutant cDNAs for rat and human CBG confirms amino acid substitutions at these locations are responsible for reduced steroid binding affinity. Codons for other amino acids suspected to contribute to the steroid binding activity of human CBG have been mutated *in vitro* and the resulting cDNAs were expressed in culture. Analysis of these products should further contribute to our understanding of the relationship between CBG structure and function.

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Abbreviations

Å	Ångstrom
A1-PI	α_1-proteinase inhibitor
AAG	α_1-acid glycoprotein
APT	*o*-aminophenylthioether
ATP	adenosine 5'-triphosphate
BB	BioBreeding
bp	base pair(s)
BSA	bovine serum albumin
°C	degree(s) Celcius
cAMP	adenosine 3',5'-cyclic monophosphate
CBG	corticosteroid binding globulin
cDNA	complementary deoxyribonucleic acid
CHO	Chinese hamster ovary
Ci	Currie(s)
CLP	cecal ligation and perforation
cRNA	complementary ribonucleic acid
CTP	cytidine 5'-triphosphate
Da	Dalton(s)
DCC	dextran-coated charcoal
DEAE	diethylaminoethyl
DNA	deoxyribonucleic acid
dNTP	deoxynucleotide 5'-triphosphate
DTT	dithiothreitol
EDTA	ethylenediaminetetraacetic acid
FBS	fetal bovine serum
FPLC	fast protein liquid chromatography
g	gravity
GTP	guanosine 5'-triphosphate
h	hour(s)

HEPES	4-(2-hydroxyethyl)-1-piperazine-ethanesulfonic acid
IPTG	isopropyl-ß-D-thiogalactopyranoside
kb	kilobase
M	molar
MEM	α-minimal essential medium
min	minute(s)
mRNA	messenger ribonucleic acid
NA	not available
ND	not detected
PAGE	polyacrylamide gel electrophoresis
PBS	phosphate-buffered saline
PCR	polymerase chain reaction
PIPES	1,4-piperazinediethanesulfonic acid
pfu	plaque forming unit(s)
PMSF	phenylmethylsulfonyl fluoride
RNA	ribonucleic acid
RT	reverse transcription
SD	standard deviation(s)
SDS	sodium dodecyl sulphate
SE	standard error
SHBG	sex hormone binding globulin
T_4	L-thyroxine
Taq	*Thermus aquaticus*
TBG	thyroxine binding globulin
TBS	tris-buffered saline
Tris	tris(hydroxymethyl)-aminomethane
tRNA	transfer ribonucleic acid
U	unit
UTP	uridine 5'-triphosphate

CHAPTER 1

Introduction

1.1 Plasma Steroid Binding Proteins

Steroid hormones are important mediators of biological activities as diverse as homeostasis, the immune response and reproduction. They exist as charged, conjugated derivatives or neutral, unconjugated compounds. The latter are found in the blood associated predominantly with plasma steroid binding proteins. These proteins reduce the metabolic clearance rate of steroids and are intimately involved in the regulation of their activity. The study of steroid binding proteins is therefore integral to our understanding of steroid hormone action.

1.1.1 Low Affinity Steroid Binding Proteins

Albumin and α_1-acid glycoprotein (AAG; also referred to as orosomucoid) are the most abundant steroid binding proteins in the blood and some of their biochemical characteristics are summarized in Table 1.1. They have relatively poor steroid binding affinity, and very little of the biologically active hormones bound by high affinity steroid binding proteins is therefore associated with albumin and AAG under normal physiological conditions (Dunn *et al.*, 1981). However, most other steroids interact poorly with the high affinity steroid binding proteins and they are therefore predominantly transported by albumin because of its very high plasma concentration (Dunn *et al.*, 1981). Orosomucoid has no known function, and it contributes negligibly to the steroid binding capacity of serum because of its low serum concentration in relation to albumin (Westphal, 1986).

Table 1.1 Physicochemical properties of human steroid binding proteins under normal physiological conditions. Values were compiled from Westphal (1971 and 1986) with the exception of 'a' (Hammond *et al.*, 1987) and 'b' (Walsh *et al.*, 1986).

	Albumin	AAG	CBG	SHBG
Serum Concentration (mg/l)	40,000	750	20 - 30	3 - 8
Molecular Weight (x 10^{-3})	66	41	~55	~100
Amino acids	585	181	383[a]	373[b]
Preferred ligands	none	progesterone	cortisol	estradiol testosterone dihydrotestosterone
Association Constant (M^{-1} at 37°C)	10^4-10^5	10^5	10^8	10^9
Binding Sites	1 - 10	1	1	1/dimer
%Carbohydrate	0	43	27	18

1.1.2 High Affinity Steroid Binding Proteins

In serum, there are two high affinity steroid binding proteins; corticosteroid binding globulin (CBG; also referred to as transcortin) and sex-hormone binding globulin (SHBG; also referred to as sex-steroid binding protein or testosterone-estradiol binding globulin). The plasma concentrations of CBG and SHBG are much lower than those of albumin and AAG, but they transport the majority of biologically active steroids and this may be attributed to their relatively high steroid binding affinities. Some of the physicochemical properties of CBG and SHBG are also summarized in Table 1.1.

Corticosteroid binding globulin has one steroid binding site/molecule which binds cortisol, corticosterone and 11-deoxycortisol with high affinity (Dunn *et al.*, 1981) and 85 - 90% of these glucocorticoids are CBG-bound under normal physiological conditions (Henning, 1978; Dunn *et al.*, 1981; Siiteri *et al.*, 1982): thus making CBG the major glucocorticoid transport protein in the blood. The affinity of CBG for androgens, mineralocorticoids and estrogens is low to negligible, but is moderately high for progestins, and it is quantitatively an important carrier of progesterone during pregnancy (Dunn *et al.*, 1981). Non-CBG-bound glucocorticoids are either albumin-bound (approximately 6-12%) or free (approximately 4-5%) in both males and females (Dunn *et al.*, 1981; Siiteri *et al.*, 1982). Because the concentration of glucocorticoids and CBG are approximately equal in blood, CBG steroid binding sites are essentially fully occupied and this ensures that physiological fluxes in cortisol concentration, for example in response to stress (Munck *et al.*, 1984), are accompanied by an increase in free steroid.

However, under homeostatic conditions, plasma cortisol and CBG concentrations parallel one another.

Sex hormone binding globulin is a dimer capable of binding one molecule of testosterone or estradiol with high affinity (Dunn *et al.*, 1981). Because the concentrations of sex-steroids and SHBG differ between males and females, the distribution of hormone between SHBG, albumin and the free fractions varies with gender (Dunn *et al.*, 1981; Siiteri *et al.*, 1982). In humans, SHBG is only approximately 50% saturated with steroid (Dunn *et al.*, 1981; Siiteri *et al.*, 1982) and may even bind other ligands, such as fatty acids (Umstot and Andersen, 1986). Therefore, alterations in steroid levels do not significantly change free hormone concentrations (Dunn *et al.*, 1981). However, the percentage of free sex-steroids decreases as SHBG concentrations increase (Siiteri *et al.*, 1982), and in contrast to CBG, alterations in SHBG levels therefore influence the bioavailability of sex-steroids.

1.1.3 Physiological Role of Plasma Steroid Binding Proteins

There are two theories concerning the impact of steroid binding proteins on steroid hormone bioavailability. In the generally accepted model, steroid binding proteins do not actively participate in the delivery of steroid to target and simply modulate the proportion of free and bound ligand in blood, and act as reservoirs of biologically-latent hormone (Tait and Burnstein, 1964). Accordingly, only non-protein-bound steroids may cross the plasma membrane of target cells by passive diffusion. In the second, more controversial model, it has been proposed that CBG

and SHBG interact directly with target cells and may therefore actively promote steroid entry into cells, as well as performing their more 'classical' functions of plasma transport and regulation of free hormone levels (Siiteri *et al.*, 1982; Rosner, 1990).

In general, the metabolic clearance rates of steroids are inversely proportional to their affinity for SHBG and CBG; *i.e.* steroids which interact poorly with these proteins are rapidly metabolized (Siiteri *et al.*, 1982). For example, as serum SHBG concentrations increase, the metabolic clearance rate of testosterone is reduced (Siiteri *et al.*, 1982; Pétra *et al.*, 1985) and this is presumably due to decreased levels of free hormone. The importance of free steroid, as the mediator of bioactivity, was suggested by a study in which increased liver glycogen was observed following cortisol but not CBG-cortisol administration to adrenalectomized mice (Slaunwhite *et al.*, 1962). Unfortunately, these mice were injected with CBG purified from human plasma, and the significance of this observation is therefore unclear. Nevertheless, the addition of CBG to culture medium reduced tyrosine aminotransferase activity in hepatoma cells and this supported the inhibition of cortisol activity by CBG (Lippman and Thompson, 1974). However, hepatic tyrosine aminotransferase activity has also been reported to increase following concurrent administration of equimolar amounts of CBG and cortisol (Rosner and Hochberg, 1972). More interestingly, estrogen-induced augmentation of both CBG and corticosterone levels increased hepatic, but not pancreatic, alanine aminotransferase activity in rats, whereas corticosterone injections increased both (Keller *et al.*, 1969). Therefore, although free cortisol is indisputably biologically active, the bioavailability

of CBG-bound cortisol may vary from tissue to tissue. While this concept remains contentious, it would seem reasonable that differences between organs with respect to sinusoidal or capillary transit time; microcirculatory pressure; steroid influx rates, tissue temperature, and possibly even increases in local CBG concentrations (see section 1.5.2) may all influence steroid bioavailability. Thus, a general rule regarding CBG's influence on glucocorticoid entry into cells may not be pragmatic.

While free concentrations (Dunn *et al.*, 1981) of cortisol and aldosterone (15 nM and 0.1 nM, respectively) are similar to the dissociation constants of the glucocorticoid (Siiteri *et al.*, 1982) and mineralocorticoid (Funder *et al.*, 1973; Matulich *et al.*, 1976) receptors (10 nM and 0.5 nM, respectively), only a fraction of the extracellular concentration would passively diffuse across the plasma membrane and be available to the intracellular receptors and it therefore seems probable that an active transport process is required. The discrepancy between free estradiol concentrations (5 - 15 pM) in non-pregnant women (Dunn *et al.*, 1981) and the dissociation constant (1 nM) of uterine estrogen receptors (Siiteri *et al.*, 1982) is even more dramatic and further highlights the need for a mechanism to increase intracellular uptake.

Steroid binding proteins would seem an obvious choice for this process since their interaction with cells would bring a large reservoir of bound steroid in close contact with target tissues, and both CBG and SHBG have been located within numerous androgen, estrogen, glucocorticoid and progesterone target tissues by immunocytochemical and biochemical criteria (Siiteri *et al.*, 1982; Rosner, 1990 and

references therein). Although the location of these proteins may either represent *de novo* synthesis or sequestration from the circulation, it suggests CBG and SHBG may facilitate increased intracellular steroid concentrations within target tissues. Cell-surface receptors for SHBG (see Rosner, 1990 for review) and CBG (see section 1.5.1) have also been described, and this further supports the concept of binding protein-mediated hormone delivery.

1.1.4 Intracellular Steroid Binding Proteins

There are two well-defined classes of intracellular steroid binding proteins; the steroid hormone receptors and metabolizing enzymes. All steroid receptors are members of a superfamily of nuclear transcription factors (Carson-Jurica *et al.*, 1990) and many of the steroid metabolizing enzymes belong to the cytochrome P450 superfamily of heme-containing, oxidative catalysts (Miller, 1988). In both instances, steroid binding activities are possessed by only some members of these protein superfamilies, and suggests that this property has been acquired to accommodate the evolution of endocrine systems.

The nuclear receptors for cortisol (glucocorticoid) and testosterone (androgen) resemble each other at the primary structure level, and this is also true for the steroid-metabolizing members of the P450 family. Furthermore, the extracellular steroid binding proteins, albumin, vitamin D-binding protein and α-fetoprotein, also appear to have evolved from a common ancestral gene (Cooke and David, 1985). One might therefore expect the extracellular transport proteins for cortisol and sex-steroids, CBG and SHBG, to be related to one another. However, they are not, nor

do they share any significant homology to any other steroid binding protein (Hammond *et al.*, 1987; Gershagen *et al.*, 1987; Bardin *et al.*, 1988). Indeed, CBG is a member of the serine protease inhibitor superfamily (Hammond *et al.*, 1987) and SHBG shares homology with protein S, a cofactor in the anticoagulation system (Gershagen *et al.*, 1987). The dissimilarity of these proteins suggests their extra-steroid-binding activities (*i.e.* regulation of steroid bioavailability) may not be mediated in a common manner, and examination of CBG and SHBG in the context of their respective gene families may reveal otherwise unsuspected biological properties.

1.2 Introduction to CBG

1.2.1 Discovery of CBG

Between 1957 and 1959, three groups of investigators described a plasma protein which bound cortisol with high affinity. Sandberg *et al.* (1957) inferred the existence of a high affinity cortisol binding protein because cortisol bound better to whole plasma than would be expected from its binding to Cohn fractions. At approximately the same time, Bush (1957) reported the results of equilibrium dialysis experiments in which increasing quantities of cortisol were added to plasma. Two substances were identified which bound cortisol; one with a high affinity and low capacity for the steroid, while the other bound cortisol with a low affinity but had a very large binding capacity (Bush, 1957). The following year, Daughaday (1958) demonstrated the migration of [^{14}C]cortisol in the α-globulin region by paper electrophoresis and this was confirmed by Slaunwhite and Sandberg (1959) who

named the cortisol binding protein 'transcortin'.

1.2.2 Distribution and Sites of Synthesis of CBG

A cortisol and/or corticosterone binding protein has been detected in serum obtained from 131 vertebrate species, representing members of the fish, amphibian, reptilian, avian and mammalian families (Seal and Doe, 1965). A corticosterone binding protein has also been identified within the unicellular eukaryote, *Candida albicans*, but it is not known if this protein is related to either CBG or the glucocorticoid receptor (Loose *et al.*, 1981; Loose and Feldman, 1982). Although there has been a single report of human CBG deficiency (Roitman *et al.*, 1984), this has never been substantiated using more sensitive assays, and a comprehensive study of 10,124 human serum samples failed to identify a total absence of CBG (Rosner *et al.*, 1973). This therefore suggests a null mutation is lethal.

Although CBG levels are generally measured in serum or plasma samples, it has been detected in other fluids and within cells. For instance, low levels of CBG have been detected in human bronchoalveolar fluid (Loric *et al.*, 1989), cerebrospinal fluid (Predine *et al.*, 1984), amniotic fluid (Challis and Bennett, 1977), lymph (Sandberg *et al.*, 1960) and milk (Rosner, 1976). Intracellular CBG has also been identified within lymphocytes (Werthamer *et al.*, 1973; Amaral and Werthamer, 1974) and tissues including: brain and pituitary (Koch *et al.*, 1976; De Kloet and McEwen, 1976; Al-Khouri and Greenstein, 1980; Perrot-Applanat *et al.*, 1984; Kuhn *et al.*, 1986), kidney (Feldman *et al.*, 1973; Funder *et al.*, 1973; Weiser *et al.*, 1979; Kuhn *et al.*, 1986), muscle (Mayer *et al.*, 1975), liver (Koblinsky *et al.*, 1972; Litwack

et al., 1973; Amaral *et al.*, 1974; Suyemitsu and Terayama, 1975; Weiser *et al.*, 1979; Kuhn *et al.*, 1986), lung (Giannopoulos, 1976), thyroid (Kuhn *et al.*, 1986) and uterus (Milgrom and Baulieu, 1970; Gueriguian *et al.*, 1974; Al-Khouri and Greenstein, 1980; Kuhn *et al.*, 1986).

Despite the identification of corticosteroid binding proteins within these tissues, liver has been long believed to be the major site of CBG biosynthesis (Westphal, 1983). Initial experiments demonstrating reduced CBG levels in rats following subtotal hepatectomy (Gala and Westphal, 1966c) and a report of low CBG concentrations in patients with liver disease (Doe *et al.*, 1964) provided initial, although indirect, evidence to support this supposition. It was not until 1979, that Weiser *et al.* reported the synthesis of a protein similar to CBG by liver slices *in vitro* and immunoselection of *in vitro* translated hepatic RNA indicated CBG was made by guinea pig liver (Perrot-Applanat and Milgrom, 1979). A similar approach demonstrated the presence of CBG mRNA in rat liver (Wolf *et al.*, 1981) and fetal rat hepatocytes secrete CBG in culture (Vranckx *et al.*, 1985; Ali *et al.*, 1986) as does the human hepatoma-derived cell line, Hep G2 (Khan *et al.*, 1984). Although the presence of CBG in non-hepatic tissues may simply represent uptake from blood, it may also be the product of local synthesis. In support of the latter, CBG mRNA has been identified in rhesus monkey testis and kidney, and a CBG cDNA has been cloned from a human lung library (Hammond *et al.*, 1987). Expression of CBG mRNA in these tissues is low by comparison to liver, and its significance is not known.

1.3 Isolation and Characterization of CBG

1.3.1 Glycoprotein Properties

Corticosteroid binding globulin was first isolated by Seal & Doe (1962) employing successive chromatographies on diethylaminoethyl-cellulose (DEAE-cellulose) and hydroxylapatite. Current CBG purification procedures utilize affinity chromatography (Le Gaillard *et al.*, 1974; Rosner and Bradlow, 1975) and this improvement facilitates the extraction of CBG from relatively large volumes of serum. Purified human CBG appears to be a single monomeric polypeptide (Le Gaillard *et al.*, 1975) and electrophoretic variants of human CBG may reflect differences in carbohydrate composition (Hammond, 1990). The physicochemical properties of human CBG have been studied extensively and are contrasted to those of rat CBG in Table 1.2.

1.3.2 Steroid Binding Properties

Corticosteroid binding globulin has one steroid binding site per molecule (Westphal, 1983), which displays a preference for biologically active corticosteroids and in some species it also binds progesterone (Dunn *et al.*, 1981). Temperature greatly influences the affinity of CBG for steroids; the association constant of human CBG for cortisol and progesterone at 4°C is at least 35-fold greater than that determined at 37°C (Mickelson *et al.*, 1981). The affinity of rat (Chader and Westphal, 1968b) and rabbit (Chader and Westphal, 1968a) CBG for corticosteroids also varies in a similar temperature-dependent manner. Although CBG heated to 46°C and cooled still retains steroid binding activity (Doe *et al.*, 1964), an

Table 1.2 Comparison of the physicochemical properties of human and rat CBG. Values for human CBG were obtained from Westphal (1986) or Le Gaillard *et al.* (1975) and rat CBG values were from Westphal (1971) or Favre *et al.* (1984).

	Human CBG	Rat CBG
Molecular Weight	49.5 - 58.5	52.6 - 75.8
% Carbohydrate	27	28
$S^{\circ}_{20,w}$	3.66 - 3.79	3.56
Partial specific volume	0.680 - 0.718	0.711
$E^{1\%}_{1\ cm}$ 280 nm	6.45 - 7.4	6.2 - 6.24

irreversible loss of steroid binding activity occurs when CBG is heated at 60°C (Daughaday *et al.*, 1962).

Van't Hoff plots indicate the enthalpy of the human CBG-cortisol complex is negative at all temperatures which indicates the protein-steroid interaction is exothermic and therefore, predominantly hydrophilic in nature (Mickelson *et al.*, 1981; Westphal, 1983). This seems reasonable in view of the relatively hydrophilic character of corticosteroids and contrasts with the comparatively hydrophobic complex observed between SHBG and dihydrotestosterone (Westphal, 1983). The steroid binding affinity of CBG is maximal between a pH of 8 and 11.5 and is greatly reduced when measured at pH 5 or 12 (Mickelson *et al.*, 1981). Furthermore, loss of steroid binding activity is permanent at pH 5 or lower (Seal and Doe, 1962; Chan and Slaunwhite, 1977). Increasing ionic strength from 1 to 3000 mM produced a minor elevation in association constants for cortisol and progesterone, while urea reduced CBG's steroid binding affinity (Chan and Slaunwhite, 1977). The latter effect is, however, reversible at concentrations less than or equal to 2 M (Chan and Slaunwhite, 1977).

Steroids are bound to CBG in a pocket approximately 25 Ångstrom (Å) deep with their C-21 side chain directed towards the exterior protein surface and the C-17 located approximately 16 Å from the binding domain entrance (Defaye *et al.*, 1980). Affinity labelling of CBG with 6ß-bromoprogesterone and subsequent hydrolysis produced progesterone-6-S-L-cysteine, and this suggested a cysteine residue was located near the C-6 position of this affinity ligand within the steroid binding

domain (Khan and Rosner, 1977). Electron spin resonance also indicated that a thiol group was located at least 15 Å from the binding pocket surface (Defaye *et al.*, 1980). Affinity labelling studies suggest methionine and histidine residues interact with the 11ß-hydroxyl and 20-keto groups of cortisol, respectively (Le Gaillard and Dautrevaux, 1977). Furthermore, Le Gaillard and Dautrevaux (1977) suggest the binding site crevice narrows in the region where the A and B steroid rings are bound. Modification of CBG with tetranitromethane reduced steroid binding activity and identified tyrosine within the binding site (Le Gaillard *et al.*, 1982). In addition, fluorescence quenching (Stroupe *et al.*, 1978; Akhrem *et al.*, 1978), ultra-violet spectra (Akhrem *et al.*, 1980) and the use of tryptophan-specific reagents (Akhrem *et al.*, 1981) suggest tryptophan is also present in the binding domain. However, the nature of the interactions between the latter two residues and bound steroids are unknown.

1.4 Factors which Influence Serum CBG Levels

1.4.1 Sex

In humans, there is no significant difference between male and female serum CBG levels (Brien, 1981; Robinson *et al.*, 1985b). However, a sexual dimorphism in rat CBG levels becomes apparent at puberty; females have approximately twice the CBG values found in males (Gala and Westphal, 1965b). Similarly, the CBG levels of mature female mice are nearly 3 times greater than their male counterparts (Gala and Westphal, 1967).

1.4.2 Age

Plasma levels of human CBG have not been well characterized with respect to ontogeny, but several trends are apparent (Brien, 1981). In umbilical cord blood they are approximately half that of normal adults, and low levels have also been observed in newborn infants. They then increase relatively rapidly and reach adult levels before 1 year of age.

The ontogeny of serum CBG levels has been studied in greater detail in the rat, rabbit and mouse. In both rat and rabbit, fetal plasma CBG concentrations reach about half the level observed in the maternal circulation after completion of approximately 70% gestation and then decline to very low levels at birth (Van Baelen *et al.*, 1977b; Gewolb and Warshaw, 1986; Seralini *et al.*, 1990b). Thereafter, rat serum CBG concentrations are low during the first weeks of life (Henning, 1978; D'Agostino and Henning, 1981) and gradually increase until adult values are attained following the onset of sexual maturity (Gala and Westphal, 1965b). In mouse, fetal serum CBG concentrations are comparatively high at approximately 85% of gestation but are only approximately 20% of maternal values (Savu *et al.*, 1977). Thereafter they rapidly decline to very low levels, and remain so for the first 2 - 3 weeks of life (Savu *et al.*, 1977). Sheep are an exception to this trend, and exhibit increasing CBG levels from mid-gestation until birth, after which they rapidly decline to low levels for the following 14 - 21 days (Ballard *et al.*, 1982).

1.4.3 Pregnancy

Changes in CBG levels associated with pregnancy were first noted in 1959

(Slaunwhite and Sandberg, 1959) and have since been studied extensively (Westphal, 1971; Westphal, 1986). In humans, CBG levels increase approximately two-fold by the end of the second trimester and remain relatively constant until term (Doe *et al.*, 1964; Moore *et al.*, 1978a), and this increase has been attributed to elevated serum estrogen concentrations during pregnancy (Brien, 1981). Minor changes in estradiol concentrations (for example during the menstrual cycle) do not influence serum CBG concentrations, and it appears a threshold estradiol concentration must be attained prior to dose-dependent increases in CBG concentrations (Moore *et al.*, 1978a). Maternal CBG levels in rats only undergo minor fluctuations (Gala and Westphal, 1965a; Van Baelen *et al.*, 1977b; Gewolb and Warshaw, 1986) and this is probably due to the relative insensitivity of female rats to estrogens (see section 1.4.4). Serum CBG levels of pregnant mice increase by at least 10-fold (Gala and Westphal, 1967; Savu *et al.*, 1977), and in both rodents, CBG levels begin a progressive, and in the case of mouse, dramatic, decline 3 days prior to delivery (Gala and Westphal, 1967; Van Baelen *et al.*, 1977b; Gewolb and Warshaw, 1986).

Human CBG usually contains 3 biantennary and 2 triantennary *N*-linked oligosaccharide chains (Akhrem *et al.*, 1982; Strel'chyonok *et al.*, 1982). However, a pregnancy-associated variant of human CBG has been identified which is composed only of triantennary, *N*-linked carbohydrates (Strel'chyonok *et al.*, 1984). The variant may be detected as early as the first month of pregnancy and its concentration increases as pregnancy progresses (Avaakumov and Strel'chyonok, 1987). By term, the concentration of this glycosylation variant represents 7 - 14% of total serum CBG levels (Avvakumov and Strel'chyonok, 1987; Strel'chyonok and

Avvakumov, 1990).

During pregnancy, total human cortisol levels rise by as much as 2-fold (Dunn *et al.*, 1981), but an accompanying increase in CBG levels ensures that free cortisol concentrations remain constant, and Cushing's syndrome-like symptoms are therefore avoided (Doe *et al.*, 1969). The biological significance of this increase in CBG concentrations is not known, but several reports link low CBG levels and poor fetal outcome. De Moor *et al.* (1966) described an association between low maternal CBG levels and increased abortion incidence. Furthermore, in 10 out of 20 pregnancies exhibiting signs of impaired fetal viability during the third trimester, maternal CBG levels were more than 3 standard deviations below those of the appropriate gestational age (De Moor *et al.*, 1966). Murao *et al.* (1986) also demonstrated that low maternal CBG levels correlated with high-risk pregnancies or those associated with an abnormal outcome.

1.4.4 Exogenous Steroid Administration

The influence of hormones on rat CBG levels has been reviewed by Westphal (1971; pages 237 - 293). Although natural and synthetic glucocorticoids reduce serum CBG levels by at least 50% (Gala and Westphal, 1966a; Yamamoto and Ohsawa, 1976), and progestins increase them by 20% or more (Gala and Westphal, 1965b) in both males and females, the effects of estrogen and androgen administration are sex-specific. Estradiol treatment increases male CBG levels by approximately 50% while female serum CBG levels are unchanged (Gala and Westphal, 1965b). Conversely, testosterone administration reduced female serum

CBG levels by approximately 25% but did not influence male CBG concentrations (Gala and Westphal, 1965b).

Estrogen-induced increases in human serum CBG levels were first recognized in 1959 (Sandberg and Slaunwhite), and prior to 1970, more than 35 reports confirmed this observation (Westphal, 1971). Corticosteroid binding globulin levels rise 2 - 3 fold in a dose-dependant manner following estrogen administration and different estrogen preparations exhibit distinct CBG-increasing potentials (Moore *et al.*, 1978b). Surprisingly, the anti-estrogen, tamoxifen, also increases serum CBG levels, and the authors suggest that this compound exerts an estrogen-like effect directly on the liver (Sakai *et al.*, 1978).

Progestogen administration to humans does not alter serum CBG levels (Moore *et al.*, 1978b). Oral contraceptives containing both progestogens and estrogens increase CBG concentrations, but this augmentation has been attributed to an estrogen effect (Moore *et al.*, 1978b). Testosterone does not alter CBG levels, although administration of several anabolic steroids (oxymethylone and methandrostenolone) slightly increases CBG concentrations (Barbosa *et al.*, 1971). Pharmacologic doses of the biologically potent glucocorticoids, dexamethasone and prednisone, depress CBG levels, while replacement glucocorticoid therapy only slightly decreases CBG binding capacity (Schlechte and Hamilton, 1987).

1.4.5 Pathophysiological Conditions

Although some reports suggest CBG levels remain constant, or increase, during

human liver disease (De Moor *et al.*, 1962; Keane *et al.*, 1969), CBG values are generally lower, and it has been suggested that the severity of disease may influence these values (Brien, 1981). Cushing's syndrome results in reduced CBG levels (Schlechte and Hamilton, 1987) and this probably reflects the increased cortisol levels associated with this disease. Reduced CBG levels have also been associated with myocardial infarction (Chazova, 1971; Zouaghi *et al.*, 1984) and Addisonian pernicious anemia (Doe *et al.*, 1982). Corticosteroid binding globulin levels in the latter could be corrected by administration of cobalamin, and vitamin B-12 may therefore be required for CBG synthesis (Doe *et al.*, 1982). Elevated CBG levels have been reported for patients suffering from lymphatic leukemia, hairy cell leukemia or non-Hodgkin lymphoma, and are associated with the presence of HLA antigens B12 and Cw5 (De Moor *et al.*, 1980; De Moor and Louwagie, 1980).

Corticosteroid binding globulin levels measured by ligand binding or radioimmunoassay decrease rapidly after the onset of septic shock, and this is accompanied by an increase in total and free cortisol levels (Savu *et al.*, 1981, Pugeat *et al.*, 1989). The cortisol binding affinity of CBG in these patients is similar to healthy controls (Pugeat *et al.*, 1989). Severe acute infection by *Candida albicans* also reduces CBG while increasing cortisol levels (Zouaghi *et al.*, 1983), and CBG values return to normal levels following recovery (Zouaghi *et al.*, 1983; Pugeat *et al.*, 1989). Patients suffering from localized inflammation or non-septic shock do not, however, exhibit reduced CBG levels (Savu *et al.*, 1981; Pugeat *et al.*, 1989). Rats subjected to turpentine-induced acute inflammation also exhibit reduced serum CBG levels with no change in its steroid binding affinity (Savu *et al.*, 1980; Faict *et*

al., 1983).

1.4.6 Diurnal Variations

There does not appear to be a significant diurnal variation in human serum CBG levels (De Moor *et al.*, 1962; Doe *et al.*, 1964). Rat circulating CBG levels may, however, vary by approximately 30%; they peak at the beginning of the nocturnal period and reach their nadir at the onset of the light period (Hsu and Kuhn, 1988). These changes correlate to the cyclic variation of corticosterone levels in rats, and are not observed following adrenalectomy (Hsu and Kuhn, 1988).

1.5 Proposed Additional Functions of CBG

1.5.1 CBG-Elastase Interactions

The determination of the primary structure of human CBG revealed that this protein was a member of the serine protease inhibitor gene family (Hammond *et al.*, 1987) and it has been subsequently shown to interact specifically with purified human neutrophil elastase (Pemberton *et al.*, 1988; Hammond *et al.*, 1990a). This results in the cleavage of CBG at a single, specific location within the carboxy-terminus of the protein and the release of bound cortisol (Pemberton *et al.*, 1988; Hammond *et al.*, 1990a).

Incubation of CBG and granulocytes from patients suffering from sepsis, also results in the specific cleavage of CBG (Hammond *et al.*, 1990a; Hammond *et al.*, 1990b). Corticosteroid binding globulin must contact the activated cells directly for proteolysis to occur and it is probably not internalized (Hammond *et al.*, 1990b).

Although the physiological significance of this event is presently unclear, the direct release of glucocorticoids to activated inflammatory cells would provide a mechanism whereby high concentrations of immunoregulatory steroid could be achieved within the inflammatory locus.

1.5.2 CBG - Receptor Interactions

Corticosteroid binding globulin complexed with cortisol or corticosterone binds to a single set of binding sites on membranes prepared from human prostate (Hryb *et al.*, 1986), rat spleen (Singer *et al.*, 1988) and rat kidney (Hsu *et al.*, 1986), and these interactions are specific, saturable and temperature dependent. Furthermore, binding sites present on human endometrium interact with CBG-bound progesterone with approximately 2 - 3 fold greater affinity than CBG-bound cortisol (Avvakumov *et al.*, 1989). Placental syncytiotrophoblasts possess two classes of CBG binding sites; one present in low concentration that binds the pregnancy-associated variant CBG with higher affinity than normal CBG, while that present in high concentrations has a preferentially affinity for normal CBG (Avvakumov and Strel'chyonok, 1988). The proposed CBG receptor has been affinity purified from human decidual endometrium and appears to be a sialoglycoprotein composed of four approximately 20 kDa subunits (Avvakumov *et al.*, 1989; Krupenko *et al.*, 1991).

Concentrations of CBG are similar to (Hryb *et al.*, 1986; Singer *et al.*, 1988; Hsu *et al.*, 1986) or greater than (Avvakumov *et al.*, 1989; Avvakumov and Strel'chyonok, 1988) the dissociation constants of the membrane CBG binding sites. In the first

instance, interactions between membrane receptors and CBG may augment local steroid concentrations and thereby increase delivery to target tissues. However, in the latter case, variations in the concentration of CBG binding sites on target cell membranes would regulate local CBG-bound steroid concentrations, and tissues could therefore modulate their exposure to extracellular steroid.

Incubation of CBG with MCF-7 (human breast cancer) and FAO (rat hepatoma) cells results in its nuclear localization (Nakhla *et al.*, 1988; Kuhn, 1988). Specific CBG binding sites have been detected on the plasma membrane of these cells, and it is possible they mediate CBG internalization (Nakhla *et al.*, 1988; Kuhn, 1988). The physiological significance of intracellular CBG has not, however, been demonstrated. Interaction of CBG with MCF-7 cells also increases cAMP production and this effect is dependent on the presence of steroid within CBG's ligand binding domain (Nakhla *et al*, 1988), but it is not known if this elicites a change in cell biochemistry.

CHAPTER 2

Materials and Methods

2.1 Materials

The general chemicals used for these studies were of analytical or molecular biology grade. Radiolabelled compounds were obtained from DuPont Canada Incorporated ([α-^{32}P]dCTP, [α-^{35}S]dATP, [α-^{35}S]UTP, [^{125}I]Bolton-Hunter reagent, L-[^{35}S]methionine, [1,2-^{3}H]cortisol and [1,2,6,7-^{3}H]corticosterone) or Amersham Canada Limited ([α-^{32}P]dCTP, [α-^{32}P]UTP and [α-^{35}S]dATP). Restriction and most modifying enzymes were obtained from Pharmacia (Canada) Incorporated or Promega Corporation. *Thermus aquaticus* (*Taq*) DNA polymerase was purchased from Promega or Perkin-Elmer (Canada) Limited and reverse transcriptase was from Life Sciences Incorporated or Gibco/Bethesda Research Laboratories. Radioinert steroids were from Steraloids Incorporated, and L-thyroxine was from British Drug House Incorporated or Sigma Chemical Company. Oligonucleotide primers were the generous gift of Allelix Biopharmaceuticals Incorporated or were obtained from the Molecular Biology Core Facility of the Medical Research Council Group in Fetal and Neonatal Health and Development. The source of additional specialized reagents are given in the appropriate section.

2.2 Isolation of CBG cDNAs

2.2.1 Rat

Approximately 5 x 10^5 plaque forming units (pfu) of a λgt11 Sprague-Dawley rat liver CDNA library (Clontech Laboratories Incorporated) were plated in semisolid Luria-Bertani medium. Bacteriophage DNA was transferred to nitrocellulose filters (Schleicher & Schuell; BA85, 0.45 μm pore size), and

hybridized *in situ* (at 37°C) with a radiolabelled (Rigby *et al.*, 1977) human CBG cDNA (Hammond *et al.*, 1987) in the presence of nitrocellulose hybridization buffer [5 x Denhardt's solution (0.1% Ficoll® 400, 0.1% polyvinylpyrrolidone, 0.1% bovine serum albumin), 5 x SSPE (0.75 M NaCl, 50 mM NaH_2PO_4, 5 mM EDTA), 0.1% sodium dodecyl sulfate (SDS), and 100 μg denatured salmon sperm DNA/ml] containing 40% formamide. Positive bacteriophage were plaque purified, amplified and their DNA was isolated (Yamamoto *et al.*, 1970). The two largest cDNA inserts were excised with *Eco*RI restriction endonuclease and subcloned into pBR322 (Bolivar *et al.*, 1977). In addition, positive bacteriophage clones were induced with isopropyl-ß-D-thiogalactopyranoside (IPTG) and the resulting ß-galactosidase fusion proteins were screened with an antibody (provided by Dr. R.W. Kuhn, University of California, San Francisco, California) against rat CBG (Raymoure and Kuhn, 1983) using the method of Young and Davis (1983), except that peroxidase-labelled protein A was used to detect antibody-antigen complexes in the presence of the chromogenic substrate, 4-chloro-1-naphthol.

2.2.2 Mouse

A λgt11 Balb/c mouse liver cDNA library was obtained from Clontech and screened at reduced stringency (nitrocellulose hybridization solution containing 40% formamide at 37°C) with a near-full length ^{32}P-labelled (Feinberg and Vogelstein, 1983) human CBG cDNA (Hammond *et al.*, 1987). Positive clones were plaque purified, amplified and purified as described previously (Yamamoto *et al.*, 1970). The phage cDNA inserts were excised with *Eco*RI restriction endonuclease, and the

hybridized *in situ* (at 37°C) with a radiolabelled (Rigby *et al.*, 1977) human CBG cDNA (Hammond *et al.*, 1987) in the presence of nitrocellulose hybridization buffer [5 x Denhardt's solution (0.1% Ficoll® 400, 0.1% polyvinylpyrrolidone, 0.1% bovine serum albumin), 5 x SSPE (0.75 M NaCl, 50 mM NaH_2PO_4, 5 mM EDTA), 0.1% sodium dodecyl sulfate (SDS), and 100 μg denatured salmon sperm DNA/ml] containing 40% formamide. Positive bacteriophage were plaque purified, amplified and their DNA was isolated (Yamamoto *et al.*, 1970). The two largest cDNA inserts were excised with *Eco*RI restriction endonuclease and subcloned into pBR322 (Bolivar *et al.*, 1977). In addition, positive bacteriophage clones were induced with isopropyl-ß-D-thiogalactopyranoside (IPTG) and the resulting ß-galactosidase fusion proteins were screened with an antibody (provided by Dr. R.W. Kuhn, University of California, San Francisco, California) against rat CBG (Raymoure and Kuhn, 1983) using the method of Young and Davis (1983), except that peroxidase-labelled protein A was used to detect antibody-antigen complexes in the presence of the chromogenic substrate, 4-chloro-1-naphthol.

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largest was subcloned into pBluescript SK^+(Stratagene Cloning Systems).

In order to obtain the entire coding region of the mouse CBG cDNA, it was necessary to isolate an additional DNA fragment which corresponded to the region 5' of the cDNA obtained above. To accomplish this, an aliquot of the cDNA library was digested with proteinase K (Kawasaki, 1990) and subjected to polymerase chain reaction (PCR) amplification using a 5'-primer (5'- GGAAGAATTCGGTAGCG ACCGGCGC) specific to a region of λgt11 located 9 - 23 bp downstream of its *Eco*RI cloning site and a 3'-primer (5'-GGGGAAAGAATTCCACTGGGGAGA **TTAAGGT**) which corresponds to nucleotides 17 - 34 of the original cDNA (bold). An *Eco*RI restriction endonuclease recognition site (underlined) was incorporated into the 3' primer to facilitate subsequent cloning and was included in the 5' primer in the event the λ *Eco*RI cloning site was destroyed during library construction. The reaction was performed in the presence of 250 μM dNTP, 100 pmol of each primer and 2.5 units (U) of *Taq* DNA polymerase for 30 cycles (cycle = denaturation for 1 min at 94°C, annealing for 2 min at 65°C and extension for 3 min at 72°C). The largest product following amplification was cloned into the *Eco*RI site of pBluescript.

2.2.3 Rabbit

A rabbit liver cDNA library was constructed to obtain a CBG cDNA. Hepatic RNA was extracted by the lithium chloride/urea method from a near-term pregnant rabbit and was enriched for poly(A)$^+$ RNA by oligo(dT)-cellulose affinity chromatography (see section 2.5). The cDNA was synthesized and *Eco*RI linkers

were added using a reagent kit obtained from Pharmacia. It was then ligated into the *Eco*RI site of bacteriophage λgt10 and packaged using the Packagene• lambda DNA packaging system (Promega). The library was propagated in *Escherichia coli* (strain C600 hfl) and screened at reduced stringency (nitrocellulose hybridization solution with 40% formamide at 37°C) with a human CBG cDNA (Hammond *et al.*, 1987) radiolabelled by random priming (Feinberg and Vogelstein, 1983) with [α-^{32}P]dCTP. Positive plaques were cloned, amplified and recombinant phage were purified (Yamamoto *et al.*, 1970). The cDNA inserts were excised with *Eco*RI, and the two largest were subcloned into pBluescript.

2.2.4 Squirrel Monkey

Hepatic RNA was extracted from an estrogen-treated squirrel monkey (*Saimiri sciureus*), enriched for poly(A)$^+$ RNA and used as a template for cDNA synthesis using a reagent kit supplied by Pharmacia. Following the addition of *Eco*RI linkers, the resulting cDNA fragments were ligated into the *Eco*RI site of λgt10 and packaged with Packagene• extract. The resulting library was propagated in *E. coli* C600 hfl and plaques were transferred to nitrocellulose and hybridized *in situ* with a radiolabelled (Feinberg and Vogelstein, 1983) human CBG cDNA at reduced stringency (nitrocellulose prehybridization buffer containing 40% formamide at 37°C). Several cDNAs were isolated which did not contain the entire open reading frame for the CBG polypeptide.

The nucleotide sequence analysis of the longest cDNA isolated from the library allowed the synthesis of 3 oligonucleotide primers (Sq5-2, Sq3-1 and Sq3-2) which

corresponded to regions within the original cDNA (Table 2.1). In addition, a fourth oligonucleotide primer (Sq5-1) was synthesized whose sequence corresponds to a region of a squirrel monkey CBG genomic clone (G.L. Hammond, personal communication) homologous to exon 1 of the human CBG gene (Underhill and Hammond, 1989). To obtain a near full length squirrel monkey CBG cDNA, primers Sq5-1 and Sq5-2 were used to generate a fragment which overlapped the library clone. This 5'-fragment was synthesized by reverse transcription (RT) and PCR (Grady and Campbell, 1989). In brief, first-strand synthesis was performed with 1 μg squirrel monkey hepatic poly(A)$^+$ RNA in the presence of 50 pmol primer Sq5-2, 0.5 mM dNTP and 2 U reverse transcriptase. Subsequent second-strand synthesis and PCR amplification (40 cycles of 1 min at 94 °C, cool over 2 min to 65°C and 3 min at 72°C) were performed in the presence of 100 pmol of primers Sq5-1 and Sq5-2, 0.25 mM dNTP and 2.5 U *Taq* DNA polymerase. A 3'-fragment was also produced with primers Sq3-1 and Sq3-2 by PCR amplification (30 cycles of 1 min at 94°C, cool over 2 min to 50°C and 3 min at 72°C) of approximately 500 ng of unligated squirrel monkey CBG cDNA (from above) in the presence of 100 pmol each of the Sq3-1 and Sq3-2 primers, 0.25 mM dNTP and 2.5 U *Taq* DNA polymerase, and was used to construct a squirrel monkey CBG cDNA for *in vitro* expression studies. The 5' and 3' PCR fragments and the original cDNA were subcloned into pBluescript for DNA sequence analysis.

2.3. Plasmid DNA Purification

Large scale cultures of bacteria transformed with CBG cDNA-containing

Table 2.1 Primer sequences used for PCR amplification of squirrel monkey CBG cDNAs. Restriction endonuclease sequences for *Eco*RI (underlined) were incorporated into all primers to facilitate subsequent cloning. Nucleotide positions given are in reference to Figure 3.6.

Primer	Sequence	Nucleotide Position
Sq5-1	5'-GGGGTTTGAATTCACAGTACACATGCTGGGTCCA	1 - 21
Sq5-2	5'-GGGGAAAGAATTCGACAGTCCCATTGCCCGCATA	complementary to 860 - 880
Sq3-1	5'-GGGGTTTGAATTCATCCGTTACCTTCATGACTCC	809 - 829
Sq3-2	5'-GGGGAAAGAATTCAGCGTTGCAATGATATTTATT	complementary to 1454 - 1474

plasmids were grown to produce quantities of cDNAs sufficient for subcloning and radiolabelling with [^{32}P]. Plasmid DNA was purified from bacterial cultures by one of two methods. In the first procedure, DNA was extracted by the alkaline lysis procedure (Birnboim and Doly, 1979), and purified with benzoylated, naphthoylated DEAE-cellulose (Gamper *et al.*, 1985). Alternatively, plasmid DNA was extracted and purified on Qiagen columns (Qiagen Incorporated) according to the manufacturer's instructions.

2.4 Sequence Analysis of DNA Fragments

2.4.1 Preparation of Single-stranded DNA Templates

The CBG cDNAs or restriction endonuclease fragments thereof were subcloned into either M13mp18 and M13mp19 (Pharmacia), pBluescript SK$^+$ or KS$^+$ or pSELECT™-1 (Promega) for the production of sequencing templates. Single-stranded M13 DNA templates were prepared essentially as described by Hindley (1983) except that bacteriophage particles were precipitated with a solution of 3 M NaCl and 20% polyethyleneglycol, resuspended in 10 mM Tris [tris(hydroxymethyl)-aminomethane], pH 8.0, containing 0.5% N-laurylsarcosine, and subjected to successive extractions with phenol, phenol/chloroform/isoamyl alcohol (25:24:1), chloroform and ether. Single-stranded pBluescript and pSELECT DNA templates were purified using the protocol supplied with pBluescript.

2.4.2 Sequencing Reactions and Gel Electrophoresis

Purified templates were sequenced by the dideoxy chain-termination method

(Sanger *et al.*, 1977) using reagents obtained from either Pharmacia (^{35}S Nucleotide Reagents), United States Biochemical (Sequenase) or Promega (T7 DNA Polymerase Sequencing System) and either M13-specific [M13 universal sequencing (Pharmacia) or M13 (-20) (United States Biochemical)] or CBG cDNA-specific primers. Reaction products were resolved by 7.9% polyacrylamide gel electrophoresis (PAGE) in the presence of 7 M urea, and gels were fixed in 10% methanol/10% glacial acetic acid (volume/volume) in water, dried under vacuum and subjected to autoradiography at room temperature using Kodak XAR5 film.

2.4.3 Alignment of cDNA-deduced Primary Structures

The mature cDNA-deduced amino acid sequences of human, rat, mouse, rabbit and squirrel monkey CBG, and the putative signal peptides of human, rat, mouse and squirrel monkey CBG were aligned and their overall similarities were determined with the CLUSTAL program of PC/Gene (IntelliGenetics Incorporated) which utilizes the method of Higgins & Sharp (1988, 1989). This program was also used to assess the evolutionary relationships between the mature proteins. The PALIGN program of PC/Gene, based on the method developed by Myers and Miller (1988), was used to align and calculate the pairwise similarity scores between all possible pairs of mature CBG polypeptide sequences.

2.5 Isolation of RNA

2.5.1 Cellular RNA Extraction

Total RNA was extracted by the method of Auffray & Rougeon (1980) with the

following modifications. Tissues (either freshly dissected or stored at -80°C) were homogenized in the presence of 3 M LiCl containing 6M urea and 50 U heparin/ml, and RNA was collected by centrifugation at 31,000 x g. Pellets were resuspended in 10 mM Tris (pH 7.5) containing 0.5% SDS (weight/volume) and 100 U heparin/ml, extracted with chloroform/isoamyl alcohol (24:1) and concentrated by ethanol precipitation.

2.5.2 Enrichment of Poly(A)$^+$ RNA

Poly(A)$^+$ RNA was selected from total RNA extracts by oligo(dT)-cellulose (Type 7, Pharmacia) chromatography (Aviv and Leder, 1972). In brief, oligo(dT)-cellulose was incubated with total RNA extracts in the presence of 10 mM Tris (pH 7.4) containing 0.5 M NaCl and 0.1% SDS. The cellulose was washed with a low salt (10 mM Tris, pH 7.4, containing 0.1 M NaCl/0.1% SDS) buffer and poly(A)$^+$ RNA was eluted with 10 mM Tris (pH 7.4) containing 0.1% SDS.

2.6 Hybrid Selection and *In Vitro* Translation of CBG mRNA

2.6.1 Hybrid Selection of Rat CBG mRNA

O-Aminophenylthioether (APT)-paper was activated with 1.2 M HCl and 3.6 mM $NaNO_2$ and allowed to react with plasmid (pBR322) DNA containing a rat CBG cDNA (Seed, 1982). Approximately 1 mg of rat hepatic RNA was resuspended in hybridization buffer [20 mM 1,4-piperazinediethanesulfonic acid (PIPES) containing 400 mM NaCl, 0.5 mM EDTA, 0.04% SDS and 60% formamide] and incubated (at 37°C) with the DNA-conjugated APT-paper for 16

h. Hybrid-selected CBG mRNA was eluted with sterile water in the presence of 100 μg yeast tRNA/ml.

2.6.2 Translation of CBG mRNA *In Vitro*

The CBG cDNA hybrid-selected and total hepatic poly(A)$^+$ RNA were translated *in vitro* with a micrococcal nuclease-treated rabbit reticulocyte lysate (Promega) in the presence of L-[^{35}S]methionine (approximately 1100 Ci/mmol). Trichloroacetic acid-precipitable radioactivity was used as an indicator of radiolabel incorporation.

2.6.3 Immunoselection of *In Vitro* Translation Products

A rabbit antiserum against rat CBG was incubated (16 h at 4°C) with *in vitro* translation products, and the immunocomplexes were separated by adsorption (1 h at 20°C) with kaolin-conjugated, donkey anti-rabbit immunoglobulins (a gift of Farmos Diagnostica, Turku, Finland). After centrifugation (5 min at 5,000 x g), the pellet was washed extensively in 10 mM phosphate-buffered saline (PBS) containing 1% Triton X-100, 1 mM phenylmethylsulfonylfluoride (PMSF) and 0.01% gelatin. Total and immunoadsorbed translation products were separated by SDS-PAGE. The gel was fixed in 10% acetic acid/5% methanol (volume/volume); treated with EN^{3}HANCE; dried under vacuum, and fluorographed (18 h at -80°C) with Kodak XAR2 film and a Dupont Cronex Hi-Plus XH intensifying screen.

2.7 Northern Blot Analysis for CBG mRNA

Before blotting experiments were performed, an aliquot of each RNA sample was subjected to 1% agarose gel electrophoresis in the presence of 2.2 M formaldehyde (Rave *et al.*, 1979), and was stained with ethidium bromide to assess the quality and relative quantity of RNA. Only intact RNA, as judged by the integrity of the 28 S and 18 S ribosomal bands, was used for subsequent analysis.

For Northern blot experiments, varying amounts of total (10-25 μg) or poly(A)$^+$ (1-20 μg) RNA were resolved by electrophoresis under similar conditions and transferred to either GeneScreen*Plus* (DuPont) or ZetaProbe (BioRad) nylon membranes by capillary blotting (Thomas, 1980). Blots were baked *in vacuo* (2 h at 80°C), incubated with either nitrocellulose (see section 2.2.1) or ZetaProbe (4 x SSPE, 1% SDS, 0.5% skim milk powder, 50% formamide and 0.5 mg denatured salmon testis DNA/ml) prehybridization solutions at 42°C, and hybridized with either nick-translated (Rigby *et al.*, 1977) or random-primed (Feinberg and Vogelstein, 1983) ^{32}P-labelled rat CBG cDNAs (Smith and Hammond, 1989) under the same conditions. Blots were washed to moderate stringency [0.2 x SSC (1 x SSC = 0.15 M NaCl and 0.015 M sodium citrate) containing 0.1% SDS at 37°C] or higher, and fluorographed at -80°C against Kodak XAR2 film using Dupont Cronex Hi-Plus XH or Lightning Plus intensifying screens. Where appropriate, signals were quantified with an LKB Ultroscan XL Enhanced Laser Densitometer.

In some instances, the blots were hybridized with a second cDNA probe for RNA common to all cell types to ensure that approximately equal amounts of intact

RNA were present in each lane. Briefly, blots were stripped by washing them 5 times in 0.1 x SSC containing 0.1 % SDS at 95 - 100°C for 5 min each, and then subjected to fluorography to ensure that there was no detectable signal. Subsequently, the membranes were hybridized with ^{32}P-labelled cDNAs for either mouse ß-actin (Minty *et al.*, 1983) or 18 S ribosomal RNA (provided by Dr. D.T. Denhardt, University of Western Ontario, London, Ontario) at reduced stringency (incubation at 37°C in hybridization buffer containing 40% formamide) and processed as described above.

2.8 Solution Hybridization Assay for CBG mRNA

2.8.1 Synthesis of Sense and Anti-sense CBG RNA

The largest rat CBG cDNA was inserted in both orientations into the *Eco*RI site of the vector, pT3/T7-mp18 (Bethesda Research Laboratories). Sense CBG RNA (mRNA standard) was transcribed *in vitro* (Melton *et al.*, 1984) with T7 RNA polymerase and unlabelled ribonucleotides, while anti-sense CBG RNA (cRNA probe) was synthesized in a similar manner with the substitution of [^{35}S]UTP (approximately 1300 Ci/mmol) for radioinert UTP. Sense CBG mRNA was passed over a Nick™ column (Sephadex G-50; Pharmacia) to ensure unincorporated ribonucleotides were removed prior to concentration determination by optical density at 260 nm.

2.8.2 Generation of a Standard Curve

Solution hybridization assays were performed as previously described (Smith

and Hammond, 1989; Smith and Hammond, 1991). Radiolabelled CBG cRNA was incubated (16 h at 70°C) in triplicate with a known amount (13 - 809 pg) of CBG mRNA in 25 μg tRNA (brewer's yeast) or 25 μg tRNA alone (to measure nonspecific binding) in 100 μl of 20 mM Tris, pH 7.5, containing 0.6 M NaCl, 10 mM EDTA, and 0.2% SDS. Subsequently, 1 ml RNA digestion buffer (10 mM Tris, pH 7.5, containing 0.3 M NaCl, 5 mM EDTA, 75 μg/ml denatured salmon sperm DNA, 25 μg/ml RNase A, and 200 U/ml RNase T_1) was added, and the incubation was continued for 30 min at 37°C. Remaining mRNA/cRNA hybrids were precipitated with 370 μl ice-cold 30% trichloroacetic acid, and washed with 1 ml 5% trichloroacetic acid/0.01% Triton X-100. The pellets were resuspended in 400 μl of 20 mM NaOH and the radioactivity was measured in the presence of 4 ml Aqueous Counting Scintillant (Amersham).

2.8.3 Measurement of Unknowns

Before solution hybridization analysis, approximately 25 μg of each RNA sample was subjected to 1% agarose gel electrophoresis in the presence of formaldehyde (Rave *et al.*, 1979), and was stained with ethidium bromide to assess the quality of RNA. Only intact RNA, as judged by the integrity of the 18 S and 28 S ribosomal bands, was used for analysis. Hepatic RNA extracts (10 - 25 μg determined by optical density at 260 nm) were measured in triplicate in the solution hybridization assay as described in section 2.8.2. In addition, 25 μg of placental RNA extract was used as a negative control.

2.8.4 Assay Parameters

In this assay, the lowest detectable amount of CBG mRNA (<0.3 pg CBG mRNA/μg RNA) was defined as the mean + 2 standard deviations (SD) of values obtained for placental RNA. The intrassay coefficient of variation (standard deviation ÷ mean × 100%) was 13.8 and interassay coefficients of variation were 10.9, 14.3 and 12.6% at high (57.5 pg/μg), medium (20.7 pg/μg) and low (14.7 pg/μg) CBG mRNA concentrations, respectively.

2.9 Nuclear Run-off Assay

2.9.1 Isolation of Nuclei

Rats treated with either dexamethasone, L-thyroxine or sesame oil as described in section 2.21 were sacrificed by CO_2 inhalation and livers were dissected and placed into ice cold physiological saline for the immediate isolation of nuclei by a modified method of Schibler *et al.* (1983). In brief, liver was finely minced, placed in 3 - 4 volumes buffer A (10 mM HEPES, pH 7.6 containing 15 mM KCl, 0.15 mM spermine, 0.5 mM spermidine, 1 mM EDTA, 2 M sucrose, 5% glycerol, 0.5 mM DTT, 0.5 mM PMSF and 0.001% trasylol), and homogenized at 0°C in a Potter model S homogeniser (Braun). The homogenate was layered over a 10 ml pad of buffer B (buffer A containing 10% glycerol) and centrifuged at 24,000 rotations per min in a Beckman SW27 rotor for 1 h at 4°C. Nuclei were resuspended in solution C [20 mM Tris, pH 7.9 containing 75 mM NaCl, 0.5 mM EDTA, 0.85 mM DTT, 0.125 mM PMSF, 50% glycerol and 100 U RNA Guard/ml (Pharmacia)] at a concentration of 10^6 nuclei/μl and stored in liquid nitrogen until use.

2.9.2 Elongation of Nascent RNA Chains

Nascent RNA chains of the purified nuclei were subjected to *in vitro* elongation in a cocktail [100 mM Tris pH 7.9 containing 300 mM $(NH_4)_2SO_4$, 4 mM $MgCl_2$, 4 mM $MnCl_2$, 200 mM NaCl, 0.4 mM EDTA, 0.1 mM PMSF, 1.2 μM DTT, 10 mM creatine-phosphate, 20 units/ml RNA Guard, 29% glycerol, approximately 10^7 nuclei, 150 μCi [^{32}P]UTP (800 Ci/mmol) and 1 mM each of GTP, ATP and CTP] at 27°C for 30 min (Schibler *et al.*, 1983). Reaction mixtures were subsequently treated (30 min at 27°C) with 2 units RQ1 DNase in the presence of 100 μg yeast tRNA and RNA was extracted with guanidinium thiocyanate-phenol-chloroform and precipitated with isopropanol (Chomczynski and Sacchi, 1987). To increase hybridization efficiency, RNA was treated with dilute NaOH (0.2 M) for 15 min on ice, and ethanol precipitated.

2.9.3 Quantification of *In Vitro* Synthesized RNA

Nitrocellulose filters were prepared (Kafatos *et al.*, 1979) for each elongation with 4 μg each of rat CBG cDNA (Smith and Hammond, 1989) in pT3/T7, ß-actin cDNA (Minty *et al.*, 1983) in pT3/T7, albumin cDNA in pBR322 (Turcotte *et al.*, 1985) and pT3/T7 alone. Filters were prehybridized in run-off hybridization buffer [50 mM HEPES, pH 7, containing 0.75 M NaCl, 0.5% SDS, 2 mM EDTA, 10x Denhardt's solution, 200 μg salmon sperm DNA/ml, 10 μg polyA/ml and 50% formamide] and then hybridized in equal volumes of run-off buffer containing radiolabelled nuclear RNA extracts (approximately 2 x 10^7 cpm/ml) for 60 h at 42°C. Filters were subsequently washed in 0.1 x SSC/0.1% SDS, exposed to XAR2

film at -80°C with a Lightning Plus intensifying screen, and resulting flourographic signals were quantified as described in section 2.7.

2.10 Reverse Transcription of Rat CBG mRNA and PCR Amplification of the Corresponding cDNA

Rat hepatic poly(A)$^+$ RNA was used to synthesize CBG cDNAs by reverse transcription (RT) and PCR amplification (Grady and Campbell, 1989) using primers listed in Table 2.2. Briefly, the cDNAs were prepared as two fragments that overlap a 123 bp sequence containing a unique *Hind*III site (Smith and Hammond, 1989). First strand synthesis was performed using 1 μg poly(A)$^+$ RNA in the presence of 50 pmol primer (either Rat5-2 or Rat3-2), 0.5 mM dNTP and 2 U reverse transcriptase. Primer Rat5-1 was used with the single-stranded cDNA produced using primer Rat5-2 for second strand synthesis, while primer Rat3-1 was used with the single-stranded cDNA produced using primer Rat3-2. Thirty cycles of PCR amplification (1 min at 94°C, 2 min at 65°C and 3 min at 72°C) was performed in the presence of 100 pmol of each primer, 0.5 mM dNTP and 2.5 U *Taq* DNA polymerase. The products were digested with *Eco*RI and *Kpn*I, resolved by low-melting-temperature agarose, recovered (Wieslander, 1979) and ligated into pBluescript for sequencing. The RT-PCR of BioBreeding rat CBG mRNA was repeated and the resulting cDNAs were sequenced to exclude errors that might have occurred during the PCR.

Table 2.2 Primer sequences used for PCR amplification of rat CBG cDNAs. Restriction endonuclease sequences (underlined) for *Eco*RI (Rat5-2 and Rat3-2) or *Kpn*I (Rat5-1 and Rat3-1) were incorporated into primers to facilitate subsequent cloning. Nucleotide positions are given in reference to Figure 3.2.

Primer	Sequence	Nucleotide Position
Rat5-1	5'-GGGGTTTGGTACCATGTCACTCGCCCTGTATACC	45 - 65
Rat5-2	5'-GGGGAAAGAATTCAAGCTTGCCCCACCTATCAATT	complementary to 872 - 893
Rat3-1	5'-GGGGTTTGGTACCCAGCTGATACAGATGGACTATG	771 - 792
Rat3-2	5'-GGGGAAAGAATTCGGCTTCTCAGGACACGTTC	complementary to 1236 - 1254

2.11 PCR Amplification of Regions of the Human CBG Gene

Genomic DNA was extracted from white blood cells by incubation (45 min at 56°C) in 10 mM Tris, pH 8.3, containing 50 mM KCl, 2.5 mM $MgCl_2$, 0.5% Tween 20 and 100 μg proteinase K/ml (Kawasaki, 1990). Following proteinase inactivation (10 min at 95°C), the coding sequences within the human CBG gene were amplified by the polymerase chain reaction (PCR) using the oligonucleotide primers listed in Table 2.3. The PCR was performed in a 100 μl reaction mixture containing approximately 1 μg genomic DNA, 100 pmol of each primer, 250 μM of each dNTP and 2.5 units of *Taq* DNA polymerase. Reaction mixtures were subjected to 30 cycles of amplification (denaturation at 94°C for 1 min, annealing at 48°C for 2 min and extension at 72°C for 3 min). The PCR-amplified DNA fragments were ligated into the *Eco*RI site of pBluescript. Each exon was amplified on two separate occasions, and three to five independent clones of each PCR reaction were sequenced.

2.12 Site-directed Mutagenesis of Rat and Human CBG cDNAS

A BioBreeding rat CBG cDNA (1,228 bp) containing the coding sequence for the CBG precursor polypeptide was constructed by combining the two fragments obtained by RT-PCR at the common internal *Hind*III site. This was done by digesting pBluescript KS containing the 5' *Kpn*I-*Eco*RI CBG cDNA fragment with *Hind*III and *Eco*RI, and directionally inserting the *Hind*III-*Eco*RI portion of the 3' *Kpn*I-*Eco*RI CBG cDNA. This insert was excised by digestion with *Kpn*I and *Eco*RI and subcloned into pSELECT-1. A human CBG cDNA (Hammond *et al.*, 1987)

Table 2.3 Primer sequences used for PCR amplification of exons 2-5 within the human CBG gene. Restriction endonuclease recognition sequences for *Eco*RI (underlined) were incorporated into all primers to facilitate subsequent cloning.

Primer	Sequence	Position
exon 2-5'	5'-GGGGTTTGAATTCCCCATTGACTCAGAGA	37 bases 5' to exon 2
exon 2-3'	5'-GGGTTGAATTCGGATGGGCCTTCAGATG	17 bases 3' to exon 2
exon 3-5'	5'-GGGGTTTGAATTCCTGCCCTACAGACCTG	17 bases 5' to exon 3
exon 3-3'	5'-GGGGTTTGAATTCTGGGGACACGTGCATT	43 bases 3' to exon 3
exon 4-5'	5'-GGGGTTTGAATTCCAGGCCCAGCCCTGGA	31 bases 5' to exon 4
exon 4-3'	5'-GGGGTTTGAATTCGAACGAACTCAGTGCCA	68 bases 3' to exon 4
exon 5-5'	5'-GGGGTTTGAATTCGACCAACGCACAGCAG	34 bases 5' to exon 5
exon 5-3'	5'-GGGAAAGAATTCGACAGTGCTGAGGCTCT	14 bases 3' to stop codon

was excised from pBluescript KS with *Hind*III and *Xba*I and inserted into the same sites of the pSELECT-1 vector.

Single-stranded DNA of each construct was prepared for site-directed mutagenesis which was performed according to the manufacturer's instructions (Promega). In brief, mutagenic (Table 2.4) and ampicillin repair oligonucleotide primers (supplied) were annealed to the single-stranded DNA, and second strand DNA synthesis was performed in the presence of T_4 DNA polymerase and ligase. A portion of the resulting DNA was transformed into a repair deficient *E. coli* strain (BMH 71-18 mut S), and cells containing mutagenized plasmids were selected in the presence of 125 μg ampicillin/ml SOC medium (Maniatis *et al.*, 1982). Plasmid DNA was purified, transformed into *E. coli* JM107 and single-stranded DNA produced from individual colonies was examined for the targeted mutagenesis by DNA sequencing. The entire cDNA of each construct was sequenced in order to confirm that no inappropriate mutations had occurred.

2.13 Expression of CBG cDNAs in Chinese Hamster Ovary Cells

The pRc/CMV vector directs the stable expression of cDNAs using the cytomegalovirus promoter and enhancer (Invitrogen Corporation). The 1,228 bp rat CBG cDNAs were excised from pSelect-1 with *Kpn*I and *Hind*III and inserted into *Hind*III-digested pRc/CMV by blunt-end ligation. The products were examined by restriction enzyme digestion to ascertain that the cDNAs were incorporated in the appropriate orientation for expression. The human CBG cDNAs were exised from pSELECT-1 as *Hind*III - *Xba*I fragments and inserted into a similarly digested

Table 2.4 Oligonucleotide primers used for site-directed mutagenesis of rat and human CBG cDNAs.

Primer	CBG cDNA	Primer Sequence	Targeted mutation
A	BB rat	5'-GGTATCAGACATGGAGAAT	Ile^{276}→Met^{276}
B	human	5'-TTGCAAAGTGTTGGTGCAG	Leu^{91}→His^{91}
C	human	5'-CAAAGGGCTGTGTAAATGTGCCTTTGAAG	Trp^{185}→Phe^{185}
D	human	5'-CGTCCACATGGAAGTTCTC	Tyr^{300}→His^{300}
E	human	5'-GAGCTCTGCGTCATGAAG	Ser^{224}→Ala^{224}
F	human	5'-CTGCACCAGCCAGCAGGGGAG	Gln^{229}→Trp^{229}
G	human	5'-CAGCTGGCTGGGGAGCT	Cys^{228}→Ser^{228}
I	human	5'-CTGTCTGCTGGCCGTTGTTGCCCAGTC	Thr insertion between residues 143 & 144
H	human	5'-GCTCTGAGTCACGAAAGTAACTGATGG	$Leu^{221}His^{222}$→ $Phe^{221}Arg^{222}$

pRc/CMV vector. A cDNA encompassing the entire coding sequence of squirrel monkey CBG was produced by inserting a 769 bp *Pst*I - *Sac*I fragment from the RT/PCR reaction (5'-fragment) (described in section 2.2.4) into a pSP72 vector (Promega) with a contiguous 494 bp *Sac*I - *Eco*RI fragment from the PCR amplified (3') cDNA (section 2.2.4). The *Pst*I - *Eco*RI fragment was subcloned into pBluescript, excised as a *Hind*III - *Xba*I cassette and inserted into a similarly digested pRC/CMV vector.

The recombinant pRc/CMV-CBG constructs were progated in *E. coli* JM107, purified with Qiagen columns (see section 2.3), and then transfected into Chinese hamster ovary (CHO pro$^-$, wild type) cells using the Polybrene®/dimethylsulphoxide technique (Chaney *et al.*, 1986). In brief, exponentially growing CHO cells (approximately 5 x 10^5) were incubated overnight in α-minimal essential medium (MEM) containing 10% fetal bovine serum (MEM/FBS), the medium was replaced with 3-4 ml MEM/FBS containing plasmid DNA (2-10 μg), followed by 5 μl Polybrene® (Aldrich Chemical Company) solution (10 mg/ml sterile water). The cells were incubated for 6 h at 37°C, after which the DNA-Polybrene® mixture was removed, and 5 ml 30% dimethylsulphoxide in MEM/FBS was added for 4 min. Transfected cells were cultured in 25 ml MEM/FBS for 48 h prior to the addition of 1.8 mg G418/ml for selection of neomycin resistance. After 10-12 days, G418 resistant colonies were trypsinized, and grown to confluency in the presence of G418 for an additional 10 days. Cells were then washed with PBS and grown in Dulbecco's modified Eagle medium for 2-3 days. The medium was then removed and stored at -20°C for subsequent analysis.

2.14 Purification of CBG from Serum

Adult rat CBG was isolated from serum prepared directly from blood samples or obtained commercially (Bioproducts for Science Incorporated). Serum obtained from 3 week old animals was used as a source of infant rat CBG, and human CBG was isolated from outdated pregnancy serum (Blood Bank, Victoria Hospital, London, Ontario). Purification procedures were employed as described previously (Seralini *et al.*, 1989). In brief, endogenous steroids were removed from the serum by exposure to dextran-coated charcoal (DCC) and the initial CBG purification was performed by affinity chromatography using 11ß-hydroxy-androst-4-en-3-oxo-17ß-carboxylic acid linked to diaminoethyl-oxirane Sepharose CL-4B. Following Reactive Blue 2-Sepharose CL-6B (Sigma) chromatography to remove contaminating albumin, additional purification was achieved by ion exchange chromatography using a 0 - 0.5 M NaCl gradient on an Fast Protein Liquid Chromatography (FPLC) MonoQ column (Pharmacia). BioBreeding and Wistar rat CBG was also subjected to size exclusion chromatography using an FPLC Superose 6 column (Pharmacia). Product purities were examined by SDS-PAGE.

2.15 Gel Electrophoresis of CBG

2.15.1 SDS-PAGE of CBG

Protein samples were incubated (100°C for 4 min) in the presence of 0.1 M ß-mercaptoethanol and 1% SDS and applied to an SDS - polyacrylamide gel consisting of a 4% acrylamide in the stacking gel and 7.5% in the separating gel (Laemmli, 1970). Following electrophoresis (approximately 17 volts/cm), gels were

either fixed in 30% methanol/10% glacial acetic acid and proteins were stained with Coomassie brilliant blue, or further processed (section 2.6.3) prior to fluorography. Pre-stained SDS-PAGE size standards were obtained from Bio-Rad Laboratories.

2.15.2 Western Blot Analysis of CBG

Protein samples were subjected to 7.5% PAGE (4% stacking gel) in the absence of SDS or ß-mercaptoethanol and were transferred electrophoretically (Towbin *et al.*, 1979) to Immobilon-P membrane (Millipore Corporation). Blots were blocked with 4% bovine serum albumin (BSA) in TBS (50 mM Tris pH 8, containing 150 mM NaCl) and subsequently incubated with antisera (diluted 1:500 in TBS/1% BSA) against either human (Robinson *et al.*, 1985b) or squirrel monkey (Kuhn *et al.*, 1988) CBG. Immunoreactive proteins were revealed using alkaline phosphatase-labelled goat antirabbit IgG (Jackson Immunoresearch Laboratories Incorporated) and Protoblot Immunoblotting reagents (Promega).

2.16 Iodination of CBG

Purified human or rat CBG (approximately 50 μg) in 0.1 M borate buffer (pH 8.0) was added to [^{125}I]Bolton-Hunter reagent and incubated at 4°C for approximately 16 h. Radiolabelled proteins were separated from low molecular weight (M_r) material on a Sephadex G-25 column (PD-10; Pharmacia), which was pre-equilibrated and eluted with 0.1 M Tris-HCl, (pH 8.0) containing 0.1 M NaCl and 0.3% gelatin. The material in the void volumne had a specific activity of approximately 1.4 μCi/μg protein.

2.17 Serum CBG Measurements

2.17.1 CBG Binding Capacity Assay

A steroid binding capacity assay (Hammond and Lähteenmäki, 1983) was used to measure CBG levels in rat and human serum. Samples were diluted 1:100 in an DCC suspension, and incubated (30 min at 20°C) to remove endogenous steroids. After centrifugation (3,000 x g for 10 min), the supernatants were further diluted as appropriate in PBS containing 0.01% gelatin, and aliquots were incubated (1 h at 20°C) with a saturating amount (10 nM) of [^{3}H]cortisol (approximately 55 Ci/mmol) in the presence and absence of 2 μM cortisol. Separation of CBG-bound steroid was achieved by incubation (10 min at 0°C) with 600 μl DCC suspension followed by centrifugation (2,000 x g for 5 min at 4°C). The supernatants were added to 4 ml Aqueous Counting Scintillant and subjected to liquid scintillation counting. Occasionally, [^{3}H]corticosterone (approximately 88 Ci/mmol) was used as labelled ligand to measure rat serum CBG levels with 2 μM corticosterone to measure non-specific binding. Under these assay conditions, dissociation of normal rat CBG-bound [^{3}H]cortisol, rat CBG-bound [^{3}H]corticosterone and normal human CBG-bound [^{3}H]cortisol is 35%, 20% and 10%, respectively during DCC separation of bound and free, and these factors were taken into account when results were calculated.

2.17.2 CBG Radioimmunoassay

Rat serum samples were diluted appropriately in PBS containing 1% horse serum and 0.01% gelatin, and incubated at 20°C with an equal volume (100 μl) of

1:5000 rabbit antiserum against rat CBG and ^{125}I-labelled rat CBG (40,000 counts per min, see above). After 16 h, 500 μl of kaolin-conjugated, donkey anti-rabbit immunoglobulin (Farmos Diagnostica) was added and allowed to incubate for 1 h at 20°C. Following centrifugation (2,000 x g, 5 min), supernatants were removed and pellets were counted. Values were standardized against an adult female serum sample containing 1,503 pmol CBG/ml when measured by steroid binding capacity assay. Interassay variability was less than 9%.

The CBG concentrations of human serum samples were determined with a solid-phase radioimmunoassay (Robinson *et al.*, 1985b) In brief, serum samples were diluted (1:500) in PBS containing 1% horse serum and 0.01% gelatin, and incubated (16 h at 20°C) with an equal volume of a slurry of anti-human CBG antiserum covalently linked to carbodiimidazole-activated cellulose (Chapman and Ratcliffe, 1982) and ^{125}I-labelled human CBG (40,000 counts per min, see above). After the addition of 0.9% NaCl and centrifugation (2,000 x g for 5 min), supernatants were removed and pellets were counted. Values were standardized against serum samples with previously determined CBG levels (Robinson *et al.*, 1985b).

2.18 CBG Steroid Binding Characteristics

2.18.1 'Apparent' Dissociation Rate Analysis

Serum samples were saturated with [^{3}H]cortisol or [^{3}H]corticosterone, as described in section 2.17.1, and the amount of CBG-bound steroid was determined after incubation with DCC for various lengths of time at 0°C (Hammond and

Lähteenmäki, 1983). The amount of CBG-bound [^{3}H]steroid present before charcoal addition was determined by extrapolation.

2.18.2 Scatchard Analysis

[^{3}H]Cortisol and [^{3}H]corticosterone were both used to determine CBG affinity constants by Scatchard analysis (Scatchard, 1949). Diluted serum (human or rat) or culture media were incubated (16 h at 4°C) with varying concentrations (approximately 0.0078 -1 pmol) of radiolabelled steroid. Radioinert cortisol or corticosterone (2 μM) was used to measure non-specific binding. Free and CBG-bound steroid were separated with DCC at 0°C as described in section 2.17.1.

2.18.3 Relative Steroid Binding Affinity Analysis

Approximately 0.1 pmol of purified Wistar or BioBreeding CBG were incubated with a saturating amount (10 nM) of [^{3}H]corticosterone and increasing amount of unlabelled steroids (corticosterone, deoxycorticosterone, cortisol, 11-deoxycortisol, progesterone and 11ß-hydroxyprogesterone). Separation of CBG-bound [^{3}H]corticosterone was achieved with DCC at 0°C. The amount of competing steroid required to displace 50% of the [^{3}H]corticosterone was used as a measure of its binding affinity for CBG relative to corticosterone (Lähteenmäki *et al.*, 1983).

2.19 Size Exclusion Chromatography of CBG

The size of CBG present in culture media was determined by size exclusion chromatography using an FPLC Superose 12 column (Pharmacia) equilibrated in

50 mM phosphate buffer, pH 7.5 containing 150 mM NaCl. Fractions (0.5 ml) were collected and immunoreactive CBG was measured by radioimmunoassay (see section 2.17.2). Gel filtration size standards (molecular weight, 1,350 - 670,000) were obtained from Bio-Rad Laboratories.

2.20 Determination of the Biological Half-life of Serum CBG

To compare the biological half-lives of CBG isolated from infant and adult rat serum, [^{125}I]-labelled CBG was injected into the tail veins of Halothane-anaesthetized 3 week old or adult rats. Blood (approximately 100 μl) was obtained from the tip of the tail at intervals between 1 and 21 h after injection, and 20 μl aliquots of serum were prepared for radioactivity measurements.

2.21 Hormonal Treatment of Rats

Intact male rats were maintained on a standard photoperiod with access to food and water *ad libitum*. Rats were lightly anesthetized (Halothane or ether) and initial blood samples were obtained in the morning by cardiac puncture. Treatment consisted of twice daily subcutaneous injections of glucocorticoids (dexamethasone, prednisolone and corticosterone), estrogens (ethinylestradiol, estradiol and estrone), progesterone, 5α-dihydrotestosterone and thyroid hormone (L-thyroxine). All steroids were administered at 25 μg/100 g body weight in sesame oil (vehicle) and L-thyroxine was given at 10 μg/100 g body weight in 0.3 mM NaOH (vehicle). At the conclusion of the treatment period, animals were sacrificed approximately 5 h after the last injection (afternoon), and blood and liver samples were obtained.

Serum was prepared and stored at -20°C and tissue was stored at -80°C until analysis.

A time course study was also performed in which rats received the same treatment regime of dexamethasone, thyroxine or sesame oil, but blood samples were collected every morning by tail clip for the preparation of serum and CBG measurement.

2.22 Concanavalin A-Chromatography of Rat Serum

Rat serum samples (approximately 100 μl) were applied to concanavalin A-agarose (Con A; Type III-AS, Sigma) columns pre-equilibrated in Con A buffer [50 mM Tris (pH 7.4) containing 0.5 M NaCl, 1 mM $CaCl_2$, 1 mM $MnCl_2$ and 1 mM $MgCl_2$], and fractions were collected until non-bound protein had eluted. Specifically-bound proteins were displaced with Con A buffer containing 5% methyl-α-D-mannopyranoside (weight/volume) and 5% dimethylformamide (volume/volume) and 1 ml fractions were collected. Aliquots of all fractions were analyzed for CBG content by radioimmunoassay (section 2.17.2), and CBG-containing samples were pooled, dialysed against PBS (16 h at 4°C), and subjected to CBG measurement by corticosterone binding capacity assay (section 2.17.1) and protein measurement by Bradford assay using bovine serum albumin as standard (Bradford, 1976).

2.23 Cecal Ligation and Perforation

Normal adult male rats were subjected to cecal ligation and perforation (CLP)

to induce sepsis (Wichterman *et al.*, 1980). All animals, including controls, were anesthetized (0.5 mg Rompun and 3.75 mg ketamine/100 g body weight) prior to any treatment, and received analgesic (1 mg Talwin/100 g body weight, every 6 h) throughout the duration of the experiment. In test animals, a midline incision was made and the cecum was removed from the body cavity, filled with stool, ligated just below the ileocecal valve and punctured either once or twice with an 18 gauge needle. The cecum was then returned to the abdomen and the incision was closed with sutures in two layers. A bolus of physiological saline (30 ml) was injected subcutaneously in the back of animals to prevent dehydration. Surgery was not performed on control animals. This protocol was approved by the University of Western Ontario Council on Animal Care.

2.24 Statistics

Data analysis involved determination of means, standard deviation (SD) and standard error of the mean (SE). Student's *t*-test was used to assess statistical differences amongst means which were considered significant when $p < 0.05$. Linear regressions were calculated by the least squares methods.

CHAPTER 3

Phylogenetic Comparisons of cDNA-deduced Corticosteroid Binding Globulin Primary Structures

3.1 Introduction

Corticosteroid binding globulin has been purified from the blood of several mammalian species, and may be generally characterized as an acidic glycoprotein with a molecular mass of 44-60 kiloDaltons (Kato *et al.*, 1988). It has been subjected to extensive physicochemical analyses (Westphal, 1971; Westphal, 1986; Nyberg and Jones, 1988; Kuhn *et al.*, 1988) and between species, differences in CBG's relative steroid binding affinity (Westphal, 1971), stoichiometry (Klosterman *et al.*, 1986; Kuhn *et al.*, 1988), and immunological properties (Kato *et al.*, 1988; Faict and De Moor, 1986) have been observed. However, no information is available with which to examine these differences in the context of CBG's primary structure. The cDNA-deduced primary structures of rat, mouse, rabbit and squirrel monkey CBG were therefore determined, and aligned with human CBG (Hammond *et al.*, 1987) in the expectation that this would reveal information about biologically important regions of CBG.

3.2 Results

3.2.1 Isolation and Characterization of Rat CBG cDNAs

Hybridization of a rat liver cDNA library with a human CBG cDNA at reduced stringency identified 14 positive clones, and upon induction with IPTG, two of these clones produced ß-galactosidase fusion proteins that reacted with an antibody against rat CBG. This is consistent with a 1 in 6 probability of cDNA insertion into λgt11 in the correct reading frame and orientation to produce immunoreactive fusion proteins. The two largest cDNA inserts were isolated, and appropriate

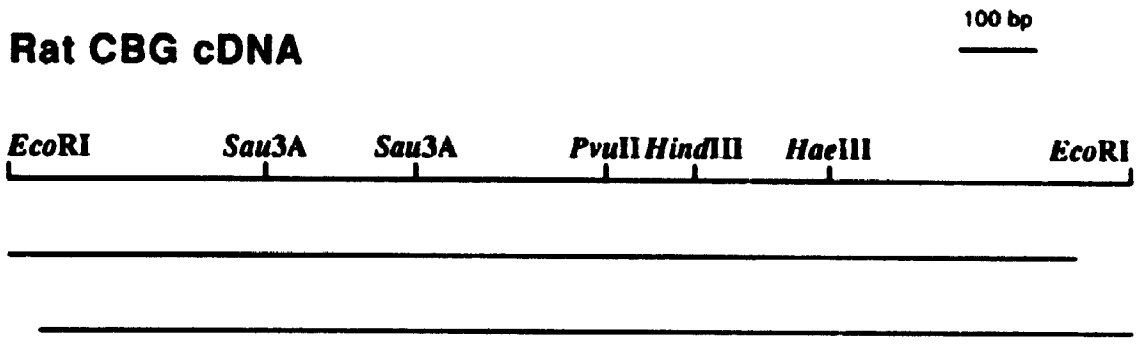
restriction fragments (Figure 3.1) were analyzed to confirm the entire sequence in both directions. Both cDNAs contained inversions at their 5'-ends, which probably represent artifacts produced during library construction (Land *et al.*, 1981). However, when overlapping sequences were compared, a sequence of 1452 nucleotides was obtained (Figures 3.1 and 3.2). Within this sequence, an initiation codon (located 44 nucleotides from the 5'-end) starts an open reading frame of 396 amino acids that terminates 196 nucleotides before the polyadenylated 3'-end. A second in-frame minicistron (predicted amino acid sequence: M-E-A-F-P-Q-S-S-Q-G-L-P-N-P-I-Y-F-stop; single letter code) is located between the CBG reading frame and a potential polyadenlyation signal (Humphrey and Proudfoot, 1988) located 12 nucleotides before the poly(A) tail.

The known amino-terminal sequence of rat CBG (Kato *et al.*, 1988) was located 22 amino acids from the initiation codon for the CBG precursor. The mature rat CBG polypeptide should therefore contain 374 amino acids and have a molecular mass of 42,229 Daltons (Da). In addition, six consensus sites (Bause, 1983) for *N*-glycosylation (Asn - X - Ser/Thr, where X is not a Pro) are present within the open reading frame (Figure 3.2).

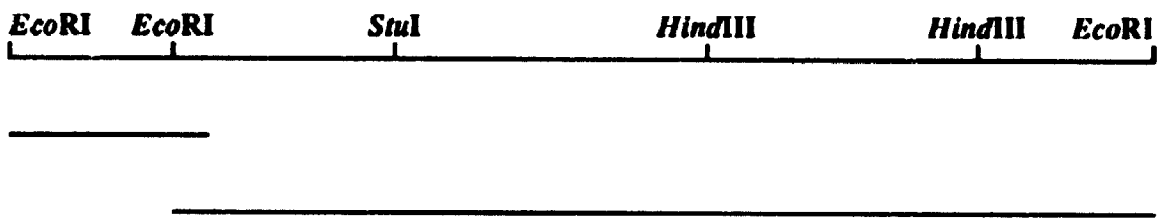
The rat CBG antibody was used to confirm the identity of proteins produced *in vitro* from CBG cDNA hybrid-selected RNA. When rat liver poly(A)$^+$ RNA was translated with a rabbit reticulocyte lysate, the antibody recognized several of the minor products; one of which was approximately 43 kDa (Figure 3.3), and the relative abundance of this immunoreactive protein was increased when a CBG

Figure 3.1 Partial restriction maps of cDNAs isolated from rat, mouse, rabbit and squirrel monkey CBG. The relative sizes of overlapping fragments, from which the combined cDNA sequences were generated, are shown below each map.

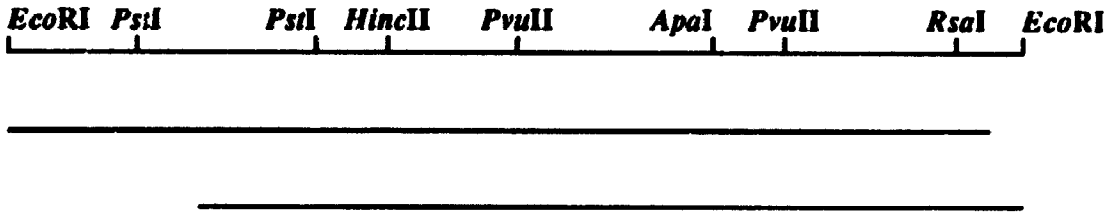
Rat CBG cDNA



Mouse CBG cDNA



Rabbit CBG cDNA



Squirrel Monkey CBG cDNA

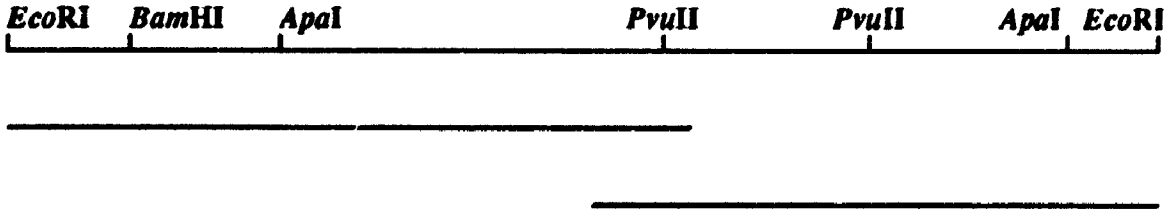
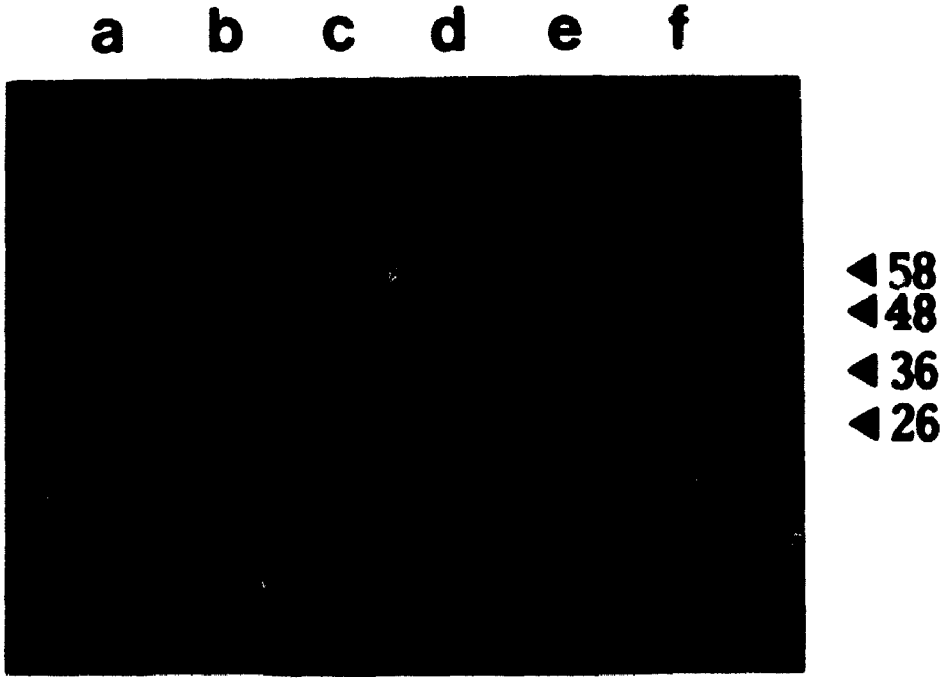


Figure 3.2 Combined nucleotide sequence of rat liver CBG cDNAs, and deduced amino acid sequence of the precursor CBG polypeptide. The amino acids in the mature peptide are numbered 1 to 374, and those in the signal peptide are -22 to -1. There are six consensus sequences for *N*-glycosylation (●). A second in-frame minicistron and a potential polyadenylation signal are underlined. Positions of amino acids are given on the *left*, and those of nucleotides on the *right*.

```
                                                                AACAGCCGGAGCCCACAGCAGCAGACGGCCTGGTCAACTGAACA   44

-22 Met Ser Leu Ala Leu Tyr Thr Cys Leu Leu Trp Leu Cys Thr Ser Gly Leu Trp Thr Ala
    ATG TCA CTC GCC CTG TAT ACC TGC CTC CTC TGG CTC TGC ACC AGT GGC CTC TGG ACT GCC  104
          -1  +1
           ●
 -2 Gln Ala Ser Thr Asn Glu Ser Ser Asn Ser His Arg Gly Leu Ala Pro Thr Asn Val Asp
    CAA GCC AGC ACT AAT GAG AGT TCA AAT TCT CAC CGA GGC CTG GCT CCC ACC AAT GTT GAC  164

 19 Phe Ala Phe Asn Leu Tyr Gln Arg Leu Val Ala Leu Asn Pro Asp Lys Asn Thr Leu Ile
    TTT GCC TTC AAC TTG TAC CAA CGC CTA GTG GCC CTA AAT CCA GAC AAG AAC ACC TTA ATC  224

 39 Ser Pro Val Ser Ile Ser Met Ala Leu Ala Met Val Ser Leu Gly Ser Ala Gln Thr Gln
    TCC CCA GTG AGC ATC TCC ATG GCT CTG GCC ATG GTA TCC CTT GGC TCT GCC CAG ACT CAG  284
                 ●
 59 Ser Leu Gln Ser Leu Gly Phe Asn Leu Thr Glu Thr Ser Glu Ala Glu Ile His Gln Ser
    TCT CTC CAG AGT CTA GGC TTC AAC CTC ACA GAG ACC TCT GAA GCT GAG ATC CAC CAG AGT  344

 79 Phe Gln Tyr Leu Asn Tyr Leu Leu Lys Gln Ser Asp Thr Gly Leu Glu Met Asn Met Gly
    TTC CAG TAC CTC AAT TAC CTT CTC AAG CAG TCC GAT ACT GGC TTA GAG ATG AAC ATG GGC  404

 99 Asn Ala Met Phe Leu Leu Gln Lys Leu Lys Leu Lys Asp Ser Phe Leu Ala Asp Val Lys
    AAT GCC ATG TTC CTC CTC CAG AAG CTG AAG CTG AAG GAC TCG TTC TTA GCA GAC GTC AAA  464

119 Gln Tyr Tyr Glu Ser Glu Ala Leu Ala Ile Asp Phe Glu Asp Trp Thr Lys Ala Ser Gln
    CAA TAC TAT GAG TCA GAG GCC TTG GCC ATC GAT TTT GAG GAC TGG ACT AAA GCC AGC CAA  524

139 Gln Ile Asn Gln His Val Lys Asp Lys Thr Gln Gly Lys Ile Glu His Val Phe Ser Asp
    CAG ATC AAC CAG CAT GTC AAG GAT AAG ACA CAG GGG AAA ATT GAG CAT GTG TTC TCA GAC  584

159 Leu Asp Ser Pro Ala Ser Phe Ile Leu Val Asn Tyr Ile Phe Leu Arg Gly Ile Trp Glu
    CTG GAT AGT CCA GCC TCC TTC ATC CTG GTC AAC TAC ATC TTC CTC AGA GGC ATA TGG GAA  644
                                                  ●
179 Leu Pro Phe Ser Pro Glu Asn Thr Arg Glu Glu Asp Phe Tyr Val Asn Glu Thr Ser Thr
    CTT CCC TTC AGC CCA GAA AAT ACT AGA GAG GAG GAC TTC TAT GTG AAT GAG ACA AGC ACC  704

199 Val Lys Val Pro Met Met Val Gln Ser Gly Ser Ile Gly Tyr Phe Arg Asp Ser Val Phe
    GTG AAG GTG CCC ATG ATG GTC CAG TCA GGC AGC ATT GGT TAC TTT CGT GAC TCA GTC TTC  764
                       ●
219 Pro Cys Gln Leu Ile Gln Met Asp Tyr Val Gly Asn Gly Thr Ala Phe Phe Ile Leu Pro
    CCC TGC CAG CTG ATA CAG ATG GAC TAT GTG GGA AAT GGA ACT GCC TTC TTC ATT CTT CCA  824

239 Asp Gln Gly Gln Met Asp Thr Val Ile Ala Ala Leu Ser Arg Asp Thr Ile Asp Arg Trp
    GAC CAG GGC CAG ATG GAC ACT GTC ATC GCT GCA CTT AGT CGG GAC ACA ATT GAT AGG TGG  884

259 Gly Lys Leu Met Thr Pro Arg Gln Val Asn Leu Tyr Ile Pro Lys Phe Ser Met Ser Asp
    GGC AAG CTT ATG ACC CCA AGG CAG GTG AAC CTA TAC ATC CCG AAA TTC TCC ATG TCT GAT  944
                                                        ●
279 Thr Tyr Asp Leu Lys Asp Val Leu Glu Asp Leu Asn Ile Lys Asp Leu Leu Thr Asn Gln
    ACC TAT GAC CTT AAA GAC GTG CTG GAA GAC CTG AAC ATT AAG GAC TTG CTC ACC AAC CAA 1004

299 Ser Asp Phe Ser Gly Asn Thr Lys Asp Val Pro Leu Thr Leu Thr Met Val His Lys Ala
    TCA GAT TTC TCA GGC AAC ACC AAA GAT GTT CCC TTG ACA TTA ACG ATG GTC CAC AAG GCC 1064
                       ●
319 Met Leu Gln Leu Asp Glu Gly Asn Val Leu Pro Asn Ser Thr Asn Gly Ala Pro Leu His
    ATG CTA CAA CTG GAT GAA GGG AAT GTG TTG CCT AAT TCT ACC AAC GGG GCT CCC CTA CAC 1124

339 Leu Arg Ser Glu Pro Leu Asp Ile Lys Phe Asn Lys Pro Phe Ile Leu Leu Leu Phe Asp
    CTG CGC TCT GAA CCA CTT GAC ATC AAG TTC AAC AAG CCC TTC ATC CTC CTG CTC TTT GAC 1184
                                                                  374
359 Lys Phe Thr Trp Ser Ser Leu Met Met Ser Gln Val Val Asn Pro Ala ---
    AAG TTC ACA TGG AGC AGC CTG ATG ATG AGC CAA GTG GTC AAT CCA GCC TAA GAACGTGTCCT 1246

    GAGAAGCCTTGGTGCCATCTGACTTTGAGGGATGGAAGCCTTTCCCCAGTCTTCTCAGGGTCTCCCCAACCCAATCTAC 1325

    TTTTAGATAACTAGCAATGAGTAGCCCTTGTTGCCACTCTCAAGACAAAGTCACTTGAGAAGGGAGCACTGAGAAAGAG 1404

    GTAACAGTTATTAAAGGATGTTTTTGCA21                                                    1452
```

Figure 3.3 Fluorograph of rat liver RNA translation products. Samples were resolved by 12% SDS-PAGE, fixed and treated with EN^{3}HANCE before fluorography (18 h at -80^{o}C). Lane a, control *in vitro* translation (water); lane b, immunoadsorption of control translation with rat CBG antibody; lane c, *in vitro* translation of rat liver poly(A)$^+$ RNA; lane d, immunoadsorption of (c) with rat CBG antibody; lane e, *in vitro* translation of CBG cDNA hybrid-selected rat mRNA; lane f, immunoadsorption of (e) with rat CBG antibody. Protein size standards (in kDa) are shown on the *right*.


a
b
c
d
e
f
58
48
36
26

cDNA was used to hybrid-select hepatic RNA prior to its translation. An approximately 36 kDa protein was the only other immunoreactive translation product of hybrid-selected mRNA.

3.2.2 Isolation and Characterization of Mouse CBG cDNAs

Four positive clones were isolated from the mouse liver cDNA library. Nucleotide sequence analysis revealed that the longest cDNA (1267 bp) was of insufficient length to encode the entire CBG polypeptide, and PCR amplification of the same library was therefore employed to isolate a cDNA which overlapped and extended the sequence in the 5' direction. A 256 bp PCR product was obtained and both it and the 1267 bp fragment were sequenced on both strands. When combined, they yielded a 1,476 bp nucleotide sequence which contained a 5'-non-coding region of 54 nucleotides followed by an initiation codon for a 397 amino acid open-reading frame and a 228 bp 3'-non-coding region (Figure 3.4). A potential polyadenylation signal was located 12 nucleotides prior to the poly(A) tail.

The amino-terminal sequence of purified mouse CBG (V-T-D-E-D-S-S-S-H-R-D-L-A-P-T-N-V-D; G.L. Hammond, personal communication) was located at residues 23-40 of the open-reading frame, and it was therefore determined that the mouse precursor CBG protein was composed of a 22 amino acid signal peptide followed by the mature polypeptide of 375 amino acids which has a molecular weight of 42,277. Six consensus sites for *N*-glycosylation (Bause, 1983) were located within the amino acid sequence.

Figure 3.4 Combined nucleotide sequence of two overlapping mouse liver CBG cDNAs, and deduced amino acid sequence of the precursor CBG polypeptide. The amino acids in the mature peptide are numbered 1 to 375, and those in the signal peptide are -22 to -1. There are six consensus sites for *N*-glycosylation (●). A potential polyadenylation signal is underlined. Positions of amino acids and nucleotides are given on the *left* and *right*, respectively.

```
                                                                    AGAGCCAGAAGGAACAGCTGGAGCCCACAGCAGACGACCTGGTCAACCAAAACA   54

-22 Met Ser Leu Ala Leu Tyr Thr Cys Leu Phe Trp Leu Cys Thr Ser Gly Leu Trp Thr Thr
    ATG TCG CTT GCC CTG TAT ACC TGT CTC TTC TGG CTC TGC ACC AGT GGC CTC TGG ACC ACC  114
        -1  +1
 -2 Gln Ala Val Thr Asp Glu Asp Ser Ser Ser His Arg Asp Leu Ala Pro Thr Asn Val Asp
    CAG GCT GTC ACT GAT GAG GAT TCA AGT TCT CAC CGA GAC CTG GCT CCC ACC AAT GTT GAC  174

 19 Phe Ala Phe Asn Leu Tyr Lys Arg Leu Val Ala Leu Asn Ser Asp Lys Asn Thr Leu Ile
    TTT GCC TTC AAC TTG TAC AAA CGC CTA GTG GCC CTG AAT TCC GAC AAG AAT ACC TTA ATC  234

 39 Ser Pro Val Ser Ile Ser Met Ala Leu Ala Met Leu Ser Leu Ser Thr Arg Gly Ser Thr
    TCC CCA GTG AGC ATC TCC ATG GCC CTG GCC ATG CTG TCC CTA AGC ACC AGG GGC TCC ACC  294
                                  ●
 59 Gln Tyr Leu Glu Asn Leu Gly Phe Asn Met Ser Lys Met Ser Glu Ala Glu Ile His Gln
    CAG TAT CTC GAG AAC CTG GGC TTC AAC ATG TCA AAG ATG TCT GAA GCT GAG ATC CAC CAG  354

 79 Gly Phe Gln Tyr Leu Asn Ser Leu Leu Gln Gln Ser Asp Thr Gly Leu Glu Met Asn Met
    GGT TTC CAG TAC CTC AAT TCC CTT CTC CAG CAG TCT GAC ACT GGC TTG GAG ATG AAC ATG  414

 99 Gly Asn Val Met Phe Leu Leu Gln Asn Leu Lys Leu Lys Asp Ser Phe Leu Ala Asp Thr
    GGC AAT GTC ATG TTC CTC CTC CAG AAC CTA AAG CTG AAA GAC TCA TTC TTA GCA GAC ACC  474

119 Lys His Tyr Tyr Glu Ser Glu Ala Leu Thr Ile Pro Ser Lys Asp Trp Thr Lys Ala Gly
    AAA CAC TAT TAT GAG TCA GAG GCC TTG ACC ATC CCT TCC AAG GAC TGG ACT AAA GCC GGC  534
                                     ●
139 Glu Gln Ile Asn Asn His Val Lys Asn Lys Thr Gln Gly Lys Ile Glu His Val Val Ser
    GAA CAG ATC AAC AAT CAT GTG AAG AAT AAG ACA CAG GGG AAA ATT GAG CAT GTG GTC TCA  594

159 Asp Leu Asp Ser Ser Ala Thr Leu Ile Leu Ile Asn Tyr Ile Phe Leu Lys Gly Ile Trp
    GAC CTG GAT AGT TCA GCC ACC CTC ATC CTG ATC AAC TAC ATC TTC CTC AAA GGA ATA TGG  654
                                                    ●
179 Lys Leu Pro Phe Ser Pro Glu Asn Thr Arg Glu Glu Asp Phe Tyr Val Asn Glu Thr Ser
    AAA CTT CCC TTC AGC CCA GAA AAT ACT AGA GAG GAG GAC TTC TAT GTG AAT GAG ACA AGC  714
                                        ●
199 Thr Val Lys Val Pro Met Met Val Gln Ser Gly Asn Ile Ser Tyr Phe Arg Asp Ser Ala
    ACA GTG AAG GTG CCC ATG ATG GTC CAG TCA GGC AAC ATT AGT TAC TTT CGT GAT TCG GCG  774
                                           ●
219 Ile Pro Cys Gln Met Val Gln Met Asn Tyr Val Gly Asn Gly Thr Thr Phe Ile Ile Leu
    ATC CCC TGC CAG ATG GTA CAG ATG AAT TAT GTG GGA AAT GGA ACT ACC TTC ATC ATT CTT  834

239 Pro Asp Gln Gly Gln Met Asp Thr Val Val Ala Ala Leu Asn Arg Asp Thr Ile Asp Arg
    CCA GAC CAG GGC CAG ATG GAC ACT GTC GTC GCT GCA CTT AAT CGG GAC ACA ATT GAT AGG  894

259 Trp Gly Lys Leu Met Ile Pro Arg Gln Met Asn Leu Tyr Ile Pro Lys Phe Ser Met Ser
    TGG GGC AAG CTT ATG ATT CCA AGG CAG ATG AAC CTG TAC ATC CCC AAA TTC TCC ATG TCT  954
                                                                              ●
279 Asp Thr Tyr Asp Leu Gln Asp Val Leu Ala Asp Val Gly Ile Lys Asp Leu Phe Thr Asn
    GAT ACC TAT GAC CTT CAA GAT GTG CTG GCA GAT GTG GGC ATT AAG GAT TTG TTC ACC AAC 1014

299 Gln Ser Asp Phe Ala Asp Thr Thr Lys Asp Thr Pro Leu Thr Leu Thr Val Leu His Lys
    CAA TCT GAT TTC GCA GAC ACC ACC AAA GAC ACT CCC TTG ACA TTA ACG GTA CTC CAC AAG 1074

319 Ala Met Leu Gln Leu Asp Glu Gly Asn Val Leu Pro Ala Ala Thr Asn Gly Pro Pro Val
    GCC ATG CTG CAA CTG GAT GAA GGG AAT GTG CTG CCT GCT GCC ACC AAT GGC CCT CCT GTA 1134

339 His Leu Pro Ser Glu Ser Phe Thr Leu Lys Tyr Asn Arg Pro Phe Ile Phe Leu Ala Phe
    CAC CTG CCC TCC GAA TCA TTC ACC CTC AAG TAC AAC AGG CCC TTC ATC TTC CTG GCC TTT 1194
                                                                  375
359 Asp Lys Tyr Thr Trp Ser Ser Leu Met Met Ser Gln Val Met Asn Pro Ala ---
    GAC AAG TAC ACA TGG AGC AGC CTG ATG ATG AGC CAA GTC ATG AAT CCA GCC TAA AAGCTTG 1255

    TCCCGGAAAACCATGGTGCCATCTGACTTTGAGAGCATAGAAGTCTTTCCCCAGTCTTCTTCAGGGTCTCCCCCCAACC 1334

    CAACCCAACCAGATGCTCCCACTTTTAGACATCTTGTAATGAATAGGCCTTGTTGCTACTCTCAAAACAAATCAATCGG 1413

    GAGGGGACCACTGAGAAAGAGGTAACAGTTATTAAAGGATGTTTTTGCA15                               1476
```

3.2.3 Isolation and Characterization of Rabbit CBG cDNAs

Two positive bacteriophage clones were obtained from the rabbit liver cDNA library and their inserts were excised and found to be 1061 and 1263 bp in length. A similar restriction map was obtained for each cDNA and subsequent sequence analysis indicated that the larger clone extended 245 bp from the 5'-end of the smaller cDNA (Figure 3.1). When the cDNAs were aligned, a combined sequence of 1306 nucleotides was obtained and confirmed by completely sequencing both DNA strands (Figure 3.5). The largest open reading frame of 369 amino acids was followed by an 195 bp non-coding region which contained a potential polyadenylation signal 12 nucleotides upstream from a poly(A) tail.

When the amino acid sequence of the open reading frame was aligned with the chemically-determined amino-terminal sequence (Seralini *et al.*, 1990b) of purified rabbit CBG (A-D-P-P-G-G-D-I-S-T-R-S-P-P-R-G-L-A-P-A-N-V-D-F) a 10 amino acid overlap was obtained. It was therefore concluded that the cDNA sequence encodes residues 15 to 383 of rabbit CBG, and that the molecular weight of the mature rabbit CBG polypeptide is 42,326. In addition, four consensus sites for *N*-glycosylation (Bause, 1983) were detected.

3.2.4 Isolation and Characterization of Squirrel Monkey CBG cDNAs

Positive clones were isolated from a squirrel monkey liver cDNA library and the largest cDNA insert was 792 bp. Reverse transcription of hepatic poly(A)$^+$ RNA and subsequent PCR amplification produced an overlapping 880 bp fragment (Figure 3.1). These two fragments were sequenced completely on both DNA

Figure 3.5 Combined nucleotide sequence of two rabbit liver CBG cDNAs, and the deduced amino acid sequence of the precursor protein. The amino acids are numbered 15-383, and potential *N*-glycosylation sites are indicated (●). A potential polyadenylation signal and the amino-terminal sequence of purified rabbit CBG (residues 1-24) are underlined. Amino acid and nucleotide positions are given on the *left* and *right*, respectively.

```
1   Ala Asp Pro Pro Gly Gly Asp Ile Ser Thr Arg Ser Pro Pro Arg Gly Leu Ala Pro Ala
                                                    C CGG GGC CTG GCT CCA GCC   19

21  Asn Val Asp Phe Ala Phe Ser Leu Tyr Arg Gln Leu Val Ser Ser Ala Pro Asp Arg Asn
    AAC GTG GAC TTT GCC TTC AGC CTG TAT CGG CAG CTC GTG TCC TCG GCT CCA GAC AGG AAC   79

41  Ile Cys Ile Ser Pro Val Ser Val Ser Met Ala Leu Ala Met Leu Ser Leu Gly Ala Ser
    ATC TGC ATC TCC CCC GTG AGC GTC TCC ATG GCC TTG GCC ATG CTG TCC CTG GGC GCC TCC  139

61  Gly His Thr Arg Thr Gln Leu Leu Gln Gly Leu Gly Phe Asn Leu Thr Glu Met Pro Glu
    GGC CAC ACT CGC ACC CAG CTT CTG CAG GGC CTG GGC TTC AAC CTC ACG GAG ATG CCC GAA  199

81  Ala Glu Ile His Gln Gly Phe Gln Tyr Leu His His Leu Leu Gly Glu Ser Asp Thr Ser
    GCT GAG ATC CAC CAG GGC TTC CAG TAC CTG CAC CAC CTC CTT GGG GAG TCG GAC ACC AGC  259

101 Leu Glu Met Thr Met Gly Asn Ala Leu Phe Leu Asp His Ser Leu Glu Leu Leu Glu Ser
    TTG GAG ATG ACC ATG GGC AAC GCC TTG TTC CTG GAC CAC AGC CTG GAG CTG CTG GAG TCC  319

121 Phe Ser Ala Asp Ile Arg Arg Tyr Tyr Glu Ser Glu Ala Leu Ala Thr Asp Phe Gln Asp
    TTC TCT GCG GAC ATC AGG CGT TAC TAC GAG TCC GAG GCC TTG GCC ACA GAT TTC CAG GAC  379

141 Trp Pro Arg Ala Cys Arg Gln Ile Asn Glu Tyr Ile Glu Asn Lys Thr Gln Gly Lys Ile
    TGG CCT AGG GCC TGC AGG CAG ATC AAC GAG TAC ATC GAG AAT AAG ACA CAG GGG AAA ATT  439

161 Ala Asp Leu Phe Leu Gly Leu Glu Asn Pro Ala Ile Leu Ile Leu Val Asn Tyr Ile Phe
    GCA GAC CTG TTT CTG GGC CTG GAG AAC CCA GCC ATC CTC ATT CTG GTC AAC TAC ATC TTC  499

181 Phe Lys Gly Thr Trp Ala His Pro Phe Asp Pro Gln Ser Thr Glu Glu Lys Ser Phe Tyr
    TTC AAA GGC ACC TGG GCA CAC CCC TTT GAC CCA CAA AGC ACC GAG GAG AAG AGC TTC TAC  559

201 Val Asp Asp Thr Thr Thr Val Met Val Pro Met Met Phe Gln Ser Ser Thr Val Lys Tyr
    GTG GAC GAT ACG ACC ACG GTG ATG GTG CCC ATG ATG TTC CAG TCG AGC ACT GTC AAG TAC  619

221 Leu His Asp Pro Val Leu Pro Cys Arg Leu Val Gln Leu Asp Tyr Val Gly Asn Gly Thr
    CTG CAC GAC CCG GTG CTC CCC TGC CGG CTG GTG CAG CTG GAT TAC GTG GGC AAT GGC ACC  679

241 Ala Phe Phe Ile Leu Pro Asp Lys Gly Lys Val Asp Thr Val Ile Ala Ala Leu Ser Arg
    GCG TTC TTC ATC CTC CCG GAC AAG GGG AAG GTG GAC ACG GTC ATC GCC GCC CTG AGC AGG  739

261 Asp Thr Ile Gln Arg Trp Ser Lys Ser Leu Thr Tyr Arg Leu Val His Leu Tyr Ile Pro
    GAC ACG ATT CAG CGC TGG TCC AAA TCC CTG ACC TAC AGG CTG GTC CAC CTG TAC ATC CCG  799

281 Lys Ala Ser Ile Ser Gly Ala Tyr Glu Leu Arg Gly Ala Leu Ala Ala Met Gly Ile Ala
    AAG GCC TCC ATC TCC GGA GCC TAC GAG CTC AGG GGC GCA CTG GCG GCC ATG GGC ATC GCA  859

301 Asp Leu Phe Thr Asn Gln Ala Asn Phe Ser Ser Ile Ser Gln Glu Gly Pro Leu Lys Val
    GAC TTG TTC ACC AAC CAG GCA AAT TTC TCA AGC ATC TCC CAG GAG GGC CCA CTG AAG GTA  919

321 Ser Lys Val Leu His Lys Ala Val Leu Gln Leu Asp Glu His Gly Gly Val Glu Val Ala
    TCG AAG GTG CTC CAT AAG GCT GTG CTG CAG CTC GAC GAG CAC GGG GGG GTG GAG GTG GCT  979

341 Ala Thr Gly Gly Pro Leu Gln Leu Val Ser Glu Pro Leu Thr Leu Asn Phe Asn Arg Pro
    GCC ACG GGA GGC CCC CTG CAG CTG GTG TCT GAA CCC CTC ACC CTG AAC TTC AAC CGG CCC 1039

361 Phe Leu Ile Leu Ile Phe Asp Asp Phe Thr Trp Ser Ser Leu Phe Leu Gly Lys Val Val
    TTC CTC ATC CTG ATC TTC GAC GAC TTC ACC TGG AGC AGC TTG TTC CTG GGC AAG GTT GTG 1099

        383
381 Ile Pro Ala ---
    ATC CCA GCC TGA GAGTCGGCCCAGCCTGGAGCCACTGCTGCATCTGACTTTGGGCGCTGGGGATCCCACGGAAGC 1174

    ATCCGTGGGAGTGGGAGGTTTTCTTGCACCCGCCAGAGCTGTTTGTACCTTTCTGCGTGTTGGAATAAATCAGTCACTG 1253

    TGA51                                                                        1306
```

strands and produced a combined nucleotide sequence of 1481 bp which contained an 88 bp 5'-non-coding region followed by a 406 amino acid open reading frame comprising 5 consensus sites for *N*-glycosylation (Bause, 1983) and an 172 bp 3'-non-coding region (Figure 3.6). A potential polyadenylation signal was located 15 bp from a 3' poly(A) tail.

3.2.5 Primary Structure Comparisons of Human, Rabbit, Rat, Mouse and Squirrel Monkey CBG

When the cDNA-deduced, mature primary structures of human, rabbit, rat, mouse and squirrel monkey CBG were aligned (Figure 3.7), it was necessary to make allowances for the different polypeptide lengths by introducing gaps at four locations. Over the resulting 384 amino acid consensus length, the residues in 40.9% of the positions are identical in all 5 polypeptides, and conservative differences are found in 41.4% of the positions. A much higher level of identity (72.7%) is observed between the signal peptides of human, rat, mouse and squirrel monkey CBG (Figure 3.8). Between pairs of mature polypeptide sequences (Figure 3.9), the level of identity between species ranges from a low of 54.9% between rabbit and mouse CBG to a high of 85.9% between human and squirrel monkey CBG.

3.3 Discussion

The availability of a human CBG cDNA enabled the isolation of cDNAs for rat, mouse, rabbit and squirrel monkey CBG. The amino-terminal sequences of rat and

Figure 3.6 Combined nucleotide sequence of overlapping squirrel monkey liver CBG cDNAs, and the deduced primary structure of the precursor polypeptide. The putative signal peptide is numbered -22 to -1, while the mature polypeptide is labelled +1 to 384. Consensus sites for *N*-glycosylation are indicted (●) as is a potential polyadenylation signal (underlined). Amino acid positions are given on the *left*, and nucleotide positions are shown on the *right*.

```
                                                                                ACAGTACAC    9
   ATGCTGGGTCCAGGACAGGAGGACTGAGCCAGCAGAAACAGCCTGAGTCCACTGCAGACTGGCCTGGCTATACTGGACA   88

-22 Met Pro Leu Leu Leu Tyr Thr Cys Leu Leu Trp Leu Leu Ser Ser Gly Leu Trp Thr Val
    ATG CCA CTC CTC CTG TAC ACC TGT CTT CTC TGG CTG CTC TCC AGT GGC CTC TGG ACC GTC  148
        -1  +1
 -2 Gln Ala Met Asp Pro Asn Ala Ala Tyr Met Asn Thr Ser Arg His His Arg Val Leu Ala
    CAG GCC ATG GAT CCT AAC GCT GCT TAT ATG AAC ACA AGT AGA CAT CAC CGG GTT CTG GCT  208

  19 Ser Val Asn Ala Asp Phe Ala Phe Ser Leu Tyr Lys His Leu Val Ala Leu Ser Pro Lys
    TCA GTC AAC GCT GAC TTT GCC TTC AGC CTG TAT AAG CAC CTA GTG GCC TTG AGT CCC AAG  268

  39 Lys Asn Val Phe Ile Ser Pro Val Ser Ile Ser Met Ala Leu Ala Met Leu Ser Leu Gly
    AAG AAC GTT TTC ATC TCC CCT GTG AGC ATC TCT ATG GCA TTA GCT ATG CTG TCC CTG GGC  328

  59 Thr Cys Gly His Thr Arg Ala Gln Leu Leu His Gly Leu Gly Phe Asn Leu Thr Glu Lys
    ACC TGT GGC CAC ACA CGG GCC CAA CTT CTC CAC GGC CTG GGG TTC AAC CTC ACT GAG AAG  388

  79 Ser Glu Ala Glu Ile His Gln Ser Phe Gln His Leu His Gln Leu Leu Ala Glu Ser Asp
    TCT GAG GCT GAG ATC CAC CAG AGT TTC CAG CAC CTC CAC CAA CTC CTT GCA GAG TCA GAC  448

  99 Ser Ser Leu Glu Met Thr Leu Gly Asn Ala Leu Phe Leu Asp Gly Ser Leu Glu Leu Leu
    AGC AGC TTA GAA ATG ACC TTG GGC AAT GCC TTG TTT CTT GAT GGC AGC CTG GAG TTG CTG  508

 119 Glu Ser Phe Ser Ala Asp Ile Lys His Tyr Tyr Glu Ser Glu Val Leu Thr Leu Asn Phe
    GAG TCA TTC TCA GCA GAC ATC AAG CAC TAC TAT GAG TCT GAG GTC TTG ACT TTG AAT TTC  568

 139 Gln Asp Trp Ala Thr Thr Ala Ser Arg Gln Ile Asn Gly Tyr Val Lys Ser Lys Thr Gln
    CAG GAC TGG GCT ACA ACA GCC AGC AGA CAG ATC AAT GGC TAT GTC AAG AGT AAG ACA CAG  628

 159 Gly Lys Ile Asp Asp Leu Phe Ser Gly Leu Asn Ser Pro Ala Val Leu Ile Leu Ile Asn
    GGG AAA ATT GAT GAC TTG TTC TCA GGG CTG AAT AGC CCA GCC GTC CTC ATC CTG ATC AAC  688

 179 Tyr Ile Phe Phe Lys Gly Thr Trp Lys Gln Pro Phe Asp Leu Ala Ser Thr Arg Glu Glu
    TAT ATC TTC TTC AAA GGC ACA TGG AAA CAG CCC TTT GAC CTG GCA AGC ACC AGG GAG GAG  748

 199 Asn Phe Tyr Val Asp Glu Thr Thr Val Val Lys Val Pro Met Met Phe Gln Ser Gly Thr
    AAC TTC TAT GTG GAC GAG ACA ACT GTG GTG AAA GTG CCC ATG ATG TTC CAG TCA GGC ACC  808

 219 Ile Arg Tyr Leu His Asp Ser Glu Leu Pro Cys Gln Leu Val Gln Leu Asn Tyr Ala Gly
    ATC CGT TAC CTT CAT GAC TCG GAG CTC CCC TGC CAG CTG GTG CAA CTG AAC TAT GCG GGC  868
   •
 239 Asn Gly Thr Val Phe Phe Ile Leu Pro Glu Lys Gly Lys Met Asn Ile Val Ile Thr Ala
    AAT GGG ACT GTC TTC TTC ATC CTT CCA GAA AAG GGG AAG ATG AAC ATA GTC ATC ACT GCG  928

 259 Leu Ser Arg Asn Thr Ile Asp Arg Trp Ser Ala Gly Leu Thr Arg Ser Gln Val Asp Leu
    CTG AGC CGG AAC ACA ATT GAC AGG TGG TCT GCA GGC CTG ACC AGA AGC CAG GTG GAC CTG  988

 279 Tyr Ile Pro Lys Val Thr Ile Ser Gly Ala Tyr Asp Phe Gly Gly Val Leu Glu Asp Met
    TAC ATT CCG AAG GTC ACC ATC TCT GGA GCC TAT GAT TTT GGA GGT GTG CTG GAG GAC ATG 1048
                                    •
 299 Gly Ile Ala Asp Leu Phe Thr Asn His Ala Asn Phe Ser Arg Ile Thr Gln Asp Ala Gln
    GGC ATT GCA GAC TTG TTC ACC AAC CAC GCA AAT TTC TCA CGC ATC ACC CAG GAT GCC CAG 1108
                                                                          •
 319 Leu Lys Leu Ser Lys Val Phe His Lys Ala Val Leu Gln Leu Ser Glu Glu Gly Val Asn
    CTG AAG TTA TCA AAG GTG TTC CAT AAG GCT GTG CTG CAA CTC AGT GAG GAG GGT GTG AAC 1168

 339 Thr Thr Gly Ser Thr Gly Val Thr Leu Asn Pro Met Ser Lys Pro Ile Ile Met Arg Phe
    ACA ACT GGC TCC ACT GGG GTC ACC CTA AAC CCG ATG TCC AAG CCT ATC ATC ATG CGT TTC 1228

 359 Asn Gln Pro Phe Leu Ile Met Val Phe Asp His Phe Thr Trp Ser Ser Leu Phe Leu Gly
    AAC CAG CCC TTC CTC ATC ATG GTC TTC GAC CAC TTC ACC TGG AGC AGC CTC TTC CTG GGC 1288
                      384
 379 Arg Val Val Asn Pro Ala ---
    AGA GTT GTG AAC CCA GCA TAA GAGACCACCCACCTGGAGCCACAGCACTATCTGACTTTGGGCACCCAGGGG 1360

    CCCTACGGAAATGTTTTGGAGAGAAGTTTCCCCCAATCTGCTCGAAGTTCTCCTCCTTCCAACCAGACTTGTATCTGCC 1439

    TTTAGGCATCCTGTAATAAATATCATTGCAACGCTA7                                          1481
```

Figure 3.7 Alignment of the cDNA-deduced mature CBG polypeptide sequences of various species. Boxes (■) represent locations where gaps were inserted to maintain the amino acid alignment, and hyphens (-) represent amino acids identical to human CBG. The position of *N*-glycosylation consensus sites (●) are indicated, as is the position of the conserved cysteine residue (*).

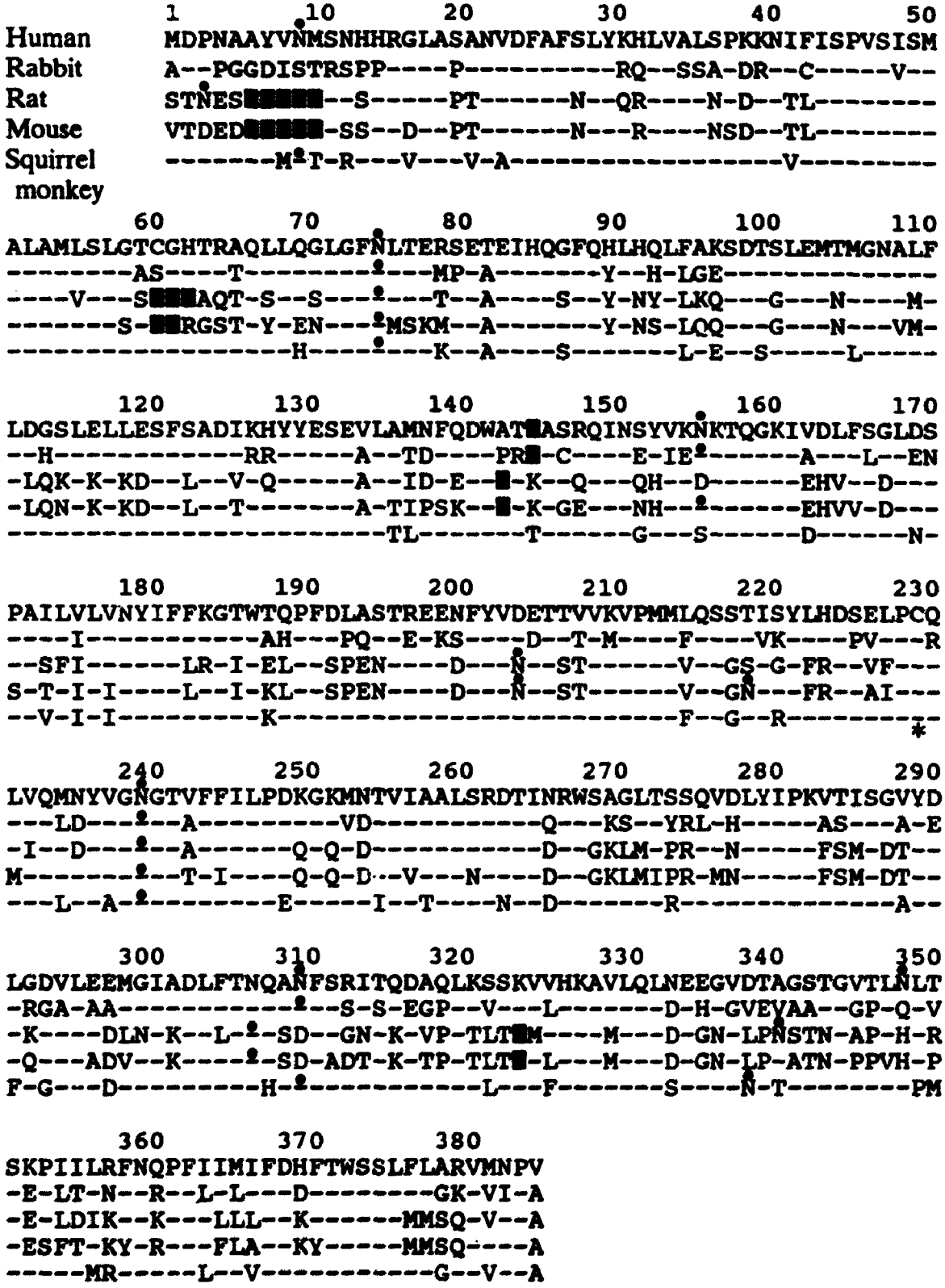

1 10 20 30 40 50
Human MDPNAAYVNMSNHHRGLASANVDFAFSLYKHLVALSPKKNIFISPVSISM
Rabbit A--PGGDISTRSPP----P----------RQ--SSA-DR--C-----V--
Rat STNES-----S-----PT------N--QR----N-D--TL--------
Mouse VTDED-----SS--D--PT------N---R----NSD--TL--------
Squirrel monkey -------M-T-R---V---V-A----------V---------
60 70 80 90 100 110
ALAMLSLGTCGHTRAQLLQGLGFNLTERSETEIHQGFQHLHQLFAKSDTSLEMTMGNALF
--------AS----T---------MP-A-------Y--H-LGE----------
----V---S----AQT-S--S----T--A----S--Y-NY-LKQ---G---N----M-
-------S--RGST-Y-EN----MSKM--A-------Y-NS-LQQ---G---N---VM-
----------H----K--A----S-------L-E--S-----L-----
120 130 140 150 160 170
LDGSLELLESFSADIKHYYESEVLAMNFQDWATASRQINSYVKNKTQGKIVDLFSGLDS
--H----------RR-----A--TD----PR-C----E-IE------A---L--EN
-LQK-K-KD--L--V-Q-----A--ID-E--K--Q---QH--D------EHV--D---
-LQN-K-KD--L--T-------A-TIPSK--K-GE---NH--------EHVV-D---
----------TL-------T------G---S------D------N-
180 190 200 210 220 230
PAILVLVNYIFFKGTWTQPFDLASTREENFYVDETTVVKVPMMLQSSTISYLHDSELPCQ
----I----------AH---PQ--E-KS----D--T-M----F-----VK----PV---R
--SFI------LR-I-EL--SPEN-----D---N--ST------V--GS-G-FR--VF---
S-T-I-I----L--I-KL--SPEN----D---N--ST------V--GN---FR--AI---
--V-I-I---------K----------F--G--R---------*
240 250 260 270 280 290
LVQMNYVGNGTVFFILPDKGKMNTVIAALSRDTINRWSAGLTSSQVDLYIPKVTISGVYD
---LD---A---------VD----------Q---KS--YRL-H-----AS---A-E
-I--D---A------Q-Q-D----------D--GKLM-PR--N-----FSM-DT--
M-------T-I----Q-Q-D--V---N----D--GKLMIPR-MN-----FSM-DT--
---L--A-E-----I--T----N--D-------R----------A--
300 310 320 330 340 350
LGDVLEEMGIADLFTNQANFSRITQDAQLKSSKVVHKAVLQLNEEGVDTAGSTGVTLNLT
-RGA-AA----------S-S-EGP--V---L-------D-H-GVEVAA--GP-Q-V
-K----DLN-K--L-SD--GN-K-VP-TLT-M----M---D-GN-LPNSTN-AP-H-R
-Q---ADV--K----SD-ADT-K-TP-TLT-L---M---D-GN-LP-ATN-PPVH-P
F-G---D---------H----------L---F-------S----N-T--------PM
360 370 380
SKPIILRFNQPFIIMIFDHFTWSSLFLARVMNPV
-E-LT-N--R--L-L---D--------GK-VI-A
-E-LDIK--K---LLL--K------MMSQ-V--A
-ESFT-KY-R---FLA--KY-----MMSQ----A
-----MR-----L--V----------G--V--A

Figure 3.8 Alignment of the putative CBG signal peptides of various species. Hyphens (-) indicate identical amino acids.

Human	MPLLLYTCLLWLPTSGLWTVQA
Rat	-S-A---------C-------A--
Mouse	-S-A------P--C-------T--
Squirrel monkey	------------LS--------

Figure 3.9 Percentage sequence identity between the mature CBG polypeptides of various species.

	Rat	Mouse	Rabbit	Human	Squirrel monkey
Rat	100				
Mouse	78.6	100			
Rabbit	58.6	54.9	100		
Human	60.7	57.9	68.9	100	
Squirrel monkey	57.8	55.7	66.3	85.9	100

mouse CBG were located within the deduced primary structures of these cDNAs, and the first 22 amino acids of each open reading frame therefore represents a signal peptide that is cleaved during cotranslational processing (Silhavy *et al.*, 1983). The rat, mouse and human CBG leader sequences exhibit features associated with typical signal peptides. They are composed of predominantly hydrophobic central core regions, are of a sufficient length for an α helix to span a membrane bilayer, and terminate with a small, neutral amino acid, alanine (Perlman and Halvorson, 1983; Boyd and Beckwith, 1990). Unlike most eukaryotic signal peptides, however, these CBG signal peptides lack positively charged amino acids at their amino termini (Boyd and Beckwith, 1990). Although the amino-terminal sequence of squirrel monkey CBG is not available, the first 22 cDNA-deduced amino acids of the human and squirrel monkey CBG precursors are nearly identical (90.9%), and it is therefore likely that this region of the squirrel monkey sequence corresponds to its signal peptide.

The cDNA-deduced amino acid composition of the rat CBG mature polypeptide compares favourably with estimates obtained by direct analysis, despite some variation in values obtained for cysteine, histidine, methionine and phenylalanine residues (Chader and Westphal, 1968b; Rosner and Hochberg, 1972; Favre *et al.*, 1984). Since rat CBG is 27.8% carbohydrate by mass (Chader and Westphal, 1968b), the molecular weight of the cDNA-deduced polypeptide chain (42,229) therefore represents 72.2% of the total glycoprotein mass (*i.e.* approximately 58 kDa) and this agrees with previous estimates of 50.5 - 62.5 kDa (Chader and Westphal, 1968b; Wolf *et al.*, 1981; Kato *et al.*, 1988). In view of the

polypeptide mass and the carbohydrate content of rat CBG, the molecular mass of rat CBG (65.9 - 75.8 kDa) determined by Favre *et al.* (1984) is probably inaccurate. Like the human CBG cDNA (Hammond *et al.*, 1987), the rat CBG cDNA also encodes an in-frame minicistron within its 3' non-coding region, but their nucleotide and predicted polypeptide sequences are very different, and it is therefore unlikely they have any biological significance.

Corticosteroid binding globulin is a member of the serine proteinase inhibitor (serpin) family and human CBG exhibits 43.2% and 44.2% identity with α_1-proteinase inhibitor and α_1-antichymotrypsin precursors, respectively (Hammond *et al.*, 1987). Therefore, to ensure the isolated rat cDNAs did not represent non-CBG members of the serpin family, hepatic RNA was hybrid selected, translated *in vitro* and products were incubated with antiserum against rat CBG. The immunoreactive product of *in vitro* translated poly(A)$^+$ RNA was of an appropriate size for a nonglycosylated CBG precursor (Wolf *et al.*, 1981), and likely corresponds to a polypeptide originating from the correct initiation codon in the CBG mRNA. The relative abundance of this protein product was increased when hepatic RNA was hybrid-selected prior to translation, and subsequent immunoselection demonstrates that the cDNA-selected RNA encodes a protein recognized by the rat CBG antibody. This, together with the identification of the amino-terminal sequence of rat CBG within the cDNA-deduced primary structure conclusively demonstrates that this cDNA encodes CBG. The only other immunoreactive, hybrid-selected RNA translation product was an appropriate size (approximately 36 kDa) to represent a protein whose translation was initiated at the second methionine codon in the CBG

mRNA.

The amino acid composition of purified mouse CBG has been reported twice by the same authors (Nyberg and Jones, 1988; Nyberg *et al.*, 1990), and the amino acid composition derived from the cDNA sequence compares more favourably with the latter report (Nyberg *et al.*, 1990). The murine CBG glycoprotein has a carbohydrate content of 20.4% (Nyberg and Jones, 1988) and the cDNA-deduced primary structure (M_r 42,277) therefore represents 79.6% of the total molecular weight of CBG (53,000). This value is essentially identical to that (52,000) previously determined by biochemical means (Nyberg and Jones, 1988). Peptide fragments of purified mouse CBG have been subjected to amino acid sequence analysis, and although the amino-terminal sequence was unobtainable, the positions of 189 residues have been reported (Nyberg *et al.*, 1990). In most instances, the cDNA-deduced amino acid sequence of mouse CBG agrees with that obtained by chemical analysis, but discrepancies occur in 20 of the 189 residues.

The mature rabbit CBG polypeptide sequence was obtained through a combination of cDNA sequence analyses and amino-terminal sequence determinations of purified protein. The amino acid composition derived from these combined data is similar to that previously obtained (Chader and Westphal, 1968a) with the exception of the values obtained for cysteine, tryptophan and methionine. When the contribution (29.2%) of carbohydrate (Chader and Westphal, 1968a) to total molecular mass is considered in addition to that calculated from the amino acid sequence, the resulting molecular weight (60,000) is in close agreement with

our (Seralini *et al.*, 1989) previous determination (55 kDa). The molecular weight of rabbit CBG has also been reported as 34,700 (Chader *et al.*, 1972) and 40,700 (Chader and Westphal, 1968a), but in view of the deduced mass of the primary structure, these values are low.

Based on the similarity between the cDNA-deduced amino acid sequences of human and squirrel monkey CBG, the open reading frame encoded by the squirrel monkey cDNA appears to comprise a 22 amino acid leader sequence and a 384 residue mature polypeptide. The predicted molecular weight of the mature protein would therefore be 42,854, and its amino acid composition is nearly identical to that previously reported (Kuhn *et al.*, 1988). Although the amino terminal sequence of squirrel monkey CBG is not available, the similarity of the amino acid compositions and the striking homology (85.9%) between the deduced primary structures of the human and squirrel monkey cDNAs strongly suggest the isolated cDNA encodes squirrel monkey CBG. Furthermore, expression of this cDNA in culture yields a protein immunoreactive with antiserum against squirrel monkey CBG (see section 7.4.3).

Although the molecular masses of human, rat, mouse, rabbit and squirrel monkey CBG polypeptides are similar, their total glycoprotein mass varies (Westphal, 1986). However, CBG electrophoretic variants have been observed within a given species (Kato *et al.*, 1988; Kuhn *et al.*, 1988; Nyberg *et al.*, 1990; Westphal, 1971), and size heterogeneity is present even within individual serum samples (Mickelson *et al.*, 1982). It is unlikely that this is due to the presence of an

additional CBG polypeptide since both rat and human CBG appear to be single copy genes (Smith and Hammond, 1989; Underhill and Hammond, 1989; Seralini *et al.*, 1990a), and it therefore appears that size variations are due to differential post-translational modification (*i.e.* glycosylation) of the CBG polypeptide.

The carbohydrate structure of human CBG has been studied and on average, five *N*-linked carbohydrate chains are incorporated into each molecule (Akhrem *et al.*, 1982; Strel'chyonok *et al.*, 1982). There are, however, six consensus sites within the polypeptide (Hammond *et al.*, 1987), and only those at asparagines 9 and 349 are known to be utilized (Hammond, 1988; Hammond *et al.*, 1990a). The only other information available on the utilization of glycosylation consensus sequences is derived from amino-terminal sequence analysis of rat CBG which suggests asparagine[3] is not linked to a carbohydrate chain (Kato *et al.*, 1988).

Phylogenetic comparisons of the positions of *N*-glycosylation consensus sequences within the cDNA-deduced primary structures of CBG may provide clues as to sites that are utilized and/or important for biological activity. For example, recognition sites for *N*-linked carbohydrate attachment are present at asparagines 74 and 239 (numbered with respect to squirrel monkey CBG) in all species examined, and these residues are located in conserved regions of the polypeptide. This suggests these consensus sites may be utilized and that carbohydrate chains at these positions may be important for the structure or function of CBG. Conversely, consensus sites for *N*-glycosylation at the amino-terminus of CBG are present in only 3 of the 5 species examined, and the rat CBG recognition sequence is not

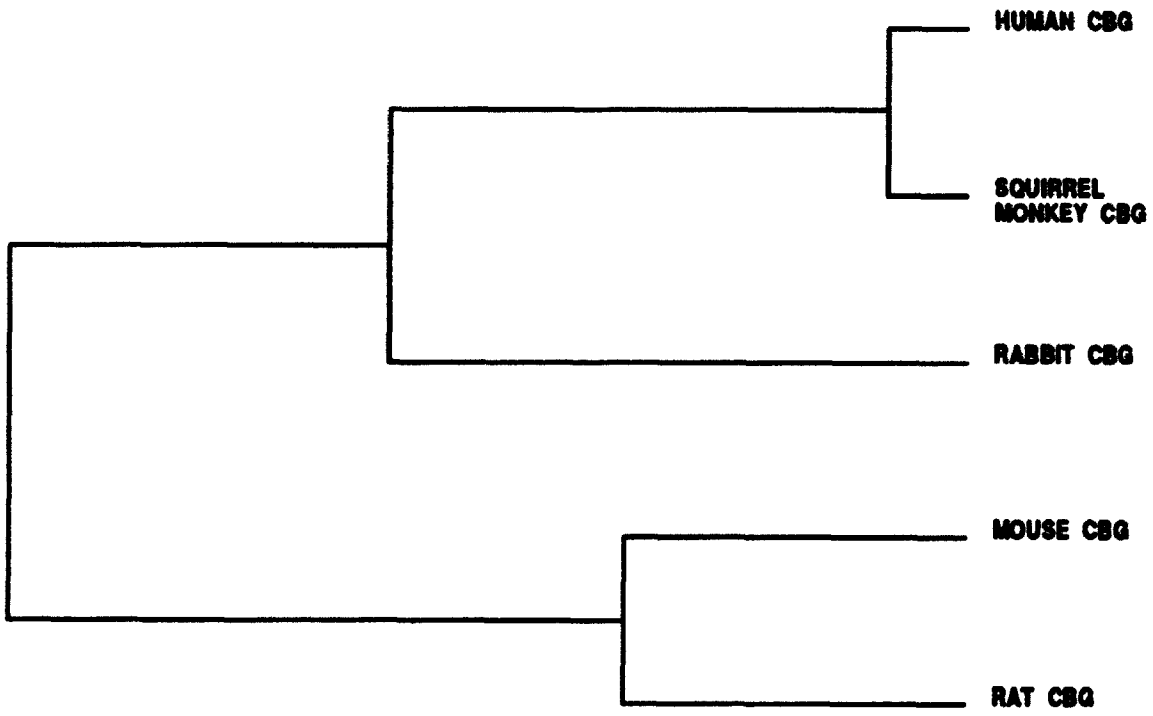
utilized. It is therefore unlikely the carbohydrate chain at the equivalent position of human CBG is functionally important. It is interesting to note that the 4 potential *N*-linked carbohydrate attachment sites of rabbit CBG are located in positions identical to human CBG (Seralini *et al.*, 1990b). Since the carbohydrate content of CBG in the rabbit is similar to human (Chader and Westphal, 1968a), all four consensus sites within rabbit CBG are probably utilized.

Although near complete deglycosylation of human CBG does not modify its steroid binding properties (Mickelson *et al.*, 1982), oligosaccharide chains probably influence CBG function. The absence of sialic acid on carbohydrate chains reduces the half-life of rat and human CBG in plasma presumably through interaction with the asialoglycoprotein receptor (Hossner and Billiar, 1981) which is responsible for the hepatic clearance of desialylated serum glycoproteins (McFarlane, 1983). Glycosylation also appears to be important for the intracellular stability of immunoreactive CBG produced by Hep G2 cells in culture, but is not essential for its secretion (Murata *et al.*, 1989). These experiments did not, however, assess the binding activity of tunicamycin-mediated, unglycosylated CBG (Murata *et al.*, 1989). A more intriguing role for the carbohydrate component of CBG is its possible involvement in the interactions with plasma membrane receptors (Singer *et al.*, 1988; Maitra *et al.*, 1990). These findings are of particular interest in view of the identification of a pregnancy-associated human CBG variant composed of 5 triantennary chains (Strel'chyonok *et al.*, 1984), which binds with higher affinity to placental syncytiotrophoblast plasma membranes than does normal CBG (for review see Strel'chyonok and Avvakumov, 1990). In addition, charge heterogeneity has also

been observed between CBG obtained from normal female and pregnant rabbits and both forms cross the fetal kidney to fetal urine and amniotic fluid, while CBG of fetal origin does not (Seralini *et al.*, 1989). Glycosylation therefore appears to regulate the distribution of maternal CBG within the fetal compartment and may influence fetal physiology.

Alignment of the primary CBG structures of the species examined in this study revealed their overall identity was low in comparison to other proteins, such as the alcohol dehydrogenases (Jörnvall *et al.*, 1987). These comparisons provided the basis for the construction of a dendrogram which illustrates the relative evolutionary distances at which the CBG sequences diverged from one another (Figure 3.10). Monoclonal and polyclonal antibodies have been previously used to examine the differences and similarities of CBG between species, and slight (Faict and De Moor, 1986) to no (Robinson *et al.*, 1985a) cross-reactivity was detected between anti-human CBG immunoglobulins and rat and rabbit CBG. Although this seems reasonable in light of the low sequence similarity between these species, no cross-reactivity was detected between anti-human CBG antibodies and serum obtained from squirrel monkeys (Robinson *et al.*, 1985a; Faict and De Moor, 1986). In addition, antiserum against squirrel monkey CBG does not recognize human CBG (Kuhn *et al.*, 1988), and in view of the homology between their primary structures, this is surprising. The only cross-reactivity observed in these studies, which included many non-primate representatives, was between Old World monkey and ape sera and anti-human CBG antibodies (Robinson *et al.*, 1985a; Faict and De Moor, 1986) and this suggests that the CBG amino acid sequences of these species must be more

Figure 3.10 Dendrogram of mature CBG polypeptides


HUMAN CBG
SQUIRREL MONKEY CBG
RABBIT CBG
MOUSE CBG
RAT CBG

similar to human CBG than that of squirrel monkey. The lack of reactivity between squirrel monkey serum and human CBG antibodies deserves special mention because amongst the species examined, the primary structures of these two proteins are most closely related, yet their physicochemical characteristics (*i.e.* steroid binding affinity and stoichiometry) are the most dissimilar. Comparisons between these two species may therefore provide the basis for studies which attempt to relate differences in primary structure to changes in function.

Monoclonal antibody studies revealed the presence of highly evolved domains within the CBG molecule as well as more conserved regions (Faict and De Moor, 1986), and alignment of the mature CBG polypeptide sequences has disclosed their distribution. Four gaps are required to align the primary structures of human, rabbit, rat, mouse and squirrel monkey CBG, and they are located in regions of relatively poor homology. Conversely, there are comparatively well conserved regions between the five species (for example, amino acids 42-58) and they are likely required to maintain structural elements essential for evolutionarily- conserved activities such as steroid binding.

Human CBG exhibits poor identity with that of other species between residues 332 and 360, and it is this region which comprises the reactive centre for specific interaction with the serine protease, human neutrophil elastase (Pemberton *et al.*, 1988; Hammond *et al.*, 1990a). Other members of the human serine protease inhibitor (serpin) superfamily, such as α_1-proteinase inhibitor (A1-PI) or thyroxine binding globulin (TBG) are also poorly conserved within this functionally important

region (Huber and Carrell, 1989) and serpins, such as CBG, may have continually modified their primary structures in order to appropriately inhibit extrinsic proteases introduced by parasitic infections (Hill and Hastie, 1987). Alternatively, it has been suggested the serine proteases are rapidly evolving, and that serpin reactive centres must therefore co-evolve to maintain appropriate inhibitory activity (Hammond *et al.*, 1991b) Indeed, the variability of serine protease activity has been illustrated by a 7-fold variation in the Michaelis constants of human and rat polymorphonuclear elastases towards a common synthetic substrate (Green *et al.*, 1991). However, within a given species, evolutionary constraints on the primary structure of this region appear to be necessary for appropriate serpin activity. For example, an amino acid substitution at residue 358 of α_1-proteinase inhibitor yields a protein which inhibits thrombin rather than elastase (Brantly *et al.*, 1988), and substitution of non-basic amino acids for arginine^{346} of plasminogen activator inhibitor I eliminates its ability to inactivate tissue plasminogen activator and urokinase (York *et al.*, 1991).

Substitution of positively charged amino acids for the acidic residue (glutamate) normally located at position 342 of A1-PI causes this protein to accumulate in the endoplasmic reticulum (McCracken *et al.*, 1991), and consequently, its serum levels are markedly reduced (Brantly *et al.*, 1988). In this context, it is interesting to note, that when the primary structures of the twenty serpins examined by Huber & Carrell (1989), including human A1-PI, TBG and CBG, are aligned with the four CBG primary structures described in this study, a glutamate residue is present at a position equivalent to residue 342 of A1-PI in all CBG structures and 18 of the 20

serpins examined. This would suggest that the presence of a glutamate residue at this position is required for the appropriate secretion of most members of the serpin family, including CBG.

The relative binding affinity of CBG for various steroid ligands differs between species (Westphal, 1986) but the most abundant glucocorticoid is the preferred CBG ligand. For example, rat CBG has a greater affinity for corticosterone than cortisol (Favre *et al.*, 1984), while human CBG preferentially binds the latter (Stroupe *et al.*, 1978). The affinities of rat and human CBG for corticosterone and cortisol, respectively, are more similar than the affinities of human and squirrel monkey CBG for cortisol (Smith and Hammond, 1991b; Smith *et al.*, 1991; Hammond *et al.*, 1991a). This finding, however, contrasts with the greater identity observed between human and squirrel monkey CBG, in comparison to the lower similarity between rat and human polypeptide sequences. This indicates a relatively small change in the primary structure of squirrel monkey CBG has resulted in a relatively large change in steroid binding affinity, and experiments which exploit the structural differences between pairs of CBG sequences may help identify the amino acids that contribute to variations in steroid binding activity.

Physicochemical studies of the human CBG steroid binding site have revealed the presence of a cysteine residue that may be affinity labelled with 6-bromo-progesterone (Khan and Rosner, 1977). There are two, unlinked, cysteine residues within the mature human CBG molecule (Hammond *et al.*, 1987), but when the primary structures of human, rabbit, rat, mouse and squirrel monkey CBG are

aligned, only one is located in the same relative position in all five species. This corresponds to cysteine^{228} of the human CBG polypeptide, and the only such residue present in mouse and rat CBG. In addition, this region of the CBG molecule is well conserved between species in comparison to the region surrounding cysteine^{60} of human CBG, and it is therefore likely that cysteine^{228} is present within the steroid binding domain (Hammond *et al.*, 1987; Smith and Hammond, 1989; Hammond *et al.*, 1991b). The same conclusion was reached based on a comparison between the homologous proteins, A1-PI and CBG. Examination of the tertiary structure of A1-PI revealed a ß-barrel characteristic of ligand binding domains (Loebermann *et al.*, 1984; Huber and Carrell, 1989), and when the primary structure of CBG was superimposed on A1-PI, cysteine^{228} was located within this ß-barrel (Huber and Carrell, 1989). Thus, two independent analyses support the conclusion that cysteine^{228} of human CBG is present within the steroid binding domain. It is therefore also probable this residue contributes to the steroid binding activity of rat, mouse, rabbit and squirrel monkey CBG.

CHAPTER 4

The Ontogeny of Rat Corticosteroid Binding Globulin and its Messenger Ribonucleic Acid

4.1 Introduction

Rat plasma CBG levels vary with respect to age, sex and pregnancy (see sections 1.4.1 - 1.4.3) and this suggests its biosynthesis is differentially influenced by these factors. For example, fetal plasma CBG concentrations are highest between 17 and 19 days of gestation and decline to very low levels at birth (Van Baelen *et al.*, 1977b; Gewolb and Warshaw, 1986). By contrast, maternal plasma CBG levels exhibit only modest decreases during the same period (Van Baelen *et al.*, 1977b; Gewolb and Warshaw, 1986). Furthermore, serum CBG concentrations are uniformly low during the first weeks of life (Henning, 1978; D'Agostino and Henning, 1981), but a sex difference becomes apparent (Gala and Westphal, 1965b) as levels increase towards adult values.

Although it is assumed that the major site of CBG synthesis is the hepatocyte, the identification of CBG in tissues other than liver (see section 1.2.2) raises the possibility of extra-hepatic CBG biosynthesis, and this question has been addressed by an assessment of the tissue distribution of CBG mRNA. Liver is the primary stie of CBG mRNA synthesis, and levels of serum CBG and hepatic CBG mRNA were therefore characterized in order to determine the biosynthetic basis for alterations in plasma CBG levels during ontogeny.

4.2 Results

4.2.1 Tissue Distribution of Rat CBG mRNA

The availability of a rat CBG cDNA allowed a comprehensive study of CBG mRNA distribution. Various tissues were obtained from animals under different

physiological conditions, and when a Northern blot of RNA extracts prepared from these samples was probed with a radiolabelled, rat CBG cDNA, an approximately 1.8 kilobase (kb) CBG mRNA was detected only in the liver (Figure 4.1). Poly(A)$^+$ RNA from various tissues was also analyzed to increase the Northern blotting sensitivity, but CBG mRNA was still undetectable in tissues other than liver (Figure 4.2).

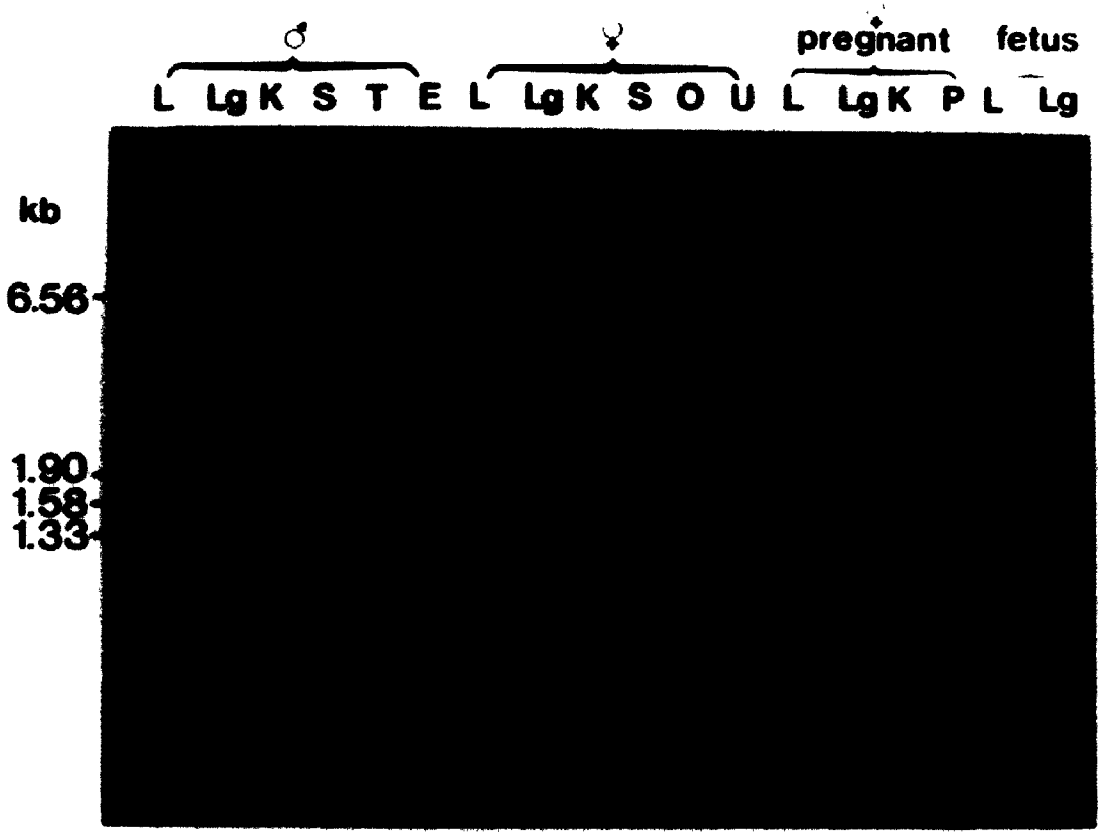
4.2.2 Quantification of Hepatic CBG mRNA Levels

It was apparent from the CBG mRNA tissue distribution studies that the relative abundance of hepatic CBG mRNA varied with the physiological status of the animal. In order to quantify this difference more accurately, liver RNA extracts obtained from animals ranging in age from day 15 of gestation (term = 21 days) until 12 weeks were further analyzed for CBG mRNA content by Northern blotting and solution hybridization assay.

4.2.2.1 *Maternal and Fetal*

The CBG mPNA levels detected by Northern blotting indicated that a higher level was present in pregnant (day 21) than in a non-pregnant female liver, and that both levels were much greater than that observed in a pooled fetal (day 21) liver RNA extract (Figure 4.1). These observations were extended when hepatic RNA, obtained from pregnant females (days 14-21 of gestation) and their corresponding fetuses (day 15-21), was examined by Northern blot analysis. A CBG mRNA of approximately 1.8 kb was present in all samples except the 21 day fetuses (Figure

Figure 4.1 Northern blot of RNA extracts obtained from rats under various physiological conditions. Twenty-five μg of RNA obtained from various tissues was probed with a radiolabelled rat CBG cDNA. Following membrane washing (0.1 x SSC/0.1% SDS at 68^{o}C) the flourograph (18h at -80^{o}C) was obtained using a Dupont Cronex Hi-Plus XH intensifying screen. Denatured DNA molecular size standards are shown on the *left* of the figure. L, liver; Lg, lung; K, kidney, S, spleen; T, testis, E, epididymis, O, ovary; U, uterus; P, placenta.



25 µg total RNA
per lane

Figure 4.2 Northern blot of RNA extracts obtained from rat tissues. Differing amounts of poly(A)$^+$ RNA were Northern blotted and probed with a radiolabelled rat CBG cDNA. Following membrane washing (0.1 x SSC/0.1% SDS at 42°C), the fluorograph (25 h at -80°C) was obtained using a Lightning Plus intensifying screen. Denatured DNA molecular size standards are shown on the *left*. Lv, liver; Lg, lung; K, kidney; S, spleen; A, adrenal; O, ovary; U, uterus; T, testis; E, epididymis.

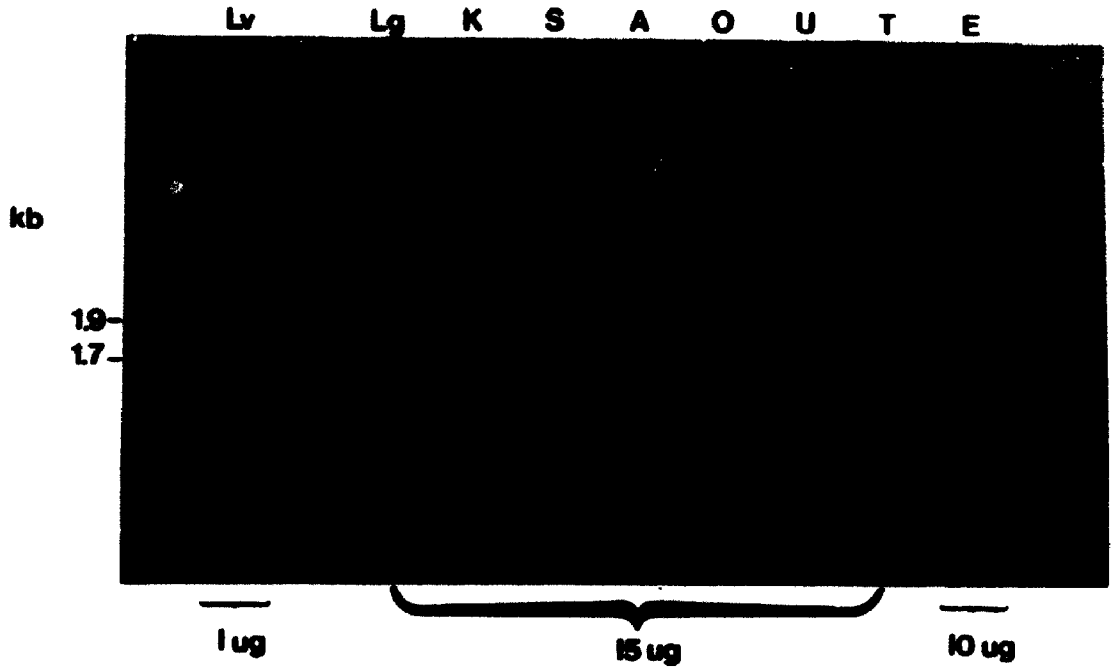
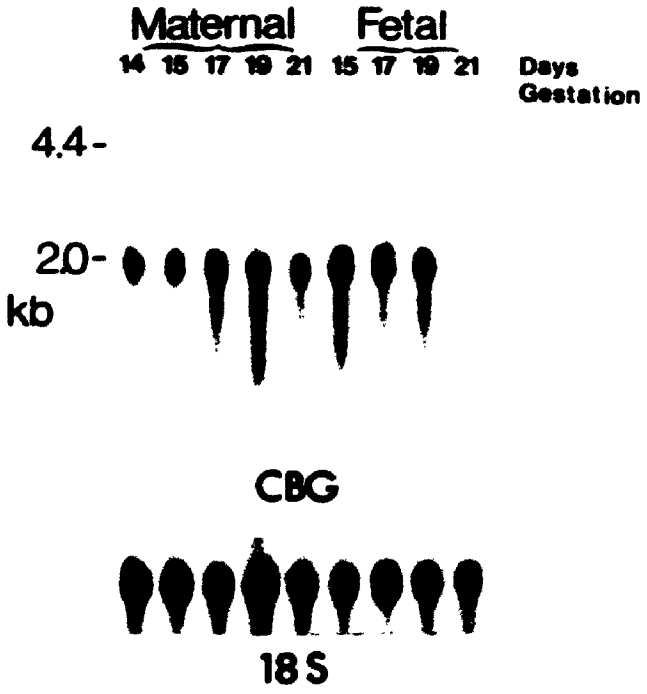

Lv
Lg
K
S
A
O
U
T
E
kb
1.9
1.7
1 ug
15 ug
10 ug
Poly (A)+ RNA

Figure 4.3 Northern blot of hepatic CBG mRNA (*top*) from mothers and their fetuses during the last third of gestation. Hepatic RNA (25 μg) was analyzed on days 14-21 for maternal samples and their corresponding pooled fetuses on days 15-21. Following membrane washing (0.2 x SSC/0.1% SDS at 37°C), the fluorogram was exposed for 3.5 h at -80°C. To show approximately equal amounts of RNA were loaded on each lane, the blots were stripped and rehybridized with a cDNA for mouse 18 S ribosomal RNA and exposed for 1.5 h (*below*). Molecular size markers are shown on the *left*.


Maternal
Fetal
14 15 17 19 21 15 17 19 21
Days Gestation
4.4-
2.0-
kb
CBG
18S

4.3).

In order to accurately quantify levels of CBG mRNA, aliquots of the hepatic RNA extracts examined in Figure 4.3 were also measured by solution hybridization assay (Figure 4.4A). The results indicate that the maternal hepatic CBG mRNA content did not change appreciably throughout the latter third of gestation. On the other hand, the hepatic CBG mRNA concentration in pooled 15 day fetuses (55.1 pg CBG mRNA/μg RNA) was greater than twice that detected in the corresponding maternal liver, and decreased progressively to relatively low levels (1.4 pg CBG mRNA/μg RNA) by day 21.

4.2.2.2 *Birth to Maturity*

A representative Northern blot of hepatic RNA from individual female rats (1-12 weeks old), revealed the presence of an approximately 1.8 kb CBG mRNA in all samples (Figure 4.5). The CBG mRNA content of samples taken at 1 and 2 weeks of age was lower than at subsequent time points, which were approximately equal. These observations were confirmed and extended by solution hybridization assay of hepatic RNA extracts obtained from male (n=2) and female (n=2) rats throughout postnatal development (Figure 4.6A). The levels of CBG mRNA measured in male and female samples (n = 4) obtained at one week were 0.4 - 0.8 pg CBG mRNA/μg RNA, and increased at least 5-fold by 2 weeks of age. After this time, CBG mRNA values doubled to reach adult levels by 3 weeks of age. Although only 2 male and female rats were used at each time point, it appears that a sex difference in hepatic CBG mRNA concentrations may already exist by 2 weeks of age, with male samples

Figure 4.4 Hepatic CBG mRNA (*A*) and serum CBG (*B*) levels in pregnant rats and their fetuses. Bars represent values obtained from mothers (n=1; open bar) and their corresponding fetuses (striped bar). Hepatic CBG mRNA was measured by solution hybridization assay and serum CBG levels were measured by a cortisol binding capacity assay. Serum was not available (NA) from day 15 fetuses.

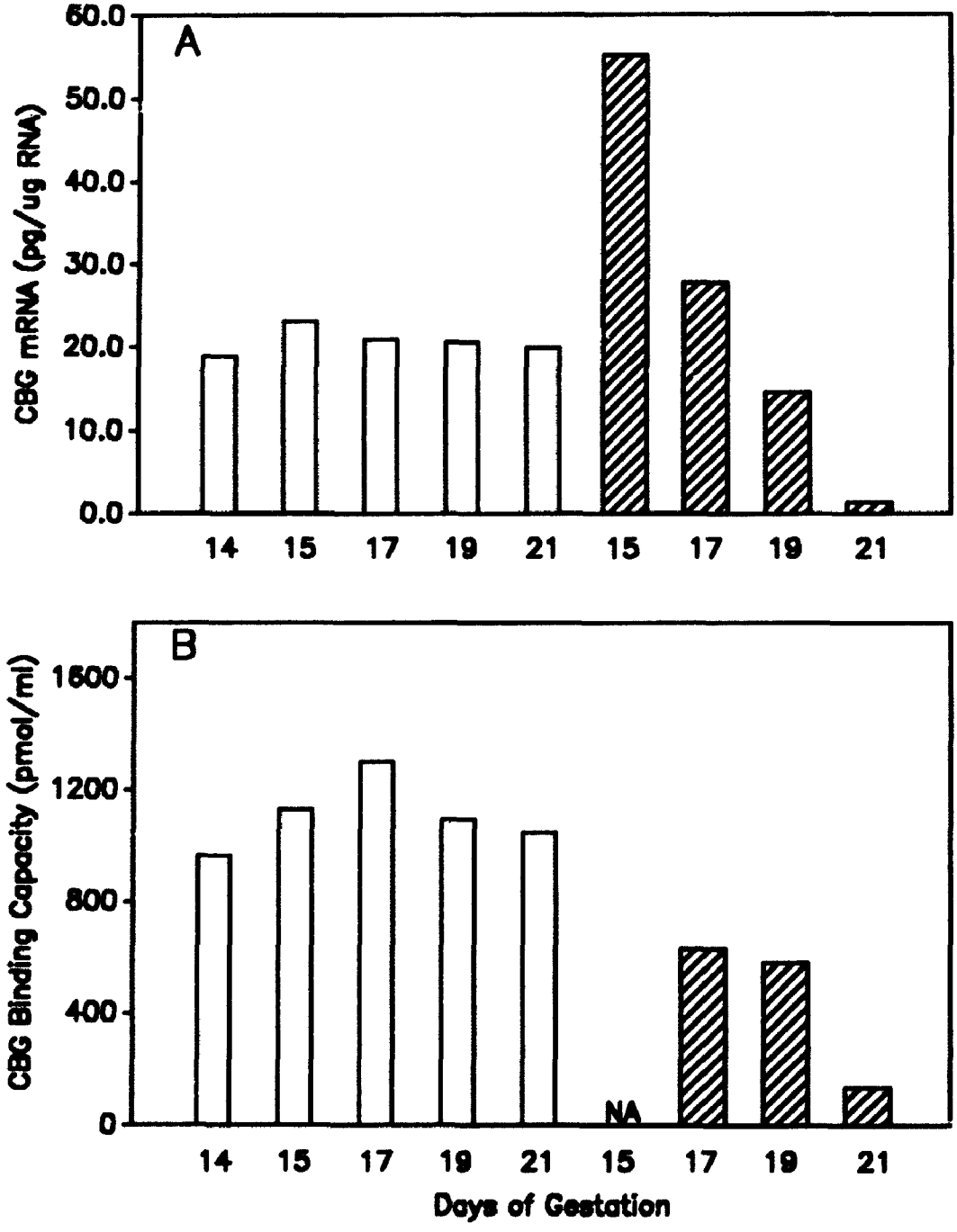

A
CBG mRNA (pg/ug RNA)
60.0
50.0
40.0
30.0
20.0
10.0
0.0
14
15
17
19
21
15
17
19
21
B
CBG Binding Capacity (pmol/ml)
1600
1200
800
400
0
NA
14
15
17
19
21
15
17
19
21
Days of Gestation

Figure 4.5 Northern blot of hepatic CBG mRNA in female rats during development (*top*). The fluorogram was exposed for 16 h. Subsequently, the blot was stripped and rehybridized with a cDNA for mouse 18 S ribosomal RNA and exposed 3 h (*below*). In both cases, blots were washed with moderate stringency (0.2 x SSC/0.1% SDS at 37°C). Molecular size markers are shown on the *left*.

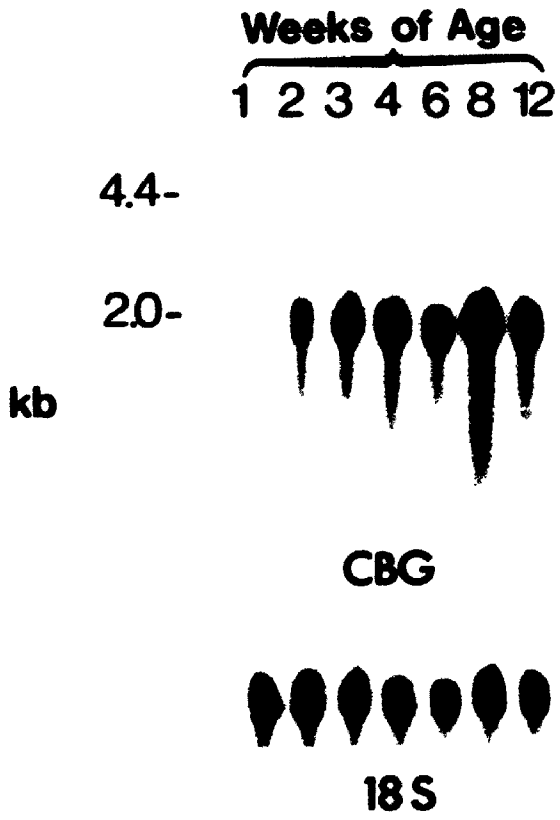
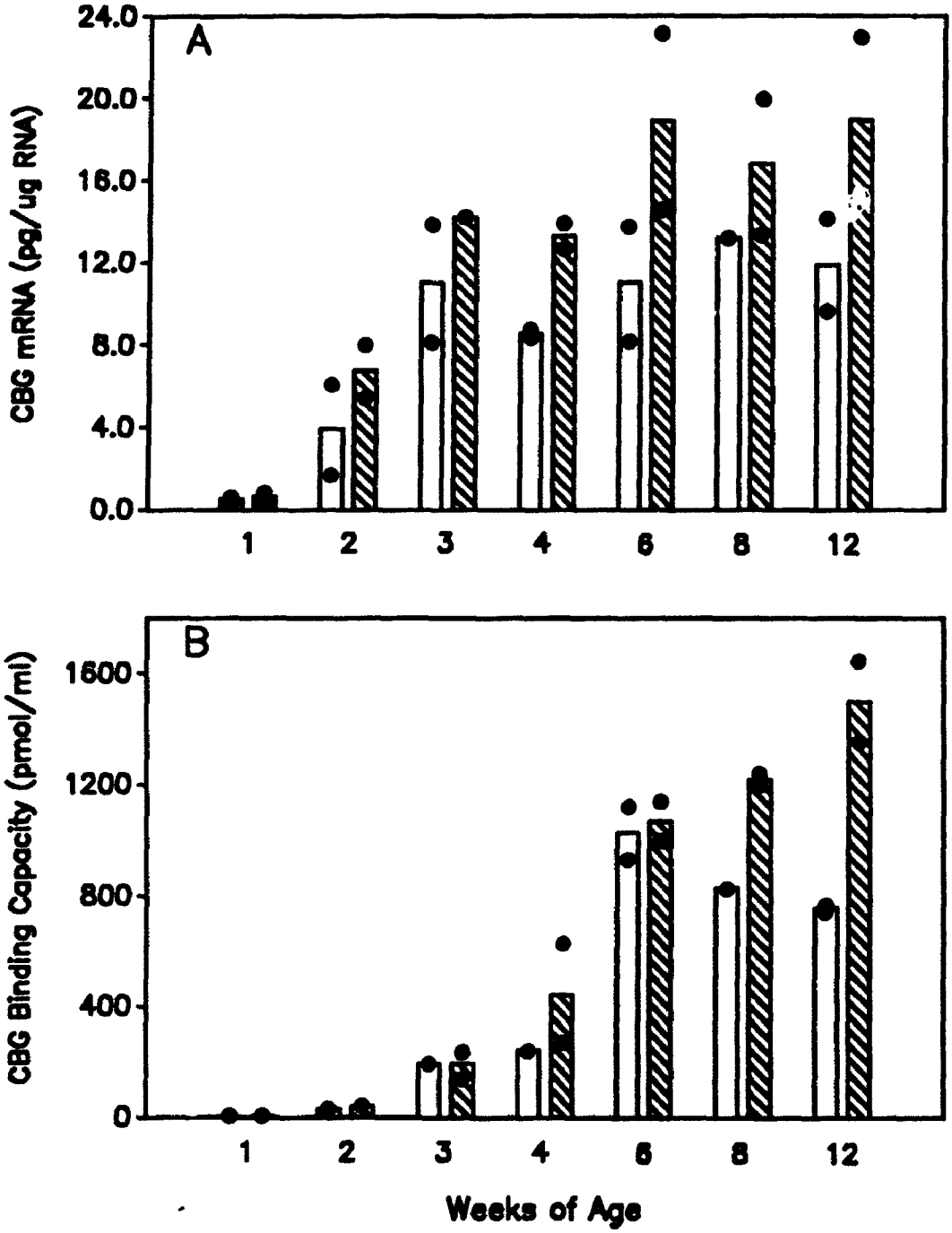

Weeks of Age
1 2 3 4 6 8 12
4.4-
2.0-
kb
CBG
18 S

Figure 4.6 Hepatic CBG mRNA (A) and serum CBG (B) levels in male (open bar) and female (stripped bar) rats. Each bar represents the mean value of 2 animals, and individual values are shown by ●. When values overlap, a single point is shown. Hepatic CBG mRNA was measured by solution hybridization assay and CBG levels were measured by cortisol binding capacity assay.


A
CBG mRNA (pg/ug RNA)
24.0
20.0
16.0
12.0
8.0
4.0
0.0
1
2
3
4
6
8
12
B
CBG Binding Capacity (pmol/ml)
1600
1200
800
400
0
1
2
3
4
6
8
12
Weeks of Age

containing on average two-thirds of the amount observed in female rat livers.

4.2.3 Serum CBG Measurements

4.2.3.1 *Maternal and Fetal*

Serum samples were obtained from individual mothers (day 14-21 of gestation) and their corresponding fetuses (days 17-21 only) and were used to measure CBG binding capacity. Serum CBG levels in maternal samples (Figure 4.4B) tended to exhibit a small increase which peaked at day 17, and then declined towards term. Although blood from the day 14 and 15 fetuses was unobtainable, the serum CBG concentrations in fetuses at days 17 and 19 (631 and 581 pmol/ml, respectively) was approximately half that found in the corresponding mothers. On the other hand, the fetal serum CBG level at day 21 of gestation (135 pmol/ml) was only approximately 13% of the value in the mother.

4.2.3.2 *Birth to Maturity*

Serum samples were also obtained from male (n=2) and female rats (n=2) at 1, 2, 3, 4, 6, 8 and 12 weeks of age. Serum CBG levels in one week old rats are very low (<8 pmol/ml) in both sexes and do not reach adult values until approximately 6 weeks of age (Figure 4.6B). However, by 8 weeks of age, there is a clear sex-difference in serum CBG levels, when adult female rats have approximately 50% higher levels when compared to age-matched males.

4.2.4 Comparison of Serum CBG and Hepatic CBG mRNA Levels

Serum CBG binding capacities were plotted against hepatic CBG mRNA levels in animals aged 3 weeks and older, and it was found that values fell into two groups that reflected their stage of maturity (Figure 4.7). In general, rats less than 4 weeks of age have much lower serum CBG concentrations in relation to their hepatic CBG mRNA content, when compared with rats greater than 6 weeks of age.

To exclude the possibility that changes in the steroid binding activity of CBG, or levels of unknown competitors during maturation might influence the binding capacity assay of infant and pubertal samples, a radioimmunoassay was used to measure CBG concentrations directly in postnatal samples. A good correlation by least squares fit analysis ($r=0.910$, $p<0.001$) was found between the values obtained by steroid binding capacity assay and radioimmunoassay (Figure 4.8).

4.2.5 Determination of the Biological Half-life of Serum CBG

In view of the discordancy between the time at which serum CBG and hepatic CBG mRNA levels reach adult values, the clearance of CBG purified from 3 week or adult rats was studied in immature and adult animals. Values were calculated as a percentage of ^{125}I-CBG present 1 h post-injection, which allows for differences in the initial redistribution volume between the two groups of animals. When either ^{125}I-labelled infant CBG ($n=2$) or adult CBG ($n=2$) was injected into 24 day old rats, the half-lives were 6.4 and 7.4 h, respectively (Figure 4.9). However, when ^{125}I-labelled CBG was injected into adult rats ($n=2$), the half-life was 14.5 h. The two-fold difference between these clearance rates therefore appears to be

Figure 4.7 Comparison of hepatic CBG mRNA levels and serum CBG binding capacities in rats aged 3 weeks (●), 4 weeks (▲) and 6-12 weeks (○).

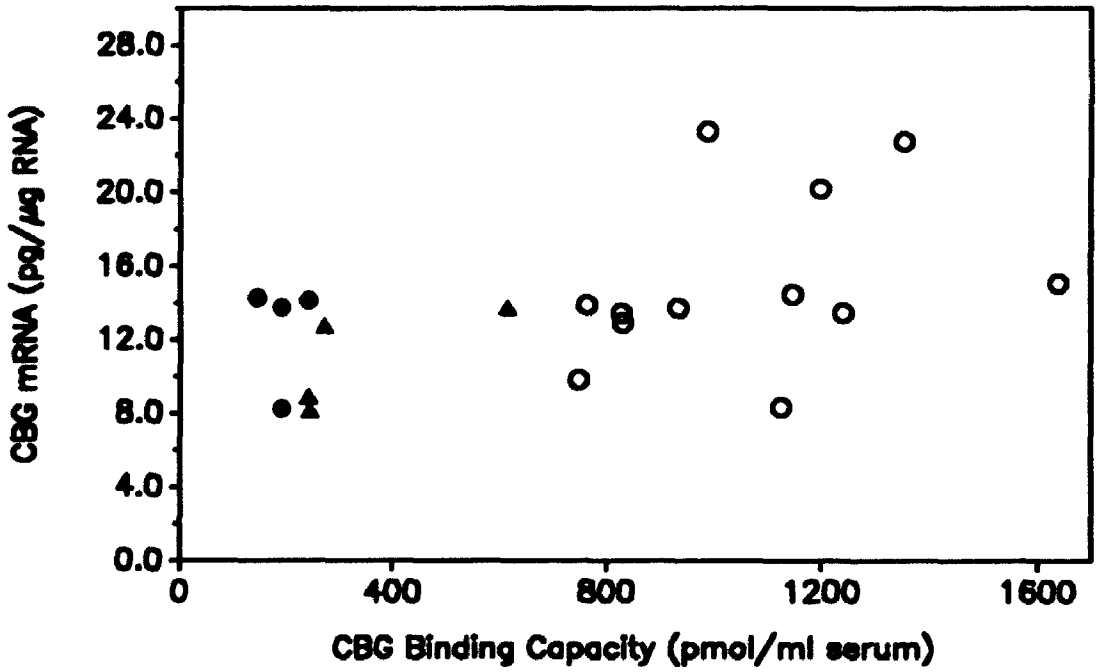

28.0
24.0
20.0
16.0
12.0
8.0
4.0
0.0
CBG mRNA (pg/µg RNA)
0
400
800
1200
1600
CBG Binding Capacity (pmol/ml serum)

Figure 4.8 Comparison of serum CBG measurements performed by cortisol binding capacity assay and radioimmunoassay. The linear regression line was obtained by least squares fit analysis and is defined as $y = 21.0\,x + 37.5$.

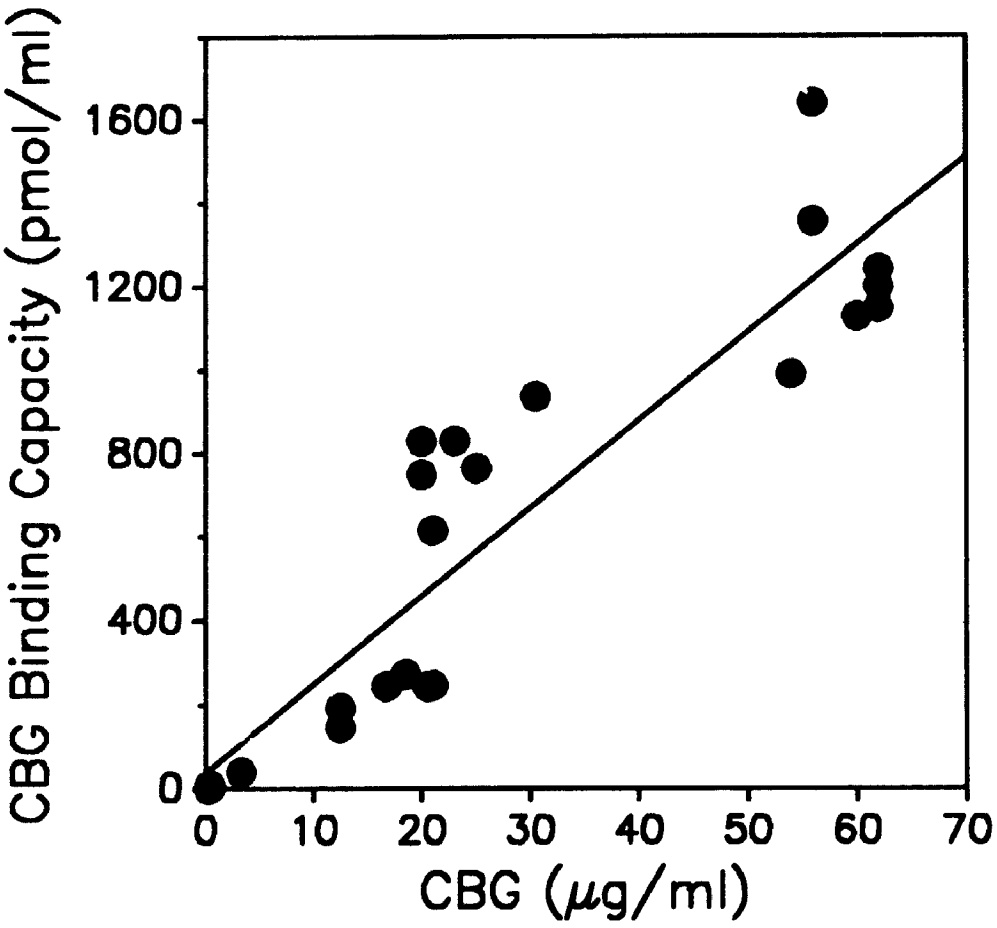
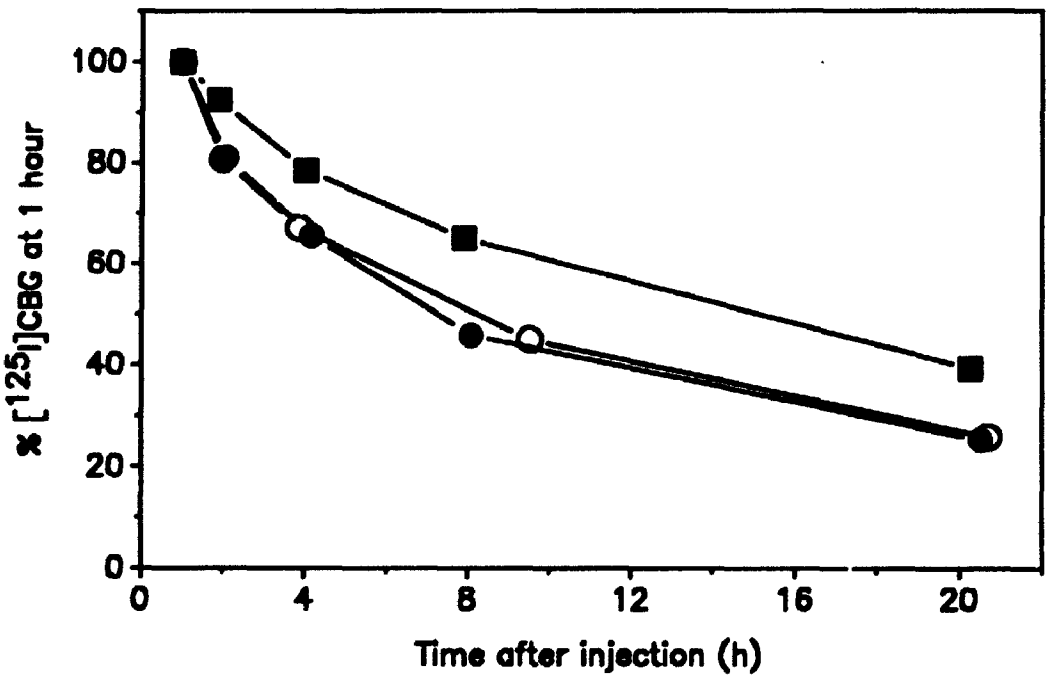

CBG Binding Capacity (pmol/ml)
1600
1200
800
400
0
0
10
20
30
40
50
60
70
CBG (μg/ml)

Figure 4.9 Serum clearance of infant (●) and adult (○) CBG in infant rats compared to adult (■) CBG in adult rats. Purified CBG preparations were radiolabelled with [^{125}I]Bolton-Hunter reagent and injected intravenously. Blood was drawn at the time points indicated and the amount of ^{125}I-CBG in serum samples is expressed as a percentage of the observed counts in samples obtained after 1 h. Each symbol represents the mean of 2 animals, and encompasses the values obtained at each time point.


100
80
60
40
20
0
0
4
8
12
16
20
% [125I]CBG at 1 hour
Time after injection (h)

dependent upon the age of the animal, rather than the origin of CBG.

4.3 Discussion

Although the Northern blot results strengthen the assumption that liver is the major site of CBG production in the rat, very small amounts of CBG mRNA have been detected in cells lining the basement membrane of rat bronchiolar epithelium by *in situ* hybridization (Hammond, 1990), and CBG-synthesizing polyribosomes have been detected in rat kidney with anti-CBG immunoglobulins (Kraujelis *et al.*, 1991). The discrepancy between these results is likely due to the different sensitivities of the techniques employed, and is consistent with the presence of CBG mRNA in a small subpopulation of cells within these given tissues. Indeed, immunochemical analysis of rat kidney slices demonstrated the presence of CBG only in cells surrounding the distal and collecting tubules (Kuhn *et al.*, 1986). The inability to detect rat CBG mRNA in extra-hepatic tissues by Northern blotting contrasts to the presence of CBG mRNA in maternal and fetal rabbit liver as well as maternal lung, spleen, ovary, and fetal kidney (Seralini *et al.*, 1990b). In addition, CBG mRNA is also present in the kidney and testis of rhesus monkey (Hammond *et al.*, 1987). It should be noted, however, that the abundance of hepatic CBG mRNA appears to be 1-3 orders of magnitude greater than in other tissues (Hammond *et al.*, 1987; Seralini *et al.*, 1990b). Although variations in Northern blotting sensitivity must be considered, these findings suggest that CBG gene expression is differentially regulated between tissues, species, and members of the same species under different physiological conditions.

Because the vast majority of CBG mRNA synthesis is located in the liver, this tissue was examined for changes in CBG mRNA levels during development. During the last third of gestation in the rat, maternal hepatic CBG mRNA levels (mean = 20.5 pg/μg RNA) do not change appreciably, and are only slightly higher than in 12 week old females (mean = 18.9 pg/μg). On the other hand, maternal serum CBG levels increased moderately at day 17 of gestation and declined towards birth, and this agrees with previous results (Van Baelen *et al.*, 1977b). These findings contrast with the striking increases in serum CBG and/or hepatic CBG mRNA levels observed in pregnant guinea pigs (Perrot-Applanat and Milgrom, 1979), hamsters (Lin *et al.*, 1990) and rabbits (Seralini *et al.*, 1990b), which are at least 3-4 times greater than in non-pregnant animals. Although it is unclear whether elevated serum CBG levels at day 17 in the pregnant rat are due to increased biosynthesis, it has been previously demonstrated that increased serum CBG concentrations in maternal rabbits during the same stage of gestation reflect an increase in production rather than a change in the clearance of the protein (Seralini *et al.*, 1989; Seralini *et al.*, 1990b).

Fetal liver taken at day 15 of gestation contains more than twice the CBG mRNA content than in any other sample and this is consistent with a previous report in which fetal rat hepatocytes removed at day 15 of gestation produced more CBG than those removed at day 18 (Vranckx *et al.*, 1985). This is, however, remarkable considering the relatively low number of hepatocytes when compared with erythropoietic cells in the liver at this stage of development (Greengard *et al.*, 1972). The production of CBG at this time may therefore play an important role in fetal

maturation, and is consistent with recent observations in fetal rabbits (Seralini *et al.*, 1990b). After day 15, the rapid decline in fetal hepatic CBG mRNA content suggests that the expression of the CBG gene is regulated independently in mother and fetus, and this could account for the 3- to 4-fold difference in maternal and fetal plasma CBG levels at term (Van Baelen *et al.*, 1977b; Martin *et al.*, 1977; Gewolb and Warshaw, 1986).

In adult rats, glucocorticoids suppress both serum CBG (Westphal, 1971) and hepatic CBG mRNA levels (see chapter 5), and dexamethasone treatment of pregnant rats produces a marked decline in maternal serum CBG levels on days 18-21 (Van Baelen *et al.*, 1977b). However, it appears that the fetal CBG gene is unresponsive to glucocorticoids prior to day 19, because fetal serum CBG levels are unaffected by maternal dexamethasone treatment at this time (Van Baelen *et al.*, 1977b). Furthermore, plasma corticosterone levels are relatively low at day 18 (Gewolb and Warshaw, 1986), and are therefore unlikely to account for the marked decrease in fetal hepatic CBG mRNA levels observed between days 15 and 17. Although the physiological significance of the decline in fetal CBG biosynthesis is unknown, the resulting decrease in circulating CBG concentrations will undoubtedly contribute to an increase in free glucocorticoid levels in the fetus (Gewolb and Warshaw, 1986), which may influence developmental events, such as lung maturation (Ballard, 1986).

The ability to detect CBG mRNA in day 21 fetal liver RNA extracts by Northern blot analysis depends upon the duration of autoradiography (see Figures

4.1 and 4.3). In addition, Northern blot analysis is subject to inconstant RNA transfer efficiencies and potential differences in RNA binding to the support matrix (Lee and Costlow, 1987). The solution hybridization assay enables the simultaneous measurement of a wide variation in CBG mRNA levels in a large number of samples with much greater precision.

Between day 18 of gestation and term, fetal rat plasma CBG levels decrease approximately 5-fold (Van Baelen *et al.*, 1977b) and subsequently increase during neonatal life (Henning, 1978; D'Agostino and Henning, 1981). In addition, a clear sex-difference is apparent by 7 weeks of age (Gala and Westphal, 1965b) which has been indirectly attributed, by measurement of *in vitro* translation products, to a difference in hepatic CBG mRNA levels (Faict *et al.*, 1985) and our results confirm these observations. The mechanisms responsible for the increase in serum CBG levels after birth are not well defined, but the administration of thyroxine appears to stimulate CBG biosynthesis in the neonate (D'Agostino and Henning, 1981; D'Agostino and Henning, 1982). Studies involving the castration of males, and androgen treatment of gonadectomized neonates, have also indicated that androgens play an important role in the imprinting of serum CBG levels in the adult male (Van Baelen *et al.*, 1977a). More recent work has indicated that this may involve a sex difference in the androgen-imprinted pulsatility of growth hormone secretion, which appears to influence CBG production (Jansson *et al.*, 1989). The fact that CBG mRNA levels in the liver approach adult values 2 weeks after birth, while serum CBG levels are still very low, demonstrates that this sexual dimorphism in CBG gene expression occurs very early during infancy.

By comparing the clearance-rate of CBG in infant and adult animals, we were also able to establish that a decrease in the biological half-life of the protein in immature rats probably accounts for the age-related difference in the profiles of hepatic CBG mRNA and serum CBG concentrations. In this context, it is important to note that the increased clearance-rate of CBG in immature animals is not related to differences in the composition of the protein, because CBG isolated from infants and adults behaves identically. Although the reason for this increase in CBG turn-over in infant rats remains to be determined, it is possible that CBG may be expended during the process of glucocorticoid delivery to developing tissues, and this may involve an interaction with a serine proteinase. An analogous situation appears to occur during inflammation when CBG-cleavage by elastase results in the release of bound steroid (Hammond *et al.*, 1990a), and may also contribute to the rapid decrease in serum CBG levels during acute inflammation (see section 1.4.5). This suggestion is not unreasonable because serine proteinases have been implicated in the normal process of tissue development and remodelling (Vassalli *et al.*, 1979; Travis and Salvesen, 1983), as well as inflammation (Travis and Salvesen, 1983), and it is therefore possible CBG may play a role in the delivery of glucocorticoids to tissues during development.

CHAPTER 5

The Influences of Hormonal Treatment and Inflammation on Rat Corticosteroid Binding Globulin and its Messenger RNA

5.1 Introduction

The levels of rat CBG vary with the age, sex and physiological condition of the animals (see introduction), and the influence of hormones and inflammation on serum CBG levels have been studied extensively (for review see Westphal, 1971 and Westphal, 1986). Although changes in serum CBG levels have been attributed to alterations in its biosynthesis (Yamamoto and Ohsawa, 1976; D'Agostino and Henning, 1982; Feldman *et al.*, 1979; Faict *et al.*, 1985), the evidence for this is indirect. In fact, age-dependent alterations in the plasma clearance of rat CBG (Smith and Hammond, 1991a) suggest that factors which regulate the biological half-life of CBG may also contribute to the maintenance of appropriate serum levels. In this context, the plasma clearance of the CBG-related protein, thyroxine binding globulin (Hammond *et al.*, 1987; Imamura *et al.*, 1991) is reduced following estrogen administration, and this has been attributed to a change in the carbohydrate moieties associated with this protein (Ain and Refetoff, 1988).

Therefore, in order to determine the biochemical basis for variations in serum CBG levels following hormone administration and inflammation, hepatic CBG mRNA levels were determined and contrasted to serum CBG values. In addition, the basis for changes in CBG mRNA levels was characterized by nuclear run-off analysis, and alterations in the carbohydrate structure of CBG were examined by concanavalin A-chromatography.

5.2 Results

5.2.1 Influences of Hormones on Hepatic CBG and Serum CBG levels

5.2.1.1 *Effects of Hormonal Treatment on Hepatic CBG mRNA Levels*

Intact male rats (6 per group) were treated with dexamethasone, prednisolone, corticosterone, 17ß-estradiol, ethinylestradiol, estrone, progesterone, 5α-dihydrotestosterone, thyroid hormone (T_4) or the vehicles, dilute NaOH (n = 3) or sesame oil (n = 6). At the conclusion of the 3 day treatment period, liver and serum samples were collected for CBG mRNA analysis and CBG measurement by cortisol binding capacity assay, respectively (see section 5.2.1.2). A representative RNA extract from each treatment group was examined for the presence of CBG mRNA by Northern blot analysis, and an approximately 1.8 kilobase CBG mRNA was detected in all samples except that obtained from a dexamethasone-treated rat (Figure 5.1). To more accurately quantify these differences, the hepatic CBG mRNA levels of all animals were examined by solution hybridization assay, and were significantly different ($p < 0.001$) from control values only in dexamethasone-treated rats (Figure 5.2A and Table 5.1) in which the hepatic CBG mRNA levels were below the limit of detection (0.3 pg/ng RNA). The CBG mRNA levels of the remaining test groups were not statistically different from control values, but several trends were observed. Prednisolone and ethinylestradiol reduced CBG mRNA levels by 14% and 24%, respectively, while T_4 increased CBG mRNA levels 39% when compared to appropriate control values.

Figure 5.1 Northern blot of CBG mRNA in 10 μg hepatic RNA obtained from intact male rats treated with 1, oil (vehicle); 2, dexamethasone; 3, prednisolone; 4, corticosterone; 5, 17ß-estradiol; 6, ethinylestradiol; 7, estrone; 8, progesterone; 9, dihydrotestosterone and 10, L-thyroxine (*top*). The blot was washed to high stringency (0.1 x SSC/ 0.1% SDS at 60°C) and exposed to XAR2 film for 21 h at -80°C. Subsequently, the blot was stripped and rehybridized with a cDNA for mouse 18 S ribosomal RNA and exposed for 16 h (*below*). Molecular size markers are shown on the *right*.

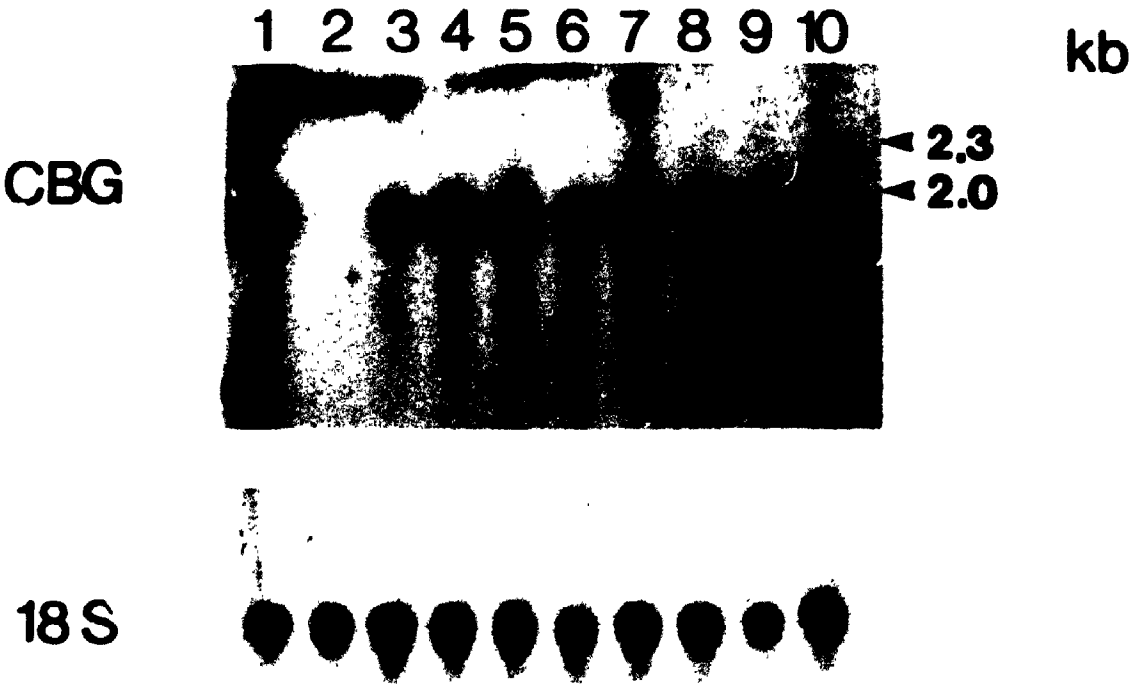

1 2 3 4 5 6 7 8 9 10
kb
CBG
2.3
2.0
18 S

Figure 5.2 Hepatic CBG mRNA (*A*) and serum CBG (*B*) levels of intact male rats treated with various hormones. Hepatic CBG mRNA was not detected (ND) in dexamethasone-treated samples. Bars represent the mean values of 6 animals, except for NaOH where n = 3, and error indicators represent standard error. Hepatic CBG mRNA was measured by solution hybridization assay and CBG levels were measured by cortisol binding capacity assay.

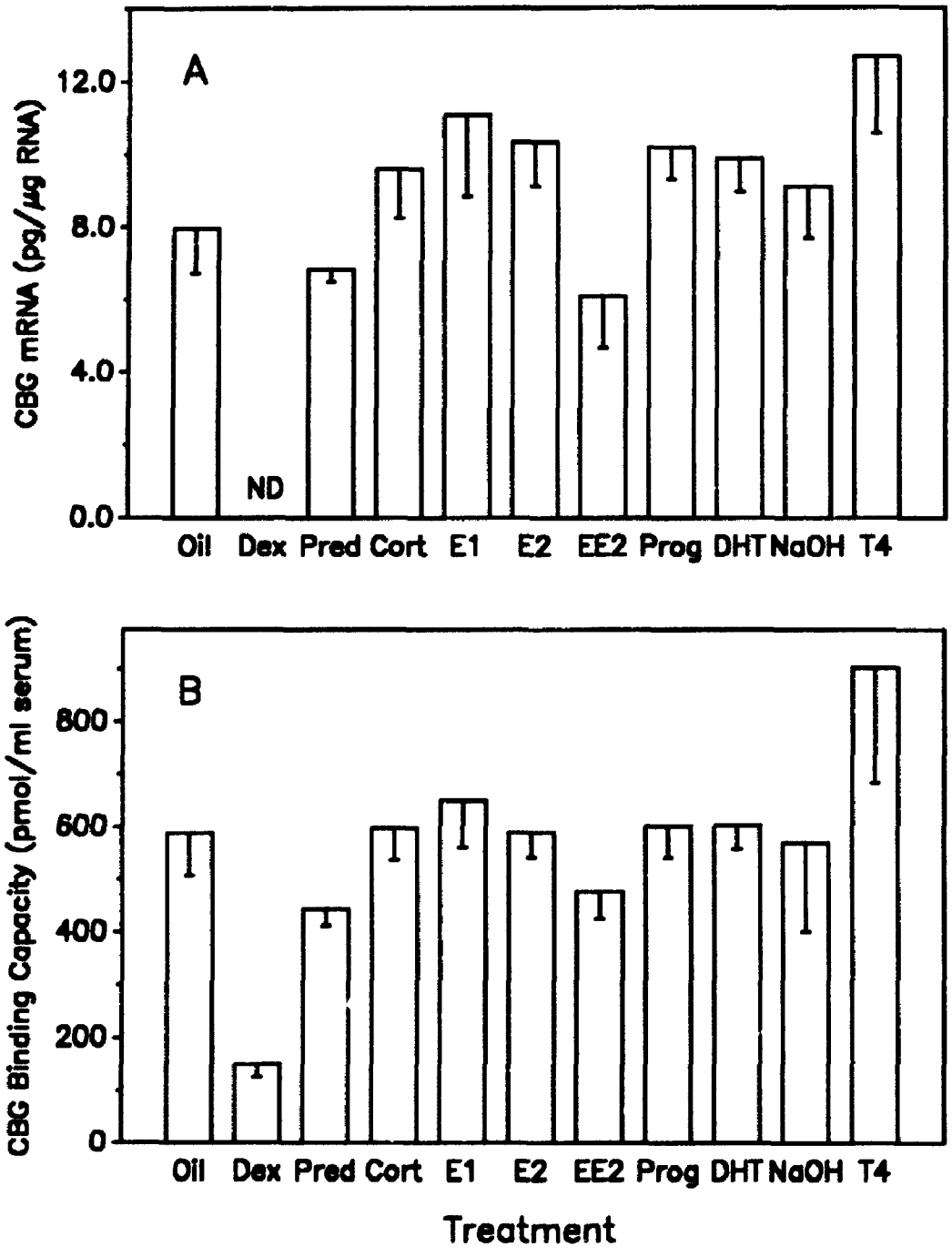

A
12.0
8.0
4.0
0.0
CBG mRNA (pg/μg RNA)
ND
Oil Dex Pred Cort E1 E2 EE2 Prog DHT NaOH T4
B
800
600
400
200
0
CBG Binding Capacity (pmol/ml serum)
Oil Dex Pred Cort E1 E2 EE2 Prog DHT NaOH T4
Treatment

Table 5.1 Hepatic CBG mRNA levels following hormone administration. Values obtained from individual rats are given, as are means and standard deviations (SD). Hepatic CBG mRNA was not detected (ND) in dexamethasone-treated rats. Values determined for samples obtained from steroid-treated rats were compared with those obtained for oil-treated animals to determine the significance (p values) between these groups. Similarly, CBG mRNA levels in T_4-treated rats were compared to those determined for NaOH-treated animals.

Hormone Treatment	Hepatic CBG mRNA (pg/μg RNA)						n	mean	SD	p
Oil	12.0	6.6	4.2	9.2	10.3	5.4	6	7.95	3.03	
Dexamethasone	ND	ND	ND	ND	ND	ND	6	0	0	<0.001
Prednisolone	6.7	7.4	7.3	6.2	5.6	7.8	6	6.83	0.83	0.404
Corticosterone	13.1	9.0	14.2	5.8	7.4	8.1	6	9.60	3.33	0.390
Estrone	12.4	15.0	9.8	1.5	17.3	10.5	6	11.08	5.47	0.248
Estradiol	11.4	10.4	13.6	5.2	12.4	9.0	6	10.33	2.97	0.199
Ethinylestradiol	11.7	7.5	3.8	6.8	5.0	1.6	6	6.07	3.48	0.341
Progesterone	12.6	8.3	12.2	7.2	11.1	9.7	6	10.18	2.16	0.172
Dihydrotestosterone	11.9	11.2	7.4	6.7	10.4	11.7	6	9.88	2.27	0.239
NaOH	10.9	10.1	6.3				3	9.10	2.46	
Thyroxine	15.6	20.3	14.5	9.5	6.1	10.0	6	12.67	5.11	0.301

5.2.1.2 *Effects of Hormonal Treatment on Serum CBG Levels*

Serum samples from both groups of control animals had similar CBG levels before and after treatment. However, dexamethasone and ethinylestradiol treatment reduced serum CBG levels to 25% ($p < 0.001$) and 81% ($p < 0.015$) of pretreatment values, respectively. No other significant changes were observed. Samples for RNA measurement were available only at the conclusion of the study, and to evaluate changes in serum CBG values with respect to CBG mRNA, the CBG levels of samples obtained at the same time (i.e. the conclusion of the study) were used for comparison. Following dexamethasone administration, rat serum CBG levels were approximately 26% of the appropriate control (sesame oil) values, and this difference was highly ($p < 0.001$) significant (Figure 5.2B and Table 5.2). Although not statistically significant ($p > 0.05$), the serum CBG levels of rats treated with T_4 increased by approximately 66% in comparison to the NaOH control group, and the average CBG levels of rats injected with prednisolone and ethinylestradiol declined to 75% and 81% of the CBG level of oil-treated rats. The serum CBG levels of the other test groups were similar to control values.

Alterations in serum CBG levels in response to hormonal treatment were greatest in rats treated with dexamethasone and T_4, and the kinetics of these changes were therefore examined further. Serum samples obtained from rats (n=3) prior to and 1, 2 and 3 days following the initiation of hormone administration were examined by CBG binding capacity assay. Control values did not change throughout the treatment period and the serum CBG levels of the test groups were not significantly different from those of sesame oil-treated rats prior to and one day

Table 5.2 Serum CBG levels following hormone administration. Values obtained from individual rats are given, as are the means and standard deviations (SD). Values obtained for samples from steroid-treated rats were compared with those determined for oil-treated animals to determine the significance (p values) between groups. Similarly, CBG levels in T_4-treated rats were compared to those determined for NaOH-treated animals.

Hormone Treatment	Serum CBG (pmol/ml)						n	mean	SD	p
Oil	844	662	386	661	662	315	6	588	199	
Dexamethasone	152	79	135	190	243	101	6	150	60	<0.001
Prednisolone	426	458	426	342	429	579	6	443	77	0.126
Corticosterone	766	636	340	573	699	571	6	597	147	0.929
Estrone	923	832	420	384	713	629	6	650	217	0.616
Estradiol	555	545	671	430	774	559	6	589	119	0.991
Ethinylestradiol	589	578	408	478	553	256	6	447	128	0.276
Progesterone	517	846	482	473	697	593	6	601	146	0.899
Dihydrotestosterone	712	710	497	673	484	541	6	603	107	0.877
NaOH	889	507	313				3	570	293	
Thyroxine	1377	1764	650	601	542	749	6	947	502	0.277

following initial hormonal treatment (Figure 5.3). However, by the second day, serum CBG levels of dexamethasone- and T_4-treated rats were significantly lower ($p < 0.002$) and higher ($p < 0.05$) than control values, respectively. At the conclusion of the hormonal treatments, serum CBG levels of dexamethasone-treated rats were approximately 29% of control values ($p < 0.001$), while serum CBG levels of T_4-treated animals were approximately 224% of controls ($p < 0.05$).

5.2.2 Hormonal Influences on CBG Glycosylation

The influence of dexamethasone and T_4 administration on the proportion of CBG molecules lacking biantennary oligosaccharide chains was examined by concanavalin A-chromatography of serum samples ($n = 2$ for each group) obtained at the conclusion of a three day treatment regime. Column fractions containing immunoreactive CBG were pooled, dialysed and their corticosterone binding capacity and protein content were determined. When expressed as fmol CBG/μg protein, there was no difference in the proportion of concanavalin A-bound and nonbound CBG between the treatment groups, and 43.2 ± 2.1% (mean ± standard error) of the CBG applied to the columns was not retained (Table 5.3).

5.2.3 Hormonal Influences on the Transcription Rate of the CBG Gene

To determine the influence of dexamethasone and thyroxine administration on the relative transcription rate of the CBG gene, nascent RNA transcripts were pulse-labelled *in vitro* and hybridized to slot blots of CBG, ß-actin and albumin cDNAs (Figure 5.4). The experiment was repeated twice, and CBG hybridization

Figure 5.3 Time course of serum CBG levels following hormonal administration. Bars represent the means of 3 animals treated with dexamethasone (solid), sesame oil (cross-hatched) and T_4 (open). Error indicators represent standard errors. Levels of CBG were measured by corticosterone binding capacity assay. Significance is indicated by * ($p < 0.05$) and ** ($p < 0.002$).

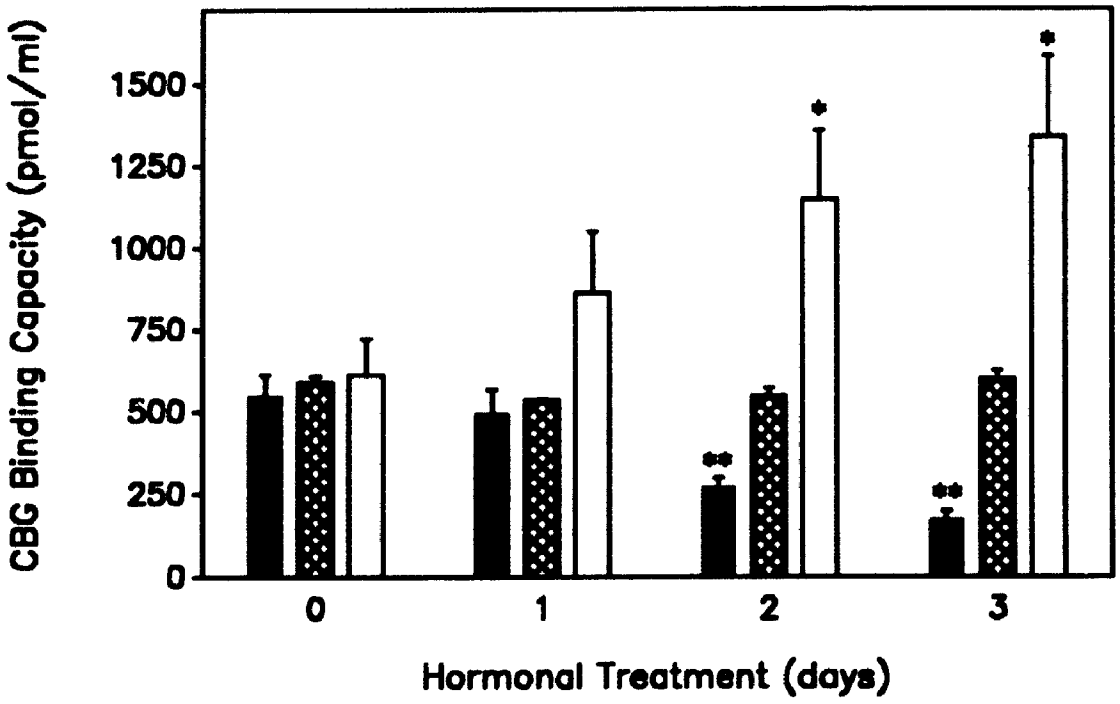
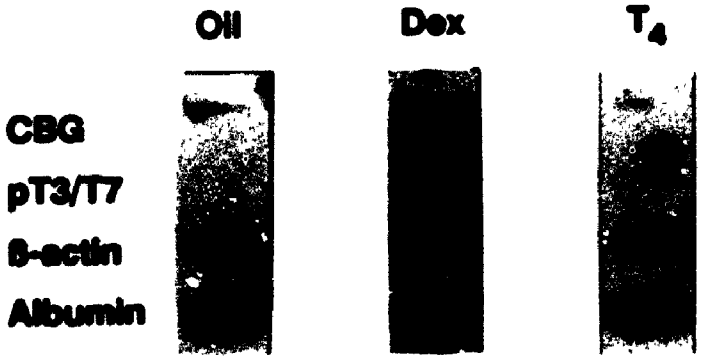

CBG Binding Capacity (pmol/ml)
1500
1250
1000
750
500
250
0
0
1
2
3
Hormonal Treatment (days)
*
*
**
**

Table 5.3 Analysis of the proportion of concanavalin A-bound rat serum CBG. Sera obtained from rats treated with sesame oil (n = 2), dexamethasone (n = 2) or L-thyroxine (n = 2) were subjected to concanavalin A-chromatography, and fractions containing immunoreactive CBG were pooled and subjected to protein measurement by Bradford assay, and CBG measurement by corticosterone binding capacity assay. Individual values are shown for all rats.

	C?		Dexamethasone		Thyroxine	
	Bound	Non-Bound	Bound	Non-Bound	Bound	Non-Bound
protein (μg)	356	1084	434	1345	400	851
	371	1368	355	1282	247	946
CBG	14.5	13.3	4.6	3.8	39.5	20.2
(pmol)	18.6	14.3	2.4	2.2	24.6	17.4
CBG/protein	40.7	12.3	10.6	2.8	98.7	23.7
(fmol/μg)	50.1	10.5	6.8	1.7	99.6	18.4
CBG/protein	76.8	23.2	79.1	20.9	80.6	19.4
(%)	82.7	17.3	80.0	20.0	84.4	15.6

Figure 5.4 Hybridization of *in vitro* ^{32}P-labelled RNA, extracted from nuclei obtained from sesame oil, dexamethasone (dex) or thyroxine treated rats, with cDNAs for CBG, ß-actin and albumin. The vector, pT3/T7, was included to assess non-specific interactions. Blots were washed at high stringency and exposed for 16 h at -80°C.


Oil
Dex
T_4
CBG
pT3/T7
β-actin
Albumin

signals were quantified by densitometry and expressed relative to the signals obtained for ß-actin mRNA in order to account for interassay differences in nuclei number and viability (Figure 5.5). Although the relative CBG mRNA signal varied between experiments, the rate of CBG gene transcription in nuclei obtained from dexamethasone-treated rats was substantially below control values in both experiments. The relative CBG mRNA signals obtained for thyroxine-treated rats were more variable, and were either less than or equal to control values.

5.2.4 Influence of Inflammation on CBG and its mRNA

5.2.4.1 *Serum CBG Levels following Induction of Inflammation*

Acute inflammation was induced in two rats by cecal ligation and perforation (CLP) at approximately 14:00 h and 5 blood samples taken from each rat over the subsequent 26 h were analyzed by CBG binding capacity assay. This revealed a gradual and progressive decline in serum CBG levels, and by the conclusion of the study period at least a 50% decrease in serum CBG was realized for both experimental animals (Figure 5.6). In contrast, the serum CBG levels of the control animal underwent a transient increase which peaked at approximately 20:00 h, and returned to pre-treatment CBG levels at the end of the experiment. Serum CBG measurements were also performed by radioimmunoassay to exclude the possibility that unknown factors produced during sepsis might influence the steroid binding activity of CBG, but no discrepancies between the binding capacity and radioimmunoassay values were observed (Figure 5.7).

Figure 5.5 Transcription rate of the CBG gene relative to ß-actin. Hybridization signals for CBG and ß-actin mRNA were obtained from two nuclear run-off experiments and quantified by scanning laser densitometry. A signal for CBG mRNA was not detected (ND) in one of the dexamethasone-treated samples.

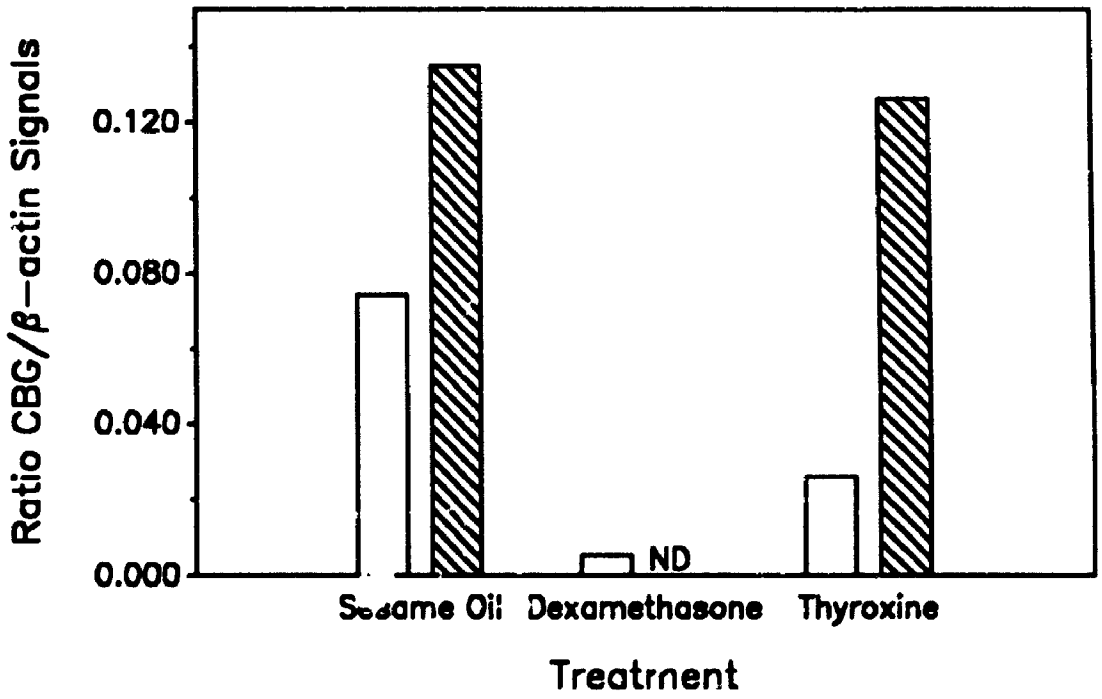

Ratio CBG/β–actin Signals
0.120
0.080
0.040
0.000
ND
Sesame Oil
Dexamethasone
Thyroxine
Treatment

Figure 5.6 Serum CBG binding capacity in rats after cecal ligation and perforation (○ = 1 hole, ● = 2 holes) when compared to a control animal (▵). The time scale is presented in terms of the 24 hour clock. The arrow represents time of CLP.

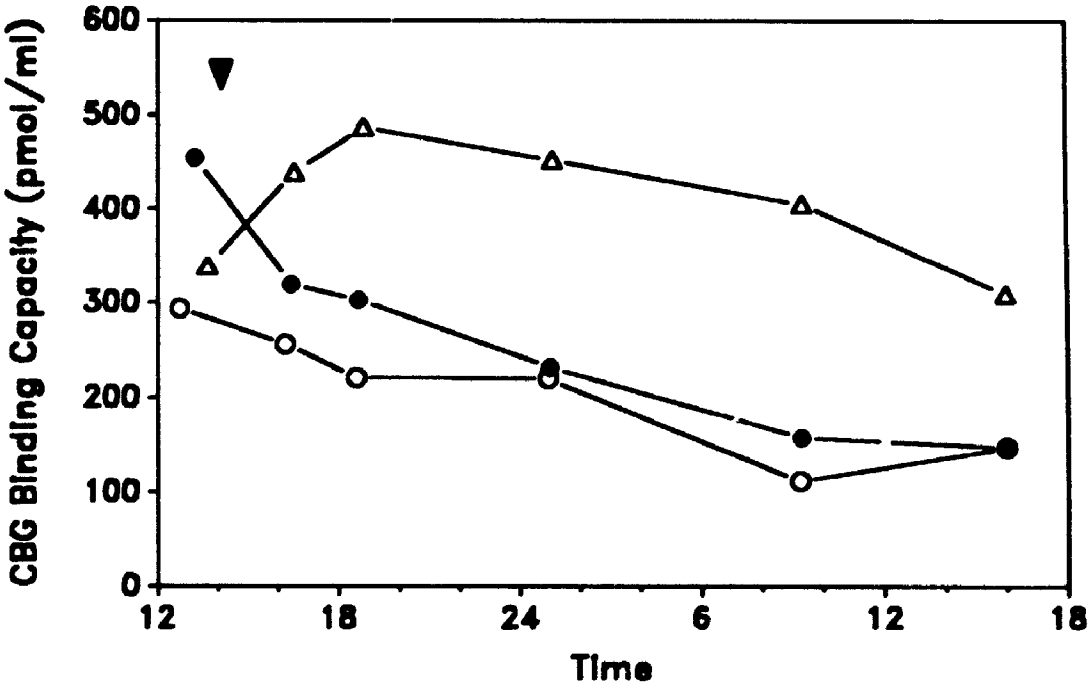
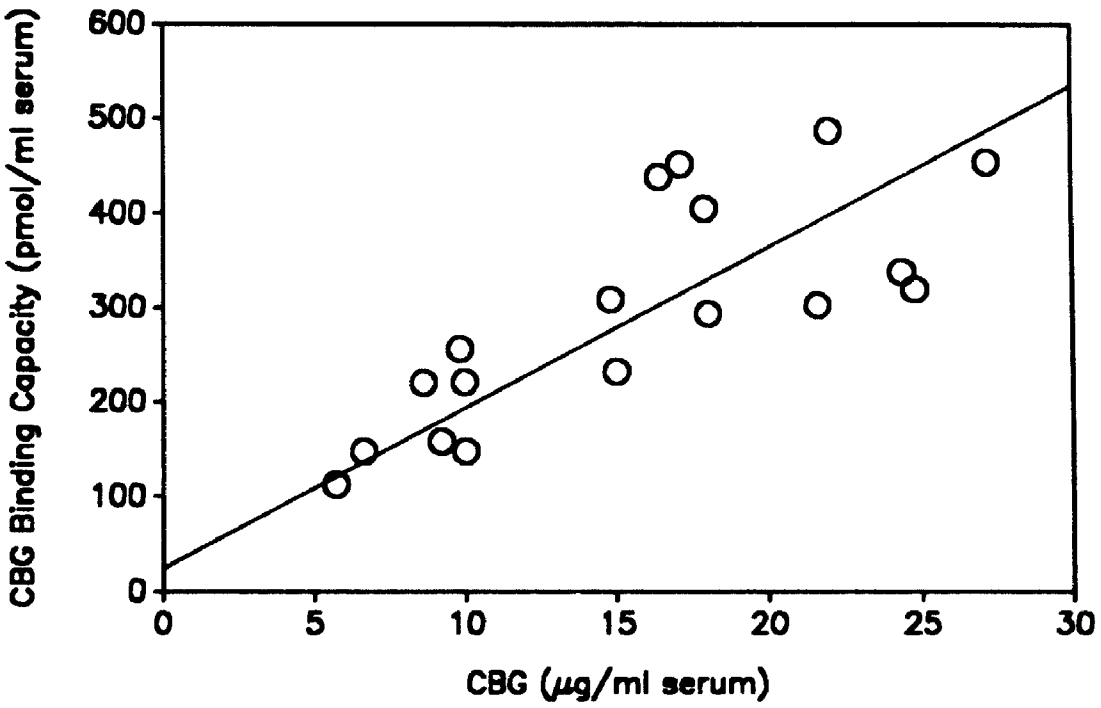

CBG Binding Capacity (pmol/ml)
600
500
400
300
200
100
0
12
18
24
6
12
18
Time

Figure 5.7 Comparison of serum CBG measurements performed by cortisol binding capacity assay and radioimmunoassay. The linear regression was obtained by least squares fit analysis and is defined as $y = 17.3\ x + 17.0$.


CBG Binding Capacity (pmol/ml serum)
600
500
400
300
200
100
0
0
5
10
15
20
25
30
CBG (μg/ml serum)

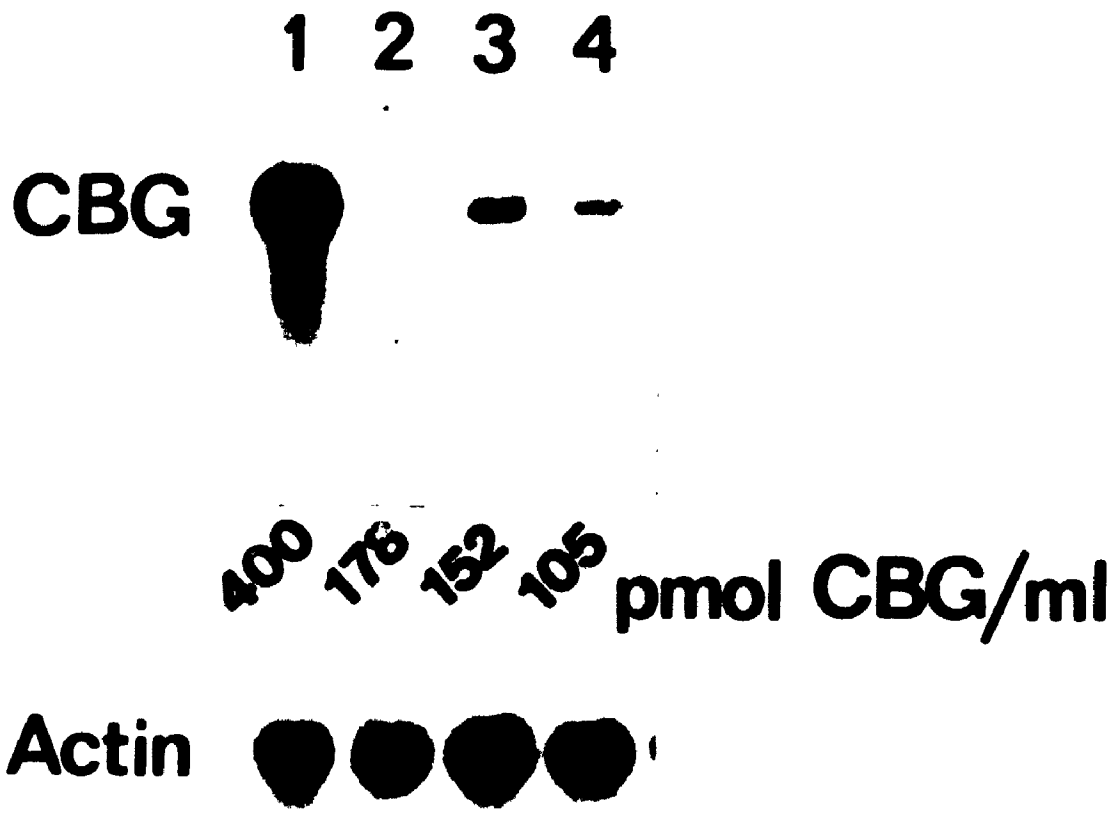
5.2.4.2 *Hepatic CBG mRNA Levels following Induction of Inflammation*

The CLP procedure was performed on an additional three rats, and at the conclusion of the 24 h study period, their livers were excised and total hepatic RNA was extracted. Northern blots and subsequent scanning laser densitometry revealed the CBG mRNA levels of septic animals to be only 11.2 ± 2.1% (mean ± standard error) of the control rat (Figure 5.8). Serum CBG levels were also determined at the conclusion of the study, and revealed characteristically low values for the septic animals (Figure 5.8).

5.3 Discussion

Based on the limits of detection of the solution hybridization assay, the inability to detect CBG mRNA in dexamethasone-treated rats represents at least a 26-fold reduction in steady-state levels when compared to control values. Previously, the *in vitro* production of CBG from isolated perfused livers obtained from dexamethasone-treated, adrenalectomized rats was noted to be reduced in comparison to that produced by the livers of untreated animals (Feldman *et al.*, 1979). Furthermore, cyclohexamide administration to rats pre-treated with dexamethasone maintains low plasma CBG levels and suggests this hormone decreases the rate of CBG synthesis rather than its clearance (Yamamoto and Ohsawa, 1976). The absence of hepatic CBG mRNA following dexamethasone treatment indicates that reduced CBG biosynthesis is largely due to decreased steady-state levels of CBG mRNA. Moreover, the greater than 14-fold reduction in the relative transcription rate of the CBG gene suggests dexamethasone represses

Figure 5.8 Northern blot of hepatic CBG mRNA (*top*) in intact male rats made septic by cecal ligation and perforation (*lanes 2 - 4*) and in a normal rat (*lane 1*). Hepatic RNA (20 μg) was hybridized with a radiolabelled rat CBG cDNA. Subsequently, the blot was stripped and rehybridized with a cDNA for mouse βactin (*below*). In both cases, blots were washed with 0.2 x SSC/0.1% SDS at 37°C. Molecular size markers are shown on the *left*. Serum CBG levels (*between blots*) were measured by cortisol binding capacity assay.


1 2 3 4
CBG
400 176 152 105 pmol CBG/ml
Actin

hepatic CBG gene expression. It is therefore remarkable that serum CBG levels persist when hepatic CBG mRNA levels are very low and this may result from a reduced CBG clearance rate, which would maintain serum CBG concentrations, and/or extra-hepatic CBG synthesis not inhibited by glucocorticoids.

In rats, glucocorticoid administration has been associated with a reduction in serum CBG levels, and removal of endogenous sources of glucocorticoids results in increased serum CBG levels (for review see Westphal, 1971 and Westphal, 1986). The serum CBG and hepatic CBG mRNA levels observed for the glucocorticoids employed in this study are inversely proportional (dexamethasone >> prednisolone > corticosterone) to their relative biological potencies (Szefler, 1989) and this likely represents the ability of a fixed dose of these steroids to interact with the glucocorticoid receptor. It is therefore reasonable that the corticosterone dosage (25 μg/100 g body weight) used in this study did not cause a significant difference in serum CBG or its hepatic mRNA levels, since a dose of approximately 2 mg/100 g body weight decreases CBG levels of adrenalectomized animals by only 50% (Westphal *et al.*, 1963).

Rats treated with estrogens exhibited no significant alterations in their serum CBG or hepatic CBG mRNA levels when compared to the control group, and this is surprising in view of previous work which demonstrated an approximately 50% increase in the serum CBG levels of intact males (Gala and Westphal, 1965b; Kawai and Kuzuya, 1972) and a 100% increase in CBG secretion from a perfused liver system (Feldman *et al.*, 1979). However, this apparent lack of response may be

related to the 3 day treatment protocol used in this study since Kawai *et al* (1972) demonstrated a significant increase only after 5 days of treatment, and Gala and Westphal (1965b) administered estradiol for 10 days. It would therefore seem likely that if treatment were continued beyond 3 days, the small increases in serum CBG and hepatic CBG mRNA levels of estrone- and 17ß-estradiol-treated rats observed in this study would increase to statistically significant levels. Furthermore, the parallel increase in serum CBG and hepatic CBG mRNA levels suggests increased CBG mRNA levels are responsible for higher protein values.

Estrogen-induced alterations in serum CBG levels have only been observed in intact male rats, and not in females or castrated males (Gala and Westphal, 1965b; Gala and Westphal, 1966c). This increase is also not observed in hypophysectomized male rats (Kawai and Kuzuya, 1972; Gala and Westphal, 1966a) and the release of thyroid-stimulating hormone from the anterior pituitary following estrogen administration (Labrie *et al.,* 1968) probably increases CBG levels via increased thyroxine secretion (Labrie *et al.,* 1968; Gala and Westphal, 1966b; Gala and Westphal, 1966c). The trend towards a reduction in serum CBG and hepatic CBG mRNA levels following ethinylestradiol administration is surprising considering the influence of estradiol, and suggests the effects of sustained, long-acting estrogen administration may directly influence hepatic CBG biosynthesis and over- ride the effects of increased thyroid-stimulating hormone production.

Treatment with progesterone did not alter serum CBG levels from controls, although higher doses (approximately 5 mg/100 g body weight for 10 days) have

been demonstrated to increase CBG levels of both intact males and females by 90 and 21%, respectively (Gala and Westphal, 1965b). The mechanism behind the progesterone-induced increase in CBG levels has been suggested to be mediated in much the same manner as post-adrenalectomy rises in CBG (Gala and Westphal, 1965b); co-incident with prolonged progesterone administration, corticosterone levels are reduced (approximately 23 - 52% of normal) and this would alleviate their negative influence on CBG levels, and subsequently increase serum CBG levels.

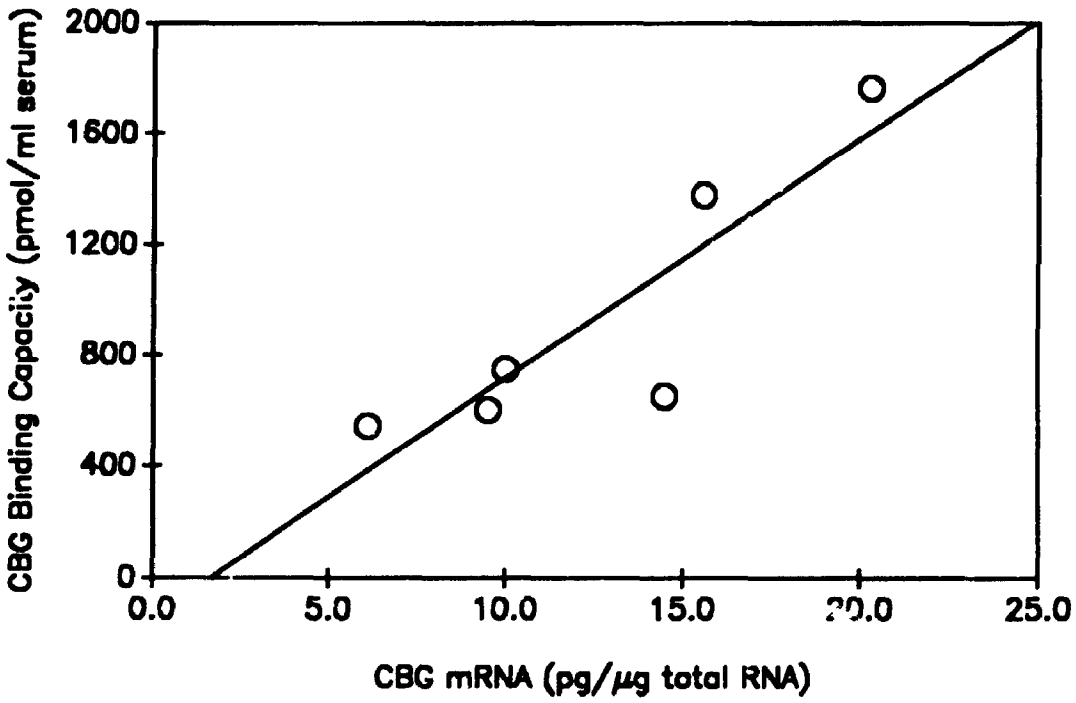
Dihydrotestosterone was administered to rats instead of testosterone because it cannot be aromatized and alterations in CBG levels would therefore reflect only androgen and not estrogen effects. Injections of dihydrotestosterone did not alter the levels of hepatic CBG mRNA or serum CBG, and this is consistent with previous studies which have demonstrated that testosterone treatment reduces serum CBG levels in intact female rats to levels associated with castrated males, and has no effect on the CBG levels of intact males (Gala and Westphal, 1965b). Thus, testosterone treatment of adult rats does not eliminate the difference in CBG levels between males and females, and may only modulate female serum CBG levels within an otherwise established sex-specific range of values. However, exposure of neonatal rats to androgens is required to establish adult male CBG levels (Van Baelen *et al.*, 1977a) and this appears to be mediated via its role in establishing pulsatile (male-specific) rather than continuous (female-specific) secretion of growth hormone, the patterns of which influence serum CBG levels (Jansson *et al.*, 1989).

Administration of thyroxine to intact male rats tends to increase both serum

CBG and hepatic CBG mRNA levels, but the response of these animals to treatment was variable and ranged from a 12 - 120% increase. The treatment of a second group of rats (Figure 5.3) resulted in a more uniform and significant increase in serum CBG levels. Thyroxine-mediated increases in serum CBG levels have been extensively documented (Westphal, 1971 and Westphal, 1986) and have been suggested to be the result of increased hepatic CBG biosynthesis (D'Agostino and Henning, 1982). The tendency towards increased hepatic CBG mRNA levels following T_4 administration suggests they mediate increased CBG biosynthesis, and comparisons of the serum CBG and hepatic CBG mRNA levels of individual rats supports this conclusion (Figure 5.9). Thyroxine-treated rats do not transcribe hepatic CBG mRNA at an increased rate, and increased steady-state levels of CBG mRNA in these rats is therefore probably mediated by reduced mRNA turn-over.

The kinetics of the decrease in serum CBG levels following dexamethasone treatment revealed that effects on CBG levels are not significant until two days following the initiation of treatment, and that responses to hormones appear to intensify with the passage of time. This may, however, simply reflect differences in the turnover of CBG mRNA in comparison to serum CBG. A similar apparent lag in the response of serum CBG concentrations to reduced CBG mRNA levels is observed for fetal rats (Smith and Hammond, 1991a) and pregnant rabbits (Seralini *et al.*, 1990b), and together this suggests the turnover of CBG mRNA is more rapid than plasma CBG. Changes in serum CBG levels with respect to time have been previously studied in dexamethasone-treated pregnant rats and although data are not available for 1 day of treatment, the overall magnitude of the decline is similar

Figure 5.9 Comparison of serum CBG and hepatic CBG mRNA levels in T_4-treated rats. Serum CBG levels were measured by cortisol binding capacity assay and CBG mRNA levels were measured by solution hybridization assay.


CBG Binding Capacity (pmol/ml serum)
2000
1600
1200
800
400
0
0.0
5.0
10.0
15.0
20.0
25.0
CBG mRNA (pg/μg total RNA)

to that demonstrated in this study (Yamamoto and Ohsawa, 1976; Van Baelen *et al.*, 1977b). The CBG levels of T_4-treated rats increased progressively and this is consistent with an accumulation of CBG mRNA leading to increased protein concentrations.

The primary structure of CBG is closely related to TBG and both are members of the serine protease inhibitor gene superfamily (Hammond *et al.*, 1987; Imamura *et al.*, 1991). Estrogen administration to humans increases the proportion of TBG possessing only triantennary oligosaccharide chains, and therefore its overall degree of sialylation (Ain and Refetoff, 1988; Ain *et al.*, 1987). The latter post-translational modification reduces the plasma clearance of TBG, and thereby increase its serum levels (Ain *et al.*, 1987). To ensure that the changes in serum CBG levels associated with T_4 and dexamethasone treatment do not reflect similar alterations in carbohydrate structure, the proportion of serum CBG decorated with only triantennary oligosaccharides was determined by exclusion on concanavalin A-chromatography (Krusius *et al.*, 1976). To compensate for column variations in glycoprotein separation, CBG values were expressed relative to total protein concentrations. The proportion of CBG bound to concanavalin A, and thus containing biantennary oligosaccharide chains, did not vary between the groups. In addition, the presence of CBG molecules possessing only triantennary oligosaccharides confirms the heterogeneity of rat CBG glycosylation previously described (Lea, 1979).

The hepatic RNA of 3 rats subjected to CLP revealed low detectable CBG

mRNA levels and this is consistent with previous indirect measurements performed by *in vitro* translation and immunoprecipitation of radiolabelled CBG following turpentine-induced inflammation (Faict *et al.*, 1985). It would appear, therefore, that CLP-induced septic shock either greatly diminishes CBG gene expression or decreases CBG mRNA stability and that one or both of these effects contribute to low CBG biosynthesis. The decrease in serum CBG levels over the 26 h period subsequent to CLP is similar to that previously observed following turpentine-induced inflammation in rats (Savu *et al.*, 1980; Savu *et al.*, 1985; Faict *et al.*, 1985; Faict *et al.*, 1983), and the rise and fall in the CBG levels of the control animal are characteristic of the normal diurnal variation of rat serum CBG values (Hsu and Kuhn, 1988). Because the CBG values of the control and septic animals change in opposing directions, the decrease in CBG levels during the first 2 - 3 h following CLP are probably even more pronounced than they would first appear. Adult rats subjected to turpentine-induced inflammation also experience a rapid initial decline in serum CBG levels, but infants subjected to the same treatment do not (Savu *et al.*, 1985) and this may be related to maturational differences in the response to inflammation. Although the magnitude of the fall in CBG levels is similar to that following dexamethasone treatment, and turpentine-induced inflammation leads to large increases in total and free corticosterone (Savu *et al.*, 1985; Szafarczyk *et al.*, 1974), it is unlikely that the reduced CBG levels are glucocorticoid-mediated because inflammation induction in adrenalectomized rats also results in low serum CBG levels (Faict *et al.*, 1983). No other steroids are known to reduce the CBG levels of intact males (Westphal, 1971), but the synthesis of CBG, like other acute

phase proteins, may be influenced by cytokines produced during sepsis. For example, albumin, fibronectin and transferrin are all negative acute phase proteins whose syntheses are regulated by interleukin 1ß (Gauldie *et al.*, 1987), interleukin-6 (Gauldie *et al.*, 1987; Castell *et al.*, 1989; Andus *et al.*, 1987) and tumor necrosis factor α (Perlmutter *et al.*, 1986) during inflammation. However, the reduction in serum CBG levels occurs too rapidly for it to be accounted for simply by decreased CBG biosynthesis, and an increase in the biological clearance of rat CBG may therefore mediate the rapid component of the response to inflammation, while low CBG mRNA levels sustain the effect.

CHAPTER 6

Identification and Characterization of

Rat Corticosteroid Binding Globulin Variants

6.1 Introduction

There have been no reports of genetic abnormalities in the plasma concentrations or steroid binding properties of CBG in rats, but the ability of this protein to modulate bioavailable steroid levels suggests that diseases influenced by inappropriate glucocorticoid action may also be influenced by inappropriate CBG activity. Our attention was therefore directed towards the Wistar-derived, BioBreeding (BB) rat because this strain represents a model of spontaneous autoimmune diabetes mellitus (Like *et al.*, 1982a); the onset of which can be delayed by treatment with glucocorticoids (Like *et al.*, 1983). Furthermore, BB rats are susceptible to other conditions that may be related in part to inadequate glucocorticoid action, such as increased neonatal mortality and respiratory tract infections (Like *et al.*, 1982a). Using an approach previously employed to identify a human CBG variant (Robinson and Hammond, 1985), serum CBG levels in BB and Wistar rats were measured by a steroid binding capacity with an assay that is sensitive to abnormalities in steroid binding affinity (Hammond and Lähteenmäki, 1983), and these results were then compared with the serum concentrations of the protein determined by radioimmunoassay (Smith and Hammond, 1991a). The CBG steroid binding characteristics of another naturally-occurring diabetic rat strain (Brattleboro) were also contrasted to those of its parent line (Long Evans) by dissociation rate and Scatchard analysis in order to determine if other rat strains afflicted with diabetes exhibit variant CBG steroid binding properties.

6.1.1 BioBreeding Rats

The BB rat was discovered in a colony of Wistar rats at the BioBreeding Laboratories in Ottawa, Ontario (Chappel and Chappel, 1983) and sublines of the original founders have been established which may vary in the incidence and severity of diabetes (Mordes *et al.*, 1987). The onset age is typically between 60 and 120 days of age, and once diabetic, rats require daily injections of insulin or death will occur within days (Nakhooda *et al.*, 1977). Prior to the destruction of the insulin-producing beta cells and the onset of hyperglycemia, the islets of Langerhans are characterized by lymphocytic infiltration (Nakhooda *et al.*, 1977) and this suggests the disease has an autoimmune pathogenesis. Indeed, administration of immunosuppressive agents such as glucocorticoids, cyclosporin A or antiserum to rat lymphocytes reduces the frequency of disease and normalizes glucose levels in diabetic animals (Like *et al.*, 1983). Neonatal thyroidectomy also prevents the onset of diabetes (Like *et al.*, 1982b).

The BB rats used in this study were obtained from the Hospital for Sick Children (Toronto, Ontario), and were monitored twice weekly until urinary ketones indicated the onset of diabetes. They were then maintained by daily doses of protamine zinc insulin. The Wistar rats were from Charles River Laboratories. The two strains were housed in different rooms of the same facility under standard conditions.

6.1.2 Brattleboro Rats

Brattleboro rats were distinguished from normal members of a Long-Evans

hooded rat colony in West Brattleboro, Vermont on the basis of their excessive intake of water (Valtin, 1982). This strain suffers from hereditary diabetes insipidus which is first manifested in weanling (approximately 3 weeks old) rats (Dlouhá *et al.*, 1982) and has been attributed to a deficiency of vasopressin synthesis (Valtin and Schroeder, 1964). The basis of the deficiency has been traced to a single base deletion in the neurophysin region of the Brattleboro vasopressin gene (Schmale and Richter, 1984).

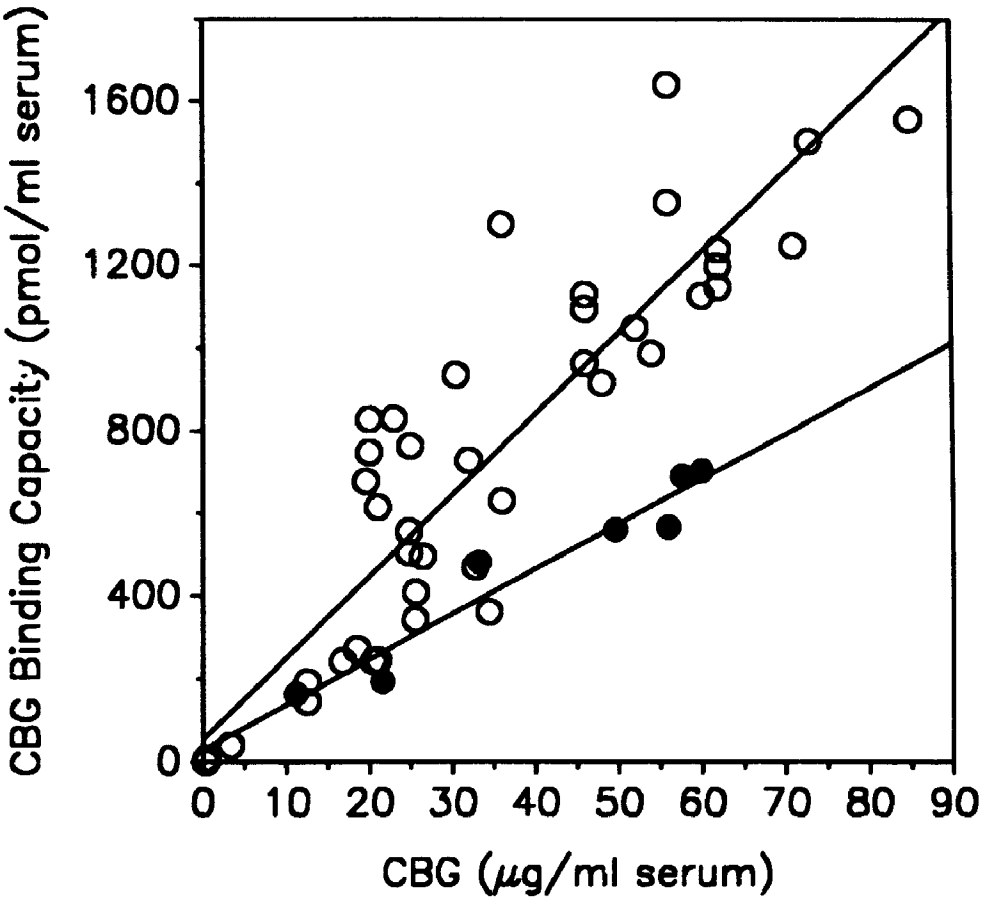
The Brattleboro rats used in these studies were obtained from Harlan Sprague Dawley as were the Long Evans black-hooded rats used as control animals. They were housed in separate rooms of the same facility under standard conditions. Neither strain of rat received any additional treatment.

6.2 Results

6.2.1 Identification of a Binding-Variant CBG in BioBreeding Rats

Serum was analyzed from adult BB rats (2 males and 5 females) and a group of 43 Wistar rats, which included infant and adult animals of both sexes, in order to obtain a wide range serum CBG levels. When the measurements of CBG binding capacity (pmol/ml) for cortisol were compared with the CBG concentrations (μg/ml) determined by radioimmunoassay (Figure 6.1) for Wistar rat serum, the equation relating the two values ($y = 19.6 x + 54.7$) is indicative of the presence of a single steroid binding site per molecule of CBG, assuming a polypeptide molecular weight of 42.000 (Smith and Hammond, 1989). In contrast, the relationship between the measurements of CBG in BB rat serum ($y = 10.9 x + 28.6$) suggests

Figure 6.1 Comparison of corticosteroid binding globulin measurements in Wistar (○) and BioBreeding (●) rats. Serum CBG levels were determined by cortisol binding capacity assay and radioimmunoassay. Linear regression lines were obtained by least squares fit analysis.


CBG Binding Capacity (pmol/ml serum)
1600
1200
800
400
0
0
10
20
30
40
50
60
70
80
90
CBG (μg/ml serum)

an apparent reduction in cortisol binding capacity with respect to its concentration; a discrepancy indicative of CBG variants with reduced affinity for steroid ligand (Robinson and Hammond, 1985).

6.2.2 Steroid Binding Characteristics of BioBreeding and Brattleboro CBGs

The steroid binding properties of BB and Wistar rat CBG were examined by measurement of the 'apparent' dissociation-rate of [^{3}H]cortisol-CBG complexes during exposure to dextran-coated charcoal (Figure 6.2A), and Scatchard analysis using [^{3}H]cortisol as ligand at 0°C (Figure 6.2B). The results indicate that the dissociation of cortisol (measured as a percentage of that present at time = 0) occurs more rapidly from BB rat CBG (6.4%/min) than Wistar CBG (4.1%/min), and this is reflected in a higher dissociation constant (K_d) of BB rat CBG (4.42 nM) than Wistar rat CBG (2.86 nM). Furthermore, this difference in binding affinity of CBG for cortisol is sufficient to account for the discrepancy between the CBG binding capacity and immunoassay measurements in BB rat serum. No detectable differences were observed when the relative steroid binding affinities of various steroids for BB and Wistar rat CBG were tested relative to corticosterone (Figure 6.3 and Table 6.1).

The steroid binding characteristics of Brattleboro and Long Evans serum CBG serum were also compared by 'apparent' dissociation rate (Figure 6.4A) and Scatchard (Figure 6.4B) analysis. The dissociation rate of [^{3}H]cortisol from Brattleboro serum CBG (5.0%/min) was lower than from Long Evans CBG (6.0%/min), and the higher affinity of Brattleboro CBG for [^{3}H]cortisol (K_d = 2.72

Figure 6.2 The 'apparent' dissociation rates (*A*) and Scatchard analysis (*B*) of Wistar (○) and BioBreeding (●) serum CBG. Dissociation rate values were determined by incubating [^{3}H]cortisol-saturated CBG with dextran-coated charcoal at 0°C for various lengths of time, and then measuring the amount of CBG-bound [^{3}H]cortisol. Scatchard analysis was performed with [^{3}H]cortisol at 0°C. Free and CBG-bound [^{3}H]cortisol were separated with dextran-coated charcoal.

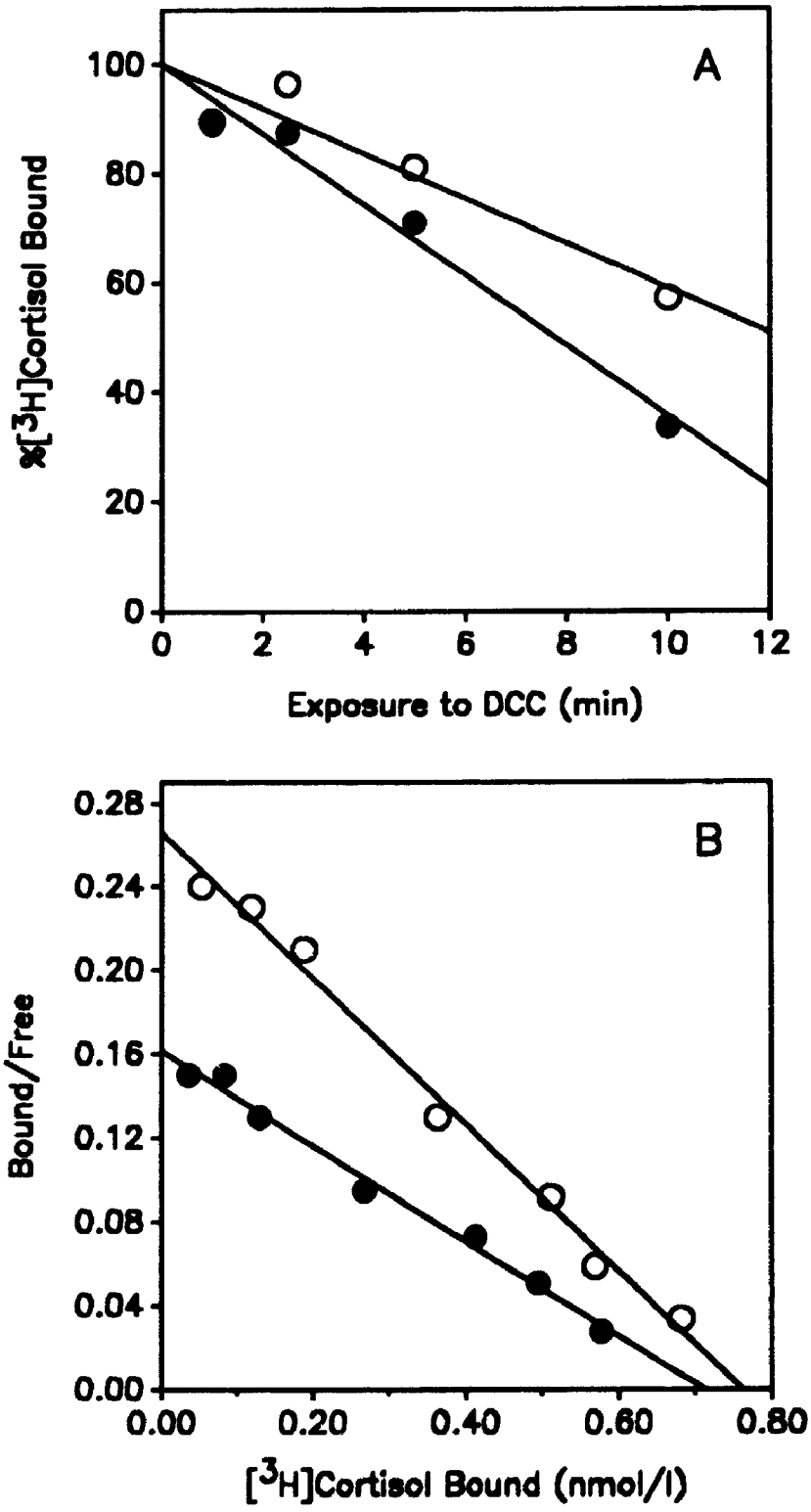

A
100
80
60
40
20
0
%[3H]Cortisol Bound
0
2
4
6
8
10
12
Exposure to DCC (min)
B
0.28
0.24
0.20
0.16
0.12
0.08
0.04
0.00
Bound/Free
0.00
0.20
0.40
0.60
0.80
[3H]Cortisol Bound (nmol/l)

Figure 6.3 Relative binding affinities of BioBreeding (*A*) and Wistar (*B*) CBG for various steroids. Appropriate dilutions of purified BioBreeding or Wistar CBG were incubated with a saturating amount of [^{3}H]corticosterone and various amounts of radioinert corticosterone (○), 11ß-hydroxyprogesterone (●), cortisol (◇), deoxycorticosterone (◆), progesterone (□) and 11-deoxycortisol (■). Non-bound steroid was removed with dextran coated charcoal at 0°C. The amount of steroid relative to unlabelled corticosterone required to displace 50% of the CBG-bound radiolabelled ligand was used as an indication of its affinity for CBG (see Table 6.1).

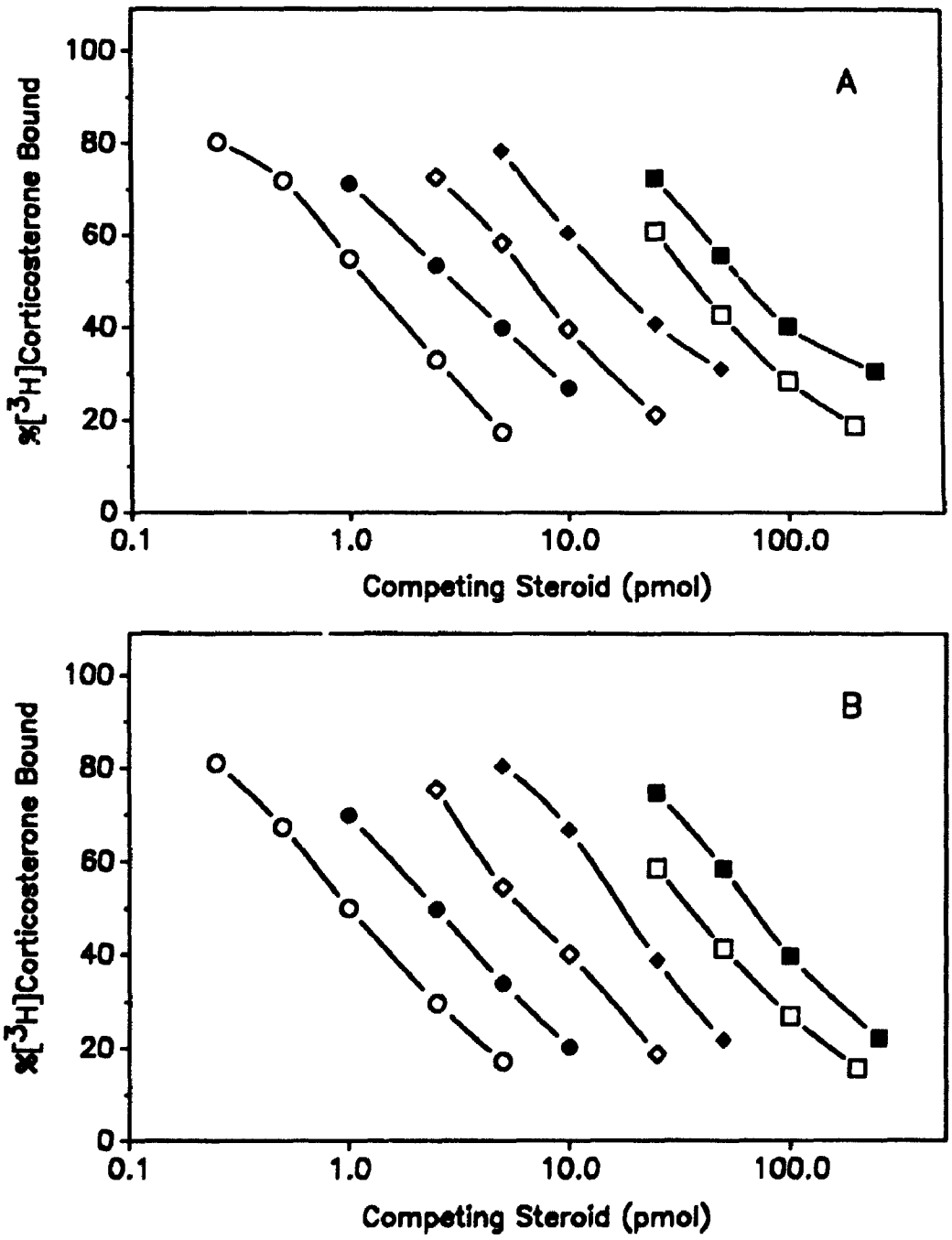
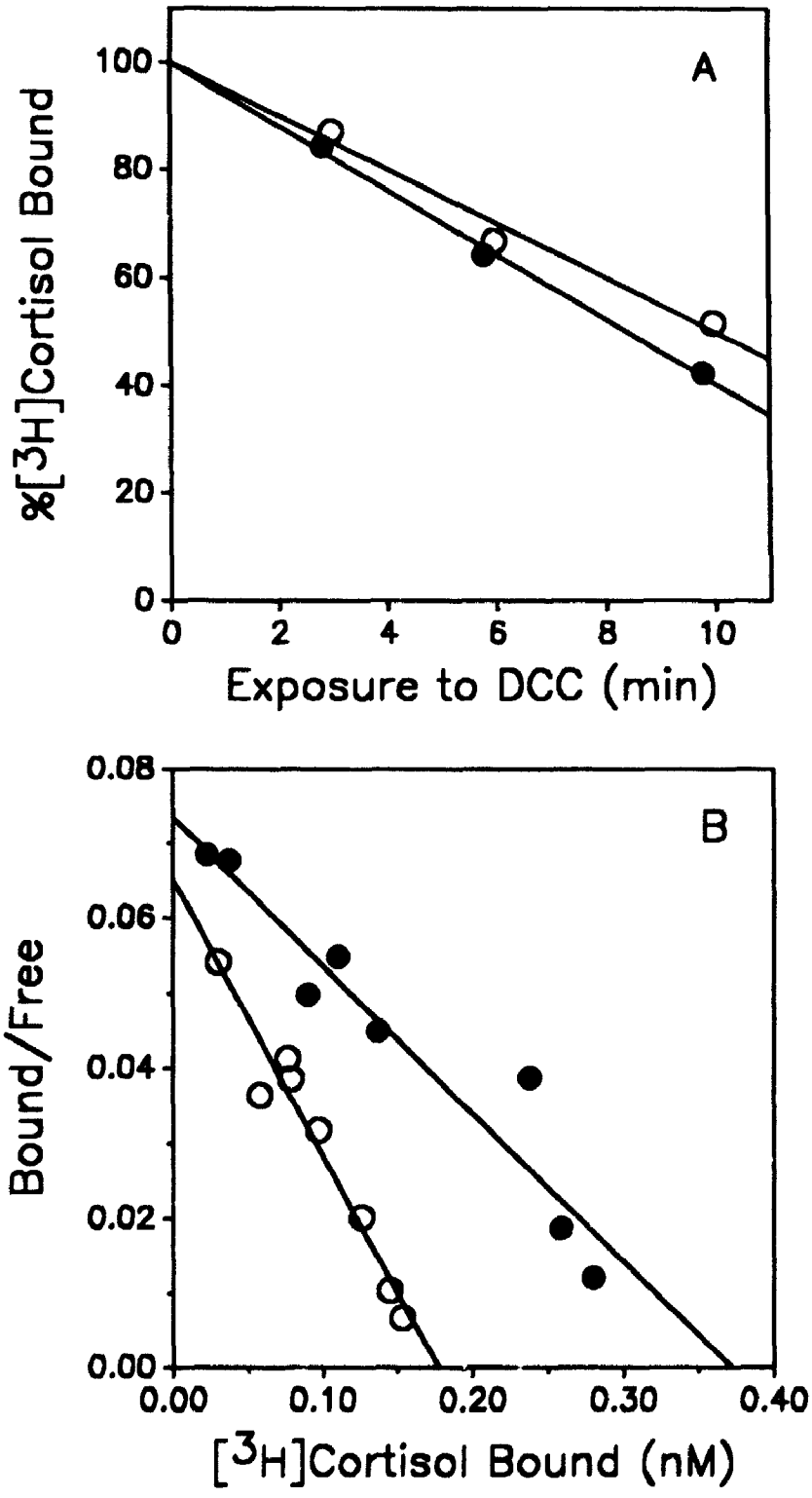

A
B
100
80
60
40
20
0
0.1
1.0
10.0
100.0
% [3H] Corticosterone Bound
Competing Steroid (pmol)

Table 6.1 Relative binding affinities of purified Wistar and BioBreeding rat CBG for various steroids. The results give means of three separate assays ± standard error.

Steroid	Relative Binding Affinities		Ratio
	BioBreeding	Wistar	BioBreeding / Wistar
Corticosterone	100.00	100.00	1.00
11ßOH-Progesterone	39.96 ± 1.59	37.67 ± 2.30	1.06
Cortisol	17.11 ± 0.40	16.70 ± 1.02	1.02
Deoxycorticosterone	6.66 ± 0.59	6.04 ± 0.42	1.10
Progesterone	2.93 ± 0.24	2.77 ± 0.20	1.06
11-Deoxycortisol	1.59 ± 0.16	1.39 ± 0.06	1.14

Figure 6.4 The apparent dissociation rates (*A*) and Scatchard analysis (*B*) of Brattleboro (○) and Long Evans (●) serum CBG. Dissociation rate values were determined by incubating [^{3}H]cortisol-saturated CBG with dextran-coated charcoal at 0°C for various lengths of time, and then measuring the amount of CBG-bound [^{3}H]cortisol. Scatchard analysis was performed with [^{3}H]cortisol at 0°C. Free and CBG-bound [^{3}H]cortisol were separated with dextran-coated charcoal.


A
100
80
60
40
20
0
%[3H]Cortisol Bound
0
2
4
6
8
10
Exposure to DCC (min)
B
0.08
0.06
0.04
0.02
0.00
Bound/Free
0.00
0.10
0.20
0.30
0.40
[3H]Cortisol Bound (nM)

nM) than Long Evans CBG (K_d = 5.06 nM) is consistent with this result.

6.2.3 SDS-PAGE of BioBreeding and Wistar CBG

When purified BB and Wistar rat CBGs were examined by electrophoresis under denaturing conditions (Figure 6.5), they exhibited similar size heterogeneity and the apparent molecular weights of the two major forms were approximately 60,500 and 55,000.

6.2.4 Isolation and Characterization of BioBreeding and Wistar CBG cDNAs

The CBG cDNA sequences for BB and Wistar rats were obtained through reverse transcription and polymerase chain reaction amplification of hepatic poly(A)$^+$ RNA prepared from both strains. Apart from two nucleotides, the 1,228 bp BB and Wistar rat CBG cDNA sequences were identical. In BB CBG, the codon for Phe^{130} was TTC, while the corresponding codon for the same amino acid in Wistar CBG was TTT. The only other difference between these two sequences resulted in an amino acid substitution at residue 276 in the mature CBG polypeptide (Smith and Hammond, 1989). The BB CBG sequence at this point (ATA) encodes for isoleucine, while the codon (ATG) for methionine is present in the Wistar rat CBG sequence (Figure 6.6).

6.2.5 Expression of cDNAs for CBG-Met^{276} and CBG-Ile^{276} *In Vitro*

In order to confirm residue 276 influences the steroid binding affinity of CBG, the codon for isoleucine^{276} in the BB rat CBG cDNA was mutated to yield the

Figure 6.5 Comparison of BioBreeding and Wistar CBG by SDS-PAGE. Purified BB (lane 1) and Wistar (lane 2) rat CBG was resolved by 7.5% polyacrylamide gel electrophoresis and stained with Coomassie brilliant blue. The positions of molecular size standards (kiloDaltons) are indicated on the *right*.

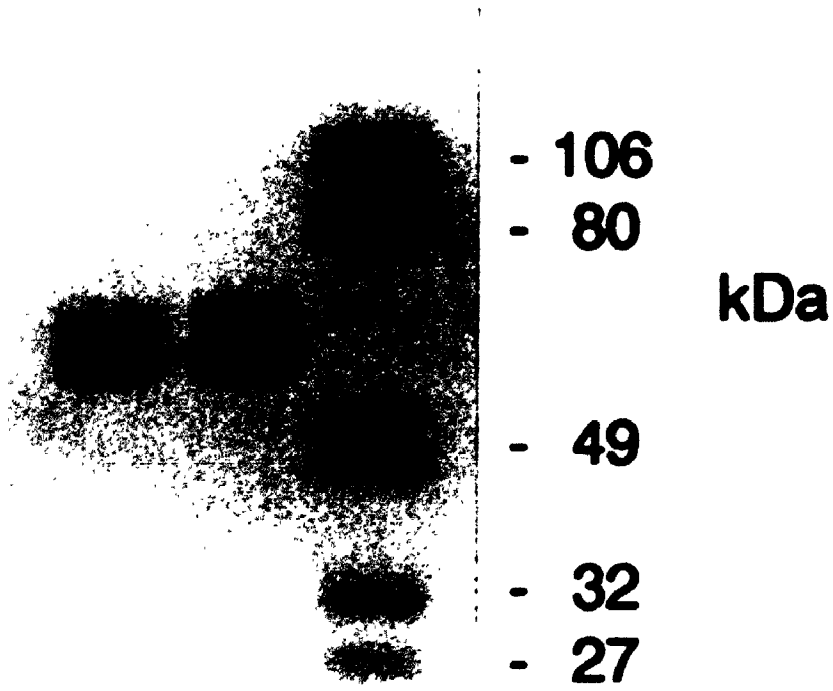
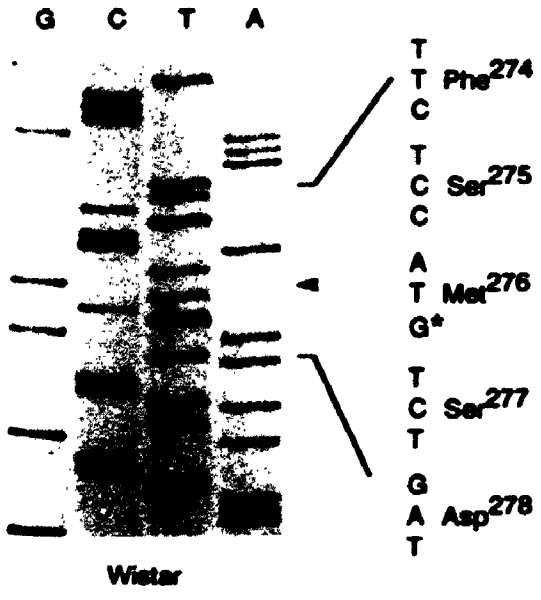
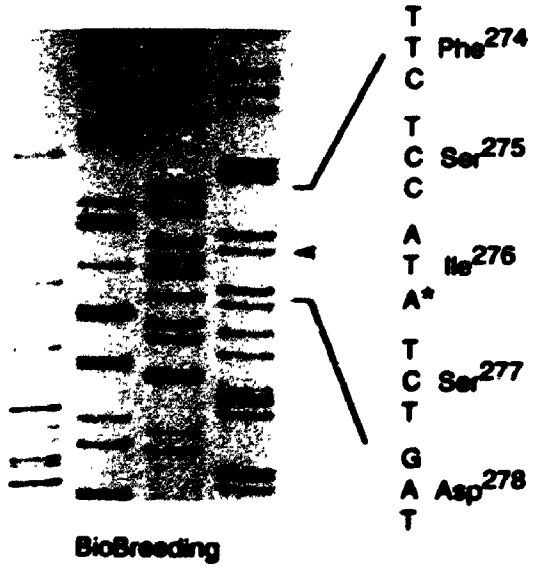

1
2
- 106
- 80
kDa
- 49
- 32
- 27

Figure 6.6 Partial sequence of Wistar and BioBreeding CBG cDNAs and their deduced amino acid sequences. The nucleotide and corresponding amino acid sequences are shown on the right for residues 274 to 278 of the mature rat CBG polypeptide (Smith and Hammond, 1989). The amino acid substitution is indicated with an asterisk, and an arrow indicates the position of the nucleotide difference between the sequences.


G C T A
T
T T Phe274
C
T
C C Ser275
C
A
T Met276
G*
T
C Ser277
T
G
A Asp278
T
Wistar


T
T Phe274
C
T
C C Ser275
C
A
T Ile276
A*
T
C Ser277
T
G
A Asp278
T
BioBreeding

codon for methionine^{276} found in the Wistar sequence, and these cDNAs were inserted into a mammalian expression vector (pRc/CMV) and transformed into Chinese hamster ovary (CHO) cells. When the recombinant products were analyzed by Scatchard analysis (at 0°C) using [^{3}H]corticosterone as ligand (Figure 6.7), the K_d of CBG-Ile^{276} (1.56 nM) was higher than that of CBG-Met^{276} (1.01 nM), and these values compare favourably with those obtained for BB (1.43 nM) and Wistar (0.95 nM) CBG in serum (Figure 6.8).

6.3 Discussion

A discrepancy between the radioimmunoassay and steroid binding capacity measurements of serum CBG levels suggested the presence of a CBG variant with reduced affinity for glucocorticoids in BB rats. The affinity constants of BB CBG for cortisol and corticosterone are within the range previously reported for CBG in other rat strains (Chader and Westphal, 1968b; Feldman *et al.*, 1973; Martin *et al.*, 1977; Bassett, 1987), but BB CBG has approximately 66% of the affinity displayed by Wistar CBG for cortisol, when measured under the same conditions. This is also reflected by an increase in the 'apparent' dissociation-rate of glucocorticoid from BB CBG when compared to Wistar CBG. Indeed, it is this increase in dissociation of cortisol from BB CBG that results in an underestimation of its binding capacity, and is indicative of the presence of a CBG variant with reduced steroid binding affinity.

The cortisol binding affinities of CBG in Brattleboro and Long Evans rats are similar to those determined for other rats stains, but the dissociation constant of

Figure 6.7 Scatchard plots of CBG-Met^{276} (○) and CBG-Ile^{276} (●) expressed in culture. Medium from cDNA-transfected CHO cells was incubated with [^{3}H]corticosterone, and unbound steroid was removed with dextran-coated charcoal at 0°C.

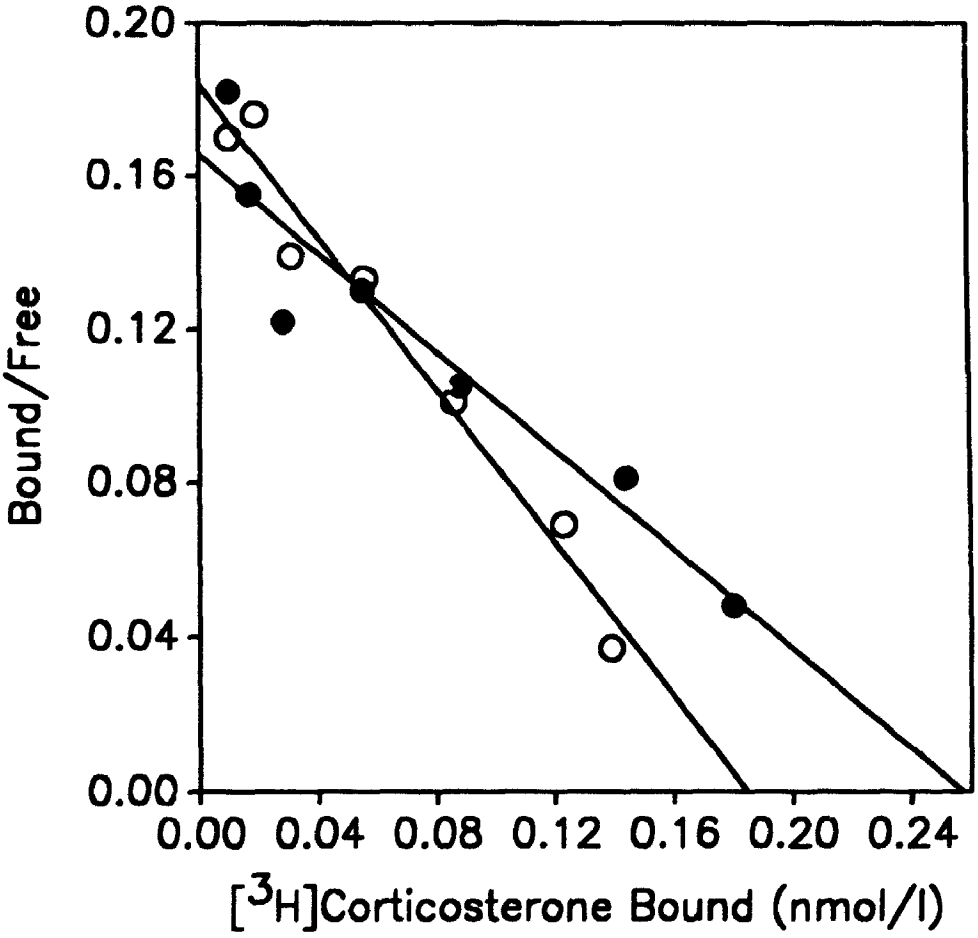
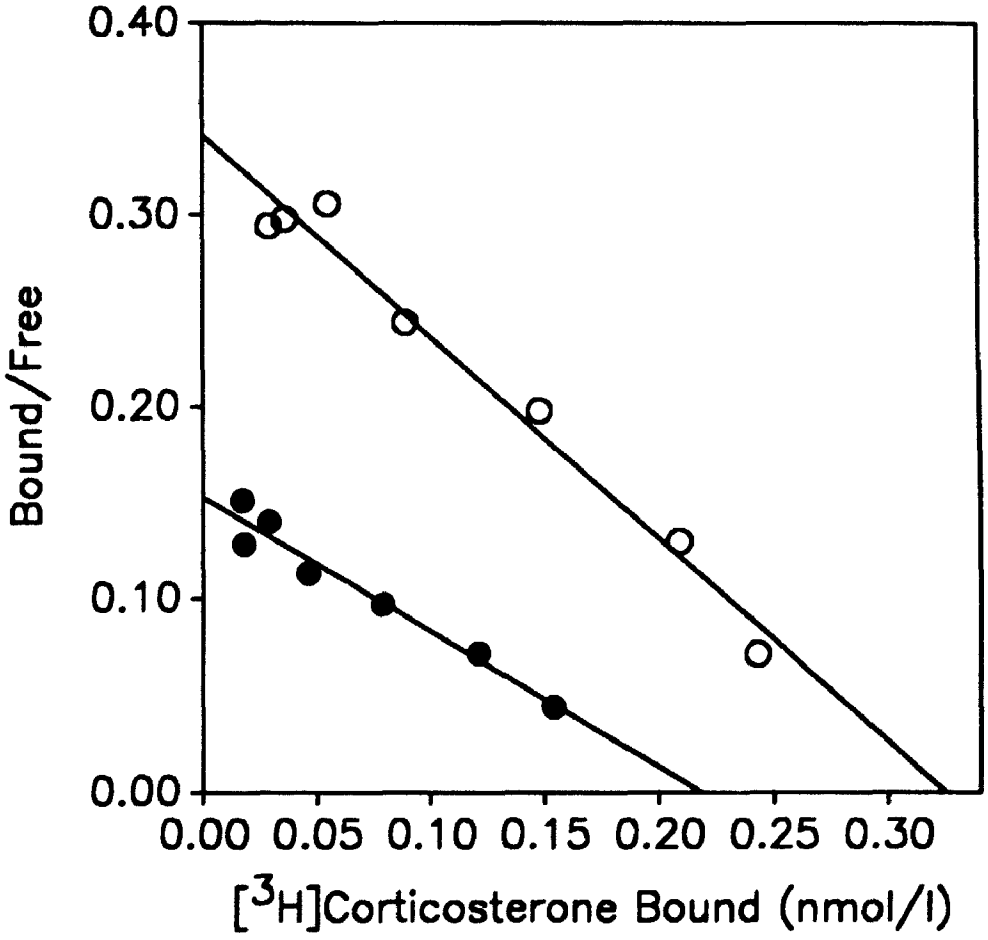

0.20
0.16
0.12
0.08
0.04
0.00
Bound/Free
0.00 0.04 0.08 0.12 0.16 0.20 0.24
[3H]Corticosterone Bound (nmol/l)

Figure 6.8 Scatchard plots of Wistar (○) and BioBreeding (●) serum CBG. Serum was diluted in PBS and incubated with [^{3}H]corticosterone, and unbound steroid was removed with dextran-coated charcoal at 0°C.


0.40
0.30
0.20
0.10
0.00
Bound/Free
0.00 0.05 0.10 0.15 0.20 0.25 0.30
[3H]Corticosterone Bound (nmol/l)

Brattleboro CBG-[^{3}H]cortisol complexes were approximately 54% of Long Evans CBG, when measured in the same assay. The affinity difference between both strains of CBG was also mirrored in the relatively rapid dissociation of Long Evans CBG-steroid complexes relative to those of Brattleboro CBG. It is interesting that of the four rat strains examined, Long Evans CBG has the lowest affinity for cortisol, and that the strain derived from it (Brattleboro) has an increased cortisol binding affinity. Future examinations of the Brattleboro and Long Evans CBG primary structures will likely identify residues which contribute to CBG's steroid binding characteristics and may allow further conclusions to be drawn on the relationship between the structure and function of this protein.

Analysis of BB and Wistar CBG mRNAs via cDNA cloning revealed a point mutation that results in a methionine to isoleucine substitution at residue 276 in the BB rat CBG sequence. To demonstrate that the BB CBG mutation is directly responsible for the reduction in its steroid binding affinity, Wistar and BioBreeding CBG cDNAs were expressed in the same cell type under similar conditions. The corticosterone binding affinities of the products were essentially identical to those determined for serum CBG in these animals. Therefore, this amino acid substitution clearly accounts for the reduced steroid binding affinity of BB rat CBG. Furthermore, BB and Wistar CBG exhibit similar size heterogeneity when examined by SDS-PAGE, and there does not therefore appear to be any obvious difference in the post-translational modification of these proteins *in vivo*.

It is surprising that a single methionine to isoleucine substitution reduces the

steroid binding affinity of rat CBG because both amino acids are non-polar and hydrophobic, and an isoleucine residue is located at the equivalent position in rabbit and human CBG (Hammond *et al.*, 1991b). Human and rabbit CBG bind cortisol with a higher affinity than corticosterone, while rat CBG binds corticosterone preferentially. It is therefore interesting that the BB mutation does not alter its binding affinity for cortisol relative to corticosterone. In addition, no differences in the relative binding affinity of BB CBG for several other natural steroid ligands were detected. However, there are numerous other natural and synthetic steroid ligands for CBG (Mickelson *et al.*, 1981) which were not tested. It is therefore possible that some of them may exhibit an abnormal affinity for BB CBG, and further studies of this nature may help define the portion of the steroid molecule that interacts with residue 276.

The primary structure of α_1-proteinase inhibitor (A1-PI) is closely related to CBG (Hammond *et al.*,, 1987). The tertiary structure of proteolytically-modified A1-PI has been determined (Loebermann *et al.*, 1984), and this has revealed a ß-barrel that is characteristic of the ligand binding domain of other proteins (Huber and Carrell, 1989). When the rat CBG amino acid sequence is superimposed on the tertiary structure of A1-PI, residue-276 lies within this ß-barrel in close proximity to cysteine^{220} which has been implicated in steroid binding (Hammond *et al.*, 1987; Smith and Hammond, 1989). It would therefore appear that residue-276 is located in a position that may directly influence steroid binding activity. Thyroxine binding globulin (TBG) is also a member of the serpin family (Flink *et al.*, 1986) and is closely related to CBG (Hammond *et al.*, 1987). A number of human TBG

mutations have been characterized (Refetoff, 1989), and it is interesting that a leucine^{283}→phenylalanine^{283} substitution in TBG-Poly is located in the vicinity of the BB CBG mutation on an adjacent ß-strand within the ß-barrel predicted by the three-dimensional structure of A1-PI (Huber and Carrell, 1989). Although the biochemical properties of this TBG mutant appear to be indistinguishable from normal TBG (Takeda *et al.*, 1989), it is not clear if this mutation results in any perturbation of thyroxine binding affinity.

Previously, CBG variants with reduced steroid binding affinity have only been identified in human serum samples, and are generally present in equimolar amounts with normal CBG (Van Baelen *et al.*, 1982; Robinson and Hammond, 1985). This may be attributed to the presence of a single gene on human chromosome 14 (Underhill and Hammond, 1989; Seralini *et al.*, 1990a) and expression of both normal and variant alleles. Homozygous carriers of this type of variant have also been identified (Van Baelen *et al.*, 1982), and they are characterized by a reduction in steroid binding affinity similar to that observed between BB and Wistar rat CBG. Unlike BB rats, these individuals do not suffer from any obvious pathological disorders, and we have no evidence for a direct connection between the BB CBG mutation and the development of autoimmune diabetes mellitus. However, glucocorticoids are important regulators of the immune system (Munck *et al.*, 1984) and their efficacy in treating autoimmune disorders has been established (Claman, 1989). Furthermore, it is becoming increasingly apparent that CBG may play a role in delivering glucocorticoids to neutrophils during inflammation (Hammond *et al.*, 1990a; Hammond *et al.*, 1990b) and a deficiency in the functional activity of this

protein may therefore contribute to this and other abnormalities observed in the BB rat (Like *et al.*, 1982a).

CHAPTER 7

Human Corticosteroid Binding Globulin Variants:

Naturally-occurring and *In Vitro* Produced

7.1 Introduction

Several human CBG variants with reduced affinity for cortisol have been examined biochemically (Robinson and Hammond, 1985; Van Baelen *et al.*, 1982). After neuraminidase treatment to remove sialic acid, they are characterized by unique isoelectric points that are indicative of primary structural abnormalities (Van Baelen *et al.*, 1982; Robinson and Hammond, 1985). Furthermore, their transmission within families follows an autosomal pattern of inheritance (Van Baelen *et al.*, 1982), consistent with the presence of a single gene on chromosome 14 (Underhill and Hammond, 1989; Seralini *et al.*, 1990a).

The amino acid sequence of human CBG has been deduced from liver and lung cDNAs (Hammond *et al.*, 1987), and confirmed by sequencing human genomic fragments that contain the coding region for CBG (Underhill and Hammond, 1989). Since tissues used to obtain these DNA sequences were taken from different individuals, and all revealed the same coding sequence for human CBG, they appear to encode the consensus primary structure of the normal protein. Analyses of the genes for CBG variants with abnormal steroid binding affinities may therefore be expected to locate amino acids that contribute to the functional integrity of the steroid binding domain.

The physicochemical properties of the human CBG steroid binding site have been reviewed with respect to phylogenetic comparisons of its primary structure (Hammond *et al.*, 1991b) and the tertiary structure of a related serine protease inhibitor (serpin), α_1-proteinase inhibitor (Huber and Carrell, 1989; Hammond,

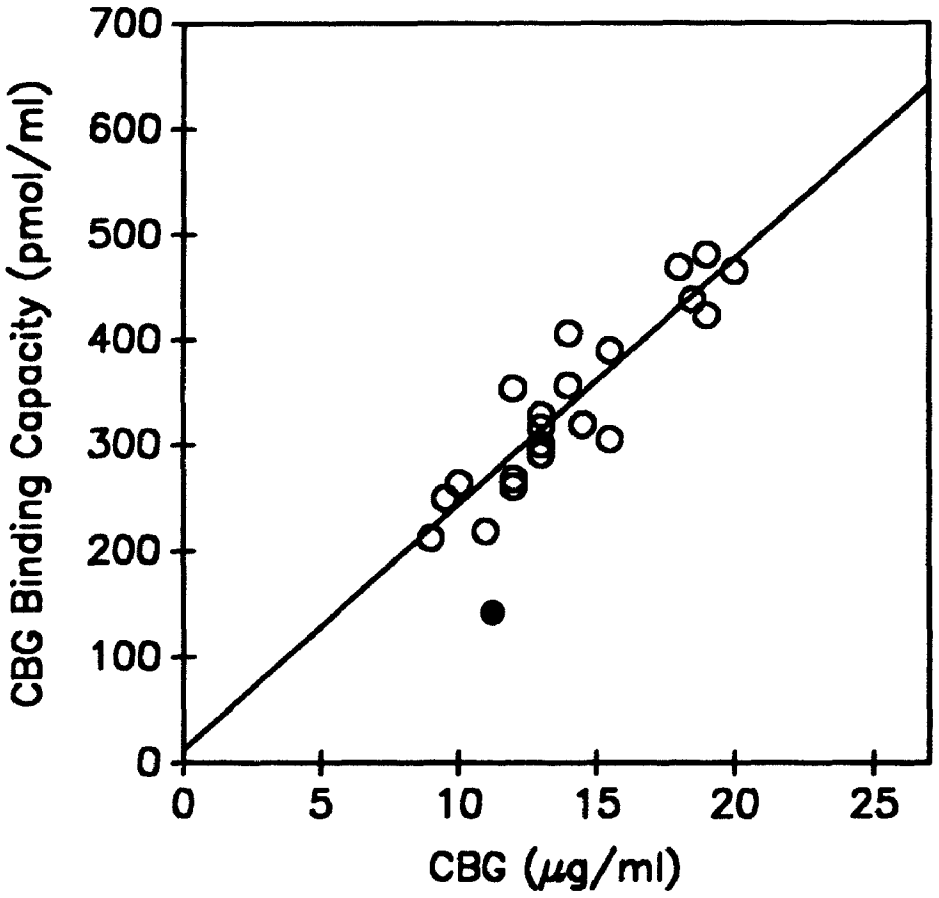
1990). In addition, localization of amino acid residues which contribute to the abnormal hormone binding properties of rat CBG (Smith and Hammond, 1991b) and human thyroxine binding globulin (Refetoff, 1989) have also allowed predictions of amino acids which may contribute to the structural and/or functional properties of human CBG. In order to test these hypotheses, mutations have been introduced separately into a human liver CBG cDNA (Hammond *et al.*, 1987) by site-directed mutagenesis, and the resulting cDNAs were expressed in Chinese hamster ovary cells to study their relative impact on the biochemical properties of the recombinant proteins.

7.2 Results: Naturally-Occurring Human CBG Variant

7.2.1 Identification of a CBG Cortisol Binding Variant

Serum samples from 22 patients in intensive care were taken for CBG analyses. With the exception of one individual, the serum CBG binding capacity and radioimmunoassay measurements in these samples ($n = 22$) were highly correlated ($r = 0.911$, $p < 0.001$). In this patient the CBG binding capacity was only approximately 50% of its immunoassayable concentration (Figure 7.1), and this is indicative of the presence of a CBG variant with reduced affinity for cortisol (Robinson and Hammond, 1985). The patient was a 73 year old female with diabetes mellitus, coronary artery disease, hypertension, osteoarthritis and gastric erosions secondary to the use of nonsteroidal anti-inflammatory drugs.

Figure 7.1 Comparison of serum corticosteroid binding globulin measurements in normal (○) and variant (●) patient samples by cortisol binding capacity and radioimmunoassay. The regression line was determined by least squares fit analysis.


700
600
500
400
300
200
100
0
CBG Binding Capacity (pmol/ml)
0
5
10
15
20
25
CBG (μg/ml)

7.2.2 Steroid Binding Characteristics of the Variant CBG

The steroid binding properties of the variant CBG were compared to those of CBG obtained from another patient sample in which the steroid binding capacity and immunoassay values were appropriately correlated. These analyses indicated that the 'apparent' dissociation of [^{3}H]cortisol from the CBG variant at 0°C occurs much more rapidly than from normal CBG (Figure 7.2A). In addition, its dissociation constant (K_d) for cortisol is higher (0.61 nM) than that (0.50 nM) of normal CBG (Figure 7.2B), and these differences are sufficient to account for its abnormally low steroid binding capacity.

7.2.3 Identification of Point Mutations within the CBG Gene

A second blood sample from the carrier of the CBG steroid binding variant was used to extract genomic DNA from her white blood cells. Amplification and analysis of CBG coding sequences of this DNA revealed two distinct alleles. The sequences obtained for exon 2 indicated the presence of the normal allele and a variant allele in which a point-mutation in the codon for residue 93 (CTC→CAC) results in a leucine→histidine substitution (Figure 7.3A). In exon 3, two variant alleles were detected with point mutations within the codon for residue 224: one allele was characterized by a single silent mutation (TCA→TCG), while the other (TCA→GCG) results in a serine→alanine substitution (Figure 7.3B). In exon 2, the codon for threonine^{104} (ACC) differed from the normal CBG cDNA sequence (ACT) in all 10 clones studied. Similarily, in exon 4, silent mutations were also detected within the codon for leucine^{290} (CTC) which differed from the normal

Figure 7.2 Cortisol binding characteristics of normal (○) and variant (●) CBG in serum samples. The 'apparent' dissociation rates (*A*) were determined by incubating [^{3}H]cortisol-saturated CBG with dextran coated charcoal (DCC) for various lengths of time, and then measuring the amount of CBG-bound [^{3}H]cortisol. Scatchard analysis (*B*) of the [^{3}H]cortisol binding affinity of CBG was measured at 0°C using DCC to separate bound and free steroid.

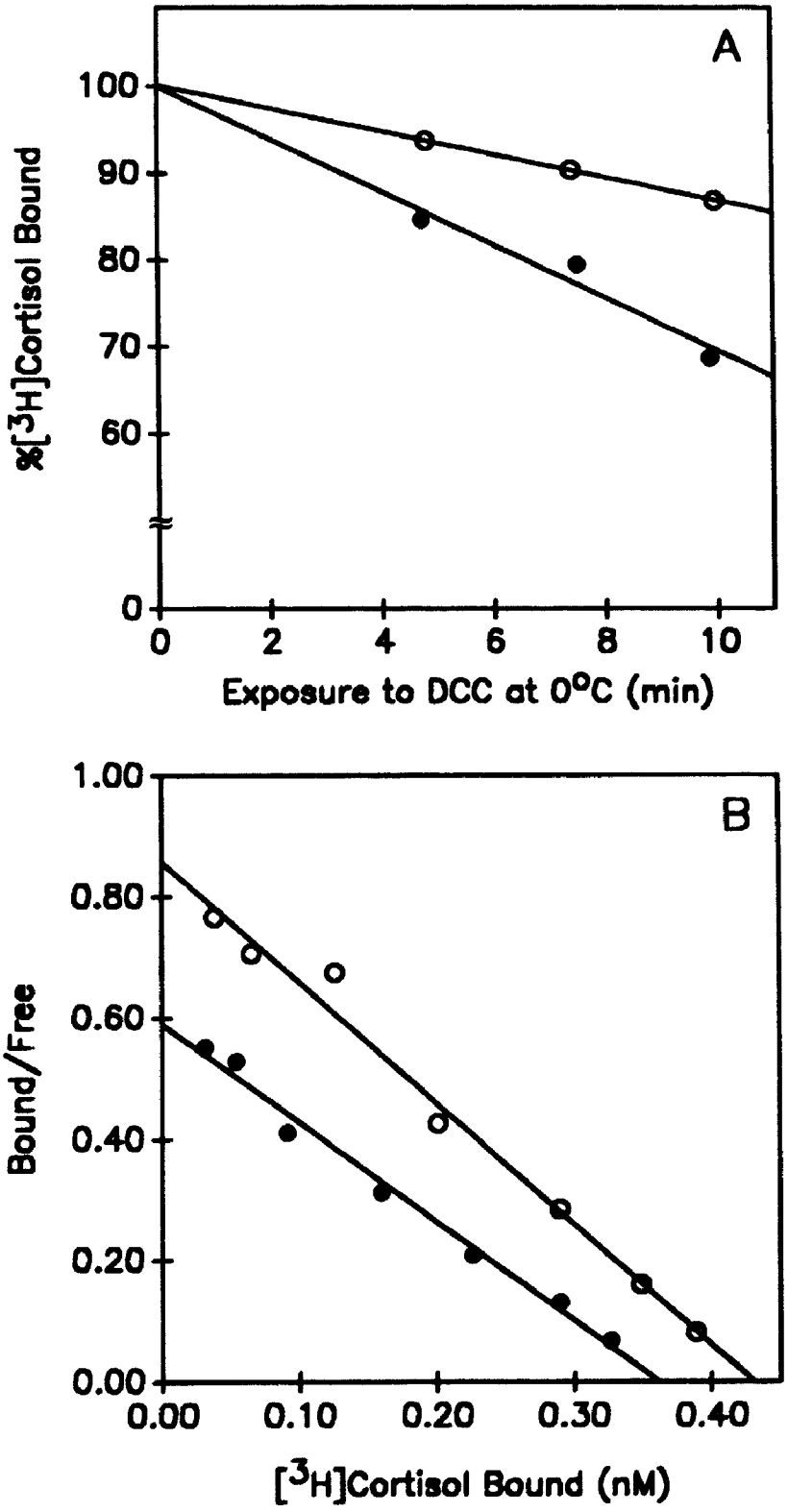
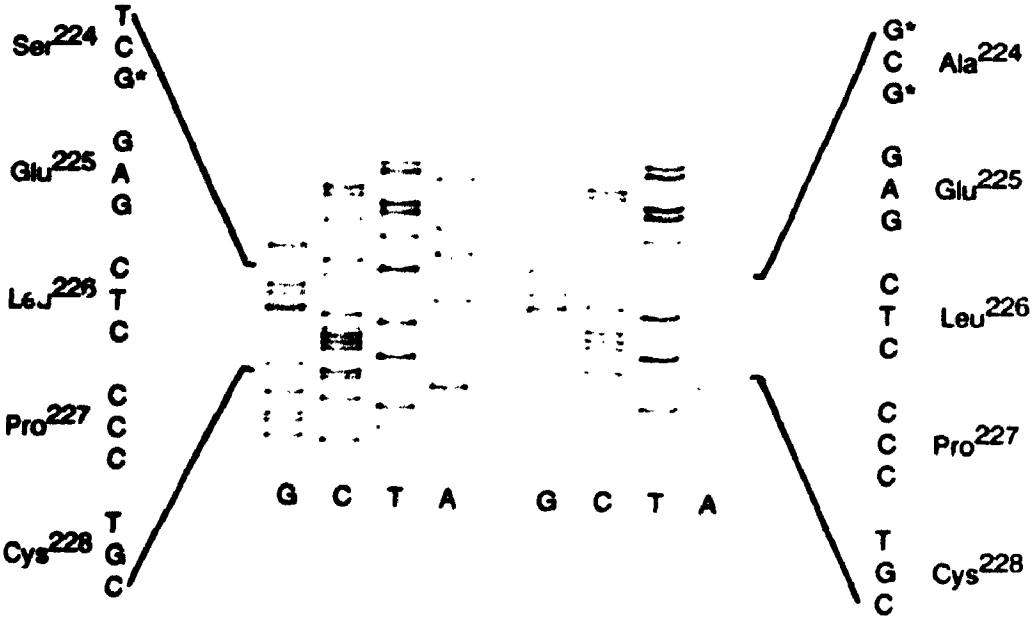

A
100
90
80
70
60
0
0
2
4
6
8
10
%[3H]Cortisol Bound
Exposure to DCC at 0°C (min)
B
1.00
0.80
0.60
0.40
0.20
0.00
0.00
0.10
0.20
0.30
0.40
Bound/Free
[3H]Cortisol Bound (nM)

Figure 7.3 Partial exon 2 (*top*) and exon 3 (*bottom*) nucleotide sequences and predicted amino acid sequences of the two alleles identified in DNA extracted from the carrier of the CBG variant. Fluorograms on the *left* depict sequences which encode for normal CBG, while those on the *right* represent the CBG mutations. The positions of point mutations (*) are indicated.


Leu93 C T C
Phe94 T T T
Ala95 G C A
Lys96 A A G
T A G C T A G C
C A* C His93
T T T Phe94
G C A Ala95
A A G Lys96


Ser224 T C G*
Glu225 G A G
Leu226 C T C
Pro227 C C C
Cys228 T G C
G C T A G C T A
G* C G* Ala224
G A G Glu225
C T C Leu226
C C C Pro227
T G C Cys228

sequence (CTT) in all 10 clones studied, and in 8 of 10 clones, the codon for aspartic acid^{315} was found to be GAT instead of GAC. Other deviations from the normal sequence in these exons, and in exon 5, were not reproducible and probably represent PCR amplification errors, which appear to have occurred with a frequency of 1 in 1053 bases analyzed.

7.3 Rationale for the Mutation of Human CBG

The influences of individual amino acids on the physicochemical properties of CBG were examined by subjecting the normal human CBG cDNA to site-directed mutagenesis, and expressing mutants in CHO cells under similar culture conditions. Eight mutations have been introduced, and the rationale for each is given below.

7.3.1 Amino Acids Mutated in the Naturally-occurring Human CBG Variant

Two mutations were identified within the alleles encoding the variant CBG which resulted in amino acid substitutions. To determine the effect of each mutation separately on the steroid binding affinity of CBG, the codons for leucine^{93} and serine^{224} within the human CBG cDNA were mutated to codons for histidine and alanine residues, respectively.

7.3.2 Amino acids Located in the Region Homologous to the ß-barrel of A1-PI

Thyroxine binding globulin (TBG), α_1-proteinase inhibitor (A1-PI) and CBG, as members of the serine protease inhibitor family, share a relatively high degree of similarity (Hammond *et al.*, 1987). The three dimensional structure of A1-PI has

been determined, and has revealed a ß-barrel that resembles a ligand binding domain (Loebermann *et al.*, 1984; Huber and Carrell, 1989), and it has been postulated that a comparable structure within TBG might form part of the thyroxine binding site (Flink *et al.*, 1986). When the human CBG polypeptide sequence is superimposed on the A1-PI tertiary structure, regions of the CBG primary structure which correspond to the ß-barrel of A1-PI may be identified. The cysteine^{228} residue previously implicated in human CBG steroid binding (Hammond *et al.*, 1987; Smith and Hammond, 1989) is located within one of these regions, and this suggests that comparisons between the ß-barrel of A1-PI and the ligand binding domain of CBG are valid. To confirm that cysteine^{228} contributes to the steroid binding characteristics of human CBG, the codon for this residue was mutated to one similar in volume (Prakash and Timasheff, 1985) and charge, serine.

Numerous studies suggest tryptophan is present in the steroid binding site (Akhrem *et al.*, 1978; Stroupe *et al.*, 1978; Akhrem *et al.*, 1980; Akhrem *et al.*, 1981). There are only four tryptophan residues within the mature human CBG polypeptide (Hammond *et al.*, 1987) and based the comparisons of CBG to the tertiary structure of A1-PI, tryptophan^{185} is most likely to be located in the steroid binding domain. For this reason, the codon for residue 185 was mutated to that of phenylalanine in order to conserve side-chain volume (Prakash and Timasheff, 1985) and hydrophobicity characteristics (Levitt, 1976).

Huber and Carrell (1989) have examined the structure of A1-PI in the context of other proteins which exhibit ligand binding properties, and concluded that, like

A1-PI, these proteins also have ß-barrel structures. However, A1-PI does not have any recognized carrier function, and it has been suggested that the presence of a "bulky" tryptophan may block the entrance to the putative ß-barrel ligand binding domain (Huber and Carrell, 1989). In support of this theory, smaller residues are found in the equivalent position of TBG and CBG (threonine and glutamine, respectively) which may allow hormones access to the barrel (Huber and Carrell, 1989). To test this hypothesis, the codon for glutamine^{229} of the human CBG cDNA was mutated to yield the codon for tryptophan.

7.3.3 Amino Acids Distinguished by Phylogenetic Comparisons of CBG

Comparisons of the primary structures of human, rat, mouse, rabbit and squirrel monkey CBG have revealed regions of high and low homology (see chapter 3 and Hammond *et al.,* 1991b) and this information, in conjunction with the results of physicochemical studies, was used to select amino acids which may influence the steroid binding or physicochemical characteristics of CBG.

Squirrel monkey and human CBG share the highest degree of identity between their primary structures, yet their physicochemical characteristics are the least similar; human CBG is a monomer with high affinity for cortisol, while squirrel monkey CBG circulates as a dimer with relatively low cortisol binding affinity (see chapter 3). The most obvious difference between their polypeptide sequences is the additional amino acid (threonine^{144}) in the squirrel monkey sequence. Therefore, a codon for an additional threonine residue was inserted into the cDNA for human CBG between the codons for residues 143 and 144 (CBG-Thr$^{143/144}$) to determine

the influence of this residue on the steroid binding affinity and stoichiometry of human CBG.

Chemical modification studies have also localized a tyrosine within the steroid binding site of human CBG (Le Gaillard *et al.*, 1982). Nine of the eleven tyrosines within human CBG (Hammond *et al.*, 1987) are conserved between the five species of CBG examined (Figure 3.7) and six of those are located within regions expected to compose the ß-barrel steroid binding pocket. The shapes of hydrophobic clusters of amino acids have been related to identifiable three-dimensional structural features (Gaboriaud *et al.*, 1987) and based on this type of analysis, limited regions of sequence identity between human CBG and the steroid receptors suggest that tyrosine^{200} and tyrosine^{288} may be involved in steroid binding (Mornon *et al.*, 1989). For this study, only one of these residues was examined, and the codon for tyrosine^{200} was mutated to a residue of similar size (Prakash and Timasheff, 1985), histidine.

In contrast to human, rabbit and squirrel monkey CBG, mouse and rat CBG have a greater binding affinity for corticosterone than cortisol (Lindenbaum and Chatterton, 1981; Favre *et al.*, 1984; Smith and Hammond, 1991b). Phylogenetic comparisons of CBG's primary structure revealed that regions of rat and mouse amino acid sequence are often identical, yet different from that conserved in human, rabbit and squirrel monkey CBG. To determine if substitution of "rodent-specific" amino acids for those conserved in the other three species would influence the steroid binding specificity of CBG, codons for leucine^{221} and histidine^{222} were

mutated to yield those of phenylalanine and arginine, respectively. These residues were chosen because they are located in proximity of cysteine^{228} which is believed to be present in the steroid binding site (Hammond *et al.*, 1987; Smith and Hammond, 1989; Huber and Carrell, 1989).

7.4 Results: CBG Variants Produced by Site-directed Mutagenesis

7.4.1 Identification of Recombinant CBG Cortisol Binding Variants

Media obtained from cDNA-transfected cells were subjected to CBG measurement by cortisol binding capacity assay and radioimmunoassay. The comparison of the values obtained for each assay revealed that the recombinant products exhibit a variable cortisol binding capacity in relation to their mass (Figure 7.4). Most notably, recombinant CBG-His^{93} and CBG-Thr$^{143/144}$ appeared to have reduced and enhanced cortisol binding affinity, respectively. Media obtained from cells transfected with cDNAs for CBG-His^{93}, CBG-Ala^{224}, CBG-Thr$^{143/144}$ and normal CBG have been subjected to additional analyses (see below), while the remaining recombinant products await further examination.

7.4.2 Cortisol Binding Affinity of CBG cDNAs Expressed in Culture

Scatchard analyses were performed on recombinant CBG-His^{93}, CBG-Ala^{224} and normal CBG present in culture media (Figure 7.5), and the cortisol binding affinity (mean ± SE) of CBG mutated at residue 93 (K_d = 2.81 ± 0.93 nM; n = 5) was much lower than CBG mutated at residue 224 (K_d = 0.80 ± 0.18 nM; n = 6) or unmutated CBG produced by CHO cells (K_d = 0.74 ± 0.12 nM; n = 10). The

Figure 7.4 Comparison of CBG measurements in media obtained from CHO cells transfected with human CBG cDNAs for 1, CBG-Ser^{228}; 2, CBG-$Thr^{143/144}$; 3, CBG-Phe^{185}; 4, CBG-Trp^{229}; 5, CBG-$Phe^{221}Arg^{222}$; 6, CBG-Ala^{224}; 7, CBG-His^{200} and 8, CBG-His^{93} by cortisol binding capacity assay (y-axis) and radioimmunoassay (x-axis). The slope is given for comparison purposes and represents unity between binding capacity and radioimmunoassay values.

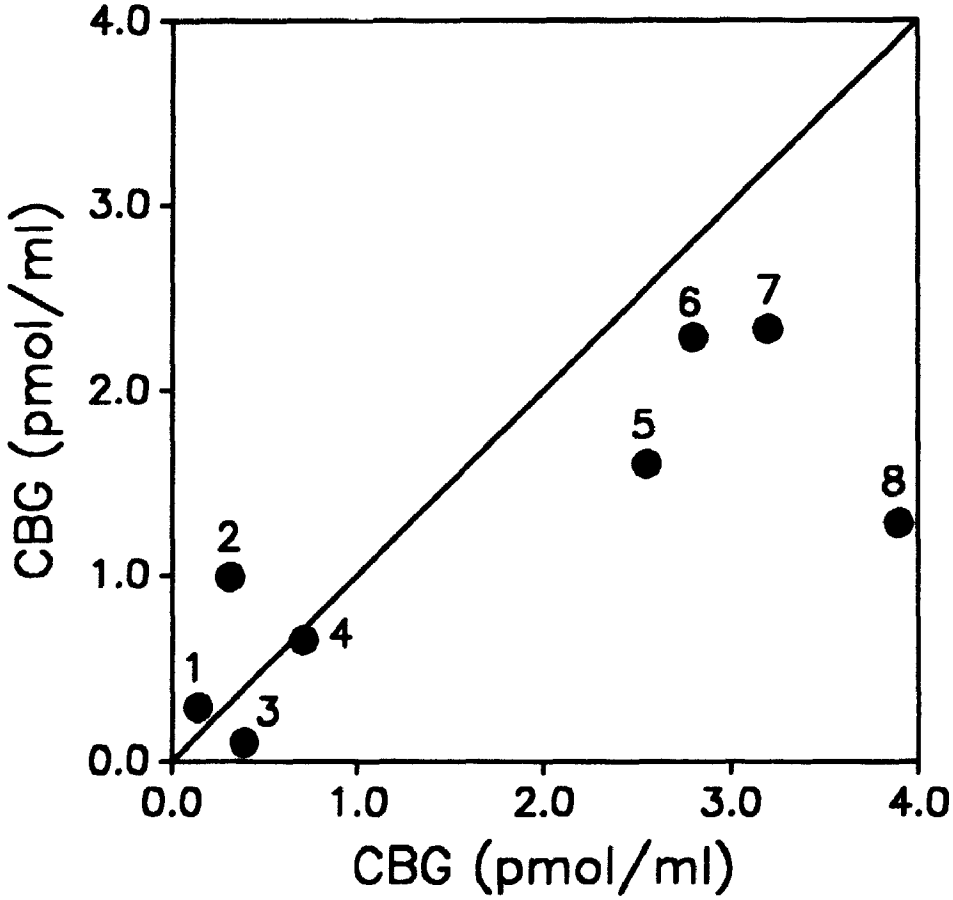
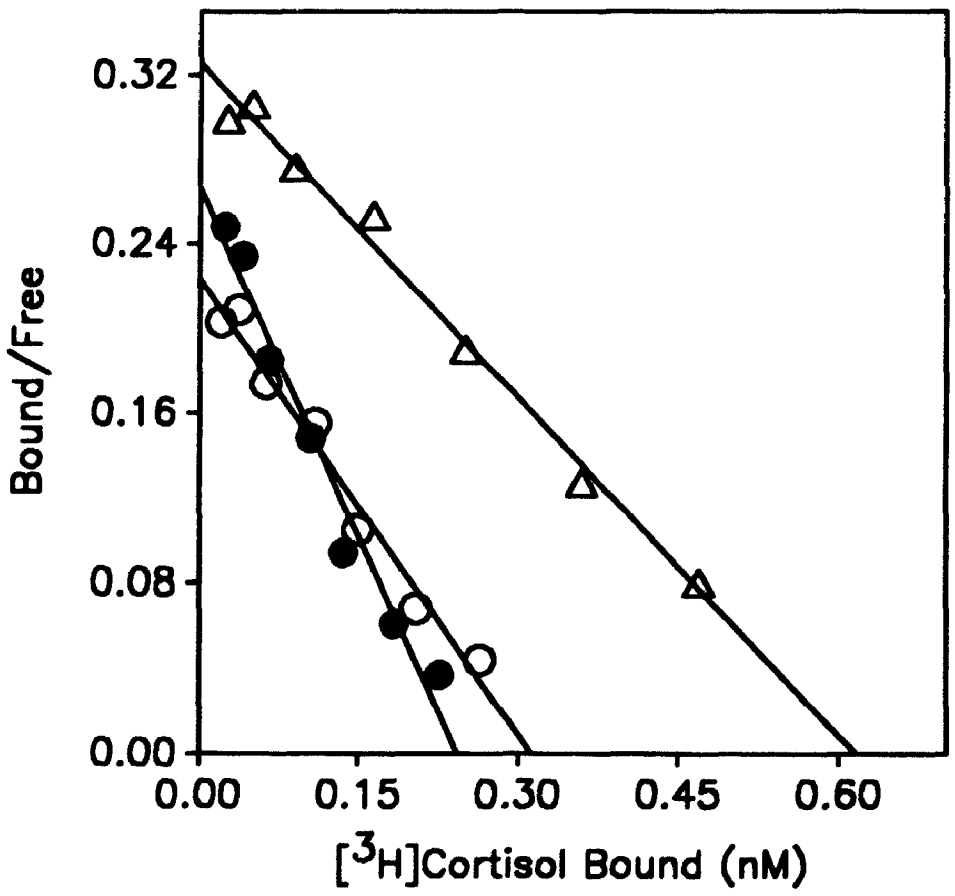

4.0
3.0
2.0
1.0
0.0
CBG (pmol/ml)
0.0
1.0
2.0
3.0
4.0
CBG (pmol/ml)
1
2
3
4
5
6
7
8

Figure 7.5 Representative Scatchard plots of normal CBG (●), CBG-His^{93} (▲) and CBG-Ala^{224} (○) produced in culture. Media obtained from transfected CHO cells were incubated at 0°C with [^{3}H]cortisol, and free steroid was removed with dextran-coated charcoal.


0.32
0.24
0.16
0.08
0.00
Bound/Free
0.00
0.15
0.30
0.45
0.60
[3H]Cortisol Bound (nM)

cortisol binding affinity of CBG-Thr$^{143/144}$ was also examined by Scatchard analysis (Figure 7.6), and found to be relatively, very high (K_d = 0.28 nM).

7.4.3 Stoichiometry of CBG-Thr$^{143/144}$

Medium obtained from CHO cells transfected with the CBG-Thr$^{143/144}$ cDNA was subjected to gel filtration on an FPLC Superose 12 column (Pharmacia), and the elution profile of immunoreactive CBG is presented in Figure 7.7. Size standards were also resolved under similar conditions, and the peak of CBG-Thr$^{143/144}$ immunoreactivity corresponds to a molecular mass of 40 to 55 kiloDaltons. To ensure that CHO cells are capable of producing a CBG dimer, the squirrel monkey CBG cDNA was expressed, and the resulting media was examined by western blotting under native conditions (Figure 7.8). Both serum and recombinant squirrel monkey CBGs migrate with a relative mobility similar to the human CBG dimer which forms spontaneously during storage of the purified protein (Mickelson *et al.,* 1982). Human serum CBG was present in monomeric form.

7.5 Discussion

It has been demonstrated that human CBG may promote the delivery of cortisol to neutrophils during inflammation (Hammond *et al.,* 1990a), and we therefore examined serum from patients with acute inflammatory diseases for abnormal CBG steroid binding activity. Patients with septicemia have much lower serum CBG concentrations than normal (Pugeat *et al.,* 1989), and 68% of the patients studied had serum CBG levels that fell below the reference range (14.9-25.2 μg/ml) defined

Figure 7.6 Scatchard plot of CBG-Thr$^{143/144}$ produced in culture. Medium obtained from a cDNA-transfected CHO cell line was incubated at 0°C with [^{3}H]cortisol, and free steroid was removed with dextran-coated charcoal.

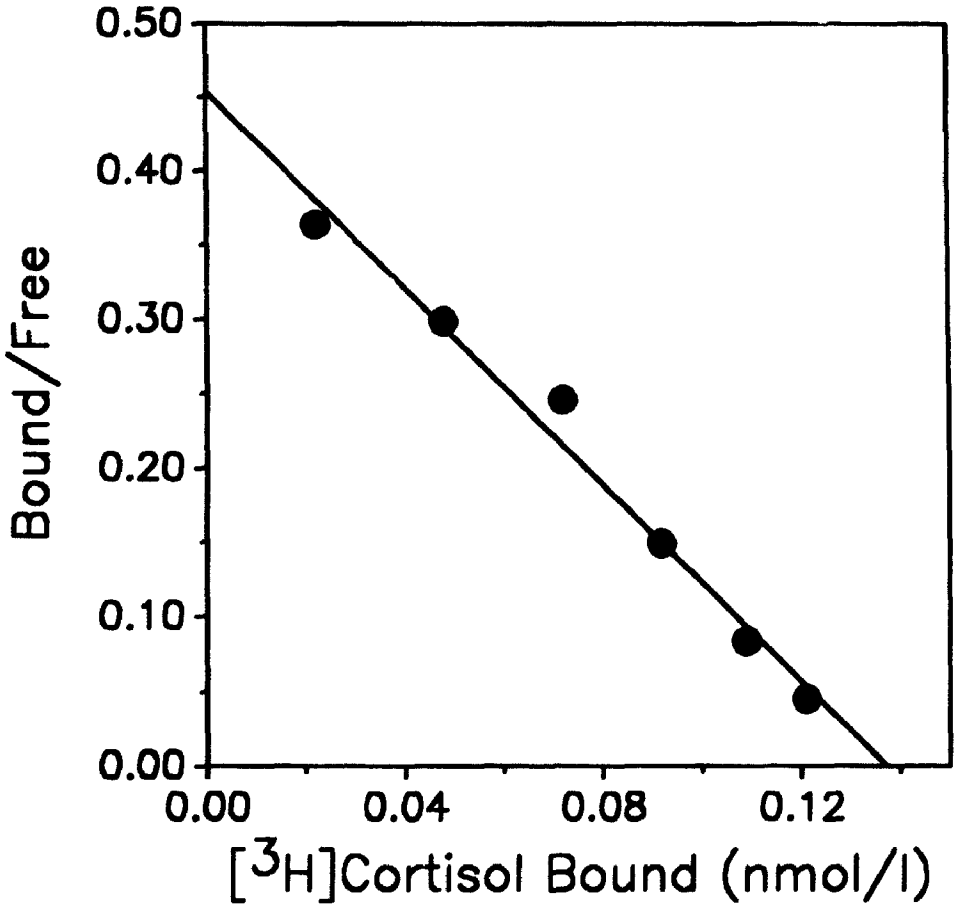

0.50
0.40
0.30
0.20
0.10
0.00
Bound/Free
0.00
0.04
0.08
0.12
[3H]Cortisol Bound (nmol/l)

Figure 7.7 Elution profile of CBG-Thr$^{143/144}$ following size exclusion chromatography. Media obtained from transfected CHO cells was applied to a Superose 12 column and fractions were subjected to CBG measurement by a human CBG radioimmunoassay. The percentages of radiolabelled CBG displaced from the solid phase antibody by column fractions are illustrated, and the positions of eluted size standards (given in kDa) are indicated *above*.

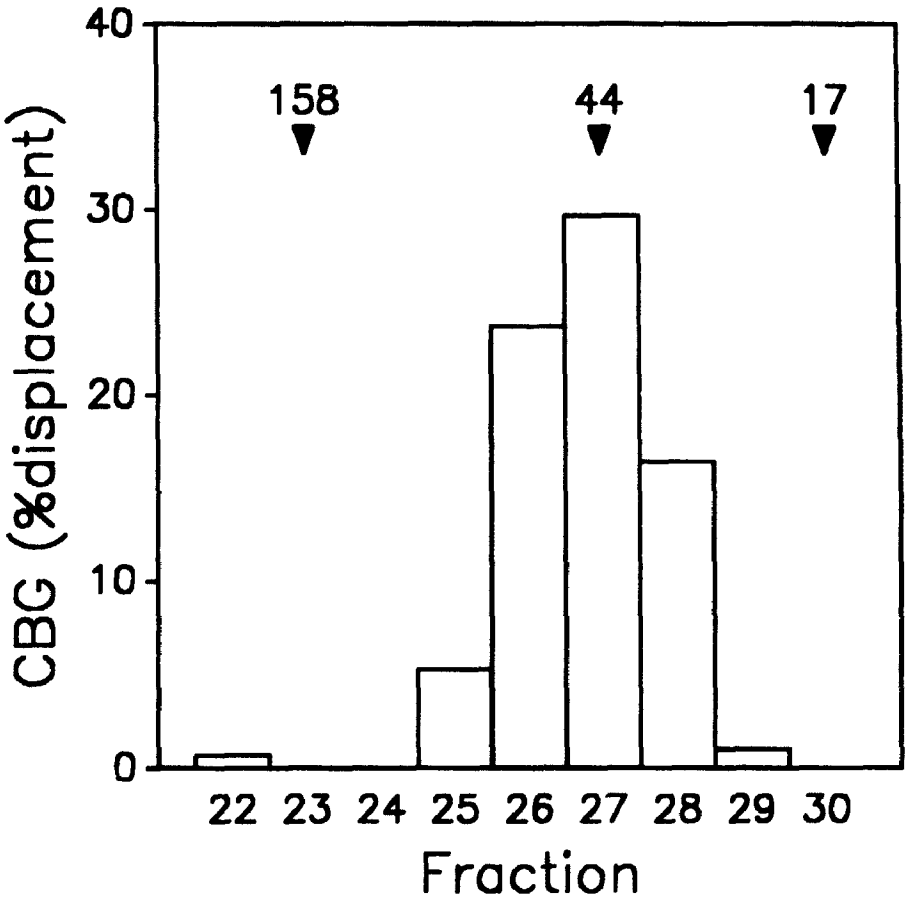
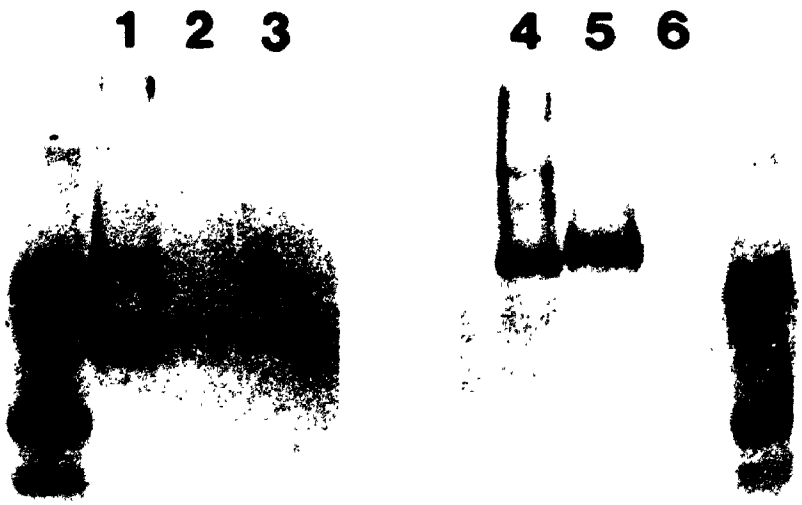

40
30
20
10
0
CBG (%displacement)
158
44
17
22 23 24 25 26 27 28 29 30
Fraction

Figure 7.8 Western blot of lane 1, purified human CBG (20 ng); lane 2, human serum (0.1 μl); lane 3, recombinant human CBG (20 μl); lane 4, squirrel monkey serum (0.1 μl); lane 5, recombinant squirrel monkey CBG (20 μl) and lane 6, conditioned Chinese hamster ovary medium (20 μl). Proteins were resolved by 7.5% separating polyacrylamide gel electrophoresis and transferred to an Immobilon membrane. Lanes 1 to 3 were probed with rabbit antisera against human CBG while lanes 4 to 6 were probed with antisera against squirrel monkey CBG. Molecular size standards are shown to the *left* of lane 1 and the *right* of lane 6.


1 2 3
4 5 6

for the radioimmunoassay (Robinson *et al.*, 1985b). When the CBG binding capacity values were compared to CBG concentrations determined by radioimmunoassay, 1 out of 22 patients was characterized by an abnormally low binding capacity value, similar to that previously associated with a CBG variant with reduced cortisol binding affinity (Robinson and Hammond, 1985). This represents a higher frequency than would be anticipated from reports in which CBG variants have been identified with a frequency of 3:500 and 1:400 within general populations (Van Baelen *et al.*, 1982; Eichner *et al.*, 1989). The carriers of these variants are known (Van Baelen *et al.*, 1982) or suspected (Eichner *et al.*, 1989) to be of Central European descent, and it is therefore interesting that the patient identified with a CBG variant originates from the same geographic area. In view of the possible involvement of CBG in the delivery of natural glucocorticoids to sites of inflammation (Hammond *et al.*, 1990a), it is tempting to speculate that these mutations may have in some way contributed to the development of these diseases and the poor control of acute infection in this patient.

Although individuals homozygous for a CBG variant with reduced cortisol binding affinity exist (Van Baelen *et al.*, 1982), most are heterozygous for this trait (Robinson and Hammond, 1985; Van Baelen *et al.*, 1982). The individual we have identified is obviously heterozygous with respect to CBG and our measurements of serum CBG binding characteristics of this patient probably represent the combined properties of both alleles, because normal and variant alleles are generally expressed in approximately equal proportions (Robinson and Hammond, 1985; Van Baelen *et al.*, 1982). In fact, the reduction in CBG steroid binding affinity in this

patient more closely resembles that of individuals heterozygous (Robinson and Hammond, 1985) rather than homozygous for this type of variant (Van Baelen *et al.*, 1982). When compared to previous analyses of sequences that encode human CBG, the coding sequences of the variant CBG alleles are relatively variable. In addition to the presence of three silent mutations, two point mutations were identified which result in amino acid substitutions. These two mutations are located on separate exons, and we are therefore unable to determine if they are present on the same allele.

Although specific residues have been identified within CBG's steroid binding domain, their location within the CBG polypeptide is not known. Investigation of naturally-occurring mutations of the CBG gene offers a viable approach for the identification of amino acids which contribute to the steroid binding ability of CBG, but the limited number of known carriers restricts the information that may be obtained from this type of analysis. In addition, this screening assay detects only steroid binding variants, and thus carriers of CBG molecules with other inappropriate physicochemical properties will not be identified. Site-directed mutagenesis and expression of the resulting cDNA enables the influence of any amino acid substitution to be examined, and is therefore a powerful tool for the study of the relationship between CBG's primary structure and its function. There are, however, limitations to the conclusions that may be drawn from the analysis of recombinant proteins. For example, mutations may reduce the steroid binding affinity of CBG because of global changes in protein structure, rather than the removal of a functional group required for appropriate steroid interaction. To

minimize this occurrence, the amino acid substitutions performed in this study were conservative in nature, and were only made after considering both phylogenetic comparisons of CBG structure and comparisons with related serpins. This represents the best available criteria with which to chose residues important for steroid binding activity rather than maintanance of the characteristic three-dimensional structure of serpins. However, the impact of mutations on structure may be assessed by analyzing the ability of normal and mutant recombinant products to displace normal, radiolabelled CBG from polyclonal antibodies against CBG. Displacement curves generated by mutant products which have lost epitopes, due to structural perturbations, will not parallel those of normal CBG.

Two amino acid substitutions were detected in the CBG gene of the CBG steroid binding variant carrier, and to more precisely determine the relative impact of each of these mutations, the normal and mutated CBG cDNAs were expressed in CHO cells. Characterization of the resulting media by the same type of analysis used to identify serum CBG variants indicates the steroid binding ability of the CBG-His^{93} mutant is low in relation to its mass, while the influence of the $serine^{224}$ to $alanine^{224}$ substitution appears to be negligible. The recombinant products were further examined by Scatchard analysis in comparison to normal CBG. As expected, the mutation at residue 93 reduced the cortisol binding affinity of CBG, while that at residue 224 produced only a slight effect.

The primary structures of CBG and A1-PI are closely related to thyroxine binding globulin (TBG), and a TBG mutation is located in the same relative

position as residue 91 of the CBG molecule (Hammond *et al.*, 1987; Mori *et al.*, 1989). This mutation (TBG-Asn^{96}) is responsible for a reduction in the thyroxine binding affinity of TBG (Mori *et al.*, 1989), and suggests that this region contributes to the ligand binding properties of those serpins which act as hormone transport proteins. The TBG-Asn^{96} variant is also characterized by reduced stability and this has been associated with the creation of an additional consensus site for *N*-glycosylation (Mori *et al.*, 1989). The histidine^{93} mutation of CBG does not appear to influence its stability because the serum CBG concentration of the carrier was increased by approximately 2-fold to within the normal range after recovery from sepsis. Moreover, levels of CBG-His^{93} expression in culture media were similar to those of normal CBG.

When compared to the tertiary structure of A1-PI, the other naturally-occurring mutation identified (residue 224) is located in close proximity to the proposed ß-barrel ligand binding domain (Loebermann *et al.*, 1984; Huber and Carrell, 1989) which also contains a residue (cysteine^{228}) previously implicated in steroid binding (Khan and Rosner, 1977; Hammond *et al.*, 1987). Therefore, although residue 224 may be present within the steroid binding domain, the relatively small influence of the serine to alanine substitution suggests this amino acid does not significantly contribute to the steroid binding affinity of CBG.

The steroid binding capacity of the CBG-Thr$^{143/144}$ mutant appears to be high for its mass, and Scatchard analysis of the recombinant protein suggests its affinity for cortisol may be significantly greater than that of normal recombinant CBG. It

would therefore appear that the additional threonine present in squirrel monkey CBG does not reduce its cortisol binding affinity. Unlike squirrel monkey CBG, human CBG-Thr$^{143/144}$ is produced as a monomer in culture, and this threonine insertion does not therefore appear to be responsible for the production of squirrel monkey CBG dimers. It is possible however, that this residue in combination with other squirrel monkey-specific residues is required to produce a CBG dimer with low steroid binding affinity.

The expression of the squirrel monkey CBG cDNA under similar culture conditions yielded a dimeric molecule, and supports previous reports of a circulating CBG dimer in squirrel monkeys (Klosterman *et al.,* 1986; Kuhn *et al.,* 1988). These results also indicate that the formation of a CBG dimer is not due to species-specific post-translational modifications since the same cell type produced monomeric human and dimeric squirrel monkey CBG. Media containing recombinant human and squirrel monkey CBG were manipulated in an identical fashion, and squirrel monkey CBG dimers are therefore not due to purification and storage artifacts, as are human CBG dimers (Mickelson *et al.,* 1982).

The glutamine^{229} to tryptophan^{229} substitution of human CBG did not appear to reduce or eliminate steroid binding activity, and it would therefore seem unlikely that the presence of a tryptophan residue in a corresponding position of A1-PI eliminates its carrier function as was suggested by Huber and Carrell (1989). This is not surprising, and it would seem likely that many residues are required to maintain an appropriate steroid binding environment.

The tyrosine^{200} to histidine^{200} and leucine^{221}histidine^{222} to phenylalanine^{221}arginine^{222} substitutions created in recombinant CBG both exhibited steroid binding activity, but comparison of radioimmunoassay and binding capacity values suggests that the affinity of these molecules may be low. Further examination by Scatchard analysis is required to substantiate this initial observation, and relative binding affinity analysis will reveal whether CBG-Phe^{221}Arg^{222} preferentially binds corticosterone instead of cortisol. Recombinant CBG-Phe^{185} and CBG-Ser^{228} residues were expressed in culture to determine if the loss of tryptophan^{185} and cysteine^{228} would affect the steroid binding affinity of CBG. The expression of these cDNAs is very low, and it will be necessary to clone cell lines that produce greater amounts of protein or to enrich the media for CBG by partial purification before further analysis can proceed.

CHAPTER 8

Conclusions

8.1 Conclusions

The isolation and characterization of cDNAs for rat, mouse, rabbit and squirrel monkey CBG has revealed their amino acid sequences and enabled a phylogenetic comparison of their primary structures with human CBG. These combined data are a valuable resource for the rational design of future investigations of CBG's physicochemical (*i.e.* steroid binding or dimerization) and biological (*i.e.* utilization of consensus sites for *N*-linked glycosylation, interaction with serine proteases and membrane receptors) properties. Indeed, the phylogenetic comparison was utilized in the design of the site-directed mutagenesis experiments of chapter 7.

Corticosteroid binding globulin concentrations change in response to a myriad of biological stimuli. However, it is not feasible to study the molecular mechanisms underlying these changes in humans, and the isolation of non-human CBG cDNAs has therefore provided the necessary molecular biological reagents for their study in animal models. For example, the rat CBG cDNA was used to examine hepatic CBG mRNA levels during ontogeny, hormonal administration and inflammation, and by comparing these values to circulating CBG concentrations, fluctuations in serum CBG levels could be attributed to either anabolic or catabolic processes. These data have therefore extended our understanding of the mechanisms that regulate serum CBG concentrations, and provide a biochemical background for the identification of the mechanisms which regulate rat CBG gene expression.

The identification and analysis of human and rat CBG steroid binding variants represents the first detailed characterization of the molecular basis of reduced

steroid binding affinity. It is interesting that the carrier of the human CBG variant was identified in a patient population suffering from sepsis, and in light of the possible role of CBG - elastase interactions in the modulation of bioavailable glucocorticoids during inflammation, it would seem reasonable to screen other patients with inflammatory diseases for variant CBG activity. Similarly, BioBreeding rats were identified as carriers of a variant CBG, and screening other rat strains which model inflammatory disease processes may also reveal variant CBG molecules. The identification and molecular characterization of new CBG steroid binding variants will further increase our understanding of relationships between structure and function.

Expression of rat and human CBG cDNAs in culture allows the impact of naturally-occurring amino acid substitutions to be assessed under controlled conditions. More importantly, this system enables the contribution of any other amino acid residue, suspected to influence steroid binding activity, to be assessed following site-directed mutagenesis and expression of recombinant products. Future analyses need not, however, be limited to the study of the steroid binding domain and the impact of structural alterations on any measurable biological property, including elastase-cleavage and membrane receptor recognition, may be assessed in a similar manner.

Glucocorticoids are essential modulators of physiological processes. The ability of vertebrate species to alter both hormone and CBG concentrations in response to endogenous and exogenous stimuli suggests the interplay between steroid and

binding protein is crucial for the regulation of glucocorticoid bioavailabilty. These studies have provided molecular reagents for the study of CBG's structure in relation to function and their future use may provide new insites into the biological activity of this protein with respect to glucocorticoid delivery.

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